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Mecanismes neuronals de la picor aguda i crònica en neurones sensorials: implicació del canal TRESK

Alba Andrés Bilbé



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Mecanismes neuronals de la picor aguda i crònica en neurones sensorials: implicació del canal TRESK

Tesi doctoral presentada per

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Doctorat de Biomedicina per la Universitat de Barcelona

Setembre 2020

Treball realitzat sota la direcció del

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Barcelona, 18 de setembre de 2020

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CERTIFIQUEN:

Que el treball d'investigació corresponent a la tesi doctoral que porta per títol "Mecanismes neuronals de la picor aguda i crònica en neurones sensorials: implicació del canal TRESK" del que és autora Alba Andrés Bilbé, ha estat realitzat sota la seva direcció al departament de Biomedicina de la Universitat de Barcelona.

I per a que així consti i als efectes oportuns, firmen el present certificat a Barcelona, el 18 de setembre de 2020.

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I. INTRODUCCIÓ

1. Què és la picor?

La primera descripció de picor o pruritus va ser proposada per Samuel Haffenreffer l'any 1660, on es va definir el pruritus com una sensació desagradable que provoca el desig de rascar-se. Aquesta definició va ser adoptada pel prestigiós dermatòleg Stephen Rothman l'any 1941 (Rothman, 1941) i segueix essent l'acceptada actualment pel Fòrum Internacional per l'Estudi de la Picor (*International Forum for the Study of Itch - IFSI*).

La principal funció associada a la sensació de picor és la posterior resposta motriu de rascat generada per eliminar agressions ambientals de la pell com, per exemple, els insectes i les plantes verinoses. Per aquest motiu es considera la picor una sensació protectora i creada com a resposta al medi extern. Tot i que la sensació pot resultar desagradable i, en canvi, la resposta de rascat gratificant, la vida dels pacients que pateixen picor patològic es veu altament afectada.

La determinació de la prevalença del pruritus en la població general és complicada ja que es tracta d'un símptoma exclusivament subjectiu i del qual només se'n poden registrar de forma objectiva els efectes secundaris. És un símptoma molt comú en la clínica, que companya diverses patologies cutànies, sistèmiques i

psicogèniques i que s'ha reportat amb més freqüència que altres símptomes molt habituals en clínica com la falta de gana, la pèrdua de pes, canvis en el gust, nàusees, vòmits, diarrea i disfunció sexual (F Dalgard et al., 2004; Silverberg et al., 2016).

La picor és el símptoma cutani més reportat, tot i que són pocs els estudis en que s'avalua la seva epidemiologia d'aquesta variable en la població general. Els valors de prevalença del pruritus en la població general varien entre el 8 i el 38% arreu del món (Mollanazar et al., 2015). Un dels primers estudis, publicat l'any 1976 i realitzat sobre la població general de Londres, va reportar una prevalença del símptoma del 8,2% sense diferenciar entre picor agut i crònic (Rea et al., 1976). Dades similars es van obtenir en un estudi transversal dut a terme a Oslo, Noruega, que va incloure un total de 18.770 adults on es va reportar una prevalença del 8,4% sobre la població general (F Dalgard et al., 2004). En un altre estudi alemany més recent, on es van enquestar 11.730 treballadors, es va obtenir una prevalença puntual de picor del 16,7% (S Ständer, Schäfer et al., 2010).

Una de les fonts més comú d'obtenció de dades sobre l'epidemiologia i prevalença de la picor són els estudis realitzats en base a pacients amb afeccions dermatològiques. Aquests grups de població solen presentar una prevalença més elevada ja que la picor és un símptoma extensament present en condicions cutànies. Un gran estudi multicèntric europeu publicat l'any 2019, mostra que aproximadament la meitat dels pacients que visiten un ambulatori dermatològic a Europa reporten presentar picor en el moment de la visita. A més, al voltant del 37% d'ells pateix picor amb una durada superior a 6 setmanes, classificat com a picor crònica (Schut, FJ Dalgard et al., 2019).

Estudiar un símptoma com la picor a nivell comunitari és important per comprendre les seves repercussions psicosocials i la seva associació amb altres factors i malalties. S'ha correlacionat el pruritus amb un empitjorament de la qualitat de vida de les persones que la pateixen, provocant efectes perjudicials tant a nivell psicològic com funcional (Weisshaar et al., 2009; Pithadia et al., 2020). Investigacions sobre la qualitat de vida han trobat que la picor afecta negativament la capacitat de concentració, el desig i l'activitat sexual, la confiança en les interac-

cions interpersonals i la qualitat i quantitat del son, entre d'altres (Silverberg et al., 2016; Pithadia et al., 2020). Altres estudis mostren una prevalença major de depressió, ansietat i idees suïcides associades amb la presència de picor en pacients dermatològics en comparació amb pacients sense picor (Marron et al., 2016; FJ Dalgard et al., 2020). Els resultats d'aquests estudis relacionen la picor amb un deteriorament general de la qualitat de vida dels pacients similar a l'associada amb el dolor. La recerca sobre les bases de la picor i els problemes relacionats pot comportar una millora en el tractament i la comprensió dels pacients.

2. Classificació del picor

Ja que la picor és un símptoma amb una elevada prevalença en la població i és sovint refractària al tractament, és important diagnosticar i classificar adequadament el tipus de picor al que ens enfrontem. No existeix una classificació oficial dels diferents tipus de picor, tot i que les tres propietats emprades per la seva distinció en la clínica són: la durada, les manifestacions clíniques associades i l'etiologia (descrites en la Figura 1) (S Ständer, 2016).

2.1. Segons la seva durada

La picor es pot distingir entre aguda o crònica depenent de la durada de la sensació. El Fòrum Internacional per l'Estudi del Picor (IFSI) descriu que si la sensació es manté present durant 6 setmanes o més, es defineix com a picor crònica (S Ständer, H Ständer et al., 2007; Yosipovitch & Bernhard, 2013).

En sentir-se de forma aguda, la picor evoca una resposta protectora innata de rascat que ajuda a l'eliminació d'agents irritants i a l'activació del sistema immunitari envers la zona de la pell afectada. Encara que la picor aguda pot resultar evolutivament útil, si aquesta sensació es cronifica, es converteix en un símptoma patològic que afecta greument la qualitat de vida de qui la pateix (Mack et al., 2018).

2.2. Segons la manifestació clínica

Una altre forma de classificar la picor, quan aquesta es presenta a la superfície de la pell, és segons la manifestació clínica amb la que apareix (S Ständer, H Ständer et al., 2007; S Ständer, 2016). Aquesta distinció, permet un cribatge ràpid inicial, que pot ajudar a diagnosticar els pacients i identificar l'etiologia de la picor per al seu possible tractament.

En un examen físic dels pacients que pateixen picor, l'observació de l'estat de la pell permet diferenciar si aquesta presenta lesions i si les lesions són primàries o secundàries. Així, es poden diferenciar tres grans grups:

- **Grup I: Picor en pell patològica.** Picor causat per una patologia cutània subjacent, en pacients amb lesions cutànies primàries (pròpies de la malaltia subjacent) i amb inflamació de la pell. En són exemples la psoriasi, la dermatitis atòpica, els limfomes cutanis de cèl·lules T i les picades d'insectes.
- **Grup II: Picor en pell no patològica.** Picor causat per una patologia sistèmica, neurològica o psiquiàtrica en pacients sense lesions cutànies primàries. En aquest grup s'inclou la picor produïda per patologies com la fallada renal crònica, l'infecció per VIH, el limfoma de Hodgkin, l'esclerosi múltiple, l'esquizofrènia i els efectes secundaris associats a l'administració d'opioides o estrògens.
- **Grup III: Lesions per rascat crònic.** Picor crònic en pacients amb lesions cutànies secundàries causades per la pròpia reacció mecànica de rascar-se o fregar-se la pell, com és el cas del prurigo nodular i del liquen simple crònic.

2.3. Segons l'etiologia de la picor

L'enfocament terapèutic en pacients que sofreixen picor s'hauria d'escollir segons els mecanismes de la malaltia subjacent. Ja que actualment no es coneixen tots els mediadors específics i no existeixen dianes terapèutiques per a la majoria de les condicions de picor cròniques, una classificació àmpliament acceptada en la clínica és la basada en l'etiologia de la malaltia subjacent que genera la picor. En aquest sentit, la picor es pot classificar en 6 subtipus diferents (Ikoma, Steinhoff et al., 2006; S Ständer, 2016; Dong & Dong, 2018):

- **Dermatològica:** causada per patologies cutànies com la dermatitis atòpica, la psoriasi, la sarna i la urticària.
- **Sistèmica:** acompanya a malalties sistèmiques d'òrgans diferents a la pell, com ara insuficiència renal crònica, malalties hepàtiques cròniques, limfoma de Hodgkin, afeccions metabòliques, l'embaràs o la picor induïda per fàrmacs.
- **Neurològica:** provinent de danys o malalties del sistema nerviós central o perifèric. Inclouen alteracions com ara tumors cerebrals, esclerosi múltiple, neuropatia perifèrica i compressió o irritació nerviosa.
- **Psicològica/psicosomàtica:** associada a trastorns psicològics o psiquiàtrics com ara trastorns obsessiu-compulsius o la picor que es relaciona amb la il·lusió de parasitosi.
- **Múltiples:** causada per la superposició i coexistència de múltiples patologies subjacents que cursen amb picor.
- **Altres:** amb origen indeterminat o causada per una malaltia no identificable.

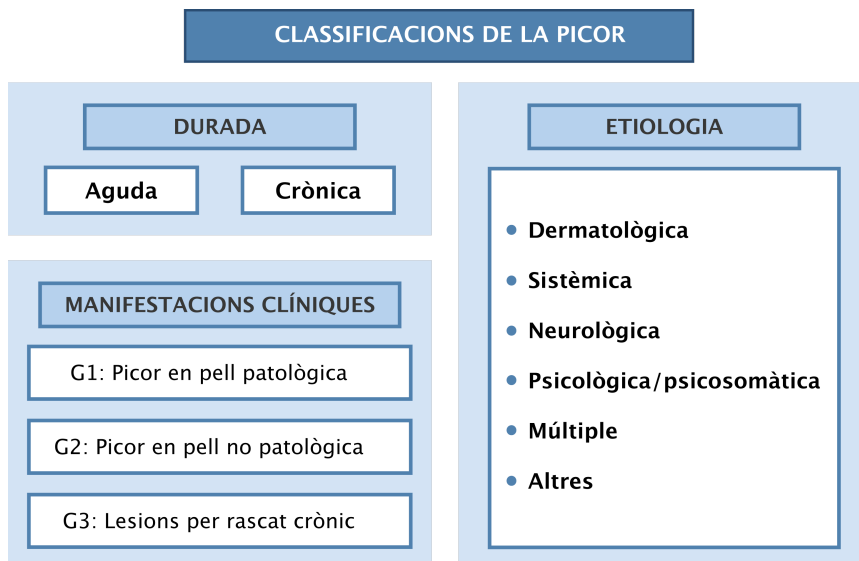


Figura 1: Les diferents classificacions de la picor. Es descriuen les tres propietats més emprades per a la classificació de la picor on s'inclou la durada, la manifestació clínica associada i l'etiologia.

2.4. Classificació neurofisiològica

Per últim, existeix un sistema de classificació proposat per Twycross et al. que es basa en els mecanismes neurofisiològics que originen la picor (Twycross et al., 2003). Tot i que aquesta classificació no és útil per l'aplicació clínica, és molt important per a la recerca neurobiològica. Se'n distingeixen les següents categories segons l'origen de la picor: Pruritoceptiva, induïda per pruritògens que activen les fibres nervioses de tipus C en els terminals nerviosos perifèrics d'un sistema nerviós sa; Neurogènica, originada per l'acció de mediadors a nivell del sistema nerviós central i sense dany nerviós; Neuropàtica, causada per danys o patologies en les neurones; Psicogènica, provocada per trastorns o causes psicosomàtiques o psiquiàtriques (S Ständer, 2016; Cevikbas & EA Lerner, 2020).

2.5. Picor contagiosa

Observar la picor en altres pot generar certa empatia amb ells. La picor contagiosa o social és un fenomen en què l'exposició visual o auditiva a comportaments de rascades en altres també pot induir un comportament de rascades i sensacions de picor reals en els observadors (Ikoma, Steinhoff et al., 2006; Papoiu et al., 2012). La picor contagiosa pot ser experimentada per humans, primats i també ha estat reportada en rosegadors (Holle et al., 2012; Schut, Grossman et al., 2015).

Inicialment es van identificar les neurones mirall com a mecanisme implicat en la generació d'aquest fenomen (Iacoboni, 2009). Recentment, diversos estudis han emprat tècniques de fMRI per investigar els mecanismes cerebrals subjacents a la picor contagiosa. Aquests han demostrat que observar comportaments de rascat en altres persones pot activar determinades regions cerebrals relacionades amb la picor inclosos el tàlem, el còrtex somatosensorial primari, el còrtex premotor, el còrtex motor suplementari, el còrtex prefrontal i l'ínsula (es descriurà més extensament els circuits cerebrals relacionats amb la picor en l'apartat 3.3.4 (Holle et al., 2012; Mochizuki et al., 2015).

La picor visualment contagiosa en ratolins es proposa que sigui transmesa a través de les neurones del sistema nerviós central mitjançant la senyalització

del neuropèptid alliberador de gastrina i el seu receptor (GRP-GRPR) (Yu et al., 2017). Cal destacar que la transmissió visual o social en la picor contagiosa es creu que està restringida només al sistema nerviós central. Tot i això, existeixen altres estudis que posen en dubte la robustesa sobre aquest resultat i ressalten la necessitat d'investigar més profundament en els mecanismes subjacents (Barry et al., 2017; Liljencrantz et al., 2017).

2.6. Perquè ens gratem?

La picor aguda i crònica pot ser induïda per substàncies exògenes com per exemple al·lèrgens, puritògens i irritants quan es posen en contacte amb la pell. Quan aquestes substàncies arriben a la part més externa de la pell, anomenada epidermis, són detectades pels terminals nerviosos perifèrics de les fibres aferents de neurones sensorials, els pruritoceptors. L'activació de determinats receptors en els terminals nerviosos perifèrics produeix una despolarització de la membrana i la generació de potencials d'acció. Aquests potencials d'acció poden passar pels cossos neuronals que es troben en els ganglis de l'arrel dorsal (DRG) o els ganglis trigèmins (TG) o viatjar directament cap a la medul·la. Els axons de les neurones sensorials fan sinapsis amb neurones de la medul·la espinal on la informació és processada per interneurons espinals que es comuniquen amb neurones de projecció. Les neurones de projecció envien el missatge, a través de les vies ascendents, a diverses àrees i circuits del cervell relacionats amb la codificació de la picor com són el tàlem i el còrtex sensorial (Figura 2) (Cevikbas & EA Lerner, 2020).

La sensació de picor, codificada per les diferents àrees del cervell, genera una resposta motora de protecció coneguda com rascat, que altera la barrera epidèrmica i facilita una cascada fisiològica per fer front a l'agressió externa. Aquesta cascada implica l'activació i comunicació multidireccional entre el sistema nerviós i el sistema immunitari presents en la pell. Si la sensació de picor perdura en el temps, pot desembocar en un procés patològic definit com a cicle de picor. El cicle de picor és un procés dinàmic on hi poden influir factors com el plaer que acompanya el rascat, que reforça l'activitat motora de rascat per incrementar la comunicació neuroimmunitària (Figura 2) (Mack et al., 2018).

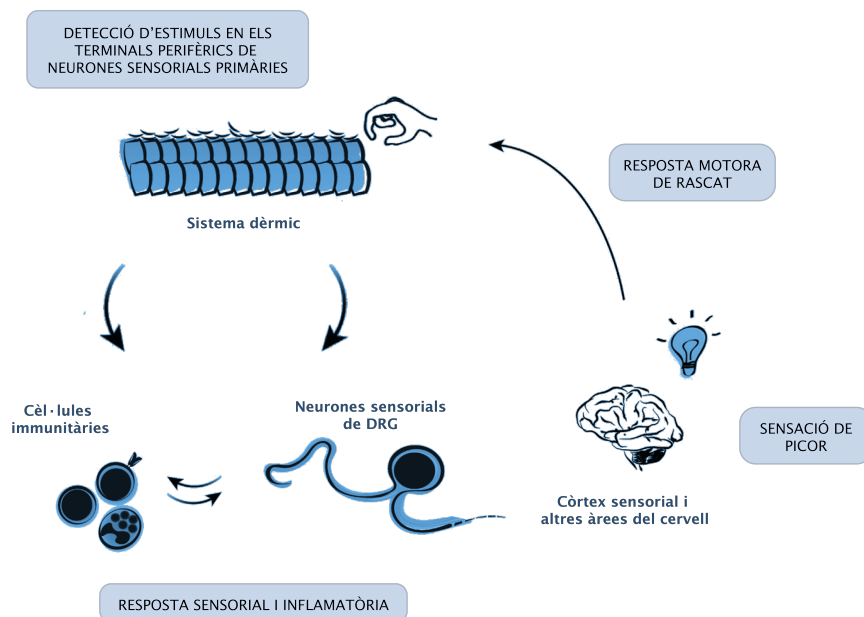


Figura 2: Esquema del cicle de la picor i el rascat. Descripció esquemàtica de les etapes que segueix la transducció, transmissió i resposta envers un estímul prurític. Adaptació de (Mack et al., 2018)

2.7. Estratègies terapèutiques en picor

Malgrat la necessitat urgent de trobar tractaments més eficaços i segurs per a la picor i el dolor crònics, la picor ha rebut poca atenció en l'àmbit de la recerca. No existeix un sol tractament antiprurític universal i els tractaments actuals per picor crònica es limiten a antihistamínics, corticosteroides tòpics o sistèmics o a certs antidepressius. No obstant això, l'eficàcia d'aquests fàrmacs és limitada i la seva aplicació està associada a molts efectes secundaris greus.

Un principi fonamental per a la gestió de la picor és que cal adaptar el tractament segons l'origen i per això és necessari identificar l'etiologia subjacent en cada cas. L'exploració dels diversos orígens del pruritus ha permès identificar noves molècules, cèl·lules i circuits implicats críticament en la generació de picor aguda i crònica que es poden convertir en noves dianes terapèutiques.

Si bé la histamina ha estat el principal focus d'interès pel tractament envers la picor, nous descobriments han revelat la poca implicació d'aquest mediador en la picor crònica. Així doncs, els mecanismes independents d'histamina s'han posat al capdavant de la teràpia antiprurítica, assenyalant els receptors de membrana, els canals iònics, els neuropèptids i les citocines com les noves dianes per als medicaments contra la picor (Stull et al., 2015; Yosipovitch, Rosen et al., 2018). A més, degut a les evidències recents, les teràpies immunomoduladores i els tractaments amb agents que regulen la hipersensibilització neuronal són de gran interès per al desenvolupament terapèutic en pacients amb picor crònica (S Ständer & Weisshaar, 2012; S Ständer, Weisshaar & Raap, 2015).

3. Sistema somatosensorial

El sistema nerviós somatosensorial té un paper clau en el control de l'estat del cos interna i externament, ens ajuda a reaccionar eficaçment davant els canvis de circumstàncies i comprèn diversos subconjunts neuronals. Les neurones sensorials es poden classificar segons el diàmetre del cos cel·lular, el grau de mielinització, les propietats electrofisiològiques, la innervació perifèrica dels òrgans sensorials, les projeccions centrals en la medul·la espinal i el patró d'expressió gènica (Basbaum & Bráz, 2010; Lallemand et al., 2012; Abaira et al., 2013).

Els subconjunts de neurones somatosensorials medien diferents modalitats funcionals, entre les que es troba el tacte, la termocepció, la propiocepció, la pruritocepció i la nocicepció. La diferenciació i la connectivitat de les diverses poblacions d'aferents sensorials es controla durant el desenvolupament a través de l'expressió de receptors neurotròfics i factors de transcripció (Marmigère et al., 2007; Abaira et al., 2013).

Les neurones sensorials inclouen fibres de petit diàmetre de tipus C no mielinitzades i de tipus A δ lleugerament mielinitzades, i fibres de mitjà i gran diàmetre de tipus A α i A β mielinitzades (Basbaum, Bautista et al., 2009; Abaira et al., 2013).

Les fibres s'estenen des de la medul·la espinal fins a la pell, les viscères, els músculs i les articulacions de tot el cos. La detecció dels diferents tipus d'estímuls de l'ambient extern o intern per part de les neurones somatosensorials es produeix mitjançant l'expressió de transductors moleculars específics als seus terminals nerviosos perifèrics (Julius et al., 2001; Basbaum, Bautista et al., 2009). Posteriorment, aquestes fibres transporten la informació sensorial a la medul·la espinal, on es pot transmetre al cervell i per processar-la (Figura 3).

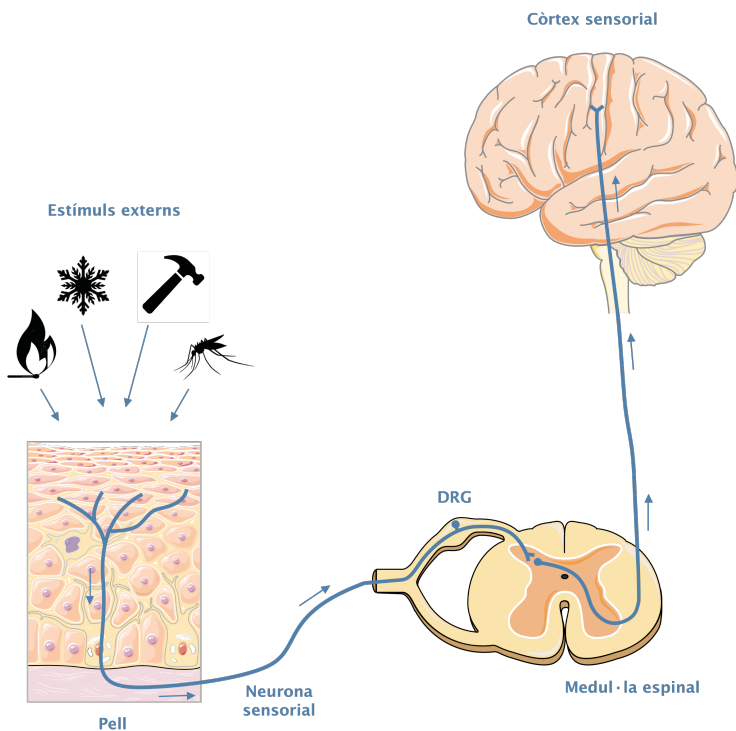


Figura 3: Organització general del sistema somatosensorial. Les neurones sensorials cutànies són activades per diversos estímuls i es projecten cap a la banya dorsal de la medul·la espinal. Les neurones de projecció envien informació de la medul·la cap al tronc cerebral i al tàlem i després es transmet a diverses regions cerebrals implicades en discriminació sensorial i emocional.

3.1. Neurones sensorials primàries

Atès l'alt grau de diversitat cel·lular del sistema somatosensorial definit a nivell de desenvolupament, anatòmic i funcional, les tècniques de seqüenciació

d'RNA de cèl·lules individuals que han sorgit recentment, permeten analitzar més profundament totes les poblacions de neurones sensorials i classificar-les específicament per entendre la seva especificació, funció normal i contribució en les malalties (Figura 4).

En els darrers anys, diversos grups han descrit els perfils transcripcionals de les neurones dels DRGs tant a nivell de cèl·lula única com a nivell poblacional mitjançant seqüenciació d'RNA (RNA-seq) (Chiu et al., 2014; Goswami et al., 2014; Reynders et al., 2015; Usoskin et al., 2015; C Li et al., 2018). Els diferents anàlisis coincideixen en línies generals i amb ells s'han assignat diferents subtipus neuronals tenint en compte altres criteris tradicionals com la mida de les cèl·lules i les propietats electrofisiològiques.

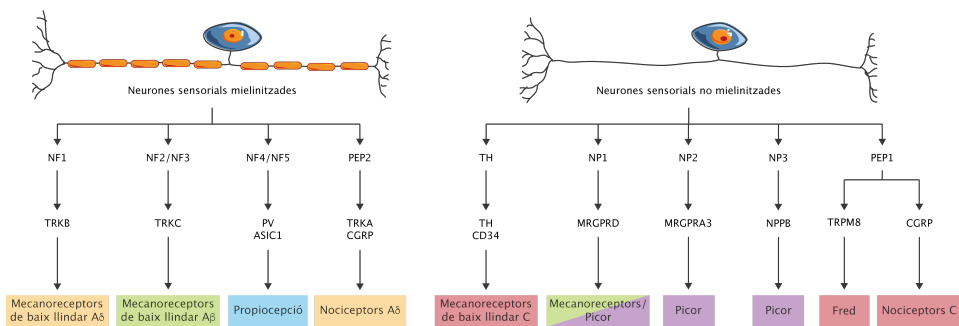


Figura 4: Classificació de les neurones sensorials primàries. Estudis de RNA-seq han identificat signatures transcriptòmiques diferents per a diverses categories de neurones mielinitzades i no mielinitzades i els seus respectius rols funcionals derivats de l'anàlisi transcripcional i de comportament.

Segons els patrons d'expressió i basant-nos en la nomenclatura designada per Usoskin et al., entre les neurones mielinitzades podem distingir les subpoblacions NF1-3 (neurofilament) corresponents a mecanoreceptors A β i A δ de baix llindar; les NF4 i 5 com a propioceptors; i les PEP2 (peptidèrgiques) que són nociceptors A δ lleugerament mielinitzats. En quant a les neurones sensorials no mielinitzades s'inclouen les subpoblacions TH que corresponen a mecanoreceptors de baix llindar de tipus C; les PEP1 que són nociceptors termosensibles; i tres tipus de nociceptors no peptidèrgics (pruritoceptors) NP1-3 que probablement són sensibles a picor (Exemplificat en Figura 4).

3.1.1. Pruritoceptors

Existeixen diversos punts de vista respecte com s'inicia el pruritus, distingint entre la picor produïda en malalties d'origen dermatològic, la picor detectada en els terminals nerviosos sensorials i la que es produeix per activació dels circuits del sistema nerviós central.

Centrant-nos en la picor aguda iniciada a nivell perifèric, aquesta pot ser d'origen mecànic o químic. Els estímuls químics i mecànics són detectats per unes neurones específiques anomenades pruritoceptors, amb els cossos cel·lulars residint en els ganglis de l'arrel dorsal i els trigèmins. Des del cos cel·lular sorgeix un axó pseudounipolar que es bifurca envers el dermatoma perifèric on rep els estímuls i cap a la medul·la espinal per a la transmissió central del missatge. Els terminals perifèrics d'aquestes fibres nervioses es troben a l'epidermis, l'estrat més extern de la pell que cobreix tot el cos i serveix de barrera contra els agents ambientals, on formen terminacions nervioses lliures ramificades (Zylka, Rice et al., 2005; Green et al., 2016). Per altre banda, la branca central d'aquestes neurones fa sinapsis amb neurones de primer ordre a la banya dorsal de la medul·la espinal (Veure en detall a la Figura 7).

Els pruritoceptors s'inclouen, majoritàriament, dins de les fibres nervioses de tipus C no mielinitzades, amb un diàmetre de 0,2-1,5 μ m de mitjana i una velocitat de conducció inferior a 2 metres per segon. També s'inclou una petita població de pruriceptors en les neurones A δ poc mielinitzades de mitjà diàmetre (Schmelz et al., 2003; S Ständer, Steinhoff et al., 2003; Ringkamp et al., 2011; LaMotte, Dong et al., 2014). Les fibres que detecten picor signifiquen menys del 10% de les fibres C de la pell i formen una població neuronal heterogènia i polimodal que distingeix les seves propietats segons els canals iònics i receptors de membrana que expressen (L Han et al., 2013).

La distinció més senzilla de les neurones sensibles a picor depèn de l'activació per histamina. Així doncs, en general es distingeixen dos grups de pruritoceptors: els histaminèrgics i els no histaminèrgics. La subpoblació histaminèrgica s'activa per histamina i per diversos neuropèptids, està implicada principalment en la ge-

neració de picor aguda i és sensible al tractament terapèutic amb antihistamínics. Els pruritoceptors no histaminèrgics poden ser induïts per pruritògens endògens i exògens i expressen gran varietat de receptors implicats en picor, a més, són les neurones encarregades de la generació de picor crònica (Ikoma, Steinhoff et al., 2006; Yosipovitch, Rosen et al., 2018). Malgrat tot, algun grup de neurones pot presentar simultàniament receptors d'histamina i d'altres pruritògens no histaminèrgics (Figura 5).

D'altra banda, els pruritoceptors que corresponen a fibres C també es poden diferenciar segons si expressen marcadors peptidèrgics o no peptidèrgics. Entre els marcadors peptidèrgics hi trobem el neuropèptid SP i CGRP que són alliberats per aquestes mateixes neurones. Els marcadors de neurones no peptidèrgiques inclouen el receptor c-Ret i l'isolectina B4 (IB4⁺) (Cevikbas & EA Lerner, 2020).

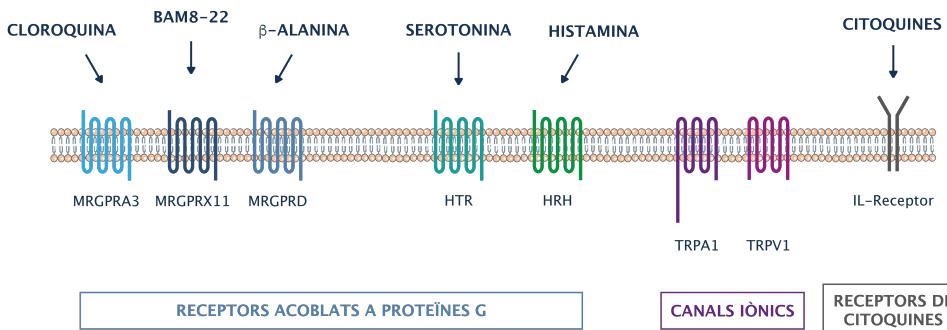


Figura 5: Receptors i canals iònics expressats en neurones sensorials implicats en la transducció de la picor. Els pruritoceptors expressen gran diversitat de receptors i canals diferents incloent receptors d'histamina (Hrh1), de serotonina (5-HTR), receptors MRGPRA3/C11/D, receptors de citoquines, canals TRPA1/V1 i canals de sodi dependents de voltatge.

Estudis recents de seqüenciació massiva de RNA de cèl·lules individuals han generat perfils del transcriptoma de les neurones dels DRG i TG, els quals han permès diferenciar i classificar més específicament les neurones sensorials (Chiu et al., 2014; Thakur et al., 2014; Goswami et al., 2014; Reynders et al., 2015; Usoskin et al., 2015; MQ Nguyen et al., 2017; C Li et al., 2018; Zeisel et al., 2018; Zheng et al., 2019). Les neurones sensorials s'han agrupat segons el patró d'expressió dels diversos receptors i canals iònics, i l'assignació dels subtipus neuronals és cohe-

rent amb els criteris de classificació emprats fins al moment basats en la mida de les cèl·lules i les propietats electrofisiològiques.

Seguint la classificació suggerida per l'estudi d'Usoskin et al., d'entre les 11 subpoblacions descrites, les que s'han relacionat amb la picor són NP1, NP2 i NP3, subtipus que corresponen a nociceptors no peptidèrgics. La subpoblació NP1 és la més heterogènia i, tot i que, s'ha implicat en picor, també s'ha associat a la percepció dolorosa, concretament al dolor i la picor neuropàtica. Aquesta expressa receptors de l'àcid lisofosfatídic (Lpar3 i Lpar5) i el receptor MRGPRD (*Mas-related G-protein coupled receptor D*). NP2 i NP3 són subpoblacions que es superposen parcialment, ambdues sensibles a histamina ja que expressen el receptor Hrh1. El grup NP2 s'activa amb cloroquina i expressa els receptors MRGPRA3 i MRGPRC11, mentre que NP3 és sensible a interleucines (IL-31), leucotriens (Cyslr2) i a la serotonina (5-HT). Aquesta última subpoblació (NP3) es considera responsable de la transducció de la picor inflamatòria (Figura 6) (Usoskin et al., 2015).

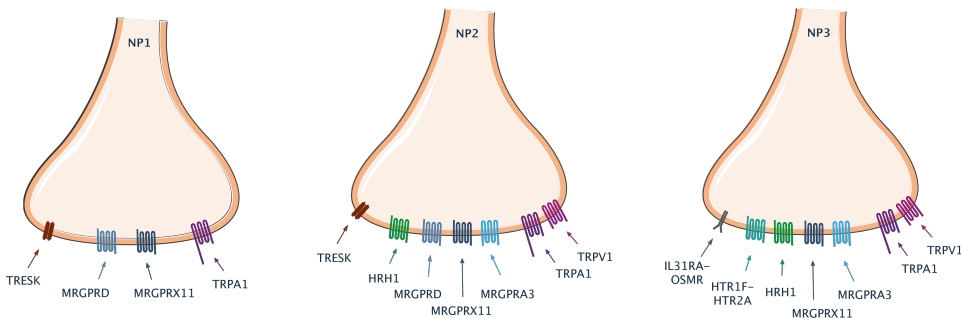


Figura 6: Perfil d'expressió dels tres subtipus de neurones sensorials dels DRG associades a picor. Les neurones NP1 es caracteritzen per l'expressió de MRGPRD, les NP2 per l'expressió de MRGPRA3 i les NP3 pel receptors de serotonina (HTR).

La majoria de les neurones associades amb la transducció de la picor coincideixen en l'expressió de receptors acoblats a proteïnes G (GPCR) que desencadenen cascades de senyalització a través de fosfolipases i proteïnes G associades. A més, l'activació dels pruritoceptors requereix l'obertura de canals iònics com TRPV1 i TRPA1 i dels canals de sodi dependents de voltatge Nav1.7 (Yosipovitch, Rosen et al., 2018). Els perfils d'expressió gènica de les neurones sensorials confereixen gran quantitat d'informació i ajuden en l'assignació de les propietats

fisiològiques de cada tipus neuronal. A continuació es descriuen amb més detall els receptors i canals iònics amb més implicació en la transducció de la picor (Figura 6).

El senyal percebut a la pell es transmet des del sistema nerviós perifèric al sistema nerviós central passant pels ganglis de l'arrel dorsal o els ganglis trigèminals. Els receptors dels pruritoceptors, quan s'activen, despolaritzen les neurones a través de l'obertura de canals iònics TRP. Quan la despolarització és suficient i arriba al llindar, es desencadena un potencial d'acció generat per l'activitat dels canals de sodi voltatge dependents Nav1.7, Nav1.8 i Nav1.9 (Wood et al., 2004; Jeffry et al., 2011).

Les neurones sensibles a picor expressen el transportador vesicular de glutamat de tipus 2 (VGLUT2), per tant s'identifiquen com a neurones glutamatergiques excitatòries (Brumovsky et al., 2007; Y Liu et al., 2010). Tot i això, la comunicació dels senyals de picor per transmissió sinàptica en algunes subpoplacions neuronals també requereix de l'alliberació de neuropèptids com és el cas del pèptid natriurètic B (NPPB) (Mishra et al., 2013) (Veure Figura 7).

3.1.1.1. Receptor d'histamina

La histamina és una amina biogènica d'acció curta amb un rol important en la inflamació associada a l'al·lèrgia. Es tracta d'una de les substàncies més estudiades relacionades amb la picor, tot i que, actualment la seva implicació en les sensacions pruritogèniques es considera limitada. Aquest pruritògen està relacionat principalment amb picor aguda en patologies com la urticària i l'alliberen cèl·lules del sistema immunitari com són els mastòcits, els basòfils i els queratinòcits.

La histamina interacciona i activa quatre receptors acoblats a proteïnes G (HRH1-HRH4), expressats en neurones histaminèrgiques dels ganglis de l'arrel dorsal (DRG) (Strakhova et al., 2009; Usoskin et al., 2015). La unió de la histamina amb els seus receptors indueix l'activació del canal iònic TRPV1 (Imamachi et al., 2009; S Kim et al., 2016) a través de les fosfolipases A2 i C i de la proteïna

quinasa C (Imamachi et al., 2009). La senyalització per TRPV1 provoca la despolarització de la membrana, l'activació del terminal nerviós i la descàrrega de potencials d'acció que desencadena l'alliberació de neuropèptids que provoquen el procés inflamatori (inflamació neurogènica) i transmeten la sensació de picor. La picor intensa provocada per histamina va acompanyada de vasodilatació local, extravasació plasmàtica i desgranulació mastocel·lular (Rosa et al., 2013).

Els antihistamínics són els fàrmacs més emprats per alleujar els símptomes de l'al·lèrgia i la picor. Tot i això, cada cop més es demostra la poca efectivitat d'aquests en moltes condicions de picor crònica i picor no histaminèrgica (Yosipovitch, Rosen et al., 2018).

3.1.1.2. Receptor de serotonina

La serotonina (5-hidroxitriptamina, 5-HT) és un neurotransmissor de monoaminèrgic que quan s'aplica a la pell provoca picor i, quan s'administra en dosis elevades, també indueix dolor (Akiyama, MI Carstens & E Carstens, 2010; Bardin, 2011). La serotonina pot ser alliberada per mastòcits, plaquetes i cèl·lules endotelials durant lesions i inflamacions. En la perifèria actua sobre neurones de DRG de petit i gran diàmetre i activa fibres A β i C; a més, també provoca efectes sobre el sistema nerviós central (Lang et al., 2006).

Es coneixen 14 receptors diferents de serotonina dividits en 7 famílies (Nichols et al., 2008; Hannon et al., 2008), tots ells són receptors acoblats a proteïnes G excepte el 5-HT₃ que és un canal iònic. S'ha demostrat l'expressió de diversos receptors en neurones dels DRG i en neurones de la medul·la espinal (T Morita et al., 2015; Usoskin et al., 2015). La picor generada pels receptors HTR es desencadena a través a l'activació de canals TRP com TRPA1 i TRPV4 i de la fosfolipasa PLC β 3 (T Morita et al., 2015; Akiyama, Curtis et al., 2016).

El sistema serotoninèrgic mostra una gran complexitat, probablement deguda a la gran diversitat de receptors. Així doncs, malgrat la gran quantitat d'investigacions sobre 5-HT i la seva vinculació amb malalties al·lèrgiques de la pell, la colèstàsis i la urèmia (Tian et al., 2016), és difícil identificar quins receptors són

importants en la transducció de la picor i el paper d'aquesta amina en la picor a nivell perifèric i central.

3.1.1.3. Mrgpr i els seus agonistes

Els receptors Mrgpr (Mas-related G protein-coupled receptors) són una família de receptors acoblats a proteïnes G compresa per desenes de membres en humans i ratolins i descoberta l'any 2001 (Dong, Sk Han et al., 2001; Zylka, Dong et al., 2003). Aquesta complexa família esta formada per 9 subfamílies que s'expressen en neurones sensorials perifèriques dels TG i DRG, en diverses regions del cervell, en el sistema nerviós entèric i en mastòcits (Dong, Sk Han et al., 2001; L Zhang et al., 2005; Avula et al., 2013; Usoskin et al., 2015). Les neurones que expressen els receptors Mrgpr també són positives pel pèptid alliberador de gastrina (GRP) (Q Liu, Tang et al., 2009) i la senyalització intracel·lular que desencadenen pot implicar els canals TRPA1 i TRPV1 (Wilson, Gerhold et al., 2011; Mishra et al., 2013).

Mrgpra3 va ser el primer membre de la família descrit com a receptor implicat en les vies de senyalització neuronal per donar lloc a la sensació de picor (Q Liu, Tang et al., 2009). S'ha descobert la cloroquina com un pruritògen que activa MrgprA3 i indueix una picor independent d'histamina que no s'alleuja quan s'administra tractament basat en antihistamínics. Així, el membre de la família MrgprC11 s'activa per un derivat de la proencefalina A, el pèptid de medul·la suprarenal bovina (BAM8-22) (Lembo et al., 2002; Q Liu, Tang et al., 2009) i també pel pèptid SLIGRL (Q Liu, Weng et al., 2011). A més, s'ha descrit la seva activació a través de l'excissió del seu extrem N-terminal induïda per diverses proteases (Reddy et al., 2015). Tanmateix, el receptor MrgprD s'expressa en neurones nociceptives no peptidèrgiques i desencadena picor induïda per la unió de l'agonista β -alanina (Zylka, Rice et al., 2005; Q Liu, Sikand et al., 2012).

Altrament, diversos membres de la família s'han relacionat amb processos de picor crònica. Models de pell seca i dermatitis al·lèrgica per contacte en ratolí han demostrat una sobreexpressió significativa dels receptors MrgprA3 i MrgprD (Zhu et al., 2017). En altres patologies cutànies que cursen amb picor com és el

cas de la psoriasis, també s'ha observat un augment de l'expressió de diversos receptors Mrgpr (Nattkemper, Tey et al., 2018).

3.1.1.4. Receptors activats per proteases

Els receptors activats per proteases (PARs) són una família de receptors acoblats a proteïnes G, codificats per quatre gens diferents (PAR-1 a PAR-4) i que presenten una expressió diferencial segons el subtipus. Així doncs, PAR-2 s'expressa principalment en neurones sensorials de petit diàmetre, mentre que PAR-1, -3 i -4 s'expressen en altres tipus neuronals, cèl·lules epitelials, astròcits i cèl·lules del sistema immunitari (Ossovskaia et al., 2004).

Agents exògens, com microbis o plantes, i diverses cèl·lules inflammatòries, poden induir la producció de proteases en nivells suficients com per provocar l'activació dels seus receptors. L'escissió d'un segment extracel·lular en l'extrem N-terminal dels receptors per part de les proteases, deixa al descobert una seqüència que actua com a lligand i que permet l'auto-activació del receptor. L'auto-activació del receptor desencadena una senyalització intracel·lular a través de proteïnes G o arrestines que provoca un augment en la transcripció de citocines, quimiocines i factors de creixement (Steinhoff, Buddenkotte et al., 2005; Akiyama, E Lerner et al., 2015).

Pel que fa a PAR-2, és el receptor més important en nocicepció i pruritocepció, el qual s'ha descrit com a mediador d'inflamació neurogènica i de picor no histaminèrgica (Steinhoff, Vergnolle et al., 2000; Steinhoff, Neisius et al., 2003). Tot i que els mecanismes implicats no són del tot clars, s'ha demostrat que la injecció intradèrmica d'agonistes de PAR-2 indueix picor independent d'histamina en humans i agreuja la picor en pacients amb dermatitis al·lèrgica (Steinhoff, Neisius et al., 2003; Kempkes et al., 2014).

3.1.1.5. Citocines

Les citocines engloben una família de proteïnes de baix pes molecular que regulen diverses funcions cel·lulars durant els processos inflamatoris i les respostes immunitàries. Les citocines activen cèl·lules immunitàries incloent mastòcits i

cèl·lules auxiliars CD4⁺, cèl·lules de la pell com queratinòcits, cèl·lules endotelials, de Langerhans i fibroblasts (Cevikbas, Wang et al., 2014). De manera paral·lela, es comuniquen amb neurones sensorials mitjançant l'activació de receptors d'alta afinitat, tot i que, el coneixement sobre els mecanismes que hi ha darrere de la seva regulació sobre la inflamació neurogènica, el dolor i la picor és escàs (Yosipovitch, Greaves et al., 2003; Sehra et al., 2008).

Les neurones sensorials implicades en el pruritus expressen receptors de citocines que al activar-se, desencadenen mecanismes de senyalització intracel·lular similars als de les cèl·lules immunitàries. Aquestes citocines permeten la coordinació entre el sistema nerviós i l'immunitari per alertar de possibles lesions i infeccions en la pell (Usoskin et al., 2015; Bando et al., 2006).

Diversos estudis assenyalen les citocines IL-31 i OSM, membres de la família IL-6, com les interleucines que indueixen la comunicació entre les cèl·lules T i les neurones sensorials (Dillon et al., 2004; Morikawa et al., 2004). Per altre banda, la limfopoietina estromal tímica (TSLP), és una citocina que indueix directament picor a través del seu receptor expressat en neurones positives per TRPA1 i es troba estretament lligada amb la dermatitis atòpica (Wilson, Nelson et al., 2013; H Han et al., 2017). També s'ha descrit el paper de IL-4 i IL-13 en la generació de picor crònica, però no aguda, al unir-se a la subunitat A del receptor de IL-4 per activar les neurones sensorials (Oetjen et al., 2017). Recentment, s'ha demostrat que la citocina IL-33, secretada pels queratinòcits, és un mediador de picor en la dermatitis al·lèrgica de contacte induïda per heura verinosa (B Liu et al., 2016) i la interleucina IL-31 s'ha associat a diverses patologies que cursen amb picor com són la dermatitis atòpica i la picor induïda per limfoma de cèl·lules T (Nattkemper, Tey et al., 2018).

3.1.1.6. Canals de potencial transitori (TRP)

Els receptors TRP constitueixen una família de canals que funcionen com sensors cel·lulars polimodals i participen en la transducció de processos sensorials com són els estímuls mecànics, tèrmics, de picor i de dolor (Clapham, 2003). Entre els membres de la família, TRPV1, TRPA1, TRPM8, TRPV3 i TRPV4 són els

canals sobre els que s'ha suggerit un paper relacionat amb la picor.

Respecte a TRPV1, va ser el primer canal identificat de la família TRP per la seva activació per capsaicina, el component actiu dels pebrots picants (chiles) (Caterina MJ et al., 2001). S'expressa principalment en nociceptors peptidèrgics i es tracta d'un canal no selectiu permeable a calci (Caterina et al., 2001; Imamachi et al., 2009). L'activitat d'aquest canal pot ser modulada per mediadors de picor, dolor i inflamació i la seva activació està associada amb la generació de potencials d'acció i l'alliberació de neuropèptids (SK Han et al., 2011). Fins al moment, només s'ha descrit un rol essencial de TRPV1 en la transducció de picor induïda per histamina (BM Kim et al., 2004; Imamachi et al., 2009).

Per altre banda, TRPA1 s'expressa en una subpoblació de neurones de petit diàmetre positives per TRPV1 dels ganglis trigèmics i de l'arrel dorsal (Jordt et al., 2004). TRPA1 s'ha associat a la transducció de picor aguda, participant en la generació de picor independent d'histamina induïda per pruritògens com la cloroquina i el BAM8-22 (Wilson, Gerhold et al., 2011). També es considera necessari en la transducció de picor crònica descrita en els models de pell seca i dermatitis al·lèrgica per contacte (Wilson, Nelson et al., 2013; T Liu & Ji, 2013; Xie et al., 2019), tot i que, aquesta implicació s'ha posat en dubte en els últims anys (Ru et al., 2017).

3.1.1.7. Neuropèptids

La substància P, de la família de les taquiquinines, és el neuropèptid més conegut amb implicació en la transducció de la picor. Entre els seus receptors, el que participa principalment en la picor a nivell de medul·la espinal és el receptor de neuroquinina-1 (NK1R) i, en canvi, en la perifèria el neuropèptid SP actua de forma més rellevant sobre receptors Mrgpr. La substància P s'expressa en terminacions nervioses sensorials, mastòcits, queratinòcits, fibroblasts i sistema nerviós central (EE Carstens et al., 2010).

Estudis de l'expressió del receptor NK1R mostren com aquest es troba en nivells profunds de la medul·la espinal i en la majoria de les neurones de projecció

ascendent de les vies implicades en el dolor i la picor (Akiyama, T Nguyen et al., 2015; H Huang et al., 2016). L'ablació de les cèl·lules positives per NK1R, ha permès descobrir un paper d'aquestes neurones en la transmissió de la picor induïda per cloroquina i serotonina i de la picor crònica associada a dermatitis al·lèrgica (Akiyama, T Nguyen et al., 2015).

3.2. Vies centrals de transmissió de la informació pruritoceptiva

3.2.1. Arribada a la medul·la espinal

Les neurones sensorials primàries projecten el missatge de la perifèria fins a la banya dorsal de la medul·la espinal. La banya dorsal és el lloc on es processa principalment tota la informació sensorial aferent nociceptiva i se'n distingeixen, bàsicament dues àrees: la superficial, que comprèn les làmines I i II i la profunda, que correspon a les làmines III, IV i V. Es tracta d'un circuit organitzat en l'espai on les fibres aferents A δ i C, que transmeten informació nociceptiva i termosensible, fan sinapsi amb neurones de la branca dorsal superficial. En canvi, per la zona profunda o ventral passen les fibres aferents A β amb informació de tacte i propioceptiva que puja per la medul·la fins al bulb (Todd, 2002).

Resultats d'investigacions moleculars recents han permès categoritzar les projeccions aferents de les fibres nociceptives, majoritàriament fibres C, i determinar-ne diferents zones on fan sinapsi dins de les làmines superficials de la banya dorsal de la medul·la espinal (Basbaum, Bautista et al., 2009; L Li et al., 2011). Les neurones peptidèrgiques es projecten a la regió exterior de les làmines I i II, mentre que les no peptidèrgiques envien la informació a la regió mitja/ventral de la làmina II (Akiyama & E Carstens, 2013)(Figura 7).

3.2.2. Circuits espinals

Les projeccions centrals de les neurones de picor que arriben a la làmina I i II de la banya dorsal, fan connexions mono-sinàptiques amb interneurons de la medul·la espinal. Tal i com s'ha mencionat anteriorment, les neurones aferents primàries alliberen el polipèptid natriurètic B que actua sobre els seus receptors (Npr1) expressats en gran part de les interneurons (Figura 7). Les interneurons

mostren característiques morfològiques heterogènies de cèl·lules d'illot, centrals, radials i verticals (Grudt et al., 2002; Zeilhofer et al., 2012; Dickie et al., 2019).

Un altre component important en el circuit espinal associat a la picor són les neurones espinals positives per al neuropèptid alliberador de gastrina (GRP) (Mishra et al., 2013; YG Sun et al., 2007). Existeix certa controvèrsia respecte aquestes neurones, ja que es coneix que transmeten el senyal rebut de la perifèria a una subpoblació d'interneurones de la làmina I que expressen el receptor de GRP (GRPR) però també s'ha proposat que les neurones aferents primàries puguin senyalitzar directament a neurones espinals GRPR positives mitjançant l'alliberament del pèptid alliberador de gastrina (Figura 7). Tot i això, els resultats dels estudis d'expressió de RNA i proteïna GRP en neurones de DRG no són concloents (YG Sun et al., 2007; Mishra et al., 2013; Nattkemper, Zhao et al., 2013; Gutierrez-Mecinas, Watanabe et al., 2014; Usoskin et al., 2015).

Les neurones espinals que contenen el receptor del pèptid alliberador de gastrina tant activades directament com indirectament per altres interneurones, semblen crítiques per a la transmissió específicament de la picor, però no dels senyals de dolor (YG Sun et al., 2007; Albisetti et al., 2019). Mitjançant la supressió genètica del receptor GRPR i l'ablació de les neurones positives per GRPR es van abolir completament els comportaments de rascat induïts per l'aplicació d'histamina, serotonina, cloroquina i SLIGRL. Cal destacar que aquestes modificacions no van afectar la transmissió de les sensacions tèrmiques, mecàniques ni doloroses, fet que demostra la necessitat de les neurones positives per GRPR per a la transmissió del senyal de picor, però no exclou que altres mediadors i receptors implicats en vies nociceptives també puguin actuar sobre les vies de transmissió de la picor (YG Sun et al., 2007; YG Sun et al., 2009; S Sun et al., 2017).

Les interneurones espinals excitadores comparteixen propietats bioquímiques ja que expressen el transportador vesicular de glutamat (VGLUT2), marcadors neuroquímics com la cinasa PKC i la somatostatina i el factor de transcripció TR4 (Wang et al., 2013; Gutierrez-Mecinas, Furuta et al., 2016; Aresh et al., 2017). Així doncs, es suggereix un paper potencial de la somatostatina (SST) en les vies de picor, la qual s'expressa tant en neurones sensorials primàries com en neuro-

nes de la medul·la espinal. Els ratolins modificats on se'ls ha eliminat la SST tant a la perifèrica com a la medul·la espinal presenten dèficits en la resposta conductual a estímuls prurítics (Stantcheva et al., 2016). A més, l'activació del receptor de SST (Sst2a) a la medul·la espinal reforça les respostes de picor evocades per Nppb o GRP per mitjà d'un mecanisme de desinhibició (J Huang et al., 2018).

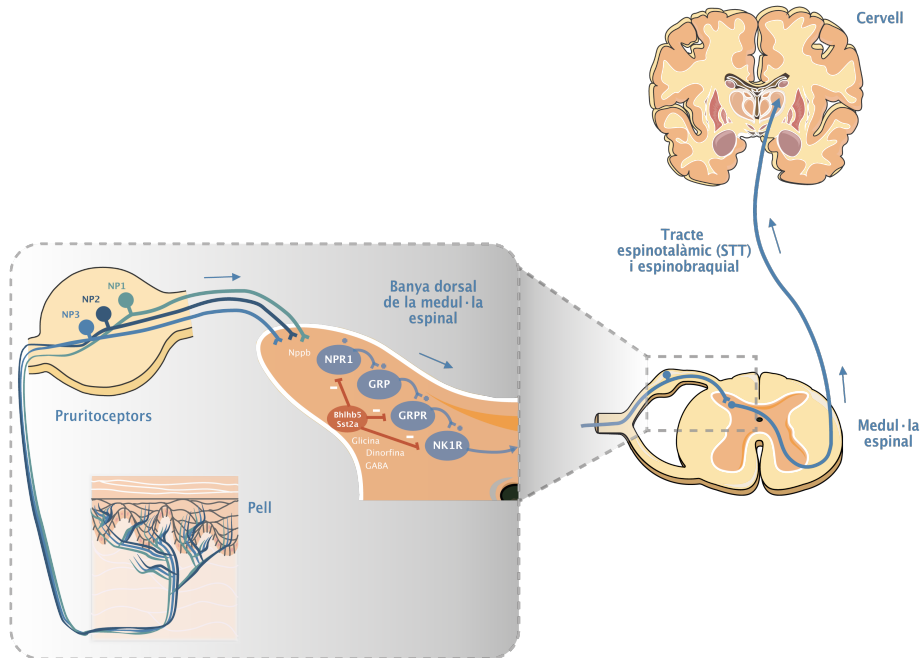


Figura 7: Circuit de les vies de la picor des de la perifèria fins al cervell. Els pruritoceptors corresponents a neurones sensorials dels subgrups NP1-3, amb el cos neuronal als DRG, detecten estímuls químics en la pell i els transmeten a la banya dorsal de la medul·la espinal. Aquest senyal es modula i transmet a través d'un complex circuit de neurones excitadores i inhibidores que expressen, entre d'altres, el neuropeptid alliberador de gastrina (GRP), el receptor del neuropeptid alliberador de gastrina (GRPR) i el factor de transcripció Bhlhb5. Posteriorment el missatge es projecta al cervell per mitjà dels tractes espinotalàmic (STT) i espinobraquial (PNB) fins al cervell.

D'altra banda, gran part de les interneurons inhibidores implicades en la transmissió de la picor representen una població complementària a les interneurons TR4 positives i tenen una expressió característica del factor de transcripció Bhlhb5 (Ross et al., 2010). La majoria de les neurones espinals inhibidores expressen galanina, dinorfina o la sintasa neuronal d'òxid nítric i alliberen els neuro-

transmissors inhibitoris GABA (àcid γ -aminobutíric) i glicina (Figura 7) (Sardella et al., 2011; Kardon et al., 2014; Foster et al., 2015). Així, s'ha demostrat que la pèrdua de les interneurons inhibidores positives per Bhlhb5 resulta en un rascat excessiu en resposta a diferents pruritògens sense provocar alteracions en la transmissió de les respostes al dolor (Ross et al., 2010).

Com ja s'ha comentat, la picor habitualment es desencadena per la unió d'un mediador químic al seu receptor en els terminals de les neurones sensorials, tot i que, també existeix un tipus de picor mecànica provocada per estímuls tàctils lleugers com els provocats per insectes o paràsits en posar-se en contacte amb la pell (Akiyama, MI Carstens, Ikoma et al., 2012; Fukuoka et al., 2013; Bourane et al., 2015). Aquest tipus de picor és independent de les neurones espinals positives pel receptor de GPR i, en canvi, s'han identificat com a components claus les interneurons excitadores que expressen urocortina 3 (Ucn3) i les que expressen el receptor del neuropèptid Y1 (NPY1R) (Pan et al., 2019; Acton et al., 2019). La via implicada en la transmissió de la picor mecànica s'inhibeix per una subpoblació de neurones espinals positives per al neuropèptid Y i es creu que actua per una via independent a la picor química ja que la supressió de cada via per separat no afecta la transmissió de l'altre tipus de picor (Kardon et al., 2014; Bourane et al., 2015).

3.2.3. Projectió ascendent

La informació prurítica que arriba de la perifèria a la medul·la espinal es transmet al cervell a través de les neurones de projectió. Aquestes neurones tenen els cossos cel·lulars en la làmina I i els seus axons creuen la línia mitja cap al costat contralateral de la medul·la on s'uneixen al tracte espinotalàmic (STT) i al tracte espinobraquial (Figura 7) (Basbaum, Bautista et al., 2009; Ikoma, Cevikbas et al., 2011; Davidson, X Zhang, Khasabov et al., 2012). Les neurones de projectió espinal tenen com a destí diverses regions cerebrals, majoritàriament projecten al nucli parabraquial (PBN) del tronc de l'encèfal i al tàlem (Figura 8) (Davidson, X Zhang, Khasabov et al., 2012; Akiyama, T Nguyen et al., 2015; Cameron et al., 2015; Hachisuka et al., 2016).

Les neurones de projecció espinal que projecten al PBN reben entrades sinàptiques directes de neurones GRPR positives espinals (Mu et al., 2017). El 80% d'aquestes neurones expressa el receptor de neuroquinina-1 (NK1R) (Todd et al., 2000; Al-Khater et al., 2009; EE Carstens et al., 2010; Akiyama, T Nguyen et al., 2015) i quan s'eliminen les neurones positives per NK1R es redueix significativament la resposta de rascat evocada per pruritògens i també la picor crònica (EE Carstens et al., 2010; Akiyama, T Nguyen et al., 2015).

Les neurones de projecció semblen ser polimodals, així doncs es suggereix la seva implicació en la transmissió de diferents modalitats somatosensorials com són el dolor, la picor i els estímuls tèrmics (Davidson & Giesler, 2010; Davidson, X Zhang, Khasabov et al., 2012; Hachisuka et al., 2016). Cal destacar que, l'ablació de les neurones de projecció que expressen NK1R no afecta la transmissió de la picor mecànica (Acton et al., 2019), però atenua les respostes davant estímuls nocius (Akiyama, T Nguyen et al., 2015). Tanmateix, els diferents tipus de pruritògens activen diferents subconjunts de neurones de projecció del tracte STT, fet que proposa que la picor histamina dependent i independent es processa per diferents vies (Davidson, X Zhang, Yoon et al., 2007).

3.2.4. Circuits cerebrals

Els senyals prurítics després d'arribar al tàlem i al nucli parabraquial (PBN), es projecten a diverses zones del cervell. El nucli parabraquial es considera un punt clau en el processament de la picor i el tàlem es coneix com l'estació de transmissió dels estímuls somatosensorials de la medul·la a les regions corticals del cervell. Les regions cerebrals activades amb més freqüència són els còrtexs somatosensorials primari i secundari, àrees que es suggereix contribueixen a la localització espacial i temporal i reconeixement de la intensitat de la sensació de picor (Figura 8) (Dhand et al., 2014; Yosipovitch & Mochizuki, 2015; Mochizuki et al., 2015). A més, els estímuls de picor generen un patró característic d'activació d'altres regions cerebrals i aquest patró es pot veure alterat en diferents patologies i trastorns relacionats amb la picor.

Es creu que les diferents àrees cerebrals processen de forma dinàmica en una

xarxa multifuncional les diferents dimensions sensorials, motrius, emocionals, atencionals, cognitivo-executives i decisives involucrades en la picor. Entre les regions que hi participen trobem les àrees motores, on s'inclou el còrtex premotor (PM), el còrtex motor primari (MI) i el còrtex motor suplementari (SMA), l'estriat i el cerebel, tots ells implicats en la planificació, execució i control del comportament de rascat induït per la picor. També es poden activar àrees associades amb el component emocional, la motivació i la recompensa com són el còrtex cingulat i prefrontal, l'amígdala i els sistemes límbics, i la ínsula, associada a sensacions desagradables (Figura 8) (Yosipovitch & Samuel, 2008; Schneider et al., 2008; Ishiiji, 2019; Mochizuki et al., 2015; Mueller et al., 2017).

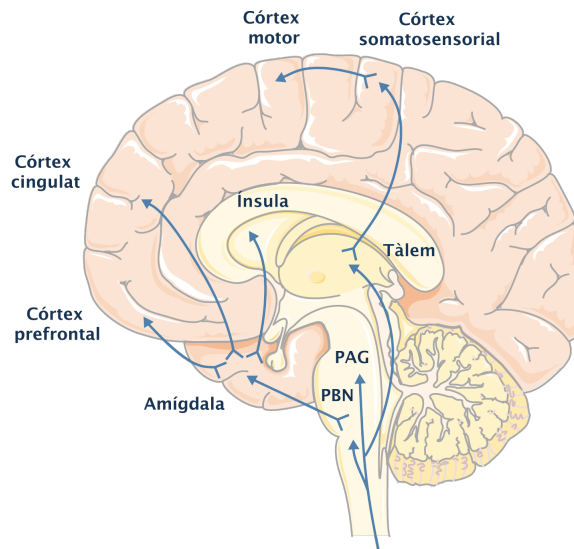


Figura 8: Regions corticals i subcorticals implicades en el processament i la modulació del pruritus. S'ha descrit tota una xarxa de regions cerebrals en el cervell involucrades en la transmissió de la picor on s'inclouen els còrtexs somatosensorials primari i secundari, el còrtex premotor (PM), el còrtex motor primari (MI), el còrtex motor suplementari (SMA), l'estriat, el cerebel, el còrtex cingulat, el còrtex prefrontal, l'amígdala, els sistemes límbics i la ínsula.

Cal destacar que, encara que durant el processament dels senyals de picor histaminèrgics i no histaminèrgics es comparteixen una sèrie d'àrees del cervell central, cada tipus activa unes regions cerebrals lleugerament diferents (Papoiu et al., 2012). La picor independent de la histamina induïda per un tipus de mongeta (*cowhage*) activa més extensament el còrtex insular, el claustrum, els ganglis

basals, el putamen, els nuclis talàmics i el pulvinar. Així doncs, la picor dependent i independent d'histamina es processa en gran mesura per xarxes cerebrals sobreposades però lleugerament diferents.

3.3. Interacció entre picor i dolor: Codificació de la informació pruritoceptiva

La picor i el dolor són sensacions similars que alerten l'organisme d'estímuls potencialment perillosos i s'associen a respostes motrius protectores. Tot i que no s'han descobert mutacions amb pèrdua de funció relacionades amb la picor amb conseqüències tan devastadores com en les associades amb la sensació de dolor, s'ha demostrat que la picor crònica és altament debilitant i resulta en una afectació de la qualitat de vida similar a la que provoca el dolor crònic. Així doncs, es suggereix que ambdues sensacions poden compartir circuits de transmissió i els mecanismes neuronals que distingeixen la picor del dolor segueixen sent un focus d'investigació en la recerca sobre el pruritus (Silverberg et al., 2016; Pithadia et al., 2020).

En l'actualitat, es coneixen diverses propietats que comparteixen les sensacions de picor i dolor com per exemple que els pacients insensibles a dolor tampoc sofreixen picor. Tant la picor com el dolor són detectats per neurones de petit diàmetre en els DRGs, indistingibles anatòmicament entre elles. A més, moltes neurones espinals responen tant a estímuls prurítics com a algògens. Cal destacar també que ambdues sensacions es transmeten a través del tracte espinotalàmic (STT) i que existeix un solapament considerable en les àrees activades durant la transmissió de la picor i del dolor (Davidson & Giesler, 2010; Akiyama & E Carstens, 2013).

No obstant això, la picor i el dolor difereixen en diversos punts. En primer lloc, mentre que el dolor es produeix tant a la superfície del cos com en teixits profunds, la picor només es genera en regions superficials com la pell, la superfície ocular (còrnia i conjuntiva) i les mucoses. A més a més, les reaccions conductuals induïdes per ambdues sensacions difereixen notablement, fet que planteja la incògnita de com el sistema somatosensorial és capaç de diferenciar entre la picor i el dolor

per desencadenar la resposta motriu adequada (Shimada et al., 2008; Akiyama, MI Carstens & E Carstens, 2010; LaMotte, Shimada et al., 2011).

Es per tots aquest motius que en la recerca dels mecanismes específics subjacents la picor s'han proposat diversos models per a descriure la codificació de la informació nociceptiva/pruritoceptiva. Aquests inclouen la teoria de l'especificitat, la teoria de la intensitat, la teoria de patrons, la teoria de porta d'accés i la teoria combinatòria. El model més estudiat per a descriure la picor és la teoria de l'especificitat, que afirma que existeixen mecanismes cel·lulars determinats per a cada modalitat sensorial. Proposa que la informació viatja a través d'una línia marcada (*labeled line*) formada per un conjunt de neurones des de la perifèria, a través de la medul·la espinal i fins al cervell, i que aquesta està especialitzada en la transducció, transmissió i percepció exclusivament pruritoceptiva.

Els anàlisis genètics i les tècniques d'anàlisi funcionals emprats en els darrers anys han permès ampliar les investigacions sobre l'existència de neurones específiques de picor. El receptor MrgprA3 expressat en unes subpoblacions de fibres C aferents no mielinitzades (NP2 i NP3) s'ha proposat com a exemple de línia marcada per a la transmissió de la picor (Han et al., 2013). Altres línies marcades descrites en terminals perifèrics inclouen les neurones que expressen la somatostatina, les positives per MrgprA3 i sensibles a la histamina i, per últim, els pruritoceptors que expressen MrgprD (NP1) (Q Liu, Sikand et al., 2012; Roberson et al., 2013; Stantcheva et al., 2016). El conjunt de les neurones on s'engloben totes aquestes línies marcades correspon a una gran proporció de les fibres C no peptidèrgiques, on s'inclouen neurones probablement polimodals que responen a estímuls mecànics i tèrmics (Zylka, Rice et al., 2005). A més, moltes de les neurones esmentades expressen receptors TRP relacionats amb el dolor com, per exemple, TRPV1 i que s'ha descrit que poden ser activades per algògens clàssics com la capsaïcina (Sikand et al., 2011).

Un fenomen extensament acceptat és que la percepció simultània del dolor o tacte inhibeix la sensació de picor. Estudis sobre la transmissió somatosensorial a nivell central han observat que l'eliminació condicional de VGLUT2 en tots els nociceptors aboleix el dolor, però augmenta significativament les respostes de picor.

Aquest fet suggereix que la inducció de picor excessiva és resultat de la desaparició de la inhibició de les interneurons Bhlhb5 positives i les interneurons que expressen NPY sobre les neurones GRP positives implicades en transmissió de la picor en la medul·la espinal (T Liu, Xu et al., 2010; Ross et al., 2010; Kardon et al., 2014; Bourane et al., 2015; Foster et al., 2015; Aresh et al., 2017). Així doncs, tenint en compte aquests descobriments, en combinació amb la teoria de l'especificitat es podria proposar la teoria de porta d'accés per descriure millor els mecanismes de transmissió de la picor.

Tot i que s'ha demostrat certa especificitat en diverses poblacions de neurones perifèriques i circuits interneuronals, és probable que la millor explicació de la transmissió somatosensorial correspongui a una superposició de diversos models. Al principal model d'especificitat se li hauria d'afegir la integració diferencial dels inputs aferents a nivell de medul·la espinal, la qual depèn de patrons espacials i temporals i de patrons d'intensitat (S Sun et al., 2017; Sharif et al., 2020). A més, degut a la modulació creuada existent entre les diferents modalitats somatosensorials a través de les interneurons espinals, s'hauria d'incorporar la teoria de la porta d'accés a les ja prèviament esmentades. Així doncs, tot i la gran quantitat de descobriments recents, els mecanismes neuronals específics i l'organització dels circuits de codificació de la picor segueixen sent força desconeguts.

3.4. Modulació i sensibilització de la picor

En el context de la medul·la espinal, la transmissió de la sensació de picor es pot veure modulada per l'activitat dels astròcits. En picor crònica s'ha descrit un circuit de retroalimentació positiva per part dels astròcits a través del receptor TLR4 (*Toll-like receptor*), que com a conseqüència, indueix l'activació de les neurones que expressen el receptor de GRP i pot desencadenar la sensació de picor (B Liu et al., 2016).

D'altra banda, la sensibilització perifèrica i central poden contribuir a l'establiment de diversos tipus de picor crònica. La picor crònica va sovint acompanyada d'*alloknesis* (picor provocada per un estímul tàtil innocu) i d'*hyperknesis* (augment de la picor envers un estímul normalment prurític) (Andersen et al.,

2018), fenòmens causats probablement per la sensibilització neuronal. La sensibilització pot ser provocada per diversos factors que actuen a nivell perifèric, en la medul·la espinal o sobre el cervell.

Un potencial mediador de la sensibilització perifèrica és el factor de creixement nerviós (NGF) que és alliberat principalment per queratinòcits epidèrmics i eosinòfils. El NGF s'uneix al receptor TrkA, sensibilitza els nervis a través de TRPV1 i augmenta la sensibilitat als neuropèptids SP, GRP i a factors neurotròfics. El mediador NGF es troba incrementat en la patologia de pell seca i s'associa a la *hyperknesis* mecànica i a la sensibilització en la picor induïda per (*cowhage spicules*) (Rukwied et al., 2013; Andersen et al., 2018). A més, s'han descrit altres molècules com a mediadors de sensibilitat perifèrica on s'inclouen la prostaglandina E2, el receptor PAR-2 i l'artemina (Murota et al., 2012; Akiyama, MI Carstens, Ikoma et al., 2012; Kawahara et al., 2015).

En relació a les vies descendents inhibidores, aquestes provenen de regions supraespinals com per exemple la substància grisa periaqueductal (PAG) i activen les interneurons inhibidores Bhlhb5 positives de la medul·la espinal a través dels receptors α -adrenèrgics. Aquestes interneurons exerceixen un efecte inhibitor sobre les neurones que expressen GPRP mitjançant l'alliberament de neurotransmissors inhibidors com la dinorfina, la glicina i el GABA (Yoshimura et al., 2006; Ross et al., 2010; Akiyama, M Carstens et al., 2011; Kuraishi, 2015). La disfunció dels circuits inhibidors descendents, on s'inclouen les neurones positives per Bhlhb5 i les que expressen el neuropèptid Y, poden contribuir a la sensibilització neuronal a nivell de medul·la espinal. Per altra banda, les vies descendents serotoninèrgiques poden agreujar la sensació de picor activant la via de senyalització GRP-GRPR a través del receptor de serotonina 5-HT1A, fenomen que s'ha observat en processos de picor crònica (Zhao et al., 2014).

A nivell central existeixen sistemes de retroalimentació negativa que actuen per mitigar la picor. Els estímuls mecànics, el dolor i el fred (transmesos per neurones que expressen TRPV1, TRPA1 i/o TRPM8) poden atenuar la sensació de picor (Yosipovitch, E Carstens et al., 2007; Palkar et al., 2018). Aquest punt es tractarà amb més profunditat en un apartat més endavant on es descriuen les

interaccions entre la picor i el dolor.

4. Interaccions amb el sistema immunitari

Noves descobertes sobre les interaccions entre el sistema nerviós, les cèl·lules epitelials i el sistema immunitari han permès una millor comprensió sobre el funcionament i la modulació de la sensació de picor (Fowler et al., 2019). Les relacions entre els sistemes prèviament mencionats són complexes, multifactorials i multidireccionals, i interactuen amb la finalitat de protegir l'organisme envers infeccions, lesions i amenaces (Figura 9).

Es suggereix que la comunicació neuroimmunitària es produeix de forma directa per proximitat entre els terminals nerviosos i les cèl·lules de Langerhans i mastòcits (Steinhoff, Schmelz et al., 2018). Tanmateix, també es proposa una comunicació indirecta mitjançant l'alliberació de mediadors i neuropèptids que contribueixen al reclutament de cèl·lules immunitàries com mastòcits i cèl·lules T CD4⁺ i a l'activació dels receptors expressats en neurones sensorials. Els mediadors implicats en picor que són alliberats pels mastòcits inclouen diverses citocines, com també les amines serotonina i histamina. Aquests molècules activen els receptors de les neurones sensorials, que al seu torn alliberen neuropèptids com el SP i el CGRP que actuen de forma creuada sobre les cèl·lules immunitàries per contribuir al cicle de picor i inflamació (Figura 9) (Cevikbas & EA Lerner, 2020).

En el cicle de picor hi intervé un altre component, les cèl·lules de la pell. Aquestes entren en joc quan es produeix un dany en la barrera epitelial causada per la resposta motora de rascat. Les cèl·lules epitelials alliberen citocines i proteases que activen les cèl·lules immunitàries i reforcen la inflamació en la zona danyada. Per altre banda, els queratinòcits modulen i activen els terminals de les neurones sensorials a través de l'acció de citocines i proteases (Mack et al., 2018).

Aquest cicle viciós de picor i rascat que desencadena la interacció neuroimmunitària es suggereix com a mecanisme distintiu darrere de la picor crònica present en algunes patologies com la psoriasi, la dermatitis al·lèrgica i el prurigen nodular (Gupta et al., 2018). En la psoriasi, s'han detectat mastòcits en l'epider-

mis, els quals interactuen directament amb els terminals nerviosos dels pruritoceptors encarregats de la percepció de la picor (Azimi et al., 2016). Les lesions cutànies per pell seca indueixen l'alliberació de citocines i proteases per part de les cèl·lules epitelials i queratinòcits, les quals poden activar directament els pruritoceptors. A més, s'ha descrit que les cèl·lules immunitàries sensibles a citocines poden modular l'activitat dels terminals nerviosos i agreujar la sensació de picor en la dermatitis atòpica (Mack et al., 2018). Així doncs, mastòcits i basòfils també s'han associat amb la picor referida en reaccions al·lèrgiques a través de l'alliberació de grànuls amb histamina que poden actuar directament sobre pruritoceptors (Steinhoff, Schmelz et al., 2018).

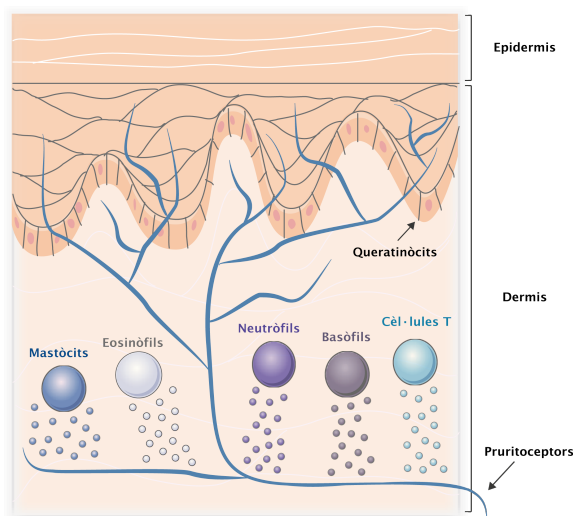


Figura 9: Cèl·lules immunes implicades en la picor. Els pruritògens exògens i els pruritògens endògens secretats per les cèl·lules immunes, com els mastòcits, les cèl·lules T, els eosinòfils, els neutròfils i els basòfils, i pels queratinòcits epidèrmics estimulen les fibres C no mielinitzades a la unió dermoepidèmica i a l'epidermis on s'origina la picor.

5. Família dels canals K_{2P}

Els canals de la família K_{2P} , també coneguts com canals de potassi amb dos dominis de porus, són importants per la seva implicació en els corrents de fuga o de *background*. Curiosament, es van identificar a partir de cerques en bases de dades de DNA sense saber les seves propietats electrofisiològiques ni funci-

onals (Lesage, Guillemare et al., 1996b). El primer canal de potassi de la família dels K_{2P} va ser descrit el 1996 (Fink et al., 1996). Entre 1996 i 2003, es van clonar, per homologia de seqüència, 14 membres més de la família codificats per diferents gens KCNK en mamífers. Els membres que formen aquesta família es distribueixen en sis subfamílies basant-se en la similitud de la seqüència i la semblança funcional: TWIK (*Two P-domain in a Weakly Inward rectifying K^+ channel*); TREK (*TWIK-RElated K^+ channel*); TASK (*TWIK-related Acid-Sensitive K^+ channel*); TALK (*TWIK-related ALkaline-sensitive K^+ channel*); THIK (*Tandem pore domain Halotane-Inhibited K^+ channel*); and TRESK (*TWIK-RElated Spinal cord K^+ channel*)(Figura 10) (Es-Salah-Lamoureux et al., 2010; Enyedi & Czirják, 2010).

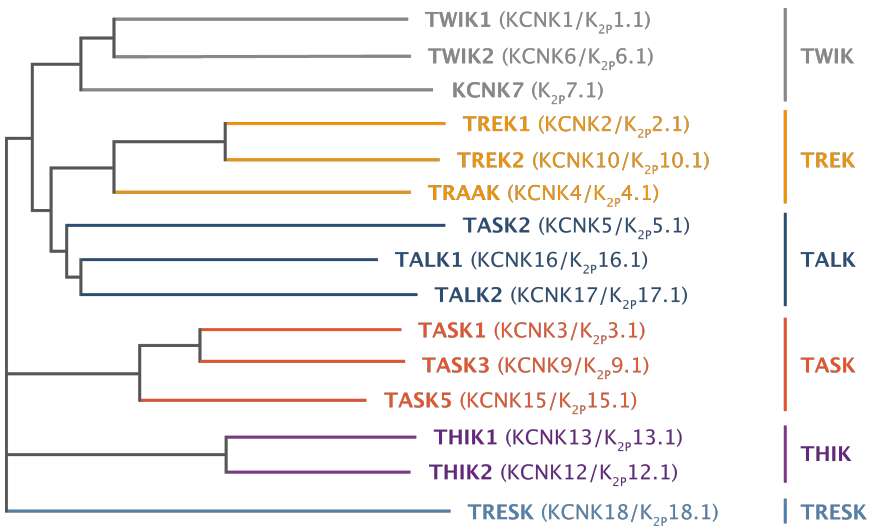


Figura 10: Diagrama en arbre de les 6 subfamílies dels canals K_{2P} amb els gens i la nomenclatura completa dels seus 15 membres. Adaptació de (Feliciangeli et al., 2015).

Els canals K_{2P} participen en el desenvolupament i l'excitabilitat de molts tipus cel·lulars (Feliciangeli et al., 2015). La distribució dels canals de fuga és àmplia i ubiqua. Alguns teixits on s'expressen són el cervell, el sistema nerviós perifèric, el cor, els pulmons, els ronyons, les artèries i les glàndules adrenals.

Val a dir que els canals de K_{2P} no estan restringits a mamífers. Els seus gens es poden trobar en organismes multicel·lulars inferiors que inclouen Drosophi-

la *melanogaster*, *Caenorhabditis elegans* i diferents tipus de plantes (Goldstein, Price et al., 1996; Perez de la Cruz et al., 2003; Czempinski et al., 1997).

5.1. Estructura dels canals K_{2P}

Tots els membres de la família de canals de potassi de fuga es caracteritzen per compartir la mateixa arquitectura molecular, tot i que, existeix una baixa similitud de seqüència entre les diferents subfamílies (Fink et al., 1996). La família rep el seu nom perquè tots els canals que la conformen presenten una tipologia distintiva en les seves subunitats.

Els canals de potassi en general contenen una breu seqüència de vuit aminoàcids molt conservada anomenada domini de porus (P) i el filtre selectiu dels ions de K^+ en un canal actiu el forma el conjunt de quatre dominis P (Doyle et al., 1998). Bona part dels canals de potassi són tetràmers, aportant cada subunitat un únic domini P formador del porus conductor d'ions. En el cas de la família dels K_{2P} , quan es va descriure per primer cop el canal TWIK1, membre prototípic de la família, es va identificar un domini P no convencional (Lesage, Guillemare et al., 1996b). Es va revelar la presència de dos dominis P en cada subunitat (P1 i P2), flanquejats per cadenes laterals hidrofòbiques que, en conjunt, formen quatre dominis transmembrana en conformació hèlix α (TM1, TM2, TM3 i TM4) (Lesage, Guillemare et al., 1996a). En conseqüència, les subunitats K_{2P} no formen tetràmers sinó que dimeritzen per constituir el filtre funcional selectiu de K^+ .

Estudis posteriors han descrit com l'estructura conservada en la família també es caracteritza per segments curts extracel·lulars entre dominis i per la presència d'una regió extracel·lular especialment llarga entre TM1 i P1. Aquesta regió extracel·lular en forma de tap crea ponts disulfur entre cisteïnes altament conservades en el mateix domini que permeten l'estabilització del dímer funcional (Sabbadini et al., 2009). Aquests canals també es distingeixen per un segon domini de porus (P2) que resideix entre TM3 i TM4 i per presentar un segment intracel·lular entre TM2 i TM3 normalment molt curt. Els extrems N i C-terminal es troben en la regió citoplasmàtica, amb un domini amino-terminal relativament curt (3-70 residus) i un terminal carboxil regulador de longitud variable (30-250 residus)

(Sabbadini et al., 2009; Enyedi & Czirják, 2010).

Les úniques variacions que existeixen en l'arquitectura entre les subfamílies són l'absència d'un enllaç disulfur en els dímers TASK1 i TASK3 i la presència d'un segment intracel·lular TM2-TM3 més llarg en TRESK, que s'ha vist implicat en la regulació de l'activitat del canal (Enyedi, Braun et al., 2012; Feliciangeli et al., 2015).

De forma general, un gen KCNK produeix una única subunitat K_{2P} . Tanmateix, existeix un inici de transcripció alternatiu en la subfamília TREK que desencadena la producció de les variants TREK1 i TREK2 amb un N-terminal citoplasmàtic més curt (Thomas et al., 2008; Simkin et al., 2008). A més, fenòmens de *splicing* alternatiu generen variants de TREK1, TREK2 i TRAAK amb nivells d'activitat variables (Fink et al., 1996; Lesage, Terrenoire et al., 2000; Lesage, Maingret et al., 2000; Gu et al., 2002; Ozaita et al., 2002; Veale, Rees et al., 2010; Rinné et al., 2014).

5.2. Propietats electrofisiològiques de la família K_{2P}

Tots els canals de la família K_{2P} comparteixen característiques electrofisiològiques similars i generen corrents de K^+ de fuga o fons (*background*). Els corrents de K^+ de fons es descriuen en la teoria com corrents independents de voltatge, amb una probabilitat d'obertura dels canals igual en tots els valors de potencial de membrana, que són independents de les concentracions de K^+ dins i fora de les cèl·lules i que no segueixen cinètiques d'activació, desactivació i inactivació (Enyedi & Czirják, 2010).

Els canals K_{2P} no compleixen perfectament tots els criteris dels corrents de K^+ de fons però s'hi aproximen. Aquests canals es mantenen oberts en tot el rang de voltatges fisiològics, la seva activitat es veu poc influenciada pels canvis de voltatge i presenten rectificació a concentracions simètriques de K^+ (Fink et al., 1996; Lesage, Guillemare et al., 1996b; Lesage, Guillemare et al., 1996a; Goldstein, Bayliss et al., 2005; Lotshaw, 2007; Bayliss et al., 2008).

L'activitat dels canals K_{2P} contribueix significativament a formar i mantenir el potencial de membrana en repòs de les cèl·lules vives prop del potencial d'equilibri de potassi (Sabbadini et al., 2009; Enyedi & Czirják, 2010). Els corrents de K^+ de fons també tenen un paper important en la regulació de l'excitabilitat, modulant la durada, freqüència i amplitud dels potencials d'acció (PA) i accelerant la repolarització en cèl·lules excitable, com neurones i cèl·lules cardiovasculars (Goldstein, Bockenhauer et al., 2001; Sabbadini et al., 2009; Enyedi & Czirják, 2014).

5.3. Regulació dels K_{2P}

Els canals KCNK són molt dinàmics i es troben regulats de forma molt estricta per diversos factors (Honoré, 2007; Steinberg et al., 2015). Una característica específica dels canals K_{2P} és que són molt poc sensibles o insensibles als bloquejadors clàssics dels canals de potassi com tetraetilamoni (TEA), Ba^{2+} , Cs^+ i 4-aminopiridina (Felicangeli et al., 2015).

La seva activitat es modula per factors fisicoquímics ambientals on s'inclou la temperatura, el pH i l'estirament de la membrana (Patel, Honoré et al., 1999; Patel & Honoré, 2001; Callejo et al., 2013). També en controlen els corrents diferents mecanismes i molècules de senyalització intracel·lular com són l'àcid araquidònic, l'anandamida, la fosforil·lació per quinases, les fosfatases i les proteïnes estructurals i adaptadores, entre d'altres (Mathie, Al-Moubarak et al., 2010; Enyedi & Czirják, 2014).

Els tractaments farmacològics també afecten l'activitat dels canals de potassi de fons, un exemple són els anestèsics locals i volàtils, com la bupivacaïna i l'isoflurà, i els estimulants respiratoris. Aquesta modulació dels corrents dels K_{2P} de forma directa o indirecta, altera el potencial de membrana i l'excitabilitat de les cèl·lules en les que s'expressen els canals. En aquest sentit, els inhibidors dels canals poden afavorir la despolarització de la membrana seguida de l'entrada de Ca^{2+} o a una resposta incrementada envers un estímul despolaritzant (Goldstein, Bockenhauer et al., 2001; Sabbadini et al., 2009; Enyedi & Czirják, 2014; Feliciangeli et al., 2015).

Els insecticides piretroides utilitzats àmpliament per al control de plagues en agricultura o en salut pública humana produeixen una inhibició important dels corrents natus de diversos K_{2P} , entre els que es troben TRESK, TRAAK, TREK-1 i TREK-2. La injecció intradèrmica de tetrametrina (TM) genera respostes noci-fensives i al·lodínia mecànica, demostrant que els efectes dels piretroides sobre els canals condueixen a comportaments associats a dolor (Castellanos, Andres et al., 2018).

5.4. Subfamília *TWIK-RElated Spinal cord K⁺ channel* (TRESK)

5.4.1. Patró d'expressió de TRESK

El canal TRESK es va clonar originalment en la medul·la espinal humana (Sano et al., 2003) i es va descriure la seva expressió en diferents àrees del sistema nerviós. El canal s'expressa més extensament en els ganglis de l'arrel dorsal i trigèmins, sobretot en neurones sensorials de petit i mitjà diàmetre. També s'ha detectat expressió de TRESK en diverses àrees del cervell, el cerebel i algunes regions del sistema nerviós perifèric com els ganglis del sistema nerviós autònom (Kang & D Kim, 2006; Dobler et al., 2007; Bautista et al., 2008; Yoo et al., 2009; Lafrenière et al., 2010; Tulleuda et al., 2011; Cadaveira-Mosquera et al., 2012).

Estudis posteriors han mostrat resultats contradictoris respecte l'expressió de TRESK. Anàlisis de seqüenciació massiva de neurones del sistema nerviós perifèric, han coincidit en una elevada expressió del canal en els ganglis trigèmins i els ganglis de l'arrel dorsal i han descrit el patró específic d'expressió del gen KCNK18.1 en les diferents subpoblacions neuronals. Els nivells més elevats d'expressió del canal es van detectar en neurones sensorials de petit diàmetre (<25 μm) probablement pertanyent a fibres C no mielinitzades que corresponen a mecanoreceptors de baix llindar i nociceptors. Concretament, s'ha detectat una expressió més extensa en els subtipus de neurones no peptidèrgiques (positives per IB4) i, en menor proporció, en neurones positives per CGRP (Usoskin et al., 2015; Zeisel et al., 2018; C Li et al., 2018; Zheng et al., 2019; Weir et al., 2019). Investigacions recents emprant aquestes tècniques de seqüenciació proposen que l'expressió de TRESK es troba restringida només a neurones aferents primàries i algunes

neurones del sistema nerviós simpàtic (Zeisel et al., 2018) i descriuen l'absència total del canal en la branca dorsal de la medul·la espinal (Häring et al., 2018).

L'ortòleg de ratolí es va identificar inicialment com TRESK-2 i va ser clonat a partir de cervell i testicles (Czirják, Tóth et al., 2004; Kang, Mariash et al., 2004). A més, en rosegadors s'ha detectat expressió del canal en teixits no nerviosos com testicles, melsa, timus, pàncrees, pulmó, ronyó i cor (Kang, Mariash et al., 2004; Enyedi & Czirják, 2014), tot i que aquestes troballes han sigut qüestionades posteriorment (Keshavaprasad et al., 2005). Dins del sistema nerviós s'ha descrit expressió en l'escorça, el cerebel, el tronc cerebral, la medul·la espinal i, especialment, en els ganglis de l'arrel dorsal (DRG) i trigemins (TG) (Kang, Mariash et al., 2004; Keshavaprasad et al., 2005; Dobler et al., 2007; Tulleuda et al., 2011; Marsh et al., 2012; Cadaveira-Mosquera et al., 2012).

5.4.2. Estructura de TRESK

El gen KCNK18.1 codifica per la seqüència del canal TRESK humà que està compost per 384 aminoàcids i que es troba a una distància força distesa de l'arbre filogenètic de la resta dels K_{2P} (Figura 6) (Enyedi & Czirják, 2014). Aquest canal forma una subfamília independent degut a les característiques estructurals úniques que presenta dins de la família dels K_{2P} .

Com és distintiu dels canals de fuga, cada subunitat de TRESK presenta els quatre dominis transmembrana, els dos dominis P formadors del porus i els extrems N i C-terminal, que es troben en la regió intracel·lular. En canvi, la subfamília de TRESK es diferencia de la resta de canals K_{2P} perquè conté un bucle intracel·lular més extens (més de 120 aminoàcids) entre els dominis TM2 i TM3 i un segment terminal carboxil curt (20-30 aminoàcids) en comparació amb altres membres de la família (Figura 11) (Kang, Mariash et al., 2004).

TRESK presenta un 67% d'homologia entre l'ortòleg humà i el de ratolí, ambdós comparteixen els trets característics com el bucle intracel·lular llarg i el segment terminal carboxil curt. Les seqüències d'aminoàcids dels canals TRESK humans i de ratolí són gairebé idèntiques dins dels dominis TM, dels dominis forma-

dors del porus i també a la part C-terminal del bucle intracel·lular on es modula l'activitat del canal per fosforilació/desfosforilació (vegeu apartat 5.4.4) (Enyedi & Czirják, 2015).

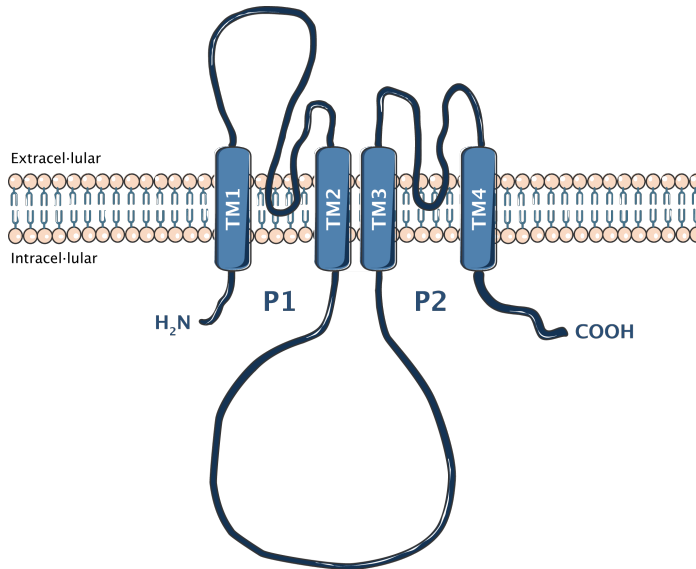


Figura 11: Estructura del canal TRESK. Estructura representativa de les subunitats del canal TRESK formades per quatre segments transmembrana (TM1-4) i dos dominis de porus (P1-2).

5.4.3. Propietats electrofisiològiques específiques de TRESK

Estudis de les propietats electrofisiològiques del canal TRESK mostren una activitat distintiva dels canals de fuga com, per exemple, l'obertura en tot el rang fisiològic del potencial de membrana i la poca o cap inactivació. El canal presenta activitat asimètrica amb una rectificació de sortida en concentracions simètriques de K^+ . Registres de canal únic han descrit en TRESK una conductància petita, d'aproximadament 16 pS, una cinètica ràpida, obertures quadrades de corrent de sortida i ràfegues d'entrada molt curtes (Kang, Mariash et al., 2004; Czirják, Tóth et al., 2004; Enyedi, Braun et al., 2012; Enyedi & Czirják, 2014).

5.4.4. Modulació de l'activitat de TRESK

Degut al rol modulador sobre el potencial de membrana, la regulació de l'activitat de TRESK és molt complexa i la duen a terme un conjunt d'estímuls físics i químics diferents. En la família dels K_{2P} la modulació de l'activitat depèn principalment de l'extensa cua a l'extrem C-terminal. En el cas de TRESK, la cua C-terminal és més curta, conseqüentment existeix una regulació diferent i una modul·lació important per part del bucle intracel·lular entre TM2 i TM3 (Enyedi & Czirják, 2014).

En TRESK, la sensibilitat al zinc està regulada pels residus His132 i Met 133, la sensibilitat al mercuri per dues cisteïnes en el segment N-terminal i la del calci es regula en el bucle intracel·lular TM2-TM3 (Sabbadini et al., 2009). TRESK també interacciona amb proteïnes de senyalització com 14-3-3 i proteïnes estructurals com la tubulina (Czirják, Tóth et al., 2004; Czirják & Enyedi, 2010; Enyedi & Czirják, 2014).

L'increment del corrent del canal està potenciat per l'augment de Ca^{2+} intracel·lular mediat per calcineurina i la interacció es duu a terme a través del lloc d'unió descrit com LQLP (Pro-Gln-Iso-Asn-Iso-Val) (Czirják, Tóth et al., 2004; Enyedi & Czirják, 2014). En repòs, el canal es troba fosforil·lat de forma constitutiva en aquests residus. Un increment de calci intracel·lular indueix l'activació del canal a través de la desforil·lació ràpida dels residus serina per part de la calcineurina. En absència del senyal de calci, el canal s'inactiva amb la refosforil·lació per mitjà de les proteïnes PKA i MARK (Braun et al., 2011; Czirják & Enyedi, 2010; Enyedi & Czirják, 2014; Andres-Bilbe et al., 2020).

Les característiques farmacològiques del canal són similars a la resta dels K_{2P} , mostrant cap o poca sensibilitat als bloquejants clàssics dels canals de potassi ni tampoc als bloquejants dels canals de potassi sensibles a ATP. En canvi, la quinina i la quinidina bloquegen eficientment el canal (Sano et al., 2003; Kang, Mariash et al., 2004). És més, alguns membres de la família K_{2P} , TRESK entre d'altres, es poden inhibir amb el compost hidroxí- α -sanshool i el derivat d'alquilamida sintètica IBA (Bautista et al., 2008; Tulleuda et al., 2011).

TRESK es pot modular amb canvis en la tensió de la membrana i/o en el volum cel·lular mitjançant estímuls mecànics i químics (Callejo et al., 2013). A més, els corrents del canal s'inhibeixen amb l'acció de l'àcid araquidònic i el pH àcid, components característics durant els processos inflamatoris o en les lesions dels teixits (Sano et al., 2003; Callejo et al., 2013). Per altre banda, l'alcalinització del pH genera un augment del corrent del 50% en el canal de ratolí (Keshavaprasad et al., 2005) però no mostra sensibilitat als canvis de temperatura (Sano et al., 2003).

Diversos estudis han descrit la influència dels anestèsics locals com la bupivacaïna sobre TRESK, els quals inhibeixen el canal tant en humans com en ratolí. Per altre banda, els anestèsics volàtils, com per exemple l'isoflurà, activen el canal amb major eficiència en el cas de TRESK humà respecte el de rata o ratolí (C Liu et al., 2004; Keshavaprasad et al., 2005).

Recentment, s'ha descobert el primer activador de TRESK anomenat cloxiquina (5-chloroquinolin-8-ol). Aquest fàrmac activa el canal de manera específica entre els canals K_{2P} per mitjà d'un mecanisme independent de Ca^{2+} i de calcineurina (Wright et al., 2013; Lengyel et al., 2017).

5.4.5. TRESK en somatosensació

TRESK és un canal de fuga amb una expressió elevada en els ganglis sensorials i amb un paper destacat en la regulació de l'excitabilitat neuronal per la qual cosa s'ha suggerit la seva implicació en la transmissió sensorial, especialment del dolor. Els corrents de TRESK, juntament amb els de TREK2, són els que contribueixen majoritàriament al corrent de fons de potassi de les neurones sensorials de DRGs de petit i mitjà diàmetre (corresponents a nociceptors) d'entre tots canals de la família dels K_{2P} (Kang & D Kim, 2006).

La pèrdua de l'activitat del canal resulta en un augment de l'excitabilitat de les neurones sensorials i una major activació de les vies del dolor (Dobler et al., 2007; Yoo et al., 2009; Tulleuda et al., 2011). Diferents estudis amb models de lesions i estudis genètics en humans suggereixen que la pèrdua de la funcionalitat

de TRESK pot contribuir a la generació de dolor patològic (Tulleuda et al., 2011; Marsh et al., 2012; Callejo et al., 2013; Zhou, CX Yang et al., 2013; Castellanos, Pujol-Coma et al., 2020). Així, s'ha descrit una important disminució de l'expressió de TRESK en els DRG després de l'axotomia del nervi ciàtic (Tulleuda et al., 2011), posterior a la inducció de dolor inflamatori per injecció de CFA (Marsh et al., 2012) i en models de dolor associat a càncer (Y Yang et al., 2018).

Tanmateix, s'ha proposat un vincle entre la pèrdua de l'expressió de TRESK i un augment de la hipersensibilitat mecànica després de lesions nervioses traumàtiques (Tulleuda et al., 2011; Zhou, CX Yang et al., 2013). Per contra, s'ha demostrat que la recuperació de l'expressió de TRESK per sobreexpressió viral del canal pot millorar la hipersensibilitat al dolor i l'alodínia mecànica induïda per aquestes lesions (Zhou, CX Yang et al., 2013; Zhou, Chen et al., 2017). És per tots aquests descobriments que aquest canal s'ha proposat com a potencial diana per al desenvolupament de fàrmacs analgèsics.

En estudis de caracterització dels ratolins TRESK KO s'ha descrit un augment significatiu de l'excitabilitat de les neurones sensorials dels ganglis de l'arrel dorsal i trigèmins respecte les neurones wildtype (WT) (Weir et al., 2019; Castellanos, Pujol-Coma et al., 2020). En concret, s'ha demostrat que l'increment de l'excitabilitat es produeix específicament en les neurones sensorials no peptidèrgiques (que expressen la isolectina B4; IB4), fet que coincideix amb la major expressió de TRESK descrita en aquesta subpoblació de neurones (Usoskin et al., 2015; Weir et al., 2019).

L'ablació de TRESK té un efecte selectiu sobre certs tipus de fibres o neurones sensorials específiques, així doncs, no produeix un augment general de l'excitabilitat sinó que només afecta algunes modalitats sensorials en concret. L'eliminació de TRESK en ratolins produeix una millora significativa de la sensibilitat mecànica i del fred, mentre que la sensibilitat al calor no es veu greument afectada (Weir et al., 2019; Guo, Qiu et al., 2019; Castellanos, Pujol-Coma et al., 2020).

Els anàlisis genètics de KCNK18 van identificar diverses mutacions en el gen que codifica per TRESK associades a la migranya familiar amb aura, una d'elles cor-

responent a una mutació negativa dominant del patró de lectura (Lafrenière et al., 2010; Andres-Enguix et al., 2012; Royal et al., 2019). La importància d'aquesta mutació en concret recau en la formació de dues proteïnes resultants a causa del canvi en el patró de lectura i de la interacció d'una d'elles amb altres canals de la família K_{2P} . Aquesta interacció desencadena la inhibició de l'activitat dels canals TREK-1 i TREK-2, amb els quals TRESK pot formar heteròmers. Conseqüentment, la mutació en TRESK produeix un augment en l'excitabilitat neuronal que facilita l'activació dels nociceptors i provoca el dolor que es manifesta durant la migra-nya amb aura (Royal et al., 2019).

II. HIPÒTESI I OBJECTIUS

1. Hipòtesi

Les sensacions de picor i dolor tenen una clara funció protectora envers estímuls nocius o prurítics de forma aguda. El problema arriba quan aquestes sensacions perduren en el temps, es coneverteixen en condicions cròniques i afecten negativament la qualitat de vida de les persones que les pateixen. Desgraciadament, la picor i el dolor crònics són condicions amb una alta prevalença en la població i els tractaments emprats en la clínica són, generalment, poc efectius i amb molts efectes adversos. L'enfocament ideal del tractament per la picor s'hauria de centrar en actuar sobre el desencadenant de la picor, és a dir, en base a l'etiologia de la picor. Tot i això, en alguns casos identificar l'origen del pruritus i tractar-lo no és fàcil.

Com s'ha esmentat anteriorment, estudis de seqüenciació massiva han proposat una classificació bàsica de les subpoblacions dels nociceptors basada en els perfils d'expressió de diferents receptors de membrana, neuropèptids i canals iònics. És més, s'ha identificat l'expressió de TRESK en diverses subpoblacions de nociceptors implicats en la transmissió de la picor. Per altre banda, TRESK s'ha relacionat amb patologies que cursen amb dolor i s'ha descrit que té un rol important en la determinació del potencial de membrana de repòs i de l'excitabilitat de

les neurones on s'expressa. Així doncs, aquesta tesi es basa en la hipòtesi de que **TRESK pot tenir un paper significatiu en la determinació de l'activació i l'excitabilitat de les neurones sensibles a la picor i, per tant, en la generació de sensacions pruments**. A més, canvis en l'expressió de TRESK en algunes malalties com la pell seca, la dermatitis al·lèrgica o la psoriasi, podrien produir hiperexcitabilitat en neurones sensorials pruritogèniques induint sensacions de picor crònica.

2. Objectius

Noves investigacions sobre el paper dels canals de fuga en el sistema nerviós somatosensorial poden contribuir a una millor comprensió de les vies de la picor i la seva implicació en diferents patologies, fets que podrien posar al descobert noves dianes específiques per al tractament de malalties cròniques. Per aconseguir-ho, en aquesta tesi vam delimitar tres objectius principals:

- Estudi dels efectes de l'ablació de TRESK sobre l'excitabilitat de les neurones sensorials implicades en la percepció de la picor.
- Investigació del paper de TRESK en picor aguda pruritoceptiva en ratolí.
- Caracterització de la participació de TRESK en models de picor crònica.

III. MATERIALS I MÈTODES

1. Animals

Tots els procediments de comportament i experimentals que inclouen animals es van dur a terme seguint les guies i recomanacions de l'Associació Internacional per l'Estudi del Dolor (IASP) i van ser prèviament aprovats pel Comitè Ètic d'Experimentació Animal de la Universitat de Barcelona i el Departament de Medi Ambient de la Generalitat de Catalunya (Catalunya, Espanya).

Els animals eren estabulats en un cicle de llum i foscor de 12/12 hores, mantinguts a una temperatura entre 20-24 °C i tenien accés il·limitat a menjar i aigua. Ambdues soques de ratolins, els salvatges (WT) i els genoanul·lats per TRESK (TRESK KO / KO), es van obtenir del Repositori Knock-Out Mouse Project (KOMP; Mouse Biology Program, University of California, Davis, CA) i les colònies s'han mantingut a l'estabulari de la Facultat de Medicina i Ciències de la Salut per mantenir la colònia.

Els procediments experimentals amb animals es van dur a terme amb ratolins mascles i femelles C57BL/6N WT i KO de 8 a 15 setmanes d'edat. En els procediments de comportament es van emprar animals de 12 a 20 setmanes d'ambdós genotips i sexes, mentre no s'indiqui el contrari.

2. Genotipatge de ratolins

Els ratolins TRESK KO van ser generats segons el protocol de *VelociGene's KOMP Definitive Null Allele Design* a partir de ratolins C57BL/6N. El gen *Kcnk18*, que codifica pel canal iònic TRESK es troba ubicat en el cromosoma 19 i per aconseguir el ratolí KO, es va substituir el gen per un casset ZEN-UB1 mitjançant recombinació homòloga. Els animals van ser sotmesos a una bateria de tests neurològics i físics per part de KOMP per descartar qualsevol tipus d'alteració abans de ser emprats per a aquest estudi. Els ratolins KO presentaven un pes i mida habituals, un comportament general normal, sense alteracions motores ni cognitives importants.

Els animals es van criar i mantenir a l'estabulari de la facultat i es van genotipar periòdicament per comprovar el fons genètic. En aquest procediment, es va extreure un fragment de cua de ratolins WT i KO d'entre 3 i 4 setmanes. Les biòpsies de cua es van processar amb l'equip de Maxwell Mouse Tail DNA Purification Kit (Promega) i se'n va extreure el DNA genòmic. El DNA aïllat es va amplificar amb la tècnica de reacció en cadena per la polimerasa (PCR) utilitzant encebadors específics per al gen *Kcnk18* i per a la seqüència del casset que substitueix el gen en el ratolí KO (ZEN-UB1) (Seqüència d'encebadors descrita en Taula 2). En la tècnica de reacció en cadena per la polimerasa, es van introduir 2 μ l de mostra, 10 μ l de preparació de PCR, 6 μ l d'aigua ultrapura i 2 μ l de preparació d'encebadors que inclou 0.5 μ l de cada encebador. Les reaccions en cadena es van dur a terme en un termociclador (Eppendorf AG) seguint el protocol descrit en la Taula 1.

Els productes de la PCR es van analitzar per electroforesis en gels d'agarosa a l'1% amb bromur d'etidi. Es van carregar 20 μ l de mostra en cada pou i 5 μ l de marcador per poder diferenciar correctament les dues bandes. Els encebadors del gen *Kcnk18* generaven com a resultat una banda de 2600 pb (parell de bases) i els encebadors del casset dels animals KO generaven una banda de 625 pb. Al finalitzar el procediment, es va identificar el genotip dels ratolins i es van distribuir els animals en caixes separades segons el genotip i el sexe per mantenir la colònia en funcionament.

Temperatura	Temps	
94 °C	4 min	
94 °C	45 seg	Repetició de 35 cicles
57 °C	1 min	
72 °C	2 min	
72 °C	10 min	
16 °C	4 manteniment final	

Taula 1: Protocol de PCR per genotipar els ratolins WT i KO. Programa de PCR utilitzat per l'amplificació del gen *kcnk18* i el casset ZEN-UB1 a partir de DNA genòmic de biòpsies de cua de ratolí.

Gen	Seqüència d'encebadors (5' - 3')		Mida de la banda
Kcnk18	For	ACCAACACCAAGCTGTCTTGTTCCTC	2600 pb
	Rev	AGACAGATGGACGGACAGACATAGATG	
ZEN-UB1	For	CTCAAGTTTACCCTACCCAAAGG	625 pb
	Rev	CCGCAGAAATAACCATCCAGAA	

Taula 2: Seqüències dels encebadors i mida dels fragments resultants de l'amplificació del gen *kcnk18* i dels casset ZEN-UB1 en el procés de genotipatge dels ratolins WT i KO.

3. Solucions

- PBS suplementat: PBS sense Ca^{+2} ni Mg^{+2} (Sigma, Madrid) suplementat amb glucosa 10 mM, HEPES 10 mM, 100 $\mu\text{g}/\text{ml}$ penicil·lina/ estreptomicina (Sigma, Madrid).
- Solució de disgregació química: es va suplementar Nutrient Mixture F-12 HAM afegint 10 $\mu\text{g}/\text{ml}$ de penicil·lina/ estreptomicina (Sigma, Madrid), 10% de sèrum fetal boví (FBS, Sigma, Madrid) i 100 de mg/ml L-glutamina (Sigma, Madrid) a HAM F-12 (Sigma, Madrid). La solució de disgregació es va aconseguir afegint 1 mg/ml d'albumina de sèrum boví (BSA, Sigma, Madrid), 1 mg/ml de col·lagenasa I (Cultek) i 5 mg/ml de dispasa II (Sigma, Madrid) a HAM F-12 suplementat.
- PBS-DEPC: Es va afegir 1 ml de DEPC (D5758; Sigma, Madrid) a 1 l de PBS sense Ca^{+2} ni Mg^{+2} . Es va barrejar enèrgicament i autoclavar per desactivar el DEPC.

- Paraformaldehid (PFA; Sigma, Madrid) 4%: En un tub es van disposar 45 ml de PBS-DEPC on es van afegir 50 μ l de NaOH 10 N i 2 g de PFA granular. Es va escalfar la solució en períodes curts fins que es va dissoldre correctament, evitant arribar a ebullició. Es van afegir 37 μ l d'HCl concentrat, es va barrejar enèrgicament i mantenir en gel fins que es necessites.
- Solució d'H₂O₂ al 3%: Es va preparar la dilució en fresc cada dia d'experiment afegint 5 ml de la solució d'H₂O₂ d'estoc (30%) a 45 ml d'aigua Mili-Q.
- Solució de rentat: Es va diluir la solució a 1X afegint 20 ml d'estoc 50X en 1 l d'aigua Mili-Q.
- Medi de cultiu: Medi Dulbecco's Modified Eagle (DMEM; Sigma, Madrid) suplementat amb 100 μ g/ml de penicil·lina/ estreptomicina (Sigma, Madrid), 100 mg/ml de L-glutamina (Sigma, Madrid) i 10% de sèrum fetal boví (FBS, Sigma, Madrid).
- HBSS suplementat: es va preparar HBSS dissolent NaCl 140 mM, KCl 4 mM, CaCl₂ 1.8 mM , MgCl₂ 1 mM i HEPES 10 mM en aigua miliQ. El dia d'experimentació es van suplementar 50 ml d'HBSS amb 150 μ l de glucosa 1M.
- Solució extracel·lular: es va dissoldre NaCl 145 mM, KCl 5 mM, CaCl₂ 2 mM , MgCl₂ 2 mM i HEPES 10 mM en aigua MiliQ. Es va emprar NaOH per calibrar el pH a 7.42 i sorbitol per aconseguir l'osmolaritat a 304 mOsm. El dia d'experimentació, es va suplementar amb glucosa 10 mM.
- Solució intracel·lular: es va dissoldre KCl 135 mM, CaCl₂ 2.1 mM , MgCl₂ 2 mM, EGTA 5 mM i HEPES 10 mM en aigua MiliQ. Es va utilitzar KOH per calibrar el pH a 7.26 i sorbitol per aconseguir l'osmolaritat a 296 mOsm. El dia d'experimentació, es va suplementar amb ATP 2.5 mM.

4. Fàrmacs, agonistes i bloquejants

- Histamina (H7250; Sigma, Madrid): es dilueix en PBS amb Ca⁺² i Mg⁺². Es va emprar una concentració de 1 μ M en els experiments *in vitro* i es van injectar 30 μ g en 10 μ l en els assajos *in vivo*.

- β -alanina (146064; Sigma, Madrid): es va diluir en PBS amb Ca^{+2} i Mg^{+2} . Es va emprar una concentració de 1 mM en els experiments *in vitro* i es van injectar 50 μg en 10 μl en els assajos *in vivo*.
- Cloroquina (CQ; C6628; Sigma, Madrid): es va diluir en PBS amb Ca^{+2} i Mg^{+2} . Es va emprar una concentració de 1 mM en els experiments *in vitro* i es van injectar 50 μg en 10 μl en els assajos *in vivo*.
- BAM 8-22 (SML0729; Sigma, Madrid): es va diluir en PBS amb Ca^{+2} i Mg^{+2} . Es va emprar una concentració de 1 mM en els experiments *in vitro* i es van injectar 100 μg en 10 μl en els assajos *in vivo*.
- Cloxiquina (5-cloroquinolin-8-ol; C47000; Sigma, Madrid): es va emprar una concentració final de 278 μM . Es va partir d'una dissolució de 5 mg de cloxiquina en 1 ml de DMSO i seguidament es va diluir una 1/10000 en PBS amb Ca^{+2} i Mg^{+2} . Es van injectar 10 μl de 278 μM en els assajos *in vivo*.
- N-methyl Leukotriene C4 (LTC_4 ; CAY-13390; Cayman Chemical, VITRO, Sevilla): el LTC_4 d'estoc en etanol es va diluir en PBS amb Ca^{+2} i Mg^{+2} per injectar 1 μg en 10 μl en els assajos *in vivo*.

5. ISH (Hibridació *in situ*)

Per comprovar que els receptors *Mrgpr* i el canal *TRESK* s'expressen en les mateixes subpoblacions de neurones sensorials i si existeix col·locació dels mateixos tal i com suggereixen diferents estudis de seqüenciació de cèl·lules individuals (Usoskin et al., 2015), es va emprar la tècnica d'hibridació *in situ* amb el mètode de RNAscope en talls de DRGs. El teixit es va extreure de DRGs de ratolins salvatges i es va incloure en medi O.C.T. per fixar-lo i evitar la degradació de RNA.

5.1. Dissecció del teixit

Els DRGs es van obtenir d'animals salvatges de 3 a 5 mesos. Per la dissecció es van sacrificar els animals per dislocació cervical sota lleu anestèsia amb isoflurà. Es va posicionar el ratolí en decúbit pron o ventral i amb l'ajuda d'unes tisores i pinces es va extreure la pell des de la cua fins a les cervicals per deixar la columna

vertebral exposada. Seguidament es va extreure la columna sencera, separant-la de la caixa toràcica i les extremitats, es van netejar les restes de teixit al voltant de l'os i es va obrir la columna per les meitats ventrals i dorsals al llarg de l'eix longitudinal d'aquesta. Es van posicionar les dues meitats de la columna sobre una placa de petri amb la medul·la exposada i es van afegir unes gotes de PBS suplementat per evitar l'assecament del teixit nerviós i la mort cel·lular. Després de col·locar la placa sota un microscopi, es va canviar a unes pinces i tisores petites i es va desplaçar la medul·la a un lateral de la columna per deixar exposats els DRG units amb els nervis que surten d'entre les vèrtebres. Es van començar a disseccionar els DRGs un per un, resseguint amb les tisores el contorn de cada gangli i tallant el més arran possible per evitar endur-se altres teixits. Un cop extrets, es van dipositar en una plaqueta amb PBS suplementat i sobre una placa de gel per mantenir-ho en fred. És important treballar amb calma però fer la dissecció el més ràpid possible per evitar la degradació de RNA.

5.2. Preparació de les seccions

El teixit es va incloure en medi O.C.T (Tissue Tek) per conservar-lo i els talls es van generar amb criòstat. Primerament es van preparar motlles d'aproximadament 1 cm de diàmetre, on es van dipositar 3-4 gotes d'O.C.T a temperatura ambient creant una capa fina al fons del motlle. Seguidament es van col·locar amb cura 4-5 DRGs per motlle, intentant situar-los junts i en un mateix pla horitzontal. Es van deixar reposar els motlles 15 minuts al congelador a -80 °C per facilitar la solidificació i posteriorment es van afegir 3-4 gotes més de medi O.C.T. fins cobrir els ganglis i arribar a 1 cm d'altura. Es van emmagatzemar els blocs en bosses de plàstic hermètiques i retolades i es van mantenir a -80 °C fins a 6 mesos.

Per obtenir els talls es va emprar un criòstat (Leica) que manté el teixit a -20 °C mentre es fan els talls amb el micròtom que inclou en l'interior. Els DRGs es van tallar en seccions de 16 µm, es manipulaven amb l'ajuda de pinzells i es van dipositar 4 seccions sobre cada portaobjectes (SuperFrost, Fisherbrand, Thermo Fisher Scientific) prèviament retolats. Es van mantenir els portaobjectes sobre una placa a 37 °C durant una hora i es van emmagatzemar a -80 °C fins el moment de realitzar la immunofluorescència.

5.3. Fixació de les seccions

Es va preparar el PFA al 4% fresc cada cop que es fixen seccions. Abans de començar el procediment assegurar-se de netejar tot el material amb solució d'H₂O₂ al 3% per evitar la degradació de RNA. Es van fixar les seccions en PFA 4% durant 15 min a 4 °C. Es van rentar les seccions dues vegades amb PBS-DEPC i es va deshidratar el teixit amb dilucions seriades d'etanol. Primer es van submergir els portaobjectes en EtOH al 50% durant 5 min a temperatura ambient. Es va repetir el pas anterior amb EtOH al 70% i dues vegades amb EtOH pur. Les seccions es van utilitzar seguidament o es van guardar submergides en EtOH pur a -20 °C fins a una setmana.

5.4. Hibridació i visualització

Es va iniciar la hibridació assecant el teixit a temperatura ambient durant 5 min i per delimitar l'àrea de tractament de les seccions, es va dibuixar una barriera hidrofòbica amb el llapis hidrofòbic Immedge repetint el mateix dibuix tres vegades i deixant assecar 1 min a temperatura ambient entre cada repetició. Amb les seccions delimitades es va començar el protocol descrit pel fabricant amb els reactius de l'equip de detecció RNAscope (ACD). El primer pas va consistir en pre-tractar el teixit cobrint les seccions amb les proteases del Pretreat IV durant 30 min a temperatura ambient.

Durant la incubació del pre-tractament es van preparar les sondes d'interès (Kcnk18, Mrgpra3 o Mrgprd). Independentment de la combinació de sondes, primerament es van afegir 200 µl de la sonda corresponent al canal 1 i seguidament es van afegir 2 µl de les sondes corresponents als canals 2 i/o 3, si es corresponia. Es va barrejar la combinació de sondes per inversió del tub, es va centrifugar breument i es va incubar durant 15 min a 40 °C en el bany humit. Es van rentar les seccions pre-tractades dues vegades amb PBS-DEPC durant 2 min i es va assecar l'excés de líquid. Es van cobrir les seccions amb les sondes i es van incubar al forn HybEZ durant 2 h a 40 °C.

Durant les incubacions, es van escalfar els reactius pertinents a temperatura ambient abans de ser utilitzats. Després de la incubació amb les sondes, es van

rentar dues vegades les seccions amb tampó de rentat durant 2 min i es va iniciar el procés d'amplificació de la senyal que consta de 4 passos. Es va retirar l'excés de líquid de les seccions, es van cobrir amb 3-4 gotes de solució d'amplificació 1 i es van incubar 30 min a 40 °C. Es va rentar el teixit dues vegades amb tampó de rentat durant 2 min. Es va cobrir el teixit amb solució d'amplificació 2 després de retirar l'excés de líquid i es va incubar durant 15 min a 40 °C. Es van repetir els rentats i es va seguir amb les incubacions de la resta d'amplificacions. Les solucions d'amplificació 3 i 4 es van incubar durant 30 i 15 min, respectivament. Per escollir la solució d'amplificació 4 més adequada per a cada experiment, es va tenir en compte el nivell esperat d'expressió de cada proteïna, l'eficiència de la sonda i la possible auto-fluorescència. Després de l'últim rentat es va muntar el cobreobjectes sobre el portaobjectes afegint 50 μ l de medi de muntatge que contenia DAPI (ProLong Diamond Antifade). Es va aplicar pressió suaument sobre el cobreobjectes per evitar l'aparició de bombolles i assegurar la correcta fixació del teixit i es van guardar les seccions a -20 °C fins a la seva visualització. Les seccions es van analitzar emprant un microscopi invertit de fluorescència i capturant imatges digitals.

6. Cultius primaris de neurones sensorials dels ganglis de l'arrel dorsal

Dies abans del cultiu es van pre-tractar cobreobjectes de 12 mm amb polilisina-L (0,1 mg/ml; Sigma, Madrid) durant 1 h i es van mantenir en sec a 4 °C fins la seva utilització. El mateix dia del cultiu, els cobreobjectes pre-tractats amb polilisina-L, es van tractar amb laminina (16 μ l/ml en PBS amb Ca^{+2} ni Mg^{+2} ; Life Technologies) durant mínim 4 h. L'obtenció de neurones sensorials dels ganglis de l'arrel dorsal es va dur a terme seguint el mateix mètode de dissecció que el descrit amb la tècnica d'hibridació *in situ*. Després de la dissecció, els ganglis en PBS suplementat fred es van traslladar a 2 ml de solució de disgregació química i es van incubar al bany a 37 °C durant 1h 45 min. Seguidament es van centrifugar els ganglis 5 min a 1000 rpm, es va canviar la solució de disgregació química per medi de cultiu i es va començar la disgregació mecànica. En aquesta, es van emprar 4 pipetes Pasteur de vidre polides amb diferents diàmetres per les quals es va fer passar la solució amb els ganglis suaument amunt i avall fins que la solució

es va homogeneïtzar. Es va tornar a centrifugar la solució 5 min a 1000 rpm i es van resuspendre les neurones en medi de cultiu nou. Es van plantar les neurones en els cobreobjectes de 12 mm pre-tractats amb polilisina-L i laminina i es van mantenir durant 48-72 h a l'incubador a 37 °C i 5% CO₂.

7. Registre del calci intracel·lular

Aquesta tècnica va permetre mesurar canvis en la concentració de calci lliure intracel·lular de les neurones després d'un estímul químic, emprant indicadors de calci com són el FURA 2-AM (F1221; Invitrogen; Thermo Fisher Scientific) o el Fluo-4 AM(F14201; Invitrogen; Thermo Fisher Scientific).

Per aquesta tècnica es van utilitzar neurones de ratolins WT i KO, cultivades durant 24 h en plaques de 24 pous (15.6 mm de diàmetre). Abans de començar l'experiment, es va canviar el medi de les neurones amb molta cura per 500 µl de medi de cultiu fresc i es van incubar amb 3 µl de FURA 2-AM o de Fluo-4 AM durant 1 h a 37 °C i 5% CO₂. Es va canviar el medi de cultiu per solució HBSS suplementat per eliminar l'excés d'indicador i es va traslladar el cobreobjectes a la cambra de registre amb HBSS suplementat. La cambra de registre estava inserida en un microscopi invertit Olympus IX70 i es va utilitzar un monocromador TILL com a font d'il·luminació. Les imatges es van captar amb un objectiu 20x i una càmera CCD (Orca II-ER, Hamamatsu Photonics). El programari que es va emprar per registrar i digitalitzar els experiments va ser l'Aquacosmos (Hamamatsu Photonics).

En els registres amb l'indicador ratiomètric FURA 2-AM, les neurones es van il·luminar cada 4 s amb llum d'una longitud d'ona de 340 nm per excitar l'indicador FURA 2-AM unit a Ca⁺² i 380 nm per excitar el FURA 2-AM lliure de Ca⁺². Es va detectar la llum que s'emetia amb una longitud d'ona de 510 nm. Es va aplicar histamina, β-alanina i BAM 8-22 de forma puntual sobre les neurones, es va deixar actuar durant 30 s i a continuació es va rentar l'agonista aplicat amb perfusió de HBSS suplementat. Els resultats obtinguts es van analitzar sostraint el senyal de fons de les imatges, es va calcular la raó de la quantitat de llum d'emissió respecte la llum d'excitació a cada longitud d'ona i es va normalitzar amb el valor

basal de cada cèl.lula. Això va permetre calcular el % d'increment per sobre del valor basal per a cada cèl.lula i també comparar els valors entre diferents experiments.

L'estimulació amb cloroquina es va dur a terme sobre les neurones durant 30 s i seguidament es va rentar l'agonista amb perfusió de HBSS suplementat. En aquest cas, les neurones es van carregar amb l'indicador Fluo-4. Aquest indicador es va estimular a 494 nm, es va registrar la quantitat de llum emesa de forma basal a 506 nm i també els possibles canvis després de l'aplicació de cloroquina. Els valors absoluts obtinguts es van analitzar sostraint el senyal de fons i es van normalitzar com descrit anteriorment. Es va considerar com a resposta positiva un increment de calci intracel·lular de més de 0.1 vegades (10%) respecte el nivell basal. Com a control positiu, només les neurones que van respondre a l'estímul de KCl 50 mM al final de l'experiment, es van considerar viables i es van incloure per l'anàlisi.

8. Electrofisiologia

8.1. Registres en neurones sensorials

Pels registres electrofisiològics es van emprar neurones sensorials de ratolins WT i KO entre 48 i 72 h després d'obtenir el cultiu primari. Els registres es van realitzar utilitzant sol·lució extracel·lular i solució intracel·lular descrites anteriorment i es van utilitzar en la configuració de *whole-cell* de la tècnica de *patch clamp*.

Els elèctrodes de registre es van generar a partir de capil·lars (HU300094; Panlab Harvard Apparatus) amb l'ajuda d'un estirador de micropipetes (P-97 Flaming/Brown micropipette puller; Sutter Instruments) i es van polir amb una microforja fins obtenir una resistència de 2-6 M Ω quan contenien solució intracel·lular.

Els experiments es van realitzar amb un amplificador de *patch clamp* (Axopatch 200B, Molecular Devices), els corrents es van registrar en configuració de

whole-cell, es van filtrar a 2 kHz, digitalitzar a 10 kHz i adquirir amb el programari pClamp 10 (Molecular Devices, CA).

Per aconseguir el registre, es va acostar l'elèctrode a la membrana de la neurona i es va aplicar pressió negativa de succió suaument fins que es va crear un segell entre la membrana de la neurona i la pipeta de l'elèctrode de més de 1 G Ω de resistència. Amb el segell creat, es va fixar el voltatge a -60 mV i es va seguir aplicant pressió negativa fins trencar la membrana de la neurona i aconseguir un continu entre la solució de la pipeta de l'elèctrode i l'interior de la neurona. En aquest punt es va canviar a mode *current clamp*, es va deixar uns segons perquè el senyal s'estabilitzes i a continuació es van aplicar els protocols. Es van incloure en l'estudi les neurones amb un potencial de membrana de repòs (RMP) inferior a -45 mV i que presentaven estabilitat durant el registre de l'aplicació de l'agonista.

Per estudiar l'excitabilitat de les neurones, es van analitzar diferents propietats electrofisiològiques basals com la capacitància (C_m) i el potencial de membrana en repòs (RMP). Seguidament, es va aplicar un protocol de polsos hiperpolaritzants i despolaritzants de corrent de 400 ms de durada partint des de -50 pA i en increments de 10 pA. Es va aplicar la llei d'Ohm, utilitzant la variació de potencial generada pel pols de -20 pA per obtenir els valors de resistència de membrana (R_m) (Figura 1).

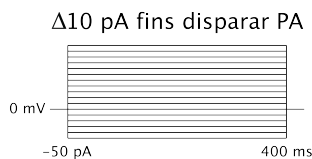


Figura 1: Esquema del protocol de polsos hiperpolaritzants i despolaritzants de corrent de 400 ms de durada partint des de -50 pA i en increments de 10pA.

Després de l'aplicació dels protocols, es va registrar el potencial de membrana en estat basal, es va administrar cloroquina 1 mM en perfusió durant 30 segons i es va rentar l'agonista amb solució extracel·lular en perfusió durant 30 segons més. Si el registre es mantenia estable, es va tornar a aplicar el protocol de polsos per obtenir més informació després de l'administració de l'agonista.

9. Comportament animal

Els procediments de comportament es van dur a terme en una sala especialitzada per evitar al màxim sorolls i pas d'altres investigadors. Tots els experiments es van realitzar en la mateixa franja horària del dia i amb els animals estabulats individualment dins de les seves pròpies caixes per disminuir l'estrès i l'ansietat dels ratolins. En l'estudi es van incloure 4 grups de ratolins per cada procediment: animals amb genotip WT i amb genotip TRESK KO i d'aquests, un grup amb condicions control i un altre amb condicions experimentals. La sensació de picor induïda en els diferents models es va quantificar per mitja de l'assaig de la galta o *cheek assay* (Shimada et al., 2008; LaMotte, Shimada et al., 2011). Aquest assaig va permetre diferenciar si els animals referien sensacions doloroses o pruritogèniques. Va consistir en injectar o tractar una part de la galta del ratolí i observar si aquest s'intentava treure la substància administrada amb la pota del darrera o la pota del davant. Si l'animal es gratava amb la pota del darrera s'anomenava *scratching* i corresponia a sensacions pruritogèniques. Si, en canvi, l'animal utilitzava la pota del davant s'anomenava *wiping* i significava que el ratolí manifestava sensacions doloroses.

9.1. Administració aguda de pruritògens

Els ratolins es van habituar a la sala 1 h abans de l'inici de l'experiment. Els animals es van anestesiàr breument amb isoflurà, es van subjectar contra una superfície plana i es va estirar la pell de la galta per exposar millor la zona a injectar. Es va utilitzar una xeringa d'insulina de 30G (Becton-Dickinson) per injectar subcutàniament 10 μ l de la solució amb agonista (Histamina, Cloroquina, BAM 8-22, β -alanina i LTC₄) o solució vehicle (PBS) a la galta dels ratolins. Els animals es van retornar a la gàbia, es va esperar fins la recuperació de l'anestèsia i es va observar el seu comportament durant 15 min. Es va comptar el número de vegades que els animals es rascaven la zona injectada (*scratching bouts*) amb la pota del davant o del darrere en intervals de 5 minuts. Només es van comptabilitzar els moviments de gratar-se o intentar treure's la substància de la galta quan es donaven de forma unilateral, diferenciant-los així de comportaments típics de neteja dels ratolins (*grooming*).

9.2. Administració aguda de pruritògens i activador de TRESK

Els ratolins es van habituar a la sala 1 h abans de l'inici de l'experiment. Es va fer un pre-tractament amb cloxiquina ($10 \mu\text{l}$ de cloxiquina $278 \mu\text{M}$) durant 30 min injectant-la subcutàniament a la galta de l'animal i seguint el mateix procediment descrit per a l'administració aguda de pruritògens. A continuació, es van injectar $10 \mu\text{l}$ de cloroquina al mateix lloc per induir picor aguda. Els animals es van retornar a la gàbia, es va esperar fins que la recuperació de l'anestèsia i es va observar el seu comportament durant 15 min. Es va comptar el número de vegades que els animals es rascaven la zona injectada amb la pota del darrere en intervals de 5 minuts.

9.3. Models animals de picor crònic

9.3.1. Model de pell seca

Els ratolins es van anestesià breument amb isoflurà i se'ls va afaitar la pell de la galta, deixant una àrea lliure de pèl d'aproximadament $0.5 \times 0.5 \text{ cm}$. Els animals es van tractar dues vegades al dia, a les 9 del matí i a les 4 de la tarda, durant 5 dies consecutius. Es va subjecta es ratolí despert i es va aplicar el tractament a la galta amb una turunda de cotó submergida en solució experimental (1:1 acetona i èter) o solució vehicle (aigua) durant 15 segons. Seguidament es va aplicar aigua amb una turunda de cotó durant 30 segons d'acord amb el protocol descrit per (Wilson, Nelson et al., 2013; T Liu, Q Han et al., 2016). Abans de la segona aplicació diària dels dies 3 i 5, els ratolins es van habituar a la sala d'experimentació. Just després de l'aplicació del tractament, es va deixar el ratolí lliure a la gàbia i es va observar el seu comportament durant 1 hora, quantificant el número de vegades que es grataven la galta de forma espontània (Figura 2).

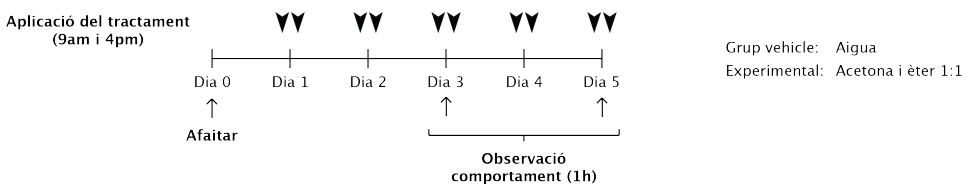


Figura 2: Esquema experimental per la generació del model de pell seca.

9.3.2. Model de dermatitis al·lèrgica

En aquest model, els ratolins van rebre una sensibilització a la zona de l'esquena i, posteriorment, l'assaig es va dur a terme a la galta del ratolí segons el protocol descrit per (Fu et al., 2014; Pall et al., 2015). Així, els animals es van anestesià breument amb isoflurà i se'ls va afaitar la pell de la galta i de la zona lumbar de l'esquena, deixant unes àrees lliures de pèl d'aproximadament 0.5 x 0.5 cm. Durant 3 dies consecutius, els animals desperts es van subjectar i es van sensibilitzar aplicant 25 μ l de SADBE 1% en acetona a la zona de l'esquena. Es va deixar un període de 5 dies per a que es generés la sensibilització sistèmica en l'animal. Al 6è dia, es van administrar 25 μ l de SADBE 1% en acetona (3,4-Dibutoxy-3-cyclobutene-1,2-dione; 339792; Sigma, Madrid) o acetona (vehicle) a la galta dels ratolins desperts per produir la reacció al·lèrgica. Es va repetir l'administració del tractament a la galta durant 2 dies consecutius. Es va observar el comportament abans de començar les aplicacions de tractament a la galta (dia 7) i durant 3 dies consecutius (Figura 3). Els animals es van habitar a la sala d'experimentació i seguidament es va observar el comportament dels ratolins durant 1 hora per quantificar el número de vegades que es grataven la galta de forma espontània.

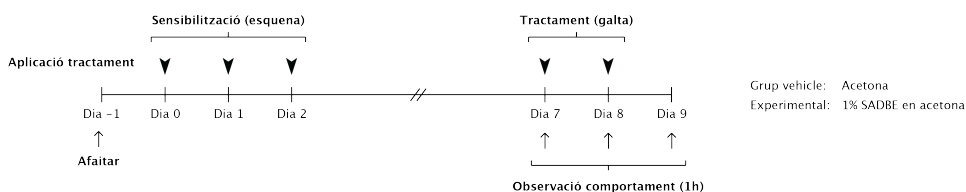


Figura 3: Esquema experimental per la generació del model de dermatitis al·lèrgica.

9.3.3. Model de picor psoriàsica induïda per imiquimod

Segons el model descrit prèviament (Follansbee et al., 2019; Sakai et al., 2016), els ratolins es van anestesià breument amb isoflurà i se'ls va afaitar la pell de la galta, deixant una àrea lliure de pèl d'aproximadament 0.5 x 0.5 cm. Els ratolins es van habitar a la sala d'experimentació a primera hora del matí durant 1 h abans de començar l'experiment. Seguidament, es va observar el comportament

espontani de cada ratolí durant 1 h, quantificant el número de vegades que es van gratar la galta amb l'extremitat posterior de forma espontània. Després de l'observació i amb el ratolí despert, es van aplicar 12.5 mg d'Aldara (Imiquimod 5%; condició experimental) o vaselina (condició vehicle), a la zona afaitada de la galta del ratolí. El tractament es va administrar durant 7 dies consecutius i sempre en la mateixa zona de la galta del ratolí. L'observació es va dur a terme el primer dia abans del primer tractament com a mesura basal i 24h després de cada aplicació del tractament o vehicle (Figura 4). Els experiments es van dur a terme en grups de 4-6 animals on es van incloure ratolins d'ambdós genotips. Per al posterior estudi histològic i per quantificar possible canvis d'expressió de RNA, es va generar el model en un grup d'animals que es van sacrificar després del 4rt dia d'observació, se'n van extreure els ganglis trigèmins i es va dissecar la zona de la pell de la galta tractada.

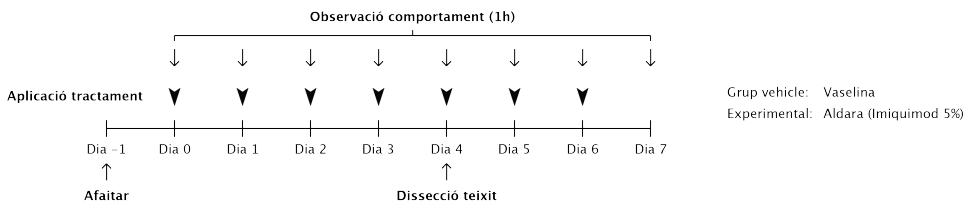


Figura 4: Esquema experimental per la generació del model de picor psoriàsica induïda per imiquimod.

10. PCR quantitativa a temps real

10.1. Obtenció del teixit

Es va extreure el RNA de 5 animals per cada condició i genotip. Per obtenir els ganglis trigèmins, es van anestesiar breument els ratolins amb isofluorà i es van sacrificar per dislocació cervical. Es va separar el crani de la resta de l'animal i es va subjectar amb unes pinces d'hemostàsia. Es va extreure la pell que cobreix el crani amb les tisores i les pinces, es va retirar la part del crani que cobreix la part superior de l'encèfal i seguidament es va extreure l'encèfal amb cura de no endur-se els nervis i ganglis que passen per sota. Amb l'ajuda d'unues pinces i tisores petites, es van subjectar els ganglis trigèmins que travessen des de la part

posterior del crani fins a les òrbites dels ulls i es van dissecionar acuradament separant-los de l'os. Es van mantenir i netejar els ganglis amb PBS suplementat fred, es van introduir en RNA later (Qiagen, Madrid) per preservar el RNA i es van congelar ràpidament submergint-los en nitrogen líquid. Es van emmagatzemar a -80 °C fins l'extracció de RNA.

10.2. Extracció de RNA i traducció a cDNA

L'extracció de RNA i síntesis de cDNA es va dur a terme el mateix dia, sempre que fos possible, per evitar al màxim la degradació de la mostra. El RNA del teixit es va extreure sonicant la mostra i seguint el protocol del producte NucleoSpin RNA (Macherey-Nagel, Alemanya). El material extret es va mantenir a 4 °C en gel si es seguia amb la traducció, o bé, es va emmagatzemar a -20 °C fins que es tornés a reprendre el protocol. El RNA aïllat, es va quantificar i es va analitzar la quantitat de material i la puresa respecte el DNA i la proteïna present en la mostra mitjançant un Nanodrop (ThermoFisher Scientific). Es van seguir els valors de puresa descrits en el protocol del fabricant per acceptar les mostres per l'estudi. Es van emprar entre 300 i 500 η g de RNA de cada mostra per la traducció a cDNA utilitzant el producte SuperScript IV, First-Strand Synthesis System (ThermoFisher Scientific). El cDNA resultant es va emmagatzemar a -20 °C.

10.3. PCR quantitativa a temps real

Per dur a terme la PCR quantitativa, cada placa experimental va contenir 4 mostres, una de cada combinació (WT o KO, vehicle o experimental) i es van agrupar les mostres que contenien la mateixa quantitat de cDNA aproximada. En aquest procediment es va utilitzar el producte Fast SYBER Green MasterMix (Applied Biosystems) i l'equip AbiPrism 7300. Es van emprar els encebadors a 1/20 (ThermoFisher Scientific) per als gens descrits en la Taula 4 i es va utilitzar el gen GAPDH a 1/100 com a gen normalitzador (*housekeeping*) per a comparar amb la resta de gens amplificats. Breument, en cada pou es van afegir 10 μ l de Master Mix, 5 μ l de cDNA de la mostra i 5 μ l de la barreja d'encebadors.

Per comprovar que l'amplificació es va realitzar correctament i que els fragments amplificats eren els esperats, es van analitzar les temperatures de dissoci-

Gen	Temperatura de fusió
Kcnk18	82 °C
Mrgpra3	82 °C
Mrgprd	81 °C
Mrgprc11	73 °C
Trpa1	75 °C
Trpv1	77 °C
Gapdh	81 °C

Taula 3: Temperatures de fusió dels fragments amplificats. Temperatures de fusió obtingudes dels diferents fragments amplificats per cada parell d'encebadors en els experiments de PCR quantitativa a temps real.

ació (*melting temp*) dels fragments amplificats mostrades en la Taula 3. L'anàlisi dels resultats de la PCR es va dur a terme per mitjà dels ΔC_t , analitzant el número de cicles per arribar al llindar obtingut (C_t , *Cycle threshold*) menys el C_t del gen de referència que en aquest cas és el GAPDH. Amb els ΔC_t de cada gen es van comparar els nivells basals d'expressió dels animals WT respecte els KO, també les condicions vehicle i experimental de cada genotip i, per últim, els nivells dels dos genotips en condicions experimentals.

11. Histologia

Les mostres de pell es van dissecionar de la galta de ratolins WT i KO després de la 4a administració de tractament experimental (Imiquimod 5%) o vehicle (Vaselina). El teixit es va fixar en paraformaldehid al 4% en PBS sense Ca^{+2} ni Mg^{+2} durant 72h a temperatura ambient. Posteriorment es va submergir en etanol al 70% i es va emmagatzemar a temperatura ambient fins al seu processament. Les mostres es van inserir en parafina i es van tallar en seccions de 5 μm , es va eliminar la parafina dels talls i es van tenyir amb la tècnica d'hematoxilina-eosina per a l'avaluació histològica. Les imatges es van capturar amb un microscopi de fluorescència Nikon Eclipse E-800. Les imatges es van analitzar per camps de visió a 20x i utilitzant el programari Image J (NIH). Els factors analitzats en les seccions van ser el gruix de l'epidermis quantificat com a número de capes epidèrmiques, el grau d'hipergranulosis representat com a percentatge de l'àrea total analitzada i el número de microabscessos per centímetre de teixit.

Gen	Nom de la proteïna	Seqüència d'encebadors (5' - 3')	
Kcnk18	TRESK	For Rev	CTCTCTTCTCCGCTGTCGAG AAGAGAGCGCTCAGGAAGG
Mrgpra3	MRGPRA3	For Rev	CTCAAGTTTACCCTACCCAAAGG CCGCAGAAATAACCATCCAGAA
Mrgprd	MRGPRD	For Rev	TTTTCAGTGACATTCTCGCC GCACATAGACACAGAAGGGAGA
Mrgprc11	TRESK	For Rev	ACTCTCTGCTACGGATCATTGA TGATTGCTGCATTGCCTAAGATA
Trpa1	TRPA1	For Rev	GTCCAGGGCGTTGTCTATCG CGTGATGCAGAGGACAGAGAT
Trpv1	TRPV1	For Rev	CCCGGAAGACAGATAGCCTGA TTCAATGGCAATGTGTAATGCTG
Gapdh	GAPDH	For Rev	TCCACTGGCGTCTTCAC GGCAGAGATGATGACCCTTTT

Taula 4: Seqüència dels encebadors per a l'amplificació dels gens en la PCR quantitativa a temps real.

12. Anàlisi estadístic

L'anàlisi estadístic es va dur a terme amb el programari GraphPad Prism 8 (GraphPad Software, Inc.). Les dades d'experiments de comportament entre genotips i grups de tractament es van analitzar emprant test no aparellats paramètrics i no paramètrics amb condició de dues cues (test de T-Student) i test ANOVA d'una via amb posterior test Sidak per la correcció de comparacions múltiples. Tots els resultats es van expressar com a mitjana amb l'error estàndard de la mitjana (SEM). En els resultats dels experiments d'imatges de calci intracel·lular, la resposta es va presentar com a percentatge i per l'anàlisi es va emprar el test de Fisher o T-Student per comparar entre genotips. L'amplitud del pic de resposta es va mostrar com a mitjana $\pm$ SEM i es va analitzar amb el test de T-Student no aparellat. Les diferències entre grups es van considerar estadísticament significatives quan presentaven un valor $p < 0.05$.

IV. RESULTATS

1. TRESK s'expressa en neurones pruritoceptives

Els diferents estímuls de picor són detectats per un tipus específic de neurones sensorials anomenades pruriceptors. Aquests, expressen múltiples tipus de receptors entre els que destaquen els receptors acoblats a proteïnes G que detecten estímuls químics. Alguns dels receptors de pruritògens coneguts per ser expressats en neurones sensorials són els d'histamina H1 i H4, els de serotonina i els Mrgpr (Nojima et al., 2003; Roßbach et al., 2008; Q Liu, Tang et al., 2009; Q Liu, Sikand et al., 2012).

El canal iònic TRESK s'expressa, entre d'altres, en neurones sensorials de petit i mitjà diàmetre com són els nociceptors (Enyedi & Czirják, 2010). S'han identificat canvis en l'expressió del canal en patologies relacionades amb el dolor, lesions del sistema nerviós i inflamació crònica (Lafrenière et al., 2010; Tulleuda et al., 2011; Marsh et al., 2012; Royal et al., 2019).

Així mateix, estudis recents de seqüenciació massiva han proporcionat informació més detallada sobre l'expressió de TRESK, mostrant que està expressat majoritàriament en subpoblacions de nociceptors no peptidèrgics i també en una de les subpoblacions de mecanoreceptors de baix llindar implicats en la sensació

de tacte (Usoskin et al., 2015; Zeisel et al., 2018; C Li et al., 2018; Zheng et al., 2019).

A fi d'analitzar més en detall les dades obtingudes per seqüenciació massiva i el patró d'expressió del canal TRESK en les diferents subpoblacions de neurones sensorials, vam realitzar un estudi comparatiu de l'expressió de TRESK amb diversos receptors específics relacionats amb la picor i el dolor. Es van escollir 5 receptors relacionats amb la picor (MrgprA3, MrgprD, MrgprX1, Cysltr2 i Hrh1) i 2 canals relacionats amb el dolor (TRPA1 i TRPV1). Els resultats de la comparació es mostren en la Figura 1, on vam observar que TRESK presenta una expressió significativa en dues de les subpoblacions de nociceptors no peptidèrgics (NP1 i NP2) i que aquestes subpoblacions expressen selectivament i de manera característica els receptors pruritogènics MrgprD (NP1) i MrgprA3 (NP2).

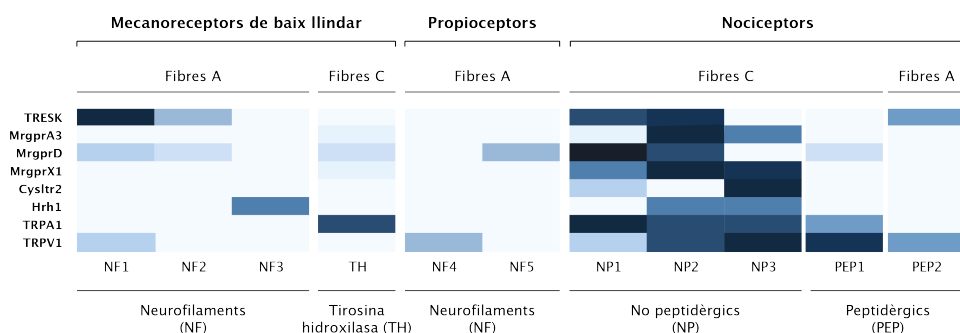


Figura 1: Patró d'expressió de TRESK, dels receptors de picor i dels canals iònics relacionats amb el dolor en les neurones de petit i mitjà diàmetre. Escala de color que representa l'expressió relativa dels gens en cada subpoblació emprant un mètode de llindar. Dades extretes de l'estudi de (Usoskin et al., 2015).

Per tal de corroborar les troballes de l'anàlisi bioinformàtic amb les dades de seqüenciació, i amb la col·laboració del grup del Dr. Mark A Hoon (NIDCR, NIH, Bethesda, EEUU), vam estudiar la coexpressió de TRESK amb aquests receptors pruritogènics per mitjà de la tècnica d'hibridació *in situ* en talls de teixit de DRG de ratolins WT. Es va utilitzar el mètode de RNAscope amb les sondes de TRESK i dels receptors MrgprA3 i MrgprD. Les imatges obtingudes de moment es va dur a terme als EEUU, ja que actualment s'està posant a punt el mètode al laboratori per complementar l'estudi amb la quantificació de les imatges. En aquests experiments preliminars, es va observar que les neurones MrgprA3 coexpressen el canal

TRESK, la qual cosa corrobora els resultats obtinguts mitjançant seqüenciació del RNA en cèl·lula única. De la mateixa manera, es va observar una coexpressió de TRESK en neurones que expressen el receptor MrgprD (Figura 2).

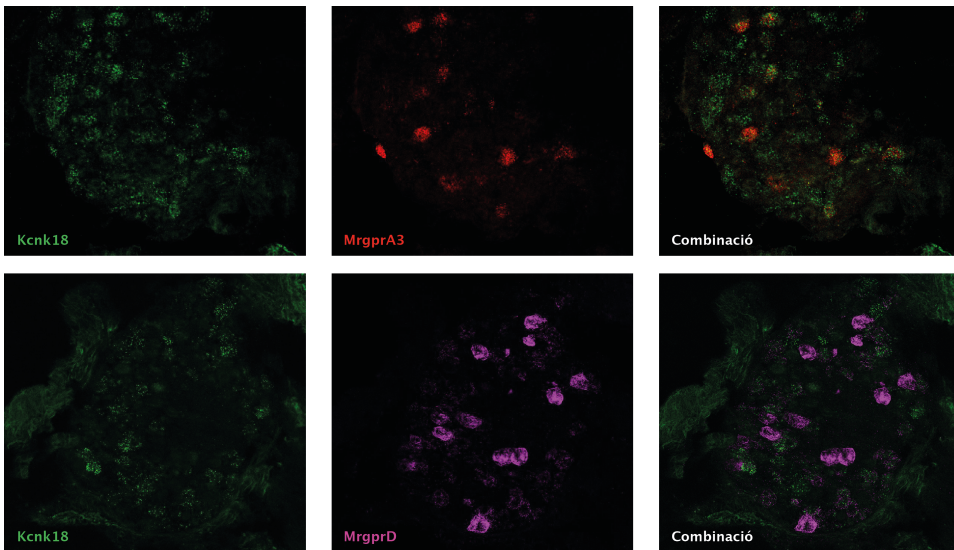


Figura 2: MrgprA3 i MrgprD es coexpressen amb TRESK en neurones sensorials. Doble hibridació *in situ* en seccions de DRG mostra coexpressió del canal iònic TRESK (verd) amb una part de les neurones MrgprA3 (vermell; panell superior) i MrgprD (magenta; panell inferior) positives.

2. Caracterització funcional de neurones pruritoceptives

Partint de la coexpressió de TRESK amb receptors implicats en picor observada en l'anàlisi bioinformàtic, corroborada amb els resultats preliminars obtinguts de les hibridacions *in situ*, vam voler comparar si les poblacions de neurones que responen a estímuls pruritoceptius actuen de forma similar en presència o absència del canal TRESK. Així doncs, vam avaluar la resposta a diferents agonistes pruritoceptius en cultius de neurones de DRG d'animals WT i KO emprant la tècnica de registre de calci intracel·lular. Per estudiar la implicació tant de la picor d'origen histaminèrgic com no histaminèrgic, es van emprar els següents agonistes: histamina (agonista del receptor Hrh1), cloroquina (MrgpA3), β -alanina

(MrgprD) i BAM8-22 (MrgprC11) (Figura 3).

Els nociceptors s'identifiquen, d'entre d'altres característiques, per formar part d'una subpoblació de neurones de petit i mitjà diàmetre, corresponent a neurones de menys de $30\text{ }\mu\text{m}$ de diàmetre (Usoskin et al., 2015). En els registres de calci intracel·lular vam utilitzar FURA 2-AM o Fluo-4 AM per carregar les neurones sensorials en cultiu 24h després de la seva dissecció. En vam estudiar un total de 2609 neurones (1221 de ratolins WT i 1388 de ratolins TRESK KO) que presentaven un diàmetre mitjà de $17.73 \pm 0.31\text{ }\mu\text{m}$ en el cas de les neurones WT, i $16.95 \pm 0.25\text{ }\mu\text{m}$ en les neurones KO. D'acord amb el seu diàmetre mitjà, podem assumir que les neurones analitzades corresponien a neurones nociceptives, on s'inclouen la subpoblació de neurones pruritoceptives.

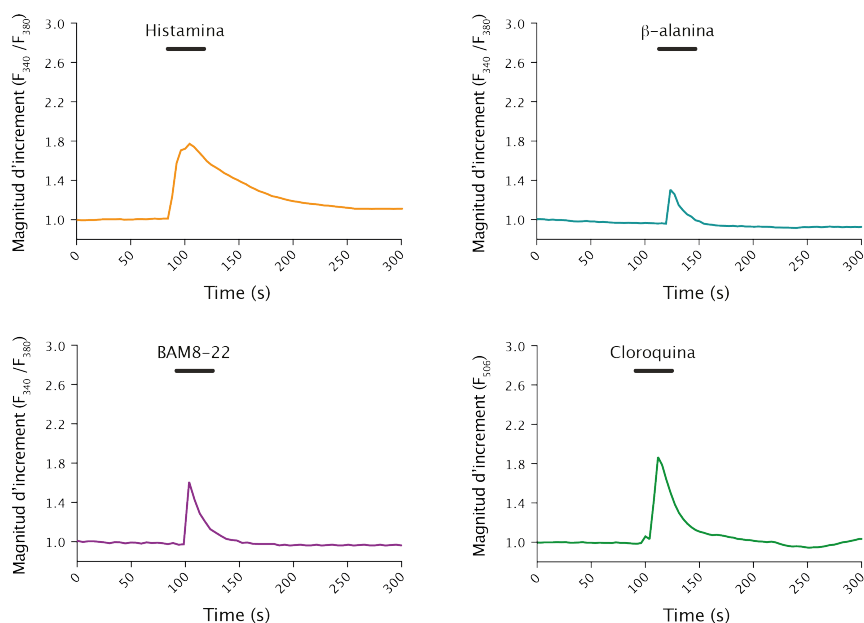


Figura 3: Exemples representatius de les mobilitzacions de calci intracel·lular en resposta als diferents agonistes pruritoceptius. Registres de calci intracel·lular de neurones en cultiu de DRG estimulades amb histamina ($1\text{ }\mu\text{M}$), β -alanina (1 mM), BAM 8-22 (1 mM) i cloroquina (1 mM).

Amb l'objectiu d'investigar la implicació de TRESK en la generació de la picor histamina dependent, vam administrar el propi agonista endogen, histamina. Tal

i com està descrit en estudis previs, només un petit percentatge de les neurones WT van respondre a l'agonista (12.02 ± 3.84 %; $n_{\text{total}}=167$), i no es van mostrar diferències significatives respecte el percentatge de les neurones KO que van respondre al mateix agonista (11.35 ± 3.85 %; $n_{\text{total}}=166$; test T-Student amb $p=0.909$ respecte WT, no significatiu; Figura 4A). L'amplitud del pic de resposta obtingut de l'administració d'histamina va ser significatiu en ambdós genotips i sense diferències significatives entre genotips (1.68 ± 0.11 vegades sobre el calci basal en neurones WT i 1.77 ± 0.19 vegades sobre el calci basal en neurones KO; test T-Student amb $p=0.653$; no significatiu; Figura 5A).

En la recerca de la participació del canal TRESK en les vies independents d'histamina que transmeten la picor, vam voler emprar tres agonistes diferents per activar els receptors pruritogènics més estudiats. El primer receptor no histamínergic en el que ens vam centrar va ser el receptor MrgprD. Per activar-lo, vam aplicar l'aminoàcid no essencial β -alanina a les neurones (288 de ratolins WT i 416 de ratolins KO), ja que el seu consum com a suplement esportiu provoca picor com a efecte secundari (Liu Q et al., 2012). L'administració de β -alanina va generar un augment de calci significatiu en un 20.25 ± 2.25 % de les neurones procedents de ratolins WT, amb una amplitud de pic mitjana de 1.33 ± 0.04 vegades sobre el calci basal. El comportament que vam observar en les neurones de ratolins KO no va mostrar diferències significatives, responnent un 16.78 ± 2.00 % (test T-Student amb $p=0.314$ respecte WT, no significatiu; Figura 4B) i un pic de 1.40 ± 0.05 vegades sobre el calci basal (test T-Student amb $p=0.281$ respecte WT, no significatiu; Figura 5B).

El segon compost avaluat *in vitro* va ser el BAM 8-22, un pèptid de medul·la adrenal bovina i un potent activador del receptor MrgprC11 (Sikand P et al., 2011). L'administració del pèptid sobre els cultius neuronals d'ambdós genotips va generar l'activació d'un petit percentatge de les neurones analitzades, sense diferències significatives entre genotips (5.88 ± 1.75 % amb una $n_{\text{total}}=416$ de neurones WT i 5.95 ± 1.86 % amb una $n_{\text{total}}=393$ de neurones KO; test T-Student amb $p=0.983$, no significatiu; Figura 4C). L'amplitud de la resposta de l'administració de BAM 8-22 tampoc va mostrar diferències significatives entre genotips (1.46 ± 0.06 vegades sobre el calci basal en grup de neurones WT i 1.41 ± 0.05 vegades sobre el

calci basal en grup de neurones KO; test T-Student amb $p=0.579$, no significatiu; Figura 5C).

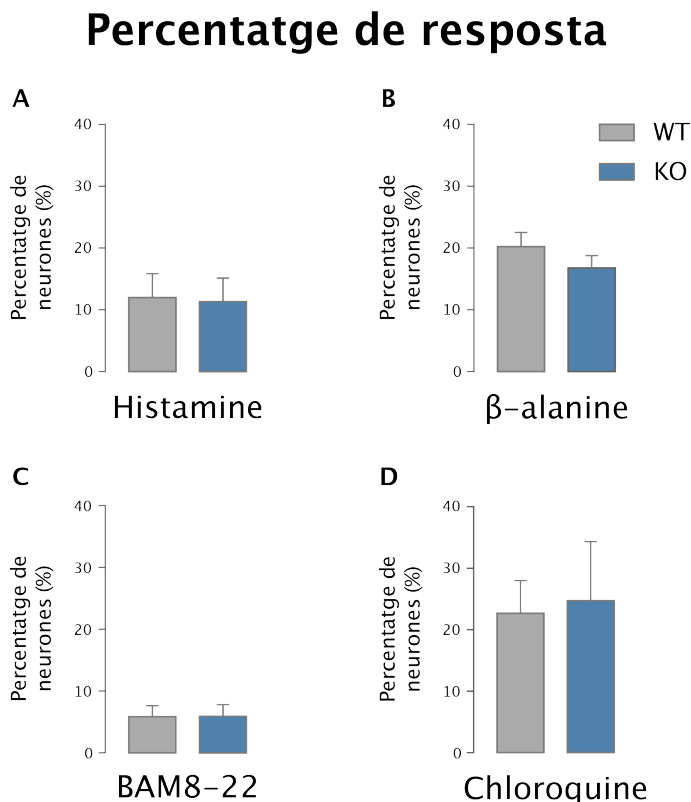


Figura 4: Agonistes pruritogènics activen poblacions similars de neurones sensorials en ratolins WT i KO. Quantificació del percentatge de resposta evocada en cultius de neurones de DRG de ratolins WT i KO per l'administració dels pruritògens histamina ($1 \mu\text{M}$; $p=0.909$, ns), cloroquina (1 mM ; $p=0.861$, ns), β -alanina (1 mM ; $p=0.314$, ns) i BAM 8-22 (1 mM ; $p=0.983$, ns). Les dades es mostren com a percentatge mitjà de diversos ratolins $\pm$ SEM. Es van analitzar 3-4 animals i >150 neurones per genotip i agonista. Resultats entre genotips comparats amb tests t-Student no aparellats. ns, no significatiu.

Per últim, vam estudiar un fàrmac anomenat cloroquina, que es prescriu per al tractament de la malària, i que activa el receptor MrgprA3. Tots els registres els vam dur a terme seguint el mateix protocol exceptuant aquest agonista, on es va emprar l'indicador de calci Fluo-4 AM. Aquest canvi va ser degut a la desaparició del senyal de fluorescència durant els registres de calci després de l'aplicació del fàrmac en neurones carregades amb Fura 2-AM. Buscant la causa d'aquest feno-

men vam descobrir que la cloroquina s'utilitza com a tractament per a malalties en peixos i que es fa passar llum ultraviolada a través dels aquaris per saber la quantitat de fàrmac que hi ha a l'aigua. Aquest esdeveniment ocorre com a conseqüència de la capacitat que presenta la cloroquina d'absorbir la llum ultraviolada. Així doncs, per buscar una alternativa en els registres de calci, vam dur a terme una corba d'absorbància de la cloroquina i en vam obtenir uns valors màxims d'absorció d'unes longituds d'ona d'entre 230 i 360 λ , que coincideixen amb el rang de longituds d'ona utilitzades per estimular l'indicador Fura 2-AM. A fi de resoldre el problema, vam canviar l'indicador de calci a Fluo-4 AM, el qual no té capacitat ratiomètrica però s'excita i emet amb una llum d'una longitud d'ona que no es veu afectada per l'absorció de la cloroquina. D'entre tots els agonistes, l'aplicació de cloroquina va provocar l'activació de la proporció més gran de neurones (22.68 ± 5.32 % d'un total de 350 neurones WT i 24.73 ± 9.62 d'un total de 413 neurones KO; test T-Student amb $p=0.861$, no significatiu; Figura 4D). La magnitud de la resposta a l'agonista va ser la més elevada d'entre els agonistes no-histaminèrgics i no es van observar diferències significatives en el patró d'activació entre genotips (1.54 ± 0.06 vegades sobre el calci basal en les neurones WT i 1.58 ± 0.06 vegades sobre el calci basal en les neurones KO; test T-Student amb $p=0.707$, no significatiu; Figura 5D).

3. L'eliminació de TRESK augmenta la sensibilitat a picor aguda

Una manera directa per determinar el paper de TRESK en la picor és la d'eliminar el canal i veure si el comportament relacionat amb la picor es veu afectat. Per això, paral·lelament als registres *in vitro*, es van realitzar experiments amb els diferents agonistes histaminèrgics i no histaminèrgics a fi de trobar un mètode adequat per estudiar-ne els efectes *in vivo* sobre ratolins WT i KO d'ambós sexes. Altres grups d'investigació en el camp del picor han desenvolupat diversos protocols per poder quantificar i analitzar la sensibilitat al picor mitjançant estudis conductuals. Algun d'aquests mètodes són el model de la galta (*Cheek assay*) (Shimada et al., 2008; Akiyama, MI Carstens & E Carstens, 2010; LaMotte, Shimada et al., 2011) i la injecció al clatell (*Nape of the neck test*) (Qu et al., 2014). Per als ex-

periments de conducta es va escollir el model de la galta (Figura 6) i es van definir unes pautes de realització per minimitzar l'estrès dels animals i procurar que les condicions fossin el més reproduïbles possibles entre grups experimentals. Aquest model permet la distinció entre els comportaments dels ratolins envers estímuls de dolor i estímuls de picor segons si per enretirar l'estímul utilitzen l'extremitat anterior o es graten amb l'extremitat posterior, respectivament (Shimada et al., 2008; LaMotte, Shimada et al., 2011).

Amplitud del pic de calci

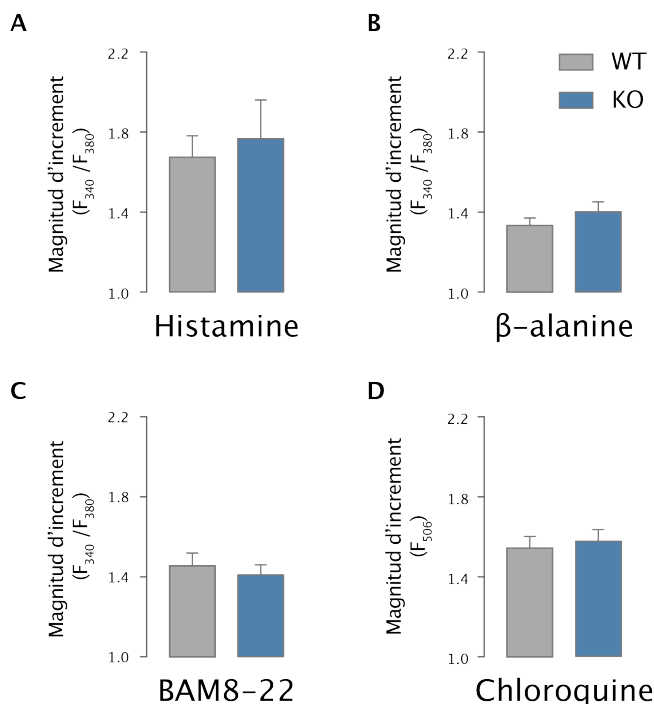


Figura 5: La magnitud d'activació de les neurones sensorials de DRG per mediadors pruritogènics és semblant entre genotips. Quantificació normalitzada de la magnitud del pic de resposta generada per l'aplicació d'histamina (1 μ M), cloroquina (1 mM), β -alanina (1 mM) i BAM 8-22 (1 mM). Valors obtinguts com a raó F₃₄₀ entre F₃₈₀ o F₅₀₆, prèvia extracció del senyal de fons i normalització. Les barres d'error representen el SEM i les comparacions s'han realitzat amb tests t-Student no aparellats.

Primerament vam utilitzar el model de la galta per estudiar els efectes de la histamina en ratolins WT i TRESK KO. La injecció d'histamina (30 μ g en 10 μ l)

va provocar un augment significatiu en el número de vegades que els ratolins WT es grataven específicament amb l'extremitat posterior en el lloc d'administració respecte el grup de ratolins WT injectats amb vehicle (28.17 ± 3.76 vegades envers 0.20 ± 0.00 vegades, respectivament). L'efecte de la injecció d'histamina en els ratolins KO no mostrava diferències significatives respecte els WT (24.67 ± 2.93 vegades; test t-Student no aparellat, $p=0.479$ respecte els ratolins WT, no significatiu; Figura 7).



Figura 6: Il·lustració que representa les diferents conductes evocades per injeccions de substàncies que generen dolor (esquerra) i picor (dreta) en ratolins. Imatge adaptada de (Yamanoi et al., 2019).

Seguidament vam voler avaluar els agonistes no histaminèrgics. Els dos primers van ser la β -alanina i el BAM 8-22 seguint el mateix protocol que amb la histamina. En aquest cas, no vam aconseguir que cap dels dos compostos generés resposta en els ratolins. Després d'una recerca en la literatura, vam provar diferents concentracions dels compostos i ampliar la finestra del temps d'observació per si els agonistes tardaven més en fer efecte (Q Liu, Sikand et al., 2012; Akiyama, Tominaga et al., 2012; Sanjel et al., 2019). Tot i amb això, no es va observar resposta després de la injecció dels compostos en cap dels dos genotips.

D'altra banda, vam administrar cloroquina de manera aguda a la galta dels ratolins salvatges i vam observar un augment significatiu dels episodis de rascat de magnitud similar al observat amb la injecció d'histamina (24.50 ± 4.03 vegades). En canvi, en els ratolins KO, la resposta que va generar la cloroquina va ser significativament major respecte els WT (48.58 ± 6.11 vegades; test p-Student no aparellat, ** $p=0.007$ entre genotips; Figura 7). Per últim, vam estudiar els efectes del mediador inflamatori Leucotriè C4 a fi de discernir més acuradament en quines subpoblacions de neurones TRESK exerceix un paper important. Aquest

mediador és l'agonista del receptor Cys1tr2 que només s'expressa en una de les subpoblacions (NP3) on s'expressa el receptor d'histamina (NP2 i NP3) i, concretament, és la subpoblació que no coincideix amb l'expressió de MrgprA3 (NP2; Figura 1). L'administració de LTC₄ va provocar una reacció comparable a la generada per la histamina i sense diferències significatives entre ambdós genotips (28.57 ± 4.24 vegades en ratolins WT respecte 31.00 ± 6.91 vegades en ratolins KO; test t-Student no aparellat, $p=0.769$, no significatiu; Figura 7).

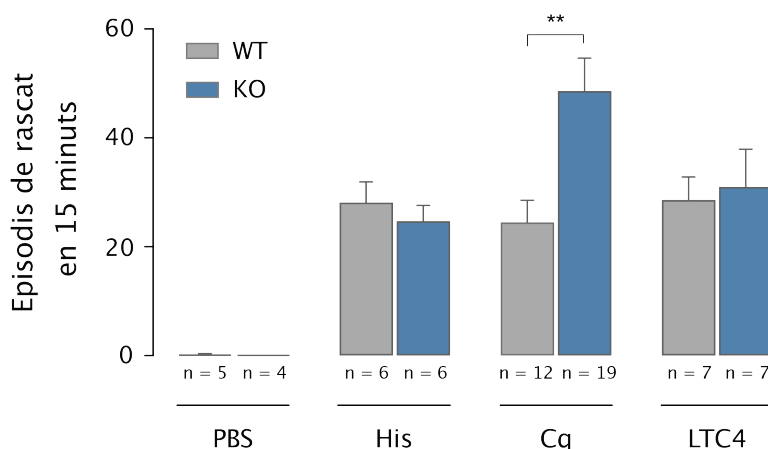


Figura 7: L'eliminació de TRESK produeix un augment en la resposta a la injecció de cloroquina però no a la d'altres pruritògens. Quantificació de la mitjana dels moviments de rascat generats amb l'extremitat posterior durant 15 minuts (episodis de rascat) específicament dirigits a la zona d'injecció en ratolins WT i KO. Injecció subcutània de 30 μ g d'histamina, 50 μ g de cloroquina i 1 μ g de LTC₄ en 10 μ l de PBS a la galta dels animals. Les barres d'error corresponen al SEM. Dades comparades amb test t-Student no aparellats, ** $p<0.01$ entre genotips.

4. L'absència de TRESK incrementa l'excitabilitat neuronal dels pruritoceptors

S'ha demostrat que l'eliminació de TRESK provoca un augment en el número de potencials d'acció generats quan s'injecta una rampa de corrent despolaritzant en les neurones en cultiu. Aquest fet es tradueix com un increment significatiu en l'excitabilitat de les neurones sensorials TRESK KO (Castellanos, Pujol-Coma et al., 2020). A banda d'això, s'ha vist que la cloroquina pot induir directament la des-

càrrega de potencials d'acció (PA) en neurones sensorials en cultiu (Q Liu, Tang et al., 2009; Wilson, Gerhold et al., 2011). Per investigar sobre les bases cel·lulars implicades en l'increment de la resposta conductual provocada per la cloroquina en ratolins TRESK KO, vam dur a terme registres de *current clamp* en neurones sensorials aplicant cloroquina en perfusió i en vam comparar les propietats de les neurones que van respondre.

Les subpoblacions de neurones sensorials involucrades en nocicepció i pruritocepció es poden identificar seguint diferents criteris. Entre els paràmetres a tenir en compte trobem l'expressió de receptors específics i el diàmetre dels somes neuronals (Lawson, 2002; Usoskin et al., 2015). Les diferències en el diàmetre del soma de les neurones en cultiu correlacionen amb variacions en la superfície de la seva membrana. D'aquesta manera, com més superfície de membrana continguin les cèl·lules, més càrrega poden acumular en aquesta, fenomen que es denomina capacitança de membrana (Cm). Per tal d'identificar aquest tipus de cèl·lules en els cultius de neurones de DRG vam emprar el criteri de capacitança de membrana, considerant per a estudi només les neurones de petit i mitjà diàmetre que presentaven una capacitança de membrana inferior a 50 pF (Ma et al., 2003). En els registres Es van enregistrar 20 neurones de ratolins WT i 22 neurones de ratolins KO, de les quals 6 i 8 van respondre a cloroquina, respectivament. Les neurones enregistrades en aquest estudi presentaven una capacitança mitjana de 22.17 ± 2.47 pF en el cas de les WT i 27.88 ± 4.08 pF en les KO (test de Mann-Whitney amb $p=0.471$, no significatiu; Figura 9A).

La membrana cel·lular consisteix en una bicapa lipídica amb proteïnes que actuen com a canals que permeten el pas de càrregues a través d'ella, permetent la reducció de la resistència que la membrana oposa al pas de les càrregues. La quantitat de corrent necessària per variar el potencial de membrana d'una neurona ve determinada per la resistència de membrana que aquesta neurona presenti. Així doncs, segons la llei d'Ohm ($V = I \times R$; on V equival a voltatge de membrana, I a corrent i R correspon a resistència de membrana), com major sigui el número de canals oberts en la membrana, menys resistència s'oposarà al pas de càrrega a través de la membrana. Seguint amb la caracterització de les neurones WT i KO, en vam calcular la resistència de membrana amb el protocol de polsos. Les neuro-

nes procedents d'animals salvatges mostraven una resistència de 925.80 ± 87.34 pF i no es van observar diferències respecte la resistència de les neurones KO (981.70 ± 209.50 pF; test de Mann-Whitney amb $p=0.237$ entre genotips, no significatiu; Figura 9B).

Els canals K_2P són extensament coneguts pel seu paper rellevant en el manteniment del potencial de repòs de la membrana en molts tipus de cèl·lules (Enyedi & Czirják, 2010). És per aquest motiu, que vam estudiar si el potencial de repòs de la membrana de les neurones es veia modificat al eliminar-ne TRESK. No vam observar diferències significatives en aquesta propietat entre genotips. Els registres de les neurones KO mostraven un potencial de membrana en repòs mitjà de -54.14 ± 4.32 mV respecte el de les neurones WT amb un potencial de membrana de -49.33 ± 2.26 mV (test t-Student no aparellat, $p=0.369$, no significatiu; Figura 9C).

L'aplicació de cloroquina en neurones de ratolins WT va generar un número de potencials d'acció mitjà de 16.17 ± 6.91 PA. Les neurones KO van mostrar un increment en el número de potencials d'acció en resposta a la cloroquina (27.63 ± 9.64 PA), tot i que no va ser estadísticament significatiu (test t-Student, $p=0.385$ entre genotips, no significatiu; Figura 9D i 8). S'ha de tenir en compte que la dispersió en els valors obtinguts en els registres va ser molt elevada i que seria necessari ampliar el número de registres per obtenir una mostra més representativa.

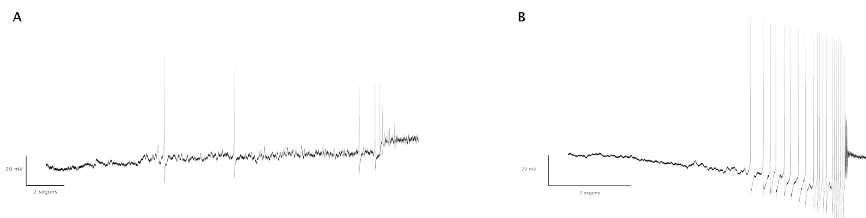


Figura 8: Exemples de registres representatius de neurones sensorials. Registres de l'excitabilitat neuronal després de l'aplicació de cloroquina en perfusió (1 mM) en neurones de ratolins WT (A) i KO (B).

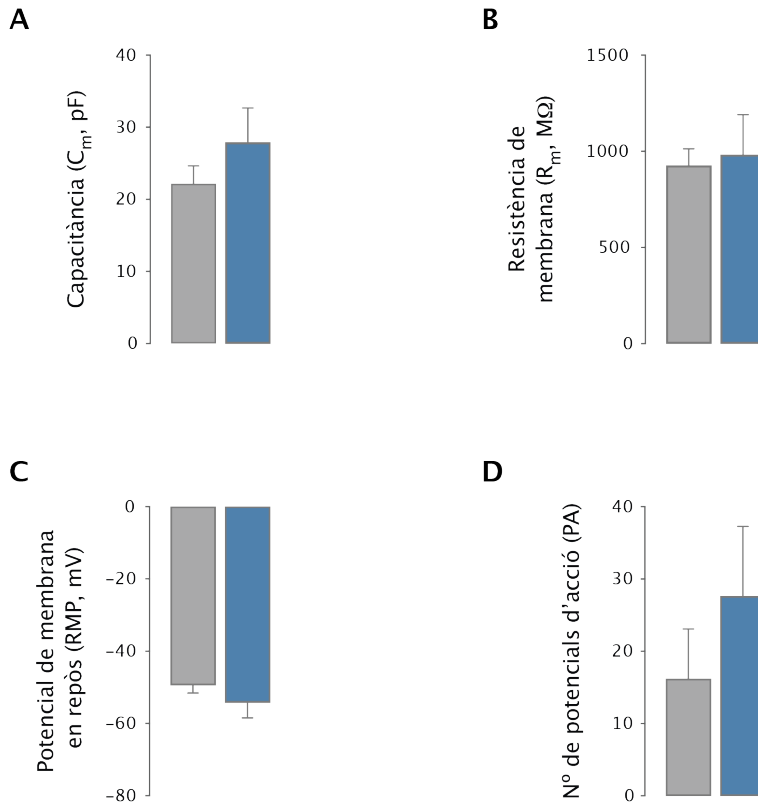


Figura 9: Les neurones sense TRESK tendeixen a disparar més potencials d'acció en resposta a cloroquina. Quantificació de paràmetres electrofisiològics analitzats activa i passivament de neurones de petit i mitjà diàmetre de DRGs en cultiu de ratolins WT i KO. (A) Capacitència mitjana de la membrana de les neurones. (B) Resistència de membrana de les neurones calculada usant un protocol de polsos de corrent hiperpolaritzants i despolaritzants de 400 ms de durada en increments de 10 pA. (C) Potencial de repòs de la membrana de les neurones. (D) Quantificació del número mitjà de potencials d'acció disparats en resposta a l'aplicació en perfusió de cloroquina (1 mM) durant 30 s. Dades mostrades com a mitjana $\pm$ SEM i comparades amb tests t-Student no aparellats

5. Exacerbació de la picor crònica amb l'absència de TRESK

Encara existeixen moltes incògnites sobre els mecanismes relacionats amb la generació i transmissió del pruritus, tot i això, ja s'hi han vist implicats un gran ventall de receptors i vies cel·lulars diferents. És per això que hem volgut investigar el possible rol del canal TRESK en els fenòmens de picor crònica a través dels

models animals de pell seca, de dermatitis al·lèrgica i de psoriasis, en els quals la generació de picor crònica es duu a terme per diferents mecanismes fisiopatològics. Aquests models pretenen mimetitzar les lesions cutànies i sensacions pruritogèniques observades en les malalties equivalents en humans, a més, generen picor espontània quantificable en els animals.

5.1. L'ablació de TRESK incrementa el fenotip generat pel model de pell seca

El model de pell seca és un dels més emprats per a l'estudi de la picor crònica. En aquests sentit, cal destacar el paper clau descrits dels receptors MrgprA3 i MrgprC11 en la transmissió del pruritus derivat d'aquest model (Zhu et al., 2017). Donat que els ratolins KO mostren un increment en l'activació del receptor MrgprA3 com a resposta a la cloroquina, vam hipotetitzar que el canal de TRESK podria exercir algun tipus de modulació en el processament de la picor crònica generada en aquest model.

El protocol que es va utilitzar en ratolins va generar una lesió cutània que pretén reproduir el fenotip de la malaltia cutània en humans anomenada xerosis. Per produir la lesió, es va seguir el protocol prèviament descrit en altres estudis (Miyamoto et al., 2002). Després de l'aplicació consecutiva del vehicle (aigua), els ratolins WT no van mostrar moviments espontanis de rascat (Dia 3: 7.43 ± 1.73 vegades; Dia 5: 11.86 ± 3.82 vegades; $n=7$). L'administració del vehicle tampoc va generar una reacció significativa en els ratolins KO (Dia 3: 2.83 ± 0.95 vegades; Dia 5: 10.71 ± 3.21 vegades; $n=7$; test ANOVA d'una via, $p>0.999$ respecte WT, no significatiu; Figura 10).

Per contra, l'administració repetida d'acetona i èter (condició experimental) en ratolins WT va provocar-los lesions cutànies i un increment significatiu dels episodis de rascat espontani que s'accentuava amb el pas del temps (Dia 3: 27.13 ± 4.94 vegades; Dia 5: 43.63 ± 6.77 vegades; $n=8$; test ANOVA d'una via, * $p=0.033$ entre dia 3 i 5, *** $p=0.0006$ respecte el vehicle; Figura 10). Tanmateix, els ratolins KO van mostrar un augment significatiu dels episodis de rascat des del primer dia d'observació respecte el vehicle (Dia 3: 44.00 ± 6.38 vegades; test ANOVA d'una

via; **** $p < 0.0001$, Figura 10) i respecte els ratolins WT (test ANOVA d'una via; $p = 0.055$; Figura 10). L'increment en els episodis de rascat dels animals KO es va mantenir en el temps sense diferències significatives respecte els ratolins salvatges (Dia 5 KO: 37.00 ± 4.83 vegades; $n = 8$; test ANOVA d'una via, $p = 0.929$ respecte WT, no significatiu; Figura 10).

Model de pell seca

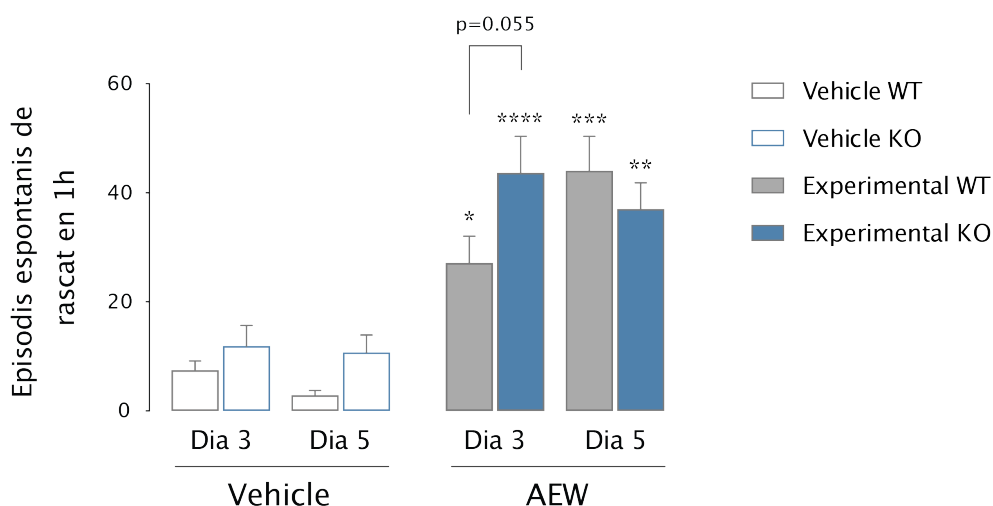


Figura 10: El model de pell seca sobre la galta de ratolins KO incrementa el número d'episodis de rascat espontani. Quantificació del número mitjà d'episodis de rascat dels ratolins durant una hora d'observació el dia 3 i 5 del model de pell seca. Tractament experimental amb 1:1 acetona-èter seguit d'aigua (AEW) o aigua (vehicle). Les barres d'error representen el SEM. Resultats analitzats estadísticament amb tests ANOVA d'una via amb correcció posterior de test Sidak per múltiples comparacions; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ entre condicions vehicle i experimental de cada genotip; $p = 0.055$ entre genotips el dia 3.

5.2. L'eliminació de TRESK augmenta la picor crònica generada en el model de dermatitis al·lèrgica

La dermatitis al·lèrgica per contacte és una malaltia inflamatòria produïda pel contacte no desitjat amb productes químics irritants o al·lèrgics. Estudis previs han mostrat com l'ablació de les neurones que expressen MrgprA3 i MrgprD provoca una reducció significativa del rascat espontani per injecció de l'al·lèrgo-

gen en ratolins prèviament sensibilitzats (Qu et al., 2014). Tenint en compte la coexpressió del canal TRESK amb aquests dos receptors pruritògens, vam reproduir el mateix model en ratolins TRESK KO per veure si se'n modificava el fenotip resultant.

Model de dermatitis al·lèrgica

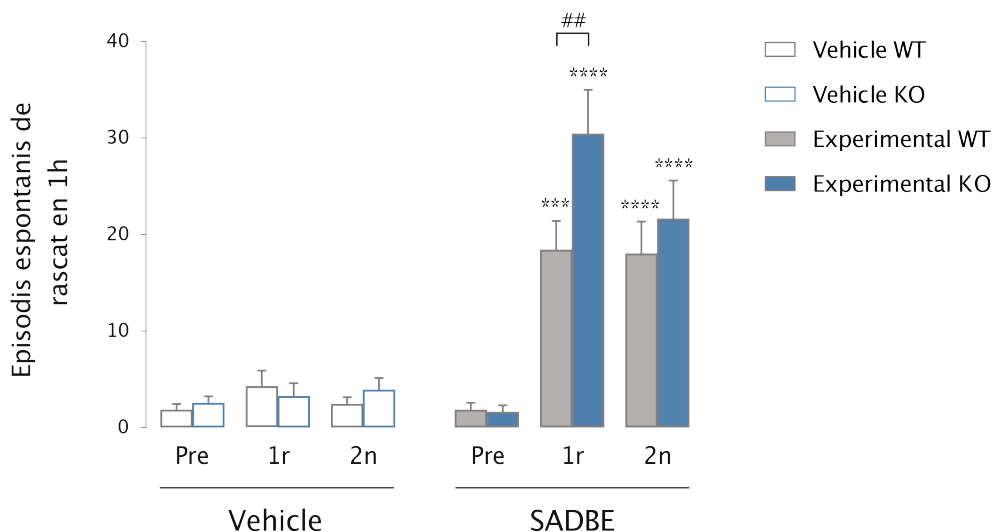


Figura 11: El model de SADBE indueix un augment inicial més pronunciat dels episodis de rascat en els ratolins TRESK KO. Quantificació de la mitjana d'episodis de rascat durant 1 hora després de l'aplicació de SADBE 1% en acetona (experimental) o acetona (vehicle) en animals WT i KO prèviament sensibilitzats. Resultats analitzats estadísticament amb tests ANOVA d'una via amb correcció posterior de test Sidak per múltiples comparacions; *** $p < 0.001$, **** $p < 0.0001$ entre condicions vehicle i experimental; ## $p < 0.01$ entre genotips. Les barres d'error representen el SEM.

Per la seva investigació, vam generar un model de dermatitis al·lèrgica de contacte mitjançant el tractament de la pell amb l'al·lèrgen SADBE. L'administració de SADBE sobre la galta dels ratolins WT, prèvia sensibilització a l'esquena, va provocar un augment significatiu dels episodis de rascat respecte els nivells basals (Basal: 1.87 ± 0.72 vegades; 1r dia: 18.44 ± 2.99 vegades; $n=16$; test ANOVA d'una via, $p < 0.0001$; No representat). També es va observar un increment significatiu respecte els ratolins tractats amb vehicle (1r dia: 4.27 ± 1.61 vegades; $n=15$; test ANOVA d'una via, *** $p=0.0005$; Figura 11). L'augment significatiu es va mantenir

durant segon dia d'observació (2n dia: 18.06 ± 3.28 vegades; test ANOVA d'una via, **** $p < 0.0001$ respecte vehicle; Figura 11). Aquests resultats concorden amb altres estudis sobre el model de dermatitis al·lèrgica (Qu et al., 2014).

De manera interessant, el mateix protocol aplicat en ratolins TRESK KO va generar una reacció espontània de rascat significativament incrementada respecte el vehicle (1r dia vehicle: 3.29 ± 1.33 vegades, 1r dia SADBE: 30.47 ± 4.54 vegades; $n=15$ i $n=17$ respectivament; test ANOVA d'una via, **** $p < 0.0001$; Figura 11). La resposta dels animals KO tractats amb SADBE també va ser significativament major a la dels ratolins WT (test ANOVA d'una via, ## $p=0.003$; Figura 11). Cal remarcar que en el segon dia d'observació, els episodis de rascat dels animals KO van disminuir, deixant de mostrar diferències significatives respecte els ratolins salvatges (2n dia: 21.69 ± 3.91 vegades; test ANOVA d'una via, $p=0.949$ respecte WT, no significatiu; Figura 11).

5.3. L'absència de TRESK provoca un augment en la severitat de la picor crònica psoriàsica induïda per imiquimod

En una altre línia de recerca sobre la possible implicació de TRESK en els mecanismes de generació del pruritus crònic, vam estudiar si el canal participa en la modulació de la picor crònica de tipus psoriàsica. Aquesta és una malaltia inflamatòria crònica de la pell amb una important implicació autoimmunitària. Per tal d'investigar-ne els mecanismes d'acció subjacents, s'han generat models animals que intenten reproduir-ne el fenotip. Nosaltres vam escollir el model de picor psoriàsica induïda per aplicació cutània d'imiquimod ja que està molt ben caracteritzat (Follansbee et al., 2019).

Tal i com s'ha descrit en la literatura (Sakai et al., 2016), l'administració repetida d'imiquimod va generar un augment en els episodis de rascat espontani dels ratolins WT des del primer dia de tractament (Figura 12). Aquest increment va arribar a ser estadísticament significatiu el dia 4, assolint el punt màxim d'episodis de rascat espontani del model (66.86 ± 18.50 vegades respecte 4 ± 2.37 vegades del vehicle; $n=7$ i $n=6$ respectivament; test ANOVA d'una via, *** $p=0.0004$; Figura 12). L'increment significatiu respecte el vehicle es va mantenir fins al fi-

nal del protocol (Dia 5: 47.57 ± 14.39 vegades, * $p=0.024$ respecte vehicle; Dia 6: 42.29 ± 9.09 vegades, $p=0.065$ respecte vehicle; Dia 7: 71.43 ± 24.28 vegades, **** $p<0.0001$ respecte vehicle; Figura 12).

Model de picor psoriàsic induït per imiquimod

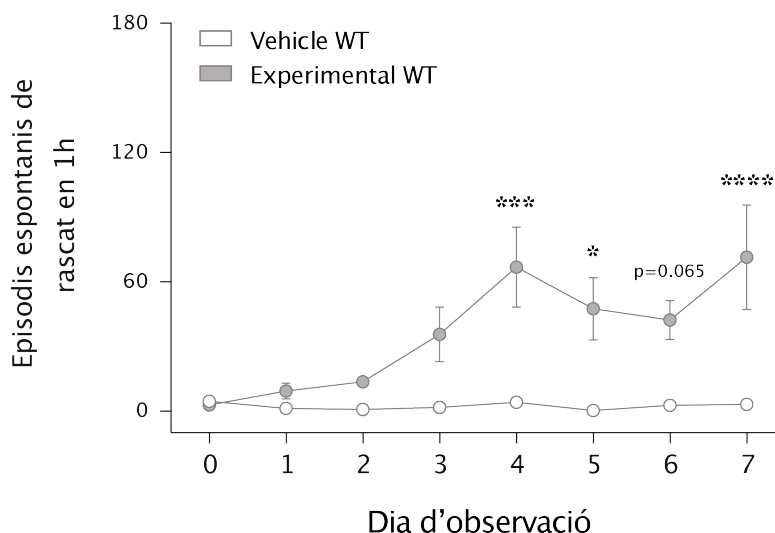


Figura 12: L'aplicació repetida d'imiquimod genera picor crònica en ratolins WT. Quantificació dels episodis de rascat en un període d'una hora després de l'aplicació repetida d'imiquimod al 5% (experimental) o vaselina (vehicle) en ratolins WT. Anàlisi estadístic amb test ANOVA d'una via amb correcció posterior amb el test Sidak per múltiples comparacions; * $p<0.05$, *** $p<0.001$, **** $p<0.0001$ entre condicions vehicle i experimental. Les barres d'error representen el SEM.

L'administració d'imiquimod també va reproduir el fenotip de picor crònica psoriàsica en ratolins TRESK KO (Figura 13). En els ratolins KO, vam observar un increment estadísticament significatiu en els episodis de rascat espontani respecte el vehicle a partir del tercer dia de tractament (97.00 ± 16.03 vegades respecte el vehicle 1.75 ± 1.03 vegades; $n=8$ i $n=4$ respectivament; test ANOVA d'una via, *** $p=0.0001$). Els valors màxims els vam observar el dia 4 (126.10 ± 24.03 vegades respecte 0.33 ± 0.33 vegades del vehicle; $p^{****} p<0.0001$) i seguidament van decaure els episodis de rascat a la meitat on es van mantenir estables fins al final del protocol (Dia 5: 43.00 ± 9.58 vegades, $p=0.502$ respecte vehicle; Dia 6: 76.63 ± 17.43 vegades, ** $p=0.009$ respecte el vehicle; Dia 7: 58.38 ± 17.11 vegades, $p=0.087$ res-

pecte vehicle).

Model de picor psoriàsic induït per imiquimod

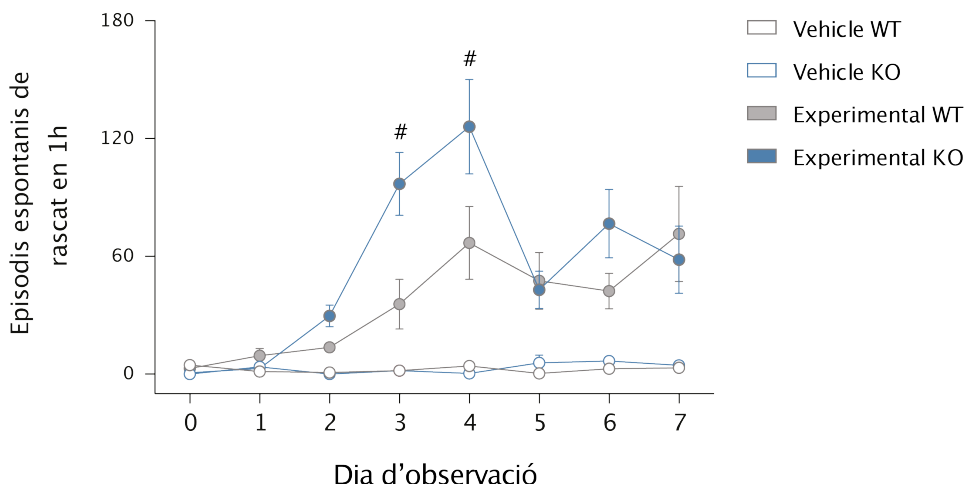


Figura 13: Els ratolins sense TRESK desenvolupen un fenotip més pronunciat en el model de picor crònica psoriàsica. Quantificació dels episodis de rascat en un període d'una hora després de l'aplicació d'imiquimod 5% (experimental) o vaselina (vehicle) durant 7 dies consecutius sobre la galta de ratolins WT i KO. Anàlisi estadístic amb test ANOVA d'una via amb correcció posterior Sidak per múltiples comparacions; # $p < 0.05$ entre grup genotips. Les barres d'error representen el SEM.

Convé destacar que vam observar un increment més ràpid en el temps i significativament major en els ratolins KO respecte els WT. Els ratolins KO van presentar un increment significatiu en els episodis de rascat a partir del dia 3 (test ANOVA d'una via, # $p = 0.018$ respecte ratolins WT; Figura 13) i aquesta resposta exacerbada es va mantenir el dia 4 (test ANOVA d'una via, # $p = 0.025$ respecte ratolins WT; Figura 13). Després d'arribar al pic màxim, els episodis de rascat espontani dels animals KO van disminuir i no es van observar diferències estadísticament significatives respecte els animals WT (Figura 13).

5.3.1. L'expressió de MrgprA3 i MrgprD disminueix en el model de picor crònica psoriàsica induïda per imiquimod

Es coneix que els receptors Mrgprs són presents en subconjunts de neurones sensorials de petit diàmetre i tenen un paper important en la picor (Q Liu, Sikand

et al., 2012). Diferents estudis posteriors han mostrat com els nivells d'expressió gènica de MrgprA3, MrgprD i MrgprC11 disminueixen en el model de picor crònica psoriàsica induïda per imiquimod (Sakai et al., 2016). A més, diversos treballs han demostrat que l'expressió de TRESK està alterada en models de dolor neuropàtic (Tulleuda et al., 2011; HY Hwang et al., 2015; Zhou, Chen et al., 2017).

Així doncs vam estudiar possibles canvis en l'expressió gènica per ampliar la caracterització del paper de TRESK en el model de picor psoriàsica induïda per imiquimod. Per quantificar canvis en els nivells d'expressió de diferents canals iònics i dels receptors Mrgprs, vam extreure el RNA dels ganglis trigèmics dels ratolins després de 4 dies de tractament, coincidint amb el pic màxim dels efectes en el picor espontani.

Per començar, vam caracteritzar els nivells basals d'expressió dels diferents canals i receptors d'interès en els ratolins KO respecte els WT. Vam observar una lleugera disminució estadísticament significativa de l'expressió del receptor MrgprD ($5.16 \pm 0.07 \Delta Ct$ respecte $4.81 \pm 0.07 \Delta Ct$ en els WT; test t-Student no aparellat, # $p=0.011$; Figura 14C) i del canal iònic TRPV1 ($6.55 \pm 0.11 \Delta Ct$ respecte $6.04 \pm 0.12 \Delta Ct$ en els WT; test t-Student no aparellat, # $p=0.014$; Figura 14F). La resta de gens analitzats no van mostrar diferències estadísticament significatives provocades per l'eliminació de TRESK.

Seguidament, vam analitzar els nivells d'expressió dels diferents RNAs en ratolins salvatges després de la inducció de picor psoriàsica amb imiquimod. Vam observar com dos dels receptors Mrgpr estudiats van mostrar una disminució significativa de la seva expressió després del tractament. En el cas del receptor MrgprA3, els valors d'expressió del grup tractament i del grup vehicle van ser de $9.69 \pm 0.07 \Delta Ct$ i $10.54 \pm 0.21 \Delta Ct$, respectivament (test t-Student no aparellat, ** $p=0.004$; Figura 15). El receptor MrgprD després de l'aplicació d'imiquimod també va mostrar una disminució significativa ($5.38 \pm 0.12 \Delta Ct$) comparat amb els nivells d'expressió després de l'aplicació de vehicle ($4.81 \pm 0.08 \Delta Ct$; test t-Student no aparellat, ** $p=0.004$; Figura 15).

Expressió gènica en gangli trigemin

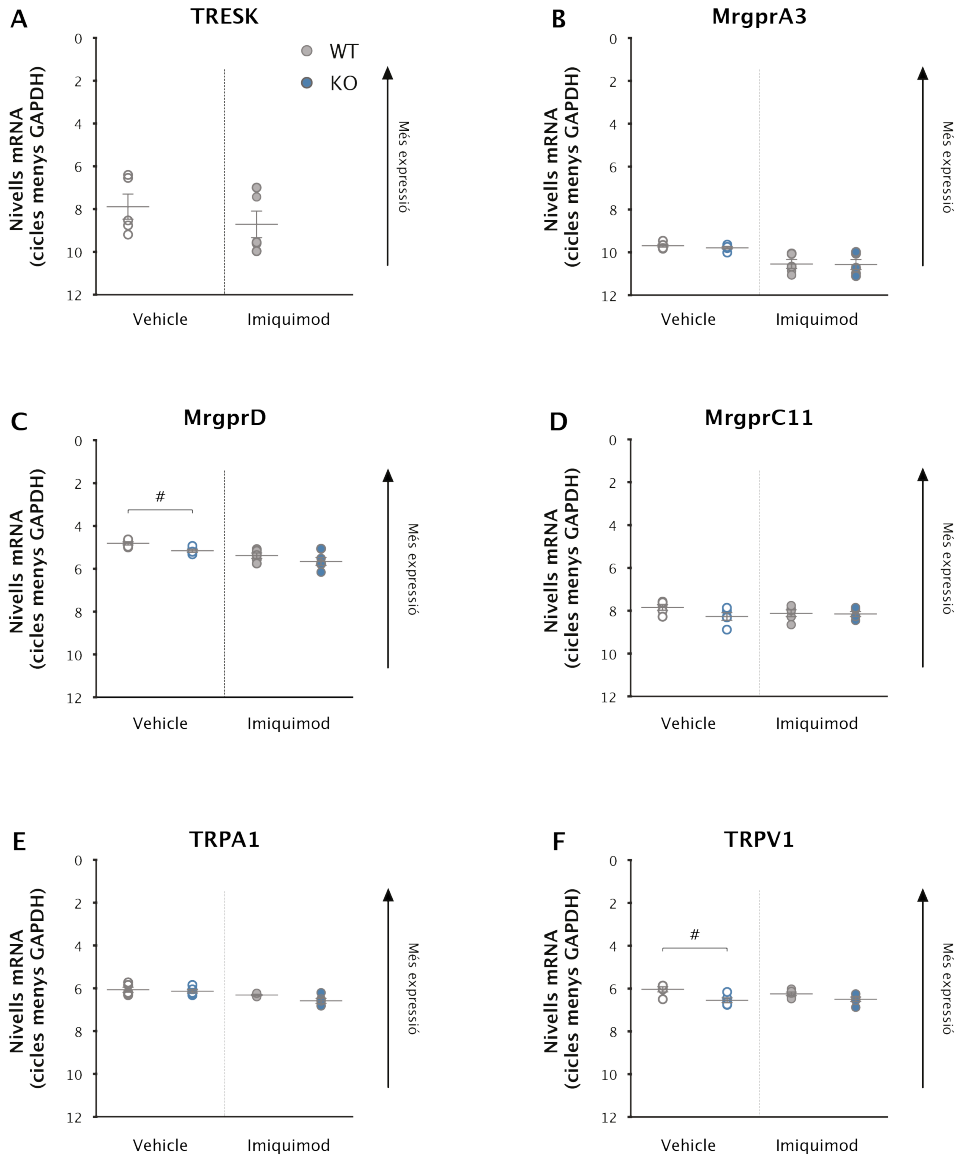


Figura 14: L'ablació de TRESK no genera grans canvis en l'expressió basal dels diferents receptors i canals. Quantificació dels nivells d'expressió dels receptors i canals iònics en els ganglis trigemin ipsilaterals de la galta tractada de ratolins WT i KO. Dissecció del teixit després del 4t dia d'aplicació d'imiquimod o vehicle. Nivells d'expressió normalitzats amb els nivells d'expressió de referència del GAPDH i comparats estadísticament amb tests t-Student no aparellats; # $p < 0.05$ entre condicions vehicle i experimental de cada genotip. Les barres d'error representen el SEM.

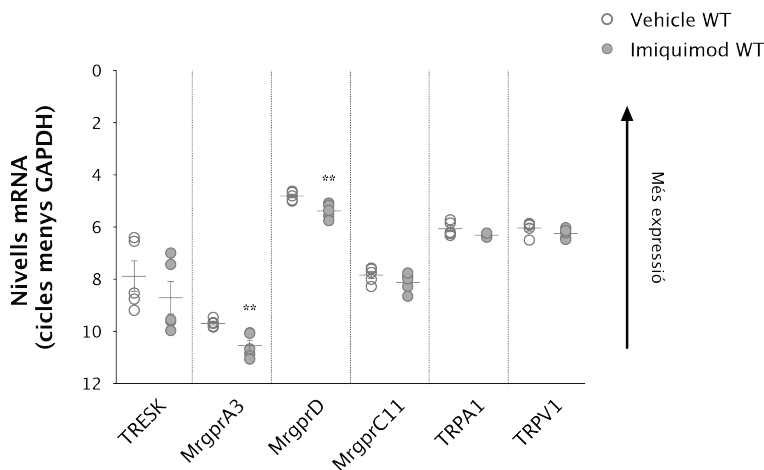


Figura 15: L'administració repetida d'imiquimod provoca una disminució de l'expressió dels receptors MrgprA3 i MrgprD en ratolins WT. Quantificació dels nivells d'expressió de RNA dels receptors i canals iònics en els ganglis trigemin de ratolins WT. Nivells d'expressió normalitzats amb els nivells d'expressió de referència del GAPDH i comparats estadísticament amb tests t-Student no aparellats; ** $p < 0.01$ entre condicions vehicle i experimental. Les barres d'error representen el SEM.

En darrer terme, vam estudiar el grau d'expressió de RNA després de la generació del model de picor psoriàsica induïda per imiquimod en animals TRESK KO. Així com en els animals salvatges, els ratolins KO tractats amb imiquimod van mostrar una disminució significativa en l'expressió del receptor MrgprA3 ($10.57 \pm 0.23 \Delta Ct$ respecte els tractats amb vehicle $9.78 \pm 0.06 \Delta Ct$; test t-Student no aparellat, * $p = 0.012$; Figura 16) i del receptor MrgprD ($5.65 \pm 0.18 \Delta Ct$ comparat amb $5.16 \pm 0.07 \Delta Ct$ dels vehicle; test t-Student no aparellat, * $p = 0.034$; Figura 16). Cal destacar també que es va observar una reducció significativa en l'expressió del canal TRPA1. Els nivells de TRPA1 van passar de $6.13 \pm 0.08 \Delta Ct$ en els ratolins KO tractats amb vehicle a $6.58 \pm 0.11 \Delta Ct$ en els tractats amb imiquimod (test t-Student no aparellat, * $p = 0.013$; Figura 16).

5.3.2. El model de picor crònica psoriàsica induïda per imiquimod genera engruiximent epidèrmic, hipergranulosi i aparició de microabscessos

Complementant la caracterització del paper de TRESK en el model de picor psoriàsica induïda per imiquimod i en col·laboració amb el grup de la Dra. Con-

cepció Soler (Departament de Patologia i Terapèutica Experimental, Facultat de Medicina, Campus de Bellvitge, Universitat de Barcelona), vam dur a terme un anàlisi histològic del teixit tractat de ratolins WT i TRESK KO (Figura 17).

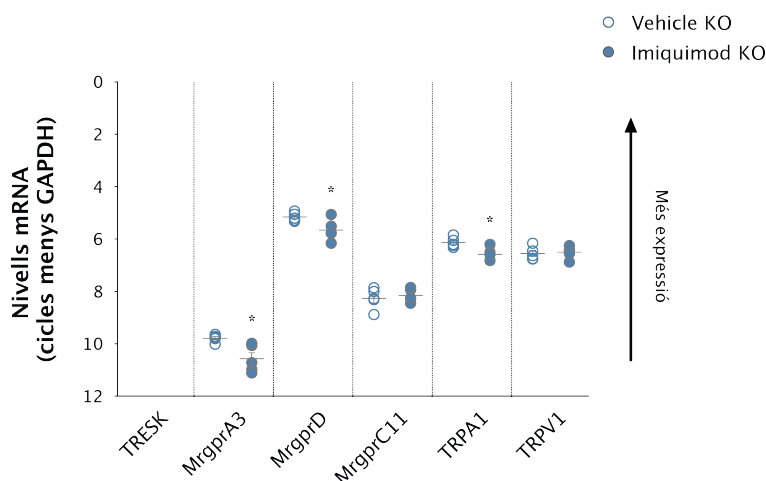


Figura 16: Els ratolins KO van mostrar una reducció en els nivells d'expressió de MrgprA3, MrgprD i TRPA1 després de l'administració repetida d'imiquimod. Quantificació dels nivells d'expressió de RNA dels receptors i canals iònics en els ganglis trigemins de ratolins KO. Nivells d'expressió normalitzats amb els nivells d'expressió de referència del GAPDH i comparats estadísticament amb tests t-Student no aparellats; * $p < 0.05$ entre condicions vehicle i experimental. Les barres d'error representen el SEM.

En condicions basals, les seccions d'ambdós genotips no van presentar diferències en les característiques histològiques. No vam observar diferències significatives en el número de capes epidèrmiques (1.98 ± 0.22 capes en ratolins WT i 1.95 ± 0.20 capes en ratolins KO; $n=6$ per genotip; test t-Student no aparellat, $p=0.912$, no significatiu; Figura 18A) i tampoc en el percentatge de superfície amb hipergranulosis (7.83 ± 3.37 % en animals WT i 22.13 ± 7.03 % en animals KO; $n=6$ per genotip; test t-Student no aparellat, $p=0.096$, no significatiu; Figura 18B). No vam observar la presència de microabscessos en cap dels genotips en condicions basals (Figura 18C).

En quant als efectes del model, els animals salvatges tractats amb imiquimod van mostrar un augment significatiu en el número de capes epidèrmiques (6.43 ± 0.15 capes; $n=8$; test t-Student no aparellat, **** $p < 0.0001$ respecte el vehicle;

Figura 18A). Aquest augment en el número de capes, correspon a un augment en el gruix de la pell, tal i com s'ha reportat en les lesions provocades per aquest model de picor crònica (van der Fits L et al., 2009). L'aplicació d'imiquimod també va provocar un increment significatiu en el grau d'hipergranulosi (34.50 ± 5.36 % ; test t-Student no aparellat, ** $p=0.002$ respecte el vehicle; Figura 18B). Cal destacar, que van formar-se microabscessos en el teixit analitzat (5.09 ± 0.84 ; test t-Student no aparellat, *** $p=0.0002$ respecte el vehicle; Figura 18C), fet que no es va observar en les seccions de pell tractades amb vehicle.

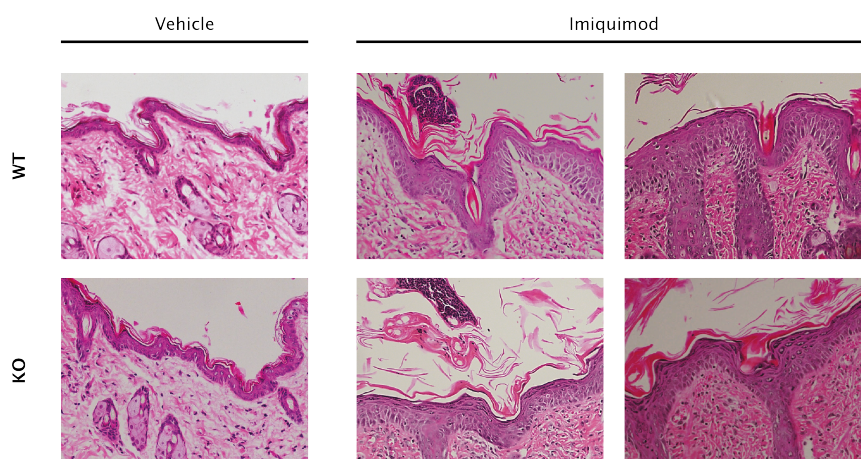


Figura 17: El tractament amb imiquimod va generar lesions de tipus psoriàtic en la galta de ratolins WT i KO. Imatges representatives de seccions tenyides amb hematoxilina i eosina de pell de la galta de ratolins tractats durant 4 dies consecutius amb imiquimod 5% i vehicle.

El tractament amb imiquimod també va produir canvis histològics en els ratolins KO. Després de l'aplicació d'imiquimod vam observar un creixement significatiu en el número de capes epidèrmiques (6.33 ± 0.16 capes; $n=8$; test t-Student no aparellat, **** $p<0.0001$ respecte el vehicle; Figura 18A). A més, les seccions de teixit de ratolins KO van mostrar un increment significatiu en els valors d'hipergranulosi, fenomen observat en el 50% de l'àrea analitzada (53.00 ± 6.48 %; test t-Student no aparellat, ** $p=0.007$ respecte els vehicle; Figura 18B). També vam observar l'aparició significativa de microabscessos (3.45 ± 0.62 %; test t-Student no aparellat, *** $p=0.0005$ respecte vehicle; Figura 18C).

La generació del model de picor crònica induïda per imiquimod va provocar efectes significatius en tots dos genotips. En la comparació entre els dos genotips només es van observar diferències significatives en els nivells d'hipergranulosi, amb un lleu increment estadísticament significatiu en els ratolins KO (test t-Student no aparellat, # $p=0.045$ entre genotips; Figura 18B). No es van observar diferències significatives en el número de capes ($p=0.662$ respecte WT; Figura 18A) o en el número de microabscessos ($p=0.139$ respecte WT; Figura 18C).

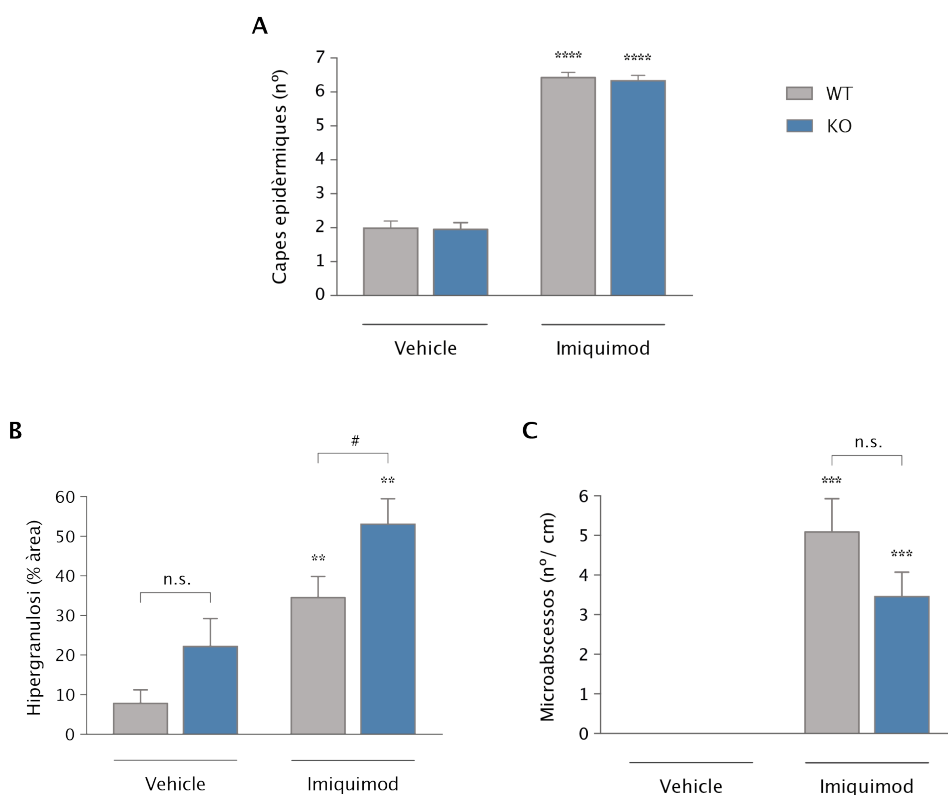


Figura 18: Els ratolins WT i KO no van presentar diferències significatives en les lesions de tipus psoriàsic induïdes per imiquimod. (A) Quantificació del gruix epidèrmic com a número de capes epidèrmiques de seccions de pell d'animals WT i KO tenyides amb hematoxilina i eosina. (B) L'hipergranulosi es va quantificar com a percentatge relatiu respecte el total de la superfície epidèrmica. (C) Quantificació del número de microabscessos per centímetre d'epidermis. Les barres d'error representen el SEM. Comparació estadística amb test t-Student no aparellat, ** $p<0.01$ *** $p<0.001$ **** $p<0.0001$ entre condicions experimental i vehicle, # $p<0.05$ entre genotips.

6. Modulació de TRESK com a antiprurític

Juntament amb les troballes d'aquesta tesi, diversos estudis han posat en rellevància la implicació del canal TRESK en la percepció del dolor induït per estímuls químics, el dolor neuropàtic i inflamatori i altres patologies relacionades amb el dolor com és la migranya amb aura (Lafrenière et al., 2010; Tulleuda et al., 2011; Marsh et al., 2012; Royal et al., 2019; Castellanos, Pujol-Coma et al., 2020). A banda d'això, recentment s'ha identificat el compost químic cloxiquina com a activador específic de TRESK entre els canals de la família K_2P (Lengyel et al., 2017), fet que suposa un pas endavant cap l'ús de TRESK com a diana terapèutica per al tractament de patologies relacionades amb el dolor i la picor. Com a mostra d'aquests avenços, l'activació de TRESK amb cloxiquina s'ha mostrat eficient per reduir l'excitabilitat dels nociceptors en un model de dolor migranyós in vitro (Pettingill et al., 2019).

És per això que vam estudiar els efectes de l'activació de TRESK per cloxiquina en el model de picor aguda produïda per injecció de cloroquina. Primerament, vam injectar conjuntament la cloroquina i la cloxiquina al mateix temps però no es va observar cap reacció de rascat en els animals (no es mostren les dades).

En un grup independent d'animals, es va fer un pretractament amb cloxiquina durant 30 min injectant-la subcutàniament a la galta de l'animal. Seguidament, vam injectar la cloroquina al mateix lloc per induir picor aguda. En els animals salvatges, vam observar episodis de rascat dirigits a l'àrea d'injecció (21.42 ± 3.05 vegades; $n=24$) sense presentar diferències significatives respecte la injecció de cloroquina sense pretractament (24.50 ± 4.03 vegades; $n=12$; test t-Student no aparellat, $p=0.555$ entre tractaments, no significatiu; Figura 19). En els ratolins TRESK KO, utilitzats com a control negatiu, la injecció de cloroquina després del tractament amb cloxiquina va produir un augment significatiu en els episodis de rascat (57.40 ± 11.80 vegades; $n=10$), sense mostrar diferències significatives respecte la injecció de només cloroquina (48.58 ± 6.11 vegades; $n=19$; tests t-Student no aparellat, $p=0.467$ entre tractaments, no significatiu; Figura 19).

Per últim, després de la publicació del treball de Pettingill et al., vam seguir

el seu protocol amb un pretractament de 30 minuts de cloxiquina més concentrada i injectada de forma intraperitoneal i la posterior injecció de cloroquina a la galta per induir picor en els ratolins (Pettingill et al., 2019). En aquest cas, no vam aconseguir dissoldre correctament la cloxiquina en PBS, així que necessitem buscar mètodes alternatius per posar a punt el protocol.

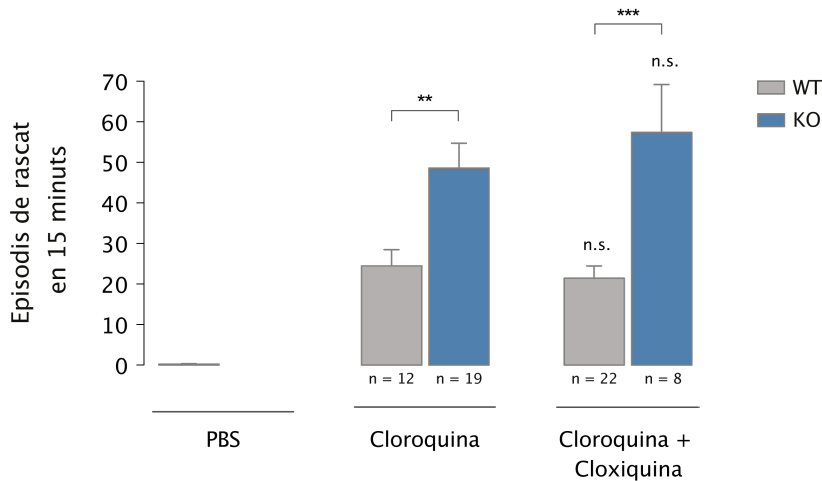


Figura 19: El pretractament amb cloxiquina no produeix diferències en la picor aguda induïda per injecció de cloroquina. Quantificació del número d'episodis de rascat en un període de 15 min induïts per la injecció de cloroquina en ratolins després de 30 min de pretractament amb cloxiquina. Les barres d'error representen el SEM. Test t-Student no aparellat, ** $p < 0.01$, *** $p < 0.001$ entre genotips; n.s., no significatiu.

V. DISCUSSIÓ

Els canals K_{2P} , degut a les seves propietats biofísiques, la interacció amb altres proteïnes i el seu perfil d'expressió, s'han relacionat àmpliament amb la percepció somatosensorial i el dolor (Mathie & Veale, 2014; Busserolles et al., 2016). Diversos estudis han ressaltat la importància de TRESK en les neurones sensorials dels ganglis de l'arrel dorsal i trigèmins, on presenta una alta expressió i és un dels contribuïdors principals al corrent de fons de K^+ , especialment en les neurones nociceptives (Dobler et al., 2007; Weir et al., 2019; Castellanos, Pujol-Coma et al., 2020).

Els canals de la subfamília TREK són els més estudiats, els que primer es van associar amb la senyalització del dolor i estan altament regulats per estímuls biofísics rellevants per a la percepció somatosensorial i la nocicepció (Kang, Choe et al., 2005). Ja que els ratolins knockout pels canals TREK presenten al·lòdina mecànica, s'ha proposat un paper dels canals regulant l'excitabilitat dels nociceptors al ser activats per estímuls mecànics (Alloui et al., 2006; Noël et al., 2009a; Pereira et al., 2014). A més, són altament termosensibles, sent els canals TREK1 i TRAAK més importants per a la sensació de fred i calor extrems, mentre que TREK2 regula la percepció dels estímuls tèrmics innocus (Alloui et al., 2006; Pereira et al., 2014).

TRESK com a membre de la família dels canals K_{2P} presenta una activitat de corrent de fuga de potassi a través de la membrana. Com a la resta de canals de la família, se li atribueix un paper en la regulació i el manteniment del potencial de membrana en repòs, en contrarestar l'efecte d'estímuls despolaritzants i en la freqüència, durada i amplitud dels potencials d'acció de diversos tipus cel·lulars incloent les neurones (Sano et al., 2003; Enyedi, Braun et al., 2012; Enyedi & Czirják, 2015). A més, s'ha descrit que TRESK juntament amb TREK-1, TREK-2 i TRAAK són els membres de la família que tenen major contribució en els corrents de fuga de les neurones sensorials dels ganglis de l'arrel dorsal i trigèmins (Kang & D Kim, 2006; Dobler et al., 2007).

Estudis previs del nostre grup han demostrat que la disfunció dels canals TRESK, causada per una reducció de l'expressió o per inhibició de l'activitat, resulta en hiperexcitabilitat de les neurones, contribuint així al dolor neuropàtic i al mal de cap associat a migranya (Tulleuda et al., 2011; Lafrenière et al., 2010; Royal et al., 2019). La falta de TRESK augmenta la sensibilitat al fred degut a que un major percentatge de fibres C s'activen per estímuls de fred moderat (Castellanos et al., 2020). D'aquesta manera, en ratolins TRESK KO, existeix un augment de la sensibilitat al fred en el *plantar cold test* durant la prova d'acetona i la presència d'al·lodínia al fred similar a la induïda pel tractament amb oxal·liplatí (Guo, Qiu et al., 2019; Castellanos, Pujol-Coma et al., 2020). La sensibilitat mecànica també està incrementada en els animals TRESK KO, que mostren una disminució important del llindar de sensibilitat mecànica avaluat amb els filaments de Von Frey (Tulleuda et al., 2011; Castellanos, Andres et al., 2018; Y Yang et al., 2018). Per contra, la sobre-expressió de TRESK en les neurones sensorials té l'efecte contrari i pot millorar condicions com l'al·lodínia mecànica en models de dolor neuropàtic.

Lafrenière et al. van descriure una mutació per canvi en el marc de lectura en el gen KCNK18 en un estudi per identificar mutacions en gens de canals iònics presents en pacients amb migranya (Lafrenière et al., 2010). La mutació (F139WfsX24) es va identificar en diversos membres d'una família que presenta migranya familiar amb aura. Recentment, estudis del grup del Dr. Sandoz amb col·laboració amb el nostre grup han descrit quin és el mecanisme d'acció d'aquesta mutació que facilita l'activació dels nociceptors i produeix dolor asso-

ciat a la migranya (Royal et al., 2019). La mutació en el gen de TRESK produeix dues proteïnes degut a l'alteració de la pauta de lectura a la creació d'un nou lloc d'inici de transcripció, la primera proteïna correspon a un canal truncat no funcional (MT1) i la segona tradueix un fragment de proteïna (MT2) que és capaç d'interaccionar amb altres membres de la família K_{2P} , TREK-1 i TREK-2, i en supprimeix la funció. La combinació del *knock-down* dels diferents canals afectats, disminueix la corrent de fre de la neurona i facilita la seva activació (Royal et al., 2019).

En el mateix sentit, s'ha descrit un cas clínic d'un pacient amb discapacitat intel·lectual i migranya amb aura del tronc cerebral portador d'una nova mutació *missense* (W101R) en el gen KCNK18 que provoca una pèrdua dramàtica de la funció del canal TRESK (Imbrici et al., 2020).

La implicació dels canals K_{2P} en la regulació de l'excitabilitat de les neurones sensorials també s'ha demostrat a través d'altres estudis, com el realitzat pel nostre grup, que va demostrar per primera vegada que la inhibició dels canals K_{2P} induïda per piretroides com la permetrina i la tetrametrina, facilita l'activació de les neurones sensorials i augmenta la seva excitabilitat, contribuint a la generació de parestèsies i dolor reportats després de l'exposició a piretroides (Castellanos, Andres et al., 2018). Per altre banda, s'ha proposat que l'alteració de l'activitat de TRESK podria estar darrere del síndrome dolorós associat a inhibidors de la calcineurina que es manifesta com a efecte secundari al tractament amb fàrmacs immunosupressors com el tacrolimus o la ciclosporina A (Smith, 2009). La hipòtesi relaciona la inhibició de la calcineurina amb una disminució del corrent de TRESK deguda a la manca de desfosforil·lació per part d'aquesta proteïna activada per calci. Com a conseqüència, els inhibidors de la calcineurina produirien un augment de l'excitabilitat neuronal que afectaria la sensibilitat al dolor. Aquesta hipòtesi també l'han suggerit altres autors per explicar el dolor associat a metàstasi òssia i la disfunció del sistema immunitari mediada per cèl·lules T (J Han et al., 2009; Y Yang et al., 2018).

Estudis recents de seqüenciació massiva d'RNA, tant a nivell de població com d'una sola cèl·lula, han identificat l'expressió de TRESK en diferents subpoblaci-

ons específiques de neurones sensorials de DRG en ratolí i en humans (Chiu et al., 2014; Usoskin et al., 2015; XY Li et al., 2015; Zeisel et al., 2018; Ray et al., 2018). També s'ha descrit un perfil d'expressió similar en neurones sensorials trigeminals (MQ Nguyen et al., 2017). En particular, TRESK mostra una expressió predominant en neurones sensorials no peptidèrgiques (NP) positives per la isolectina B4 (IB4) i també en una de les subpoblacions de mecanoreceptors de baix llindar implicats en la sensació de tacte (Usoskin et al., 2015; Zeisel et al., 2018; C Li et al., 2018; Zheng et al., 2019).

Els perfils d'expressió gènica de les neurones sensorials confereixen gran quantitat d'informació i ajuden en l'assignació de les propietats fisiològiques de cada tipus neuronal. La majoria de les neurones implicades en la detecció d'estímuls prurítics (NP1, NP2 i NP3) expressa receptors acoblats a proteïnes G (GPCR) com els de la família MRGPR, receptors d'histamina (HRH) o receptors de serotonina (HTR). Les subpoblacions NP1 i NP2 són les que mostren una expressió més important del canal TRESK, expressen selectivament i de manera característica els receptors pruritogènics MrgprD (NP1) i MrgprA3 (NP2) i presenten un patró d'expressió característic que suggereix la seva participació en la transmissió del dolor i la picor (Usoskin et al., 2015). Encara que preliminars, els resultats de les nostres hibridacions *in situ* corroboren la coexpressió de TRESK en neurones MRGPRA3 i MRGPRD positives (Figura 2) i és per això que en aquesta tesi hem hipotetitzat que TRESK podria tenir un paper significatiu en la determinació de l'activació i l'excitabilitat de les neurones sensibles a la picor.

1. Participació de TRESK en la sensibilitat a la picor no histaminèrgica mediada per cloroquina

La picor aguda té un paper protector en l'eliminació dels agents irritants per evitar danys addicionals i darrerament s'han dut a terme una quantitat considerable d'investigacions per desxifrar els mecanismes moleculars i cel·lulars subjacents. Aquí hem escollit diversos pruritogens que activen la via histaminèrgica (histamina) o no histaminèrgica i que presenten receptors en subpoblacions de neurones sensorials on també s'expressa TRESK (Figura 1).

Els resultats de l'activació de les neurones sensorials pels diferents prurítogens utilitzats mostren que existeix una proporció similar en el nombre de neurones que presenten respostes de calci intracel·lular en animals WT i TRESK KO i que l'absència del canal no modifica l'expressió dels receptors en les diferents subpoblacions les poblacions dels DRGs. A més, l'absència de TRESK tampoc va mostrar diferències significatives en els pics de resposta de calci, la qual cosa manifesta que l'increment de calci intracel·lular es manté similar en les neurones WT i TRESK KO i que probablement es tracta d'un mecanisme independent a la presència de TRESK.

Quan l'efecte dels prurítogens es va estudiar *in vivo* per tal d'abordar si TRESK contribueix en la sensibilitat a la picor aguda, els resultats van mostrar que mentre que l'administració aguda d'histamina i LTC₄ generen una resposta de rascat similar en ratolins WT i TRESK KO, la injecció de cloroquina és l'única que provoca una resposta incrementada per l'absència del canal i per això proposem els següents mecanismes involucrats.

Liu et al. han demostrat que MRGPRA3 és el receptor principal involucrat en la resposta a la cloroquina d'entre els 12 receptors MRGPRs estudiats (Q Liu, Tang et al., 2009). Els autors no descarten la possible participació d'altres receptors de la família en la senyalització de CQ en les neurones dels DRG tot i que estableixen, amb experiments de guany i pèrdua de funció del receptor, que MRGPRA3 és necessari per a la resposta a la CQ en ratolins (Q Liu, Tang et al., 2009; L Han et al., 2013). TRESK és un dels membres de la família amb major contribució en les corrents de fuga, les quals poden actuar com a fre en la generació dels potencials d'acció. Així doncs, l'absència de TRESK podria augmentar l'excitabilitat neuronal, fenomen descrit prèviament per altres autors (Tulleuda et al., 2011; Guo, P Liu et al., 2014; Castellanos, Pujol-Coma et al., 2020), i que concordaria amb l'increment de la resposta de rascat a la cloroquina que mostren els ratolins TRESK KO (Figura ??). El receptor MRGPRA3 s'expressa predominantment en la subpoblació de neurones sensorials NP2 i, en menor mesura, en NP3. Com ja hem mencionat anteriorment, TRESK s'expressa abundantment en NP2 i és per això que proposem que TRESK tingui una participació important en la modulació de la sensibilitat de la picor específicament mediada per la subpobla-

ció NP2. Tanmateix, suggerim que TRESK no participa en la detecció de l'estímul de picor (cloroquina) sinó que contribueix en la modulació de l'excitabilitat de les neurones que detecten aquesta picor.

Tenint en compte que la CQ pot induir la generació de potencials d'acció en neurones de DRG dissociades (Q Liu, Tang et al., 2009), a través de registres electrofisiològics hem observat que l'estimulació amb cloroquina produeix un increment del nombre de potencials d'acció disparats per neurones de DRG de ratolins TRESK KO respecte els WT. Aquests resultats corroboren la hipòtesi de que l'absència de TRESK pot provocar una hiperexcitabilitat en les neurones NP2, ja que aquesta subpoblació inclou gran part de les neurones MRGPRA3⁺, i que es traueix en un augment de la picor induïda per cloroquina (Figura 2).

Per tal de completar l'estudi electrofisiològic, proposem tenir en compte la gran heterogeneïtat de les neurones sensorials, que es podria reduir utilitzant marcadors de neurones no peptidèrgiques com l'IB4 o virus que marquen específicament les neurones que expressen MRGPRA3. A més, s'ha descrit una reducció de l'expressió de diversos receptors GCPR, inclòs MRGPRA3, en les neurones en cultiu respecte l'expressió en DRG intacte (Wangzhou et al., 2020) i, segons Tulleuda et al., s'ha identificat una disminució de l'expressió de TRESK en neurones en cultiu que s'intensifica amb el pas del temps i és comparable a la disminució de l'expressió del canal observada després d'una axotomia (Tulleuda et al., 2011). Per tant, seria molt convenient trobar un protocol adequat on l'expressió dels canals i els receptors sigui estable i es modifiqui el mínim possible per tal de poder estudiar amb més detall el paper de TRESK en l'excitabilitat de la subpoblació de neurones que expressa MRGPRA3.

En quant als mecanismes cel·lulars associats a l'activació del receptor MRGPRA3 mediada per CQ, s'ha suggerit que l'activació del receptor MRGPRA3 requereixi la cascada de senyalització a través de la proteïna Gq-GPCR amb una estimulació posterior de la fosfolipasa C (PLC) emprant registres extracel·lulars de l'activitat nerviosa perifèrica en preparacions de *skin-nerve* (SK Han et al., 2002; Ru et al., 2017; Ajayi, 2018). També s'ha descrit que l'activació de les neurones MRGPRA3 per l'aplicació de CQ indueix un augment de [Ca²⁺] intracel·lular

(Than et al., 2013), com s'ha observat en aquest estudi. Per altre banda, el mecanisme d'activació depenent de calci és específic per a TRESK entre els canals de la família K_{2P} i la calcineurina n'és un component indispensable (Czirják, Tóth et al., 2004). Segons el model regulador, el canal TRESK està fosforil·lat de manera constitutiva en condicions de repòs i un increment de calci intracel·lular indueix un augment de l'activació del canal a través de la desforil·lació ràpida dels residus serina (S252, S262, 264, 267) per part de la calcineurina (Czirják, Tóth et al., 2004; Czirják & Enyedi, 2006). En absència del senyal de calci, cessa l'activitat fosfatasa i s'inactiva de nou el canal amb la refosforil·lació per part de les proteïnes PKA i MARK (Braun et al., 2011; Czirják & Enyedi, 2010; Enyedi & Czirják, 2014).

Així doncs, és possible que TRESK moduli, en condicions basals, l'excitabilitat de les neurones MRGPRA3⁺ que pertanyen a la subpoblació NP2 actuant com a fre envers la despolarització provocada per estímuls com pot ser la cloroquina. Donada la modulació de TRESK per calcineurina, l'activitat del canal podria veure's incrementada a través de la desforil·lació per calcineurina com a conseqüència de l'augment de calci intracel·lular provocat per l'activació del receptor MRGPRA3 i l'augment de calci produït per l'activació de la cascada de senyalització que inclou la formació de IP_3 (Figura 1). Tot plegat explicaria perquè en els animals TRESK KO veiem un increment de la resposta a la cloroquina, ja que s'ha perdut l'efecte de fre que fa el canal així com l'augment de la seva activitat a través de Ca^{2+} /calcineurina en les neurones de la subpoblació NP2 (Figura 2).

En un altre ordre de coses, la histamina és el pruritogen histaminèrgic per excel·lència i en els nostres ratolins ha generat una resposta de rascat similar en els animals WT i KO per TRESK, el que fa pensar que TRESK no participa activament en la modulació de la sensibilitat d'aquest tipus de picor.

La histamina s'ha relacionat extensament amb la sensació de picor, és alliberada pels mastòcits quan els teixits s'inflamen o són estimulats per al·lèrgens i actua excitant un conjunt de fibres nervioses de tipus C no mielinitzades (Tani et al., 1990). D'entre les fibres C, la histamina activa preferentment les fibres no sensibles a estímuls mecànics (Schmelz et al., 2003) i ho pot fer a través de 4 receptors diferents (H1-H4), que s'han emprat com a dianes terapèutiques i sobre

els que s'han desenvolupat bloquejadors, els antihistamínics (Strakhova et al., 2009; Bongers et al., 2010).

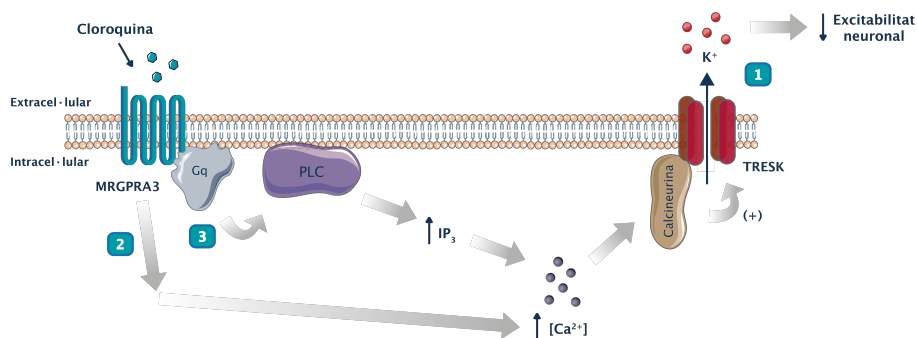


Figura 1: Proposta dels mecanismes subjacents a la modulació de TRESK en les neurones NP2 que expressen MRGPRA3. En els ratolins WT, (1) l'activitat basal de TRESK actua com a fre envers l'activació neuronal provocada per la unió de cloroquina al seu receptor MRGPRA3. Addicionalment, (2) l'augment de calci intracel·lular induït per la cloroquina i (3) l'increment de calci provocat per la cascada de senyalització derivada de l'activació de MRGPRA3, incrementa encara més l'activitat de TRESK i l'atenuació de l'excitabilitat neuronal.

Els receptors d'histamina presenten un perfil d'expressió diferencial, el receptor HRH1 és el que presenta major expressió en neurones sensorials dels DRG i és el que s'ha associat més directament amb la picor induïda per la injecció d'histamina, ja que antagonistes selectius d'aquest receptor inhibeixen la resposta de picor (Denham et al., 2003; Usoskin et al., 2015). Concretament, HRH1 s'expressa en NP2 i NP3 mentre que TRESK està present majoritàriament en NP1 i NP2 (Figura 1) (Usoskin et al., 2015). El fet de que el receptor HRH1 s'expressi en una població neuronal que no presenta o expressa molt poc TRESK (NP3) podria ser el motiu principal pel que no observem diferències en la resposta de rascat en absència de TRESK. Així doncs, la injecció local d'histamina activa principalment les neurones de NP2 i NP3 però els nostres estudis indiquen que TRESK només tindria un paper rellevant sobre NP2 ja que gairebé no s'expressa en NP3. Per tant, malgrat que TRESK podria estar contrarestant l'increment de l'excitabilitat de les neurones de NP2 provocada per estímuls histaminèrgics, el canal no té cap efecte sobre l'activació per histamina en la subpoblació de neurones NP3. Conseqüentment, encara que l'ablació de TRESK podria generar un increment en l'excitabilitat de les neurones NP2 que es traduiria en un augment en la respos-

ta de rascat quan s'administra histamina, aquest efecte estaria emmascarat per l'activació "normal" sobre NP3, amb la qual cosa l'efecte general de l'administració local d'histamina és que no es veu alterat per l'absència de TRESK (Figura 2).

Per tal de caracteritzar-ho més en detall, vam utilitzar el leucotriè C_4 (LTC_4) ja que el seu receptor, anomenat Cyslr2, s'expressa majoritàriament en NP3 i, per tant, ens permetria estudiar de forma específica si TRESK participa en la transmissió de la picor en aquest subgrup de neurones (Usoskin et al., 2015; Solinski et al., 2019). El LTC_4 és un agonista que ha estat àmpliament emprat per a l'estudi de l'asma i l'al·lèrgia, és secretat pels mastòcits i s'ha associat a la picor induïda per mastòcits (Di Gennaro et al., 2012). La manca de diferències en la resposta de rascat envers la injecció de LTC_4 entre els ratolins TRESK KO i els WT confirma que TRESK no està implicat en la transmissió del pruritus generat per l'activació de les neurones NP3 ja que no hi està pràcticament expressat (Figura ??). Aquests resultats concorden amb la nostra hipòtesi de que el canal podria exercir un efecte modulador exclusivament en el subgrup de neurones NP2 i, consegüentment, només en els tipus de picor transmesos per aquestes neurones (Figura 2).

Considerant que TRESK s'expressa en NP1 i NP2 i que el receptor MRGPRX11, associat a somatosensació i picor s'expressa en tres de les subpoblacions de neurones sensorials no peptidèrgiques (NP1, NP2 i NP3), vam suggerir que l'absència del canal podria incrementar la picor induïda per l'agonista no histaminèrgic de MRGPRX11 (Akiyama, MI Carstens, Ikoma et al., 2012) anomenat pèptid de la medulla adrenal o BAM8-22 (Lembo et al., 2002). Es tracta d'un derivat escindit proteolíticament de la proencefalina A i que s'ha relacionat amb la generació de picor independent d'histamina i la inducció de sensacions nociceptives, com coïssor i cremor en humans (Sikand et al., 2011). També s'ha suggerit que la picor colestàtica podria ser atribuïda, en part, a un increment de BAM8-22 en la pell i del seu receptor MRGPRX11 en les neurones sensorials (Sanjel et al., 2019). Sorprenentment, no només no hem observat diferències en el comportament dels ratolins TRESK KO respecte el WT sinó que no hem observat pràcticament cap comportament de rascat en resposta a la injecció intradèrmica de BAM8-22. Encara que els efectes de l'agonista BAM8-22 en humans estan descrits, existeixen molt pocs estudis on s'hagi emprat aquest pèptid per induir picor *in vivo*, fet que

ha limitat molt el desenvolupament dels experiments i la cerca d'alternatives en el protocol d'administració.

Els pocs estudis existents també suggereixen que l'activació dels terminals nerviosos induïda per BAM8-22 sembla ser d'una magnitud molt menor respecte la que generen altres pruritogens, com són la CQ o la histamina (Pradhananga et al., 2015; Pall et al., 2015). Tot i això, atribuïm la manca de resposta a l'agonista principalment a factors experimentals, doncs al disposar de literatura limitada, variables com el temps d'observació, la procedència i concentració del pèptid o el vehicle d'injecció podrien alterar considerablement els resultats dels experiments. Al tractar-se d'un pèptid, existeix la possibilitat de que el mètode d'aplicació o al injectar-se en un teixit es pugui ocasionar una degradació parcial d'aquest, atenuant l'activació del receptor i conseqüentment la resposta de rascat associada, fenomen que probablement no ocorre quan es secreta de forma endògena local o a nivell de SNC. En definitiva, malgrat que el receptor MRGPRX11 s'expressa considerablement en subpoblacions de neurones que expressen TRESK, no podem extreure conclusions sobre la implicació del canal en l'excitabilitat i sensibilitat d'aquestes neurones ja que els efectes de l'agonista *in vivo* són insuficients.

Per últim, el resultat més sorprenent ha estat la falta de resposta a l'administració local de β -alanina. Aquest pruritogen es va escollir per estudiar la participació de TRESK en la picor no histaminèrgica degut a la seva acció sobre el receptor MRGPRD, que es coexpressa notablement amb el canal TRESK principalment en la subpoblació de neurones sensorials NP1 (Figura 1, 2). Malgrat el potencial d'aquest agonista per estudiar la picor no histaminèrgica i els possibles canvis en l'excitabilitat de les neurones en absència de TRESK, no hem aconseguit reproduir els experiments *in vivo* publicats per altres autors (Q Liu, Sikand et al., 2012; Pradhananga et al., 2015).

Estudis sobre la picor provocada per β -alanina han revel·lat una heterogeneïtat i diversitat funcional de les neurones MRGPRD⁺ (Q Liu, Sikand et al., 2012), les quals engloben gran part de totes les neurones sensorials no peptidèrgiques (Zylka, Rice et al., 2005). Curiosament, només un subconjunt de les neurones que expressen MRGPRD s'activen per β -alanina, i presenten propietats fisiològiques

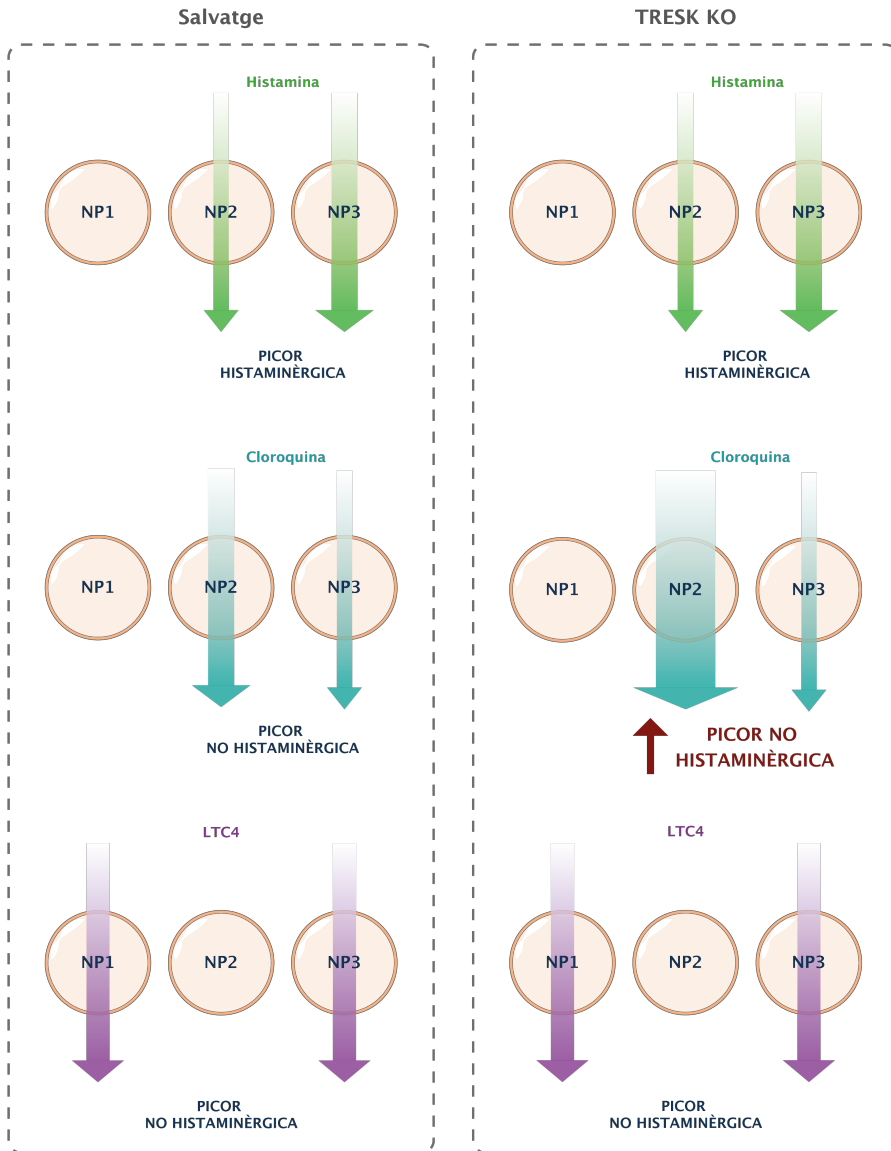


Figura 2: Hipòtesi plantejada per explicar l'increment en la sensibilitat a cloroquina en absència de TRESK. La picor induïda per histamina o LTC₄ activa les subpoblacions de neurones NP1 i NP3 majoritàriament, on l'expressió de TRESK és escassa o inapreciable. En aquests casos l'absència de TRESK no provoca canvis en l'excitabilitat neuronal i, per tant, la sensibilitat a la picor no es veu afectada. Quan la picor és induïda per cloroquina, la subpoblació de neurones que s'activa notablement és NP2, on TRESK s'expressa en abundància. L'absència de TRESK en aquesta subpoblació produeix un increment en l'excitabilitat neuronal que es tradueix en un increment en la sensibilitat a la picor induïda per cloroquina.

diferents a les neurones insensibles a β -alanina però positives per MRGPRD. Per aquest motiu s'ha plantejat la qüestió de si una part de les neurones que expressen MRGPRD es dedica a la transducció d'estímuls prurítics mentre que d'altres s'encarreguen de mediar estímuls mecànics (Cavanaugh et al., 2009; Q Liu, Sikand et al., 2012; Abraira et al., 2013).

Respecte la vessant prurítica dels efectes de la β -alanina activant el receptor MRGPRD, podrien haver factors experimentals com són la concentració i procedència del pruritogen, el temps d'observació o el vehicle d'injecció, que expliquin la manca de resposta de rascat, tot i que s'han utilitzat els procediments i concentracions descrites en altres estudis. D'altra banda, és important destacar que altres grups ens han manifestat els mateixos problemes durant la reproducció d'aquest model de picor aguda quan se'ls ha contactat buscant indicacions o consells. A més, s'ha suggerit la possibilitat de que la injecció de β -alanina com a model de picor aguda solament generi resposta de rascat en animals que prèviament presenten una lesió cutània com pot ser pell seca.

Recentment, Christensen et al. ressalten les limitacions dels models actuals per a l'estudi de la picor induïda per β -alanina en animals i proposen un nou mètode d'administració per aconseguir establir amb èxit un model fiable de picor no histaminèrgica mediada per MRGPRD (JD Christensen et al., 2019). Per aquests motius, proposem que s'hauria de canviar completament l'enfocament actual quan s'utilitzi la β -alanina com a pruritògen per a l'estudi de la picor mediada per MRGPRD i, per extensió, mediada per les neurones de les subpoblacions NP1 i NP2. Suggerim que en experiments futurs s'utilitzin (1) animals genèticament modificats que expressin fluorescència en les neurones que presenten el receptor MRGPRD, (2) protocols d'administració diferents o (3) es busquin altres activadors endògens o sintètics que permetin activar eficientment el receptor i generar respostes de picor aguda suficientment intenses. Val la pena mencionar que, si bé la manca de resposta conductual a la β -alanina no ha permès estudiar la contribució de TRESK en la sensibilitat a la picor, seria interessant estudiar si la coexpressió de TRESK en les neurones MRGPRD⁺ (NP1 i NP2) es pot relacionar amb l'alteració en la sensibilitat mecànica descrita per l'ablació de TRESK (Weir et al., 2019; Castellanos, Pujol-Coma et al., 2020).

Si considerem que l'absència de TRESK provoca un augment en l'excitabilitat de les neurones NP2, que es tradueix en un increment en la resposta de rascat induïda per cloroquina, l'activació directa i selectiva del canal podria revertir o atenuar aquests efectes. En aquest sentit, Wright et al. van descriure la cloxiquina (5-cloro-8-quinolinol) com un activador de TRESK (Wright et al., 2013). La cloxiquina és un fàrmac que pertany a la família dels 8-hidroxiquinolina i que antigament s'ha utilitzat com a antifúngic, antiamebic i antibacterià (Gärtner et al., 1977; Gershon et al., 2004; Hongmanee et al., 2007; Oliveri et al., 2016). Recentment s'ha proposat que la cloxiquina també és un compost desacoblador mitocondrial i que té propietats cardioprotectores (J Zhang et al., 2016).

La cloxiquina sembla que presenta certa selectivitat envers TRESK ja que no afecta altres canals de potassi, on s'inclouen Kir1.1 o Kv11.1, ni tampoc altres canals de la família dels K_{2P} (Wright et al., 2013; Lengyel et al., 2017). Aquest compost activa TRESK en el seu estat de repòs mitjançant un mecanisme independent de calci intracel·lular i calcineurina (Lengyel et al., 2017). Pettingill et al. han demostrat que l'activació de TRESK per part de la cloxiquina redueix l'activació espontània dels nociceptors en un model de dolor humà in vitro. A més, demostren que la cloxiquina és capaç de reduir la hipersensibilitat mecànica i tèrmica en un model de migranya crònica induïda per nitroglicerina (Pettingill et al., 2019).

En el present estudi es va voler avaluar si la participació de TRESK en la sensibilitat a la picor induïda per cloroquina s'accentua amb l'activació per cloxiquina, partint de la hipòtesi de que l'activació de TRESK amb cloxiquina pot provocar una disminució en l'excitabilitat de les neurones MRGPRA3⁺, sobretot les que pertanyen a la subpoblació NP2, i per tant, l'atenuació de la resposta de rascat generada per la injecció de cloroquina.

Sorprenentment, ni la injecció local de cloroquina amb cloxiquina ni el pretractament amb cloxiquina i la posterior injecció del pruritogen han provocat diferències en el nombre d'episodis de rascat dels nostres ratolins respecte la injecció de només cloroquina (Figura 16). Malgrat que, com era d'esperar, el pretractament amb cloxiquina no ha mostrat efectes per si sol sobre el comportament

de rascat induït per cloroquina en els ratolins TRESK KO, hem experimentat un seguit d'obstacles durant la seva manipulació i administració.

En primer lloc convé destacar que en el moment en que es van dur a terme els experiments, gairebé no existia literatura publicada on s'administrés la cloxiquina *in vivo*. Així doncs el protocol i la concentració emprats primerament van ser una adaptació de les dades publicades per altres autors en experiments *in vitro* (cel·lulars) i dels pocs estudis que, fins al moment, existien en animals. En el protocol inicial es van administrar conjunta i localment la cloxiquina i la cloroquina. Considerant que l'activador potser necessita una finestra de temps major que la cloroquina per ser efectiu, es va intentar corregir el protocol implementant un pretractament amb l'activador 30 minuts abans de la injecció amb el pruritogen, però tampoc va resultar efectiu (Figura 16).

En segon lloc, la cloxoquina és un compost altament insoluble en solvents aquosos. Mentre que no hem detectat inconvenients en la dissolució de l'activador a baixes concentracions, l'increment de la concentració per al seu ús en experiments *in vivo* ha resultat en una dramàtica disminució de la solubilitat. En la literatura recent, la majoria dels estudis utilitzen el dissolvent orgànic DMSO a altes concentracions (X Zhang, 2015; A Morita et al., 2018). Ja que s'han descrit una gran quantitat d'efectes indesitjats en l'ús del DMSO com a vehicle en experimentació animal (Kelava et al., 2011), l'intent de dissoldre la cloxiquina amb una concentració de DMSO <1% per evitar els efectes adversos, no ha estat possible.

Recentment, Pettingill et al. han utilitzat el compost per generar el model de migranya, la nitroglicerina, com a vehicle per a l'administració de cloxiquina. Tot i que aquest mètode no genera problemes en la solubilitat de la cloxiquina, no és una opció plausible per als nostres estudis (Pettingill et al., 2019). Altres estudis publicats fa poc, han utilitzat l'oli d'oliva, un altre dissolvent orgànic lipòfil, com a vehicle de la cloxiquina per la seva administració intraperitoneal (Z Zhang et al., 2019). Atès que no s'han descrit efectes adversos importants sobre aquest dissolvent, proposem que podria ser el millor candidat per utilitzar en experiments futurs.

2. Implicació dels canals TRESK en el desenvolupament de la picor crònica

Tot i que s'han identificat diversos factors que medien la transducció de la picor aguda en les neurones sensorials primàries i el SNC emprant models animals enlloc de subjectes humans, els models animals de picor crònica són limitats. En aquest sentit es coneix molt poc sobre l'origen de la picor crònica i els mecanismes que promouen la cronificació de les sensacions de picor. Encara menys es coneix sobre les vies de senyalització, ni en les neurones ni en la pell, que inicien i mantenen les condicions de picor crònica. S'ha demostrat que diversos canals iònics contribueixen en la transducció dels estímuls prurítics de forma aguda i també són necessaris en condicions de picor crònica (Wilson, Gerhold et al., 2011; Wilson, Nelson et al., 2013). Considerant el paper dels canals TRESK en la sensibilitat de la picor aguda induïda per cloroquina, és lògic pensar que també podrien estar implicats en la cronificació de la picor en diferents patologies.

En alguns dels models de picor crònica que s'utilitzen habitualment, s'ha descrit l'activació específica de diferents subpoblacions neuronals depenent del model i el protocol emprat. D'entre els més estudiats, el model de dermatitis al·lèrgica de contacte, el de pell seca i el de psoriasis induïda per imiquimod són dels models dels que es disposa més informació pel que fa a les subpoblacions neuronals implicades (Qu et al., 2014; Sakai et al., 2016; Zhu et al., 2017).

La dermatitis al·lèrgica de contacte (ACD) és un trastorn freqüent de la pell que afecta milions de persones cada any i s'associa a una disminució de la qualitat de vida. Es caracteritza per una resposta immune generada per l'activació específica d'un al·lergen i mediada principalment per cèl·lules dendrítiques dèrmiques i cèl·lules T efectores que produeixen inflamació cutània localitzada al lloc de contacte amb l'al·lergen (AD Christensen et al., 2011; Alikhan et al., 2014). Per estudiar l'ACD experimentalment es generen models emprant sensibilitzadors de contacte com el dinitroclorobenzè i l'àcid esquàric dibutilester (SADBE). Aquest últim es basa en una petita molècula haptè que s'utilitza habitualment en el tractament de l'alopecia areata i les berrugues (Pall et al., 2015).

Existeixen pocs estudis sobre les alteracions sensorials associades a aquesta malaltia. En un d'ells, Qu L et al. han descrit un augment en l'excitabilitat de les neurones MRGPRA3⁺ i MRGPRD⁺ i un increment de les descàrregues de potencials d'acció en resposta a estímuls nocius mecànics i de calor després de la generació del model. Aquestes troballes indiquen la possibilitat de que l'hiperexcitabilitat de les neurones MRGPRA3⁺ i MRGPRD⁺ podria contribuir a la picor i el dolor associats a l'ACD (Qu et al., 2014). En les regions afectades per la dermatitis al·lèrgica de contacte, s'ha descrit que hi ha una intensificació de la picor independent d'histamina i que el dolor evocat químicament s'acompanya de picor (Fu et al., 2014). Recentment, s'ha descrit que el perfil transcripcional de les neurones MRGPRA3⁺ canvia després de la inducció d'un model de dermatitis al·lèrgica de contacte, tot i que no es menciona si existeixen canvis en l'expressió de TRESK (Xing et al., 2020).

La pell seca (clínicament anomenat xerosis) és un problema cutani comú en adults d'edat avançada i es defineix com una disrupció de la superfície de la pell a causa d'un contingut inadequat d'aigua o d'oli de la pell. La pell seca sovint causa picor o sensació de cremor i la resposta de rascat associada resulta en un augment en el risc d'infecció secundària a la pell (Hurlow et al., 2011). D'entre els models animals que s'utilitzen per induir sequedat a la pell i alterar la barrera cutània, només el tractament amb acetona, èter i aigua (AEW) augmenta significativament els episodis de rascat espontani (Miyamoto et al., 2002). Akiyama et al. han descrit un augment en els episodis de rascat induïts per diversos pruritogens, acompanyats de l'increment en la resposta de les neurones primàries dels DRG i de les neurones secundàries de la medul·la espinal, juntament amb l'aparició d'*alloknesis* en aquest model de picor crònica (Akiyama, MI Carstens & E Carstens, 2010; Akiyama, M Carstens et al., 2011). En conseqüència, s'ha proposat que la sensibilització perifèrica de les fibres aferents primàries podria ser un dels possibles mecanismes que contribueixin a l'increment del comportament de rascat en el model de pell seca (Akiyama, MI Carstens & E Carstens, 2010).

En quant als mecanismes cel·lulars i moleculars subjacents, s'ha demostrat que el canal iònic TRPA1 és necessari tant per a la traducció dels senyals de picor crònic cap al SNC com per als canvis fisiopatològics derivats de la picor i el rascat

que es manifesten en el model de pell seca (Wilson, Nelson et al., 2013). Alguns estudis han descrit que la condició de pell seca incrementa l'expressió dels receptors de picor MRGPRA3 i MRGPC11 en neurones de DRG, estén l'expressió de MRGPRA3 a una fracció de neurones sensorials més àmplia i indueix un augment de la innervació de les fibres MRGPRA3⁺ a l'epidermis (Wilson, Nelson et al., 2013; Zhu et al., 2017).

Zhu et al. han demostrat que l'ablació dels 12 gens MRGPR suprimeix el comportament de rascat en els models d'ACD induït per SADBE i el de pell seca provocat per l'aplicació de AEW (Zhu et al., 2017). Concretament, s'ha identificat una disminució significativa en els episodis de rascat espontanis associats a pell seca en ratolins on s'han suprimit les neurones MRGPRA3⁺ (L Han et al., 2013).

La psoriasi és una malaltia autoinflamatòria i autoimmunitària crònica de la pell que afecta aproximadament del 2% al 3% de la població mundial i que sovint presenta la picor crònica com a símptoma altament freqüent (MP Schön, 2005; Schon et al., 2009; Reszke et al., 2019). Aquesta patologia es caracteritza per la presència de plaques escamoses en la pell amb uns trets histològiques particulars, on s'inclouen un engruiximent de l'estrat corni (hiperqueratosi), retenció de nuclis dins dels corneòcits (paraqueratosi) i infiltració de cèl·lules inflamatòries (Bowcock et al., 2005).

L'aplicació tòpica de manera repetida del fàrmac imiquimod (IQ) s'utilitza per estudiar la patogènesi de la psoriasi en models humans i animals. Aquest model indueix unes lesions cutànies similars a les plaques presents en humans i caracteritzades per eritema, engruiximent de la pell, alteracions epidèrmiques i infiltració de cèl·lules inflamatòries i és capaç d'induir episodis de rascat associats a picor (Fanti et al., 2006; Fits et al., 2009). L'imiquimod (IQ) és un anàleg purí amb propietats anticancerígenes i antivíriques. S'utilitza àmpliament per tractar diverses malalties de la pell com el mol·lusc contagiós, les berrugues genitals causades pel virus del papil·loma humà, el carcinoma de cèl·lules basals i la malaltia de Bowen (Syed, 2001). Tot i que es desconeix el mecanisme d'acció exacte, es creu que l'IQ modula les respostes immunes activant cèl·lules dendrítiques, macròfags o altres cèl·lules mitjançant el receptor de tipus Toll 7 (TLR7) (Dahl, 2000; M Schön et al.,

2007). A més, s'ha descrit que el compost desencadena potencials d'acció en neurones sensorials de ratolí mitjançant l'activació de TLR7 (T Liu, Xu et al., 2010) i que també provoca respostes independents de TLR7 a la pell, induint un augment de $[Ca^{2+}]$ intracel·lular a través de l'activació directa del receptor d'IP3 (SJ Kim et al., 2011; Lee et al., 2012; Walter et al., 2013; H Hwang et al., 2014). Sakai et al. han descrit que els nivells de mRNA de MRGPRA3, MRGPC11 i MRGPRD disminueixen gradualment amb el pas del temps en les neurones dels DRG en el model de picor psoriàsica induïda per imiquimod (Sakai et al., 2016). Els autors proposen que les neurones que expressen els receptors MRGPR podrien presentar hiperexcitabilitat associada al model, fenomen que també s'ha observat en un model de dermatitis de contacte (Qu et al., 2014).

Curiosament, en els tres models emprats en aquest estudi, els ratolins TRESK KO segueixen un patró similar ja que tots exhibeixen un augment significatiu dels episodis de rascat respecte els WT només a l'inici del desenvolupament dels models, mentre que en estadis més crònics de la patologia, no existeixen diferències significatives entre els animals salvatges i els que no expressen el canal (Figures 10, 11, 13). Degut a que s'ha proposat una alteració en l'excitabilitat de les subpoblacions de neurones que expressen els receptors MRGPR com a causa de la picor crònica observada en aquests models, suggerim que l'expressió de TRESK en algunes d'aquestes subpoblacions estaria contribuint a la modulació de l'excitabilitat d'aquestes (Usoskin et al., 2015; Xing et al., 2020). A més, donat que la implicació de les neurones MRGPRA3⁺ coincideix en els tres models (Qu et al., 2014; Sakai et al., 2016; Zhu et al., 2017) i que les nostres troballes indiquen la participació de TRESK en la sensibilitat de les neurones de la subpoblació NP2 que expressa MRGPRA3, hipotetitzem que els corrents de TRESK estarien limitant l'activació de les neurones sensorials durant les fases inicials de la picor crònica que es desenvolupa en aquests models.

D'aquesta manera, en absència de TRESK augmentaria l'excitabilitat neuronal i, en conseqüència, s'incrementaria l'activació neuronal envers els estímuls prurítics que generen els models, cosa que es traduiria en un major nombre d'episodis de rascat. Aquests efectes semblen més importants a l'inici del procés (dies inicials) mentre que quan la lesió cutània està més establerta, el fenotip es similar

entre animals WT i KO (Figures 10, 11, 13), cosa que indica una menor contribució de TRESK sobre l'excitabilitat en etapes més avançades.

Per altre banda, el model d'ACD induït per SADBE reproduïx la inflamació de la pell similar a l'observada en humans (Z Zhang et al., 2019) i el model de psoriasi induïda per imiquimod promou la infiltració de cèl·lules inflammatòries a la pell (Fanti et al., 2006; Fits et al., 2009). Després d'una lesió o d'un procés d'inflamació tissular, la pell afectada s'infiltra amb cèl·lules inflammatòries i s'hi alliberen mediadors inflamatoris que formen un exsudat amb un contingut àcid i hiperosmòtic (Vakili et al., 1970).

Diversos estudis han reportat la modulació d'alguns canals de la família K_{2P} per part de la "sopa inflamatòria", contribuint a la sensibilització perifèrica de les neurones sensorials en condicions inflammatòries (Alloui et al., 2006; Noël et al., 2009b). Els corrents de TRESK, en particular, s'inhibeixen per mediadors inflamatoris com el pH àcid o l'àcid araquidònic (Sano et al., 2003) i el canal presenta una sensibilitat intrínseca als canvis en la tensió de la membrana, com per exemple, els provocats per canvis osmòtics durant lesions o inflamacions dels teixits (Callejo et al., 2013). D'altra banda, una reducció en l'expressió dels canals K_{2P} , entre els que es troba TRESK, s'ha associat en múltiples ocasions amb una hiperexcitabilitat de les neurones sensorials. De manera similar a la d'altres canals iònics de potassi, s'ha descrit que en lesions perifèriques com l'axotomització i en models de dolor crònic neuropàtic es redueix l'expressió de TRESK, ocasionant un augment en l'excitabilitat de les neurones sensorials (Tulleuda et al., 2011; Tsantoulas et al., 2014; Y Yang et al., 2018). Marsh B et al. van demostrar que durant la inflamació es redueix l'expressió dels canals TASK-1, TASK-3 i TRESK, que va resultar en un increment de les descàrregues de PA en fibres C i del dolor espontani en rates (Tulleuda et al., 2011; Marsh et al., 2012; Tsantoulas et al., 2014; Y Yang et al., 2018).

Així, podem hipotetitzar que TRESK podria atenuar l'excitabilitat de les neurones NP2 positives per MRGPRA3 durant la primera fase de la picor crònica. En canvi, a mesura que els models van avançant i els processos inflamatoris s'intensifiquen, la contribució de TRESK en la modulació de l'excitabilitat de les neurones

que expressen els receptors MRGPR seria menor. Una altre possibilitat seria que la manca de diferències en els episodis de rascat en les fases avançades del model siguin degudes a una transformació dels ratolins WT cap a un fenotip TRESK KO. Aquest canvi podria ser mediat per una inhibició de TRESK per part de diferents mediadors inflamatoris com l'àcid araquidònic i el pH àcid (Sano et al., 2003) i per una possible disminució de l'expressió del canal a causa de la inflamació/lesió i la regulació transcripcional dependent de l'activitat neuronal (Jung et al., 2008; Flavell et al., 2008; Marsh et al., 2012). Com a resultat, en els ratolins WT es produiria un augment de l'activació neuronal i un increment de la resposta a estímuls prurítics i mecànics similars a la descrita en els ratolins TRESK KO (Figures 10, 11, 13).

Malgrat la hipòtesi de la reducció de l'expressió de TRESK com una de les causes de l'hiperexcitabilitat de les neurones MRGPR⁺ implicades en la generació de la picor crònica, no hem observat diferències significatives en els nivells d'expressió del canal en el model de picor psoriàsica induïda per imiquimod (Figures 14 i 15). Tot i que pot ser que enlloc d'una reducció de l'expressió del canal el que succeeix és que disminueixi l'activitat d'aquest, cal esmentar que els anàlisis d'expressió es van dur a terme durant el pic màxim de picor (dia 4 del model) i utilitzant totes les neurones del TG (Dia 4). Es possible que els efectes sobre l'expressió de TRESK siguin posteriors en els animals salvatges i que per aquest motiu no s'observin diferències significatives respecte als controls. A més, el fet d'extreure l'mRNA de tot el gangli enlloc de només de les neurones que innerven la galta, que són les afectades pel model, podria emascarar part de l'efecte sobre la expressió de TRESK, ja que la proporció de neurones no afectades dins el gangli és molt major. Així doncs, seria molt interessant poder marcar específicament les neurones que innerven la galta i repetir l'extracció al principi i en etapes més tardanes del model per poder fer una comparació més completa i precisa dels canvis d'expressió en les neurones sensorials implicades en la picor induïda pel model.

Seguint amb el model de picor psoriàsica induïda per imiquimod, postulem que els efectes directes de l'imiquimod també poden interferir amb l'activitat de TRESK i la seva participació durant el desenvolupament del model. Kim et al. han descrit diverses accions de l'IQ sobre les neurones, incloent la despolarització de

la membrana, la descàrrega de potencials d'acció, l'increment de la concentració de calci intracel·lular i el comportament de rascat associat a picor (SJ Kim et al., 2011). Els mateixos autors han demostrat que l'IQ incrementa l'excitabilitat de les neurones sensorials dels DRGs bloquejant directament diversos canals de K^+ , com els K_v i algun dels K_{2P} (TREK-1 i TRAAK), la qual cosa podria contribuir a la generació de picor (Lee et al., 2012). Tot i que, TREK-1 i TRAAK són els canals sobre els que s'ha demostrat el bloqueig directe per IQ (Lee et al., 2012), en experiments no publicats del nostre grup hem observat que els corrents de TRESK també s'inhibeixen per IQ *in vitro*, fet que podria contribuir a l'increment de l'excitabilitat neuronal d'una manera similar a la que succeeix amb el bloqueig de TRESK per piretroides (Castellanos, Andres et al., 2018).

Així doncs, en aquest model proposem l'existència de dos efectes en paral·lel en els que TRESK es veuria implicat. Per una banda, la lesió psoriàtica induïda per IQ produeix picor per activació dels pruritoceptors i la presència de TRESK actua com a fre envers aquesta activació. Per una altra banda, la inhibició directa de TRESK per part de l'IQ (només present en ratolins salvatges), atenua l'acció del canal sobre la modulació de l'excitabilitat neuronal i afavoreix el fenotip de picor crònica. Per tant, tot i que observem diferències significatives en els episodis de rascat a l'inici del model en absència de TRESK, aquestes diferències podrien ser encara més pronunciades si no hi hagués un efecte directe de l'IQ.

3. Activació de TRESK com a nova estratègia terapèutica antiprurítica

La picor crònica és una condició multidimensional i debilitant que afecta greument la qualitat de vida de qui la pateix. Tot i que amb els anys s'han desenvolupat diversos tractaments enfocats a la resposta immunitària present en la picor crònica, les teràpies existents per tractar aquest símptoma són molt limitades, sovint poc eficients o directament inexistents. No obstant això, els avenços en la comprensió del component neuronal que media la picor està conduint al descobriment de noves dianes terapèutiques i al desenvolupament de nous fàrmacs antiprurítics.

Atès el paper de TRESK en la sensibilitat dolorosa, s'ha proposat el canal com a diana molt interessant per al desenvolupament de nous fàrmacs analgèsics i antiprurítics (J Han et al., 2009; Gohlke et al., 2013). A més, ja que TRESK s'expressa abundantment en les neurones dels DRG i TG, s'espera que la modulació del canal alteri l'excitabilitat de les neurones sensorials en els ganglis TG i DRGs, però pugui presentar efectes mínims en altres teixits. Tanmateix, com els ganglis on s'expressa TRESK es troben fora de la barrera hematoencefàlica, si es desenvolupen fàrmacs adequats es podria modular l'activitat del canal a nivell perifèric sense intervenir en la seva funció en el sistema nerviós central.

En aquesta tesi s'ha estudiat per primer cop la possible implicació del canal TRESK en les sensacions de picor i proposem la participació del corrent de TRESK en la regulació de l'excitabilitat de determinades subpoblacions de neurones sensorials implicades en la detecció d'estímuls pruritoceptius no histamínèrgics. L'absència del canal agreuja els comportaments de rascat associats a diversos models de picor crònica, com la pell seca i la psoriasi induïda per imiquimod. A més, entre els possibles mecanismes cel·lulars subjacents, suggerim que els canvis en la concentració de calci intracel·lular que impliquin l'activitat de la calcineurina podrien ser rellevants per a la contribució de TRESK i seria interessant abordar el seu estudi en projectes posteriors.

Així doncs, des del punt de vista terapèutic, l'objectiu és buscar mol·lècules petites que actuïn directament sobre TRESK i activin del canal de manera específica per disminuir l'excitabilitat de les neurones sensorials i atenuar les sensacions de picor (Bruner et al., 2014) (Figura 3). S'han descrit moduladors de l'activitat de TRESK com és el sanshool, els anestèsics locals i volàtils, l'isobutilalquil amida (IBA), la lamotrigina, els ions zinc i mercuri, la loratadina, els piretroides, l'àcid araquidònic i l'àcid flufenàmic (Sano et al., 2003; Czirják & Enyedi, 2006; Bautista et al., 2008; Tulleuda et al., 2011; T Liu & Ji, 2013; Bruner et al., 2014; Veale & Mathie, 2016; Monteillier et al., 2016; Castellanos, Andres et al., 2018). Lamentablement, molts d'aquests compostos són compostos poc específics ja que poden afectar altres canals K_{2P} i/o altres dianes farmacològiques. Recentment, s'ha identificat la cloxiquina com a activador "relativament" selectiu de TRESK ja que no

afecta altres canals de potassi, ni tampoc altres canals de la família dels K_{2P} i s'està treballant en la caracterització dels seus efectes *in vivo* (Wright et al., 2013; Lengyel et al., 2017; Pettingill et al., 2019).

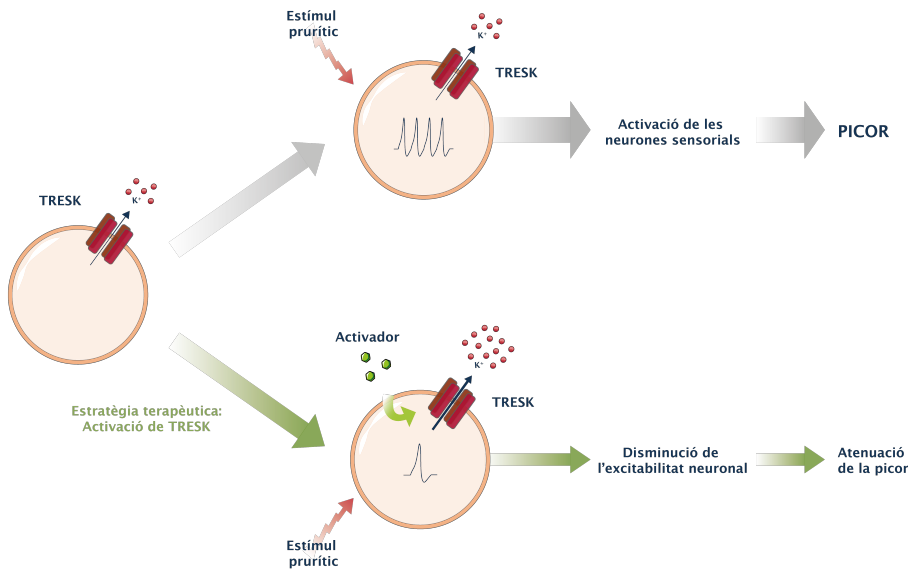


Figura 3: Esquema representatiu de l'activació de TRESK per la seva acció sobre l'activació neuronal i la generació d'efectes antiprurítics.

Malgrat que en aquest estudi no hem pogut demostrar que l'activació de TRESK atenuï la picor a causa de dificultats tècniques, plantegem que el canal té un gran potencial com a nova diana terapèutica en el tractament de la picor i que la cerca de nous activadors específics és essencial per poder avançar en aquest àmbit. Tanmateix, ja que la picor patològica és una condició crònica amb la que els pacients han de conviure de per vida, suggerim que seria molt rellevant disposar de nous fàrmacs alternatius a la utilització d'antihistamínics especialment per a la picor crònica d'origen no histaminèrgic, pel qual pràcticament no es disposa d'eines farmacològiques en l'actualitat.

VI. CONCLUSIONS

1. El canal TRESK s'expressa selectivament en determinades subpoblacions de neurones sensorials no peptidèrgiques implicades en la detecció d'estímuls de picor o pruritoceptors, on té un paper rellevant en la regulació de la seva excitabilitat.

2. TRESK contribueix en la modulació de la sensibilitat a la picor aguda no histaminèrgica mediada per la subpoblació neuronal no peptidèrgica (NP2) que presenta una expressió característica del receptor MRGPRA3, mentre que el seu paper en altres subpoblacions (NP3) no és important. La seva absència provoca un increment de les respostes de rascat induïdes per cloroquina, agonista del receptor MRGPRA3.

3. L'absència de TRESK no modifica el percentatge de neurones que responen a diferents pruritògens histaminèrgics i no histaminèrgics, indicant que TRESK participaria en la regulació de la excitabilitat neuronal, però no en la regulació dels mecanismes de detecció dels estímuls de picor.

4. La picor crònica induïda en diferents models (dermatitis al·lèrgica de contacte, pell seca i psoriasis) està incrementada per l'absència del canal TRESK, cosa que indica la participació de les subpoblacions que expressen aquest canal en la fisiopatologia de la picor crònica d'origen dermatològic.

5. La participació de TRESK és més rellevant en les primeres fases del desenvolupament de la picor crònica, mentre que la seva participació es més lleu en fases més tardanes de la patologia, quan la lesió dermatològica està més establerta.

6. Els resultats de l'estudi indiquen que TRESK és una diana farmacològica interessant per al tractament de la picor d'origen no histaminèrgic mitjançant el desenvolupament d'activadors selectius per aquest canal.

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A. LLISTA D'ACRÒNIMS

ACD: *Allergic contact dermatitis*, Dermatitis al·lèrgica de contacte

AEW: *Acetone, Eter and Water*, Cetona, èter i aigua

C-LTMRs: *Low-threshold C mechanoreceptors*, Mecanoreceptors de baix llindar de tipus C

CFA: *Complete Freund's Adjuvant*

CGRP: *Calcitonin Gene-Related Peptide*, Pèptid relacionat amb el gen de la calcitonina

CQ: Cloroquina

DRG: *Dorsal Root Ganglia*, Ganglis de l'arrel dorsal

MRGPR: *Members of the Mas-related G Protein-coupled Receptor*, Receptor de la família Mas acoblat a proteïnes G

NF: Neurofilament

NP: Neurones no peptidèrgiques

Npr1: *Natriuretic Polypeptide Receptor 1*

PA: Potencial d'acció

PEP: Neurones peptidèrgiques

SADBE: *Squaric acid dibutylester*, Àcid esquàric d'èster dibutílic

SNC: Sistema nerviós central

SNP: Sistema nerviós perifèric

TG: *Trigrminal Ganglia*, Ganglis trigèmins

TH: Tirosina hidroxilasa

TRESK: *TWIK-RElated Spinal cord Potassium channel*

TRPA1: *Transient Receptor Potential cation channel, subfamily A, member 1*,
Receptor de potencial transitori A1

TRPV1: *Transient Receptor Potential cation channel, subfamily V, member 1*, Re-
ceptor de potencial transitori V1

B. ARTICLES PUBLICATS EN RELACIÓ AMB LA TESI

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Pyrethroids inhibit K_{2P} channels and activate sensory neurons: basis of insecticide-induced paraesthesias

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Abstract

Pyrethroid insecticides are widely used for pest control in agriculture or in human public health commonly as a topical treatment for scabies and head lice. Exposure to pyrethroids such as permethrin or tetramethrin (TM) causes sensory alterations such as transient pain, burning, stinging sensations, and paraesthesias. Despite the well-known effects of pyrethroids on sodium channels, actions on other channels that control sensory neuron excitability are less studied. Given the role of 2-pore domain potassium (K_{2P}) channels in modulating sensory neuron excitability and firing, both in physiological and pathological conditions, we examined the effect of pyrethroids on K_{2P} channels mainly expressed in sensory neurons. Through electrophysiological and calcium imaging experiments, we show that a high percentage of TM-responding neurons were nociceptors, which were also activated by TRPA1 and/or TRPV1 agonists. This pyrethroid also activated and enhanced the excitability of peripheral saphenous nerve fibers. Pyrethroids produced a significant inhibition of native TRESK, TRAAK, TREK-1, and TREK-2 currents. Similar effects were found in transfected HEK293 cells. At the behavioral level, intradermal TM injection in the mouse paw produced nocifensive responses and caused mechanical allodynia, demonstrating that the effects seen on nociceptors in culture lead to pain-associated behaviors in vivo. In TRESK knockout mice, pain-associated behaviors elicited by TM were enhanced, providing further evidence for a role of this channel in preventing excessive neuronal activation. Our results indicate that inhibition of K_{2P} channels facilitates sensory neuron activation and increases their excitability. These effects contribute to the generation of paraesthesias and pain after pyrethroid exposure.

Keywords: Nociceptor, K_{2P} channels, Paraesthesia, Background current, Membrane potential, Mechanical sensitivity, Insecticide

1. Introduction

Pyrethroid insecticides have been highly used since the 1970s for domestic pest control and in public health, commonly as topical treatments for scabies and head lice. Their use is still very important for control of mosquito populations and malaria spread

despite the increasing incidence of resistance.²³ They are more than 2000 times more toxic to insects than to humans because of insects' increased sodium channel sensitivity, lower body temperature, and smaller size.⁴² High doses of pyrethroids can cause paralysis, ataxia, convulsions, hypersensitivity, choreoathetosis, tremor, and in extreme cases, death by provoking neuronal hyperexcitability.^{6,37,47,48} More commonly, local effects are seen after skin contact or ingestion. Tactile, painful, and paraesthetic effects including tingling, itching, burning, and stinging sensations of the skin have been associated with repetitive activity in skin nerve terminals because of the known effects of pyrethroids on Na^+ channels,^{41,43} where a slowing of the kinetics of channel opening and closing results in delayed and prolonged openings. These effects diminish action potential threshold and cause repetitive firing of sensory neurons, which is thought to underlie the reported abnormal sensations. At higher doses, the effects on sodium tail currents are large enough to prevent the generation of further action potentials, thus producing a conduction block.^{6,29,30} Inhibitory effects on chloride, calcium, and GABA-induced chloride currents have also been described.^{6,30,37}

Direct effects of permethrin (PM), deltamethrin (DM), and tetramethrin (TM) have been partially reported in neurons but they were mainly attributed to modulation of Na^+ channels, where they increased the duration of action potentials and produced spontaneous discharges.^{21,43} Similar effects of pyrethroids across disparate types of nociceptors with quite different

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complements of Na_v channels (sensitive or resistant to tetrodotoxin) suggest that Na_v channels are not solely responsible for nociceptor activation at body temperature.^{21,43}

Two-pore domain potassium channels (K_2P) carry most of the “leak” current and are expressed in different subpopulations of sensory neurons, including nociceptors. Their electrophysiological properties allow them to carry K^+ currents over a wide range of membrane potentials and as such they are key determinants of neuronal excitability, contributing to the likelihood of depolarizing stimuli to achieve action potential threshold, as well as shaping the neuron firing response.^{18,35} TREK-1, TREK-2, TRAAK, and TRESK make a major contribution to leak currents in trigeminal and dorsal root ganglion (DRG) neurons,^{15,22,45,50} where they have been implicated in pain induced by mechanical, thermal and chemical stimuli, perception of warmth and cold, and neuropathic and inflammatory pain.^{1,3,28,31,36,45}

Because of their important role in setting neuronal excitability, we studied whether $\text{K}_{2\text{P}}$ channels are implicated in the excitation of sensory neurons by pyrethroids. Here, we report that pyrethroids inhibit $\text{K}_{2\text{P}}$ channels, activate sensory neurons and peripheral nerve fibers, producing aversive behavioral responses in mice. Our results reveal a new target of pyrethroid action, suggesting that their $\text{K}_{2\text{P}}$ channel blocking effects contribute to the mechanisms underlying a persistent activation of sensory neurons that can lead to painful and paraesthetic sensations.

2. Methods

2.1. Animal behavior

2.1.1. Animals

All behavioral and experimental procedures were performed in accordance with the recommendations of the International Association for the Study of Pain (IASP) and were reviewed and approved by the Animal Care Committee of the University of Barcelona and by the Department of the Environment of the Generalitat de Catalunya, Catalonia, Spain (Ref. 8468, 8548, 6595). Male C57BL/6N mice between 8 and 15 weeks old were used in all experimental procedures (cellular cultures, electrophysiology, calcium imaging, and behavior), except for single nerve fiber recordings, where CD1 mice were used due to higher availability. Mice were housed at 22°C with free access to food and water in an alternating 12-hour light and dark cycle. Animals were housed individually for all behavioral assays. Behavioral measurements were taken in a quiet room, taking great care to minimize or avoid discomfort of the animals. TRESK (Kcnk18) knockout (KO) mice and wild-type (WT) littermates were obtained from the KOMP Repository (Mouse Biology Program, University of California, Davis, CA). The TRESK KO mouse was generated by replacing the complete Kcnk18 gene by a ZEN-UB1 cassette according to the VelociGene's KOMP Definitive Null Allele Design.

At 3 weeks of age, WT or KO newborn mice were weaned, separated, and identified by ear punching. Genomic DNA was isolated from tail snip samples with Maxwell Mouse Tail DNA Purification Kit (Promega, Madison, WI). Polymerase chain reaction (PCR) was performed with primers to detect the Kcnk18 gene: forward primer (TUF) 5'-GAGGAGAACCCTGAGTTGAAGAAG-3' and reverse primer (TUR) 5'-GCACCTCCGAGGCAGTAAC-3' or the inserted cassette in the KO mice: forward primer (REG-Neo-F) 5'-GCAGCCTCTGTTCCACATACACTTCA and reverse primer (gene-specific) 5'-AGACTTCTCCAGGTAACAACCTCTGC-3'. The PCR mixture contained 1- μL DNA sample, 2.5- μL PCR buffer (10 $\times$ concentration), 2- μL dNTP

mixture (2.5 mM), 0.5- μL (20 μM) forward and reverse primers, 0.2- μL Taq DNA polymerase (5 U/ μL), 1.7- μL MgCl_2 (25 mM), 6.5- μL betaine (5 M), 0.325- μL dimethyl sulfoxide [DMSO], and 9.7- μL water. Polymerase chain reaction amplifications were performed with 31 cycles in a programmable thermal cycler (Eppendorf AG, Hamburg, Germany). The program used was 94°C for 5 minutes and cycles of 94°C for 15 seconds, 60°C for 30 seconds, 72°C for 40 seconds, and with a final extension at 72°C for 5 minutes. Polymerase chain reaction products were analyzed by electrophoresis on 1% agarose gels.

2.1.2. Evaluation of nocifensive behavior

Using a 30-g needle, 10 μL of a solution containing 100- μM TM or vehicle (control; DMSO 0.1% in phosphate buffered saline [PBS]) was administered intradermally in the plantar surface of the hind paw of 2 independent groups of mice for each genotype. Mice behavior was observed and the number of flinches and lickings of the paw were counted for a 15-minute period starting immediately after the injection. On the day previous to testing, animals were habituated to the testing room and to the handling procedure. The experimenter and the person analyzing data were blind to the mice genotype. After evaluation of nocifensive behavior, mechanical and thermal sensitivity of each mouse was assessed as detailed below.

2.1.3. Mouse mechanical sensitivity

To assess mechanical sensitivity, the withdrawal threshold to punctate mechanical stimuli of the hind paw was determined 30 minutes before and 15 minutes after TM injection in the hind paw (as previously described) by the application of calibrated von Frey filaments (North Coast Medical, Inc, Morgan Hill, CA). The von Frey filaments (size: 2.44, 2.83, 3.22, 3.61, 3.84, 4.08, 4.17, 4.31, and 4.56; equivalent to [in grams] 0.04, 0.07, 0.16, 0.4, 0.6, 1, 1.4, 2, and 4, respectively) were applied perpendicular to the plantar surface of the hind paw and gently pushed to the bending point for 5 seconds. The 50% withdrawal threshold was determined using the up-down method as previously described.¹¹ A brisk hind paw lift in response to von Frey filament stimulation was regarded as a withdrawal response.

2.1.4. Mouse thermal sensitivity

The heat sensitivity of mice was assessed by measuring hind paw withdrawal latency from a radiant infrared source (Hargreaves' Method) using the Ugo Basile (Italy) Model 37370 Plantar test. Mice were acclimatized to the testing room for at least 30 minutes and each measurement was the mean of 3 trials. Withdrawal latency was measured 30 minutes before injecting TM in the hind paw and 15 minutes after injection.

2.1.5. Blinking test

To assess the mouse nocifensive response to noxious stimulation, 5 μL of vehicle in saline solution or the drug of interest were topically applied to the anterior ocular surface of an eye. Mice were lightly restrained (by grasping the scruff between the thumb and forefinger), so that the solution remained on the corneal surface and the number of blinks (complete lid closure) counted over a 2-minute period. After the observation time, the ocular surface was abundantly washed with PBS solution, and mice were returned to their home cages.

2.2. Single nerve fiber recording

2.2.1. Animals

Adult outbred CD1 female mice ($n = 18$, mean body weight 32 ± 1.8 g) bred at the University Animal House were used (obtained from Harlan, Spain). European Union and State legislation for the regulation of animal experiments was followed. All experimental protocols were approved by the University of Alcalá Committee on Animal Research and the Regional Government (project license: ES280050001165).

2.2.2. Surgical extraction and maintenance of the skin flap

Mice were killed by cervical dislocation, and the saphenous nerve together with the skin flap was excised and pinned down to a Sylgard based recording chamber, corium side up. The preparations were superfused with oxygenated synthetic interstitial fluid (SIF, composition [in mM]: 108 NaCl, 3.48 KCl, 0.7 MgSO_4 , 26 NaHCO_3 , 1.7 NaH_2PO_4 , 1.53 CaCl_2 , 9.6 sodium gluconate, 5.55 glucose, and 7.6 sucrose) at a rate of 5 mL per minute. Recording temperature was monitored and maintained at $32^\circ\text{C} \pm 1^\circ\text{C}$ by means of a Peltier device (Warner Instruments, Hamden, CT). A second preparation from the same animal was stored in oxygenated SIF at 4°C and used if required, on the same day. There were no detectable differences in results between the first and second preparations studied.

2.2.3. Electrical recording and stimulation

The electrical activity from sensory fibers was recorded by means of suction microelectrodes obtained from glass pipettes (20 to 30 μm external tip diameter) filled with SIF as previously described.³⁸ The microelectrode tip was carefully placed in contact with the proximal end of the nerve trunk under visual guidance by using a micromanipulator and negative pressure was applied to isolate thin nerve filaments. Controlled electrical pulses of supramaximal intensity for C-fibers (0.5 ms pulse width, maximum strength up to 10 mA) were delivered to the nerve trunk via a thin bipolar tungsten electrode (WPI, World Precision Instruments, Sarasota, FL) to identify the fibers present in the filament on the basis of their conduction velocity (CV). The experiments only proceeded when a single fiber was recorded or at least could be clearly differentiated from background discharges and other units on the basis of spike amplitude and shape. The presence of mechanosensitivity was explored by gently touching the skin-flap with a smooth-tipped glass rod (diameter = 0.5 mm); on location of the receptive field (RF), mechanical thresholds were established by means of gravity-driven von Frey hairs as described in detail.^{51,52}

Electrical signals were recorded by means of a Dagan EX4-400 amplifier (Dagan, Minneapolis, MS), digitized at 20 KHz (Power 1401, CED) and stored for offline analysis. Fibers were classified according to their CV into A-units ($\text{CV} > 1$ m/s) or C-units ($\text{CV} < 0.8$ m/s) on the basis of pilot experiments using whole nerve recordings, which showed a CV of ~ 0.8 m/s for the C-fiber volley. After electrical and mechanical characterization, the RF of the fiber under study was isolated by means of a hollow steel cylinder (internal diameter ~ 6 mm) placed on top of the skin and medical grease (WPI, Germany) was added to improve isolation. To reproduce mechanical stimulation in both intensity and location, the von Frey probes were mounted on a micromanipulator and moved down until the probe tip (0.6 mm diameter) contacted perpendicularly the most sensitive spot of the RF. The following probe above threshold intensity was used during the

experimental protocol, in which stimuli (~ 5 seconds duration) were applied at ~ 5 -minute intervals.

Tetramethrin was dissolved in DMSO at 10 mM and stored at 4°C . Tetramethrin was diluted in SIF to final concentrations of 25, 50, 100, or 200 μM immediately before use. Tetramethrin ($\sim 300 \mu\text{L}$) was directly applied inside the cylinder and maintained for ~ 10 minutes and the mechanical responsiveness was tested at least twice. A change in baseline firing was taken as an indication of excitatory response. For mechanoinensitive fibers, electrical stimulation was applied to assess for the presence of the fiber. The magnitude of the mechanical response was quantified as the total number of spikes counted during the 5-second stimulation. Mean values during control and those obtained during exposures to each TM concentration were used for analysis and graphical representations. The maximum discharge frequency during the stimuli was also noted.

Waveforms were analyzed off-line with Spike 2 software (CED, Cambridge Electronic Design, Cambridge, United Kingdom). Autocorrelograms were performed for each of the recorded fibers to evaluate the success of the spike sorting. Statistical analyses were performed in Graphpad Prism 5 (GraphPad Software, San Diego, CA) on the raw data using Wilcoxon matched pairs test. The level of statistical significance was set at $P < 0.05$. Values are quoted as mean $\pm$ SEM.

2.3. Culture of dorsal root ganglion neurons

Mice were euthanized by decapitation under anesthesia (isoflurane) and thoracic, lumbar, and cervical DRG were removed for neuronal culture as previously described.^{9,45} Briefly, DRG were collected and maintained in cold (4°C – 5°C) Ca^{2+} - and Mg^{2+} -free PBS solution (Sigma) supplemented with 10-mM glucose, 10-mM Hepes, 100-U.I./mL penicillin, and 100- $\mu\text{g}/\text{mL}$ streptomycin until dissociation. Subsequently, ganglia were incubated with collagenase CLS I (1 mg/mL; Boehringer AG, Berlin, Germany) for 1 hour 45 minutes followed by 15-minute trypsin treatment (0.25%; Sigma) before mechanical dissociation with fire-polished Pasteur pipettes of decreasing diameter.

Neurons found in the pellet were suspended in 2-mL Dulbecco's Modified Eagle's medium [DMEM] + 10% fetal bovine serum (FBS; Sigma), centrifuged at 1000 rpm for 5 minutes and resuspended in the culture medium (DMEM + 10% FBS, 100- $\mu\text{g}/\text{mL}$ penicillin/streptomycin, and 100-mg/mL L-glutamine). Cell suspensions were transferred to poly-L-lysine/laminin-coated 12-mm diameter glass coverslips and incubated at 37°C in humidified 5% CO_2 for up to 1 day before being used for patch-clamp electrophysiological recordings or calcium imaging experiments. Nerve growth factor or other growth factors were not added.

2.4. Calcium imaging

Dorsal root ganglion neurons obtained as described above were loaded with 5- μM fura-2/AM (Calbiochem, San Diego, CA) for 45 to 60 minutes at 37°C in culture medium. Coverslips with fura-2 loaded cells were transferred into an open flow chamber (0.5 mL) mounted on the stage of an inverted Olympus IX70 microscope equipped with a TILL monochromator as a source of illumination. Pictures were taken with an attached cooled CCD camera (Orca II-ER, Hamamatsu Photonics, Shizuoka, Japan) and were digitized, stored, and analyzed on a PC computer using Aquacosmos software (Hamamatsu Photonics). After a stabilization period, image pairs were obtained alternately every 4 seconds at excitation wavelengths of 340 (λ_1) and 380 nm (λ_2 ; 10 nm

bandwidth filters) to excite the Ca^{2+} bound and Ca^{2+} free forms of this ratiometric dye, respectively. The emission wavelength was 510 nm (120-nm bandwidth filter). Typically, 20 to 40 cells were present in a field and $[\text{Ca}^{2+}]_i$ values were calculated and analyzed individually for each single cell from the 340- to 380-nm fluorescence ratios at each time point. Only neurons that produced a response $>10\%$ of the baseline value and that, at the end of the experiment, produced a Ca^{2+} response to KCl-induced depolarization (50 mM) were included in the analysis. Several experiments with cells from different primary cultures were used in all the groups assayed. The extracellular (bath) solution used was 140-mM NaCl, 4.3-mM KCl, 1.3-mM CaCl_2 , 1-mM MgCl_2 , 10-mM glucose, and 10-mM HEPES, at pH 7.4 with NaOH. In extracellular Ca^{2+} -free experiments, the bath solution used was 145-mM NaCl, 5-mM KCl, 3-mM MgCl_2 , 10-mM glucose, 1-mM EGTA, and 20-mM HEPES, at pH 7.4 with NaOH (nominally Ca^{2+} free, estimated <10 nM).

2.5. Electrophysiological recording

2.5.1. Recordings in sensory neurons

Electrophysiological recordings were performed as previously described.^{8,9} Briefly, recordings were performed with a patch-clamp amplifier (Axopatch 200B; Molecular Devices, Union City, CA) and restricted to small and medium DRG neurons ($<30\text{-}\mu\text{m}$ diameter; <45 pF), which largely correspond to nociceptive neurons.²⁷ Patch electrodes were fabricated in a Flaming/Brown micropipette puller P-97 (Sutter instruments, Novato, CA). Electrodes had a resistance between 2 and 4 M Ω when filled with intracellular solution (in mM): 140 KCl, 2.1 CaCl_2 , 2.5 MgCl_2 , 5 EGTA, 10 HEPES, and 2 ATP at pH 7.3. Bath solution (in mM): 145 NaCl, 5 KCl, 2 CaCl_2 , 2 MgCl_2 , 10 HEPES, and 5 glucose at pH 7.4. The osmolality of the isotonic solution was 310.6 ± 1.8 mOsm/Kg. In experiments using extracellular high K^+ , the solution used was (in mM) 15 NaCl, 135 KCl, 2 CaCl_2 , 2 MgCl_2 , and 10 HEPES at pH 7.4. Membrane currents were recorded in the whole-cell patch clamp configuration, filtered at 2 kHz, digitized at 10 kHz, and acquired with pClamp 10 software. Data were analyzed with Clampfit 10 (Molecular Devices) and Prism 5 (GraphPad Software, Inc, La Jolla, CA). Series resistance was always kept below 15 M Ω and compensated at 70% to 80%. All recordings were performed at room temperature (22°C – 23°C), 18 to 24 hours after dissociation. To study the effects of pyrethroids on sensory neuron excitability, after achieving the whole-cell configuration in the patch clamp technique, the amplifier was switched to current-clamp bridge mode. Only neurons with a resting membrane voltage below -50 mV were considered for the study.

2.5.2. Electrophysiology in transfected cells

HEK293T cells, cultured in DMEM supplemented with 10% FBS, 100- $\mu\text{g}/\text{mL}$ penicillin/streptomycin, and 100-mg/mL L-glutamine at 37°C in humidified 5% CO_2 , were seeded on 12-mm diameter poly-L-lysine coated glass coverslips 24 hours before transfection. Cells were transiently transfected using X-tremeGENE 9 transfection reagent (Roche, Mannheim, Germany) according to the manufacturer's instructions. Rat TRESK (kindly provided by S. Yost, University of California-San Francisco) and Mouse TRESK (kindly provided by P. Enyedi, Semmelweis University, Budapest, Hungary) and Human TRESK (kindly provided by Y. Sano, Astellas Pharma, Japan) were subcloned into the pIRES2-EGFP vector (Clontech) to form the rTRESK-pIRES2-GFP,

mTRESK-pIRES2-GFP, and hTRESK-pIRES2-GFP constructs, respectively. The vectors pCD8-hTREK-2 and pCD8-mTRAAK (kindly provided by F. Lesage, Institut de Pharmacologie Moléculaire et Cellulaire-CNRS, Valbonne, France) were cotransfected with pEGFP-C3 vector to allow identification of transfected cells. hTREK-1-pIRES-EGFP was kindly provided by S. Bhattacharya (Bascom Palmer Eye Institute, University of Miami). Cells were used for electrophysiological recordings 24 to 48 hours after transfection. Patch clamp recordings were performed as described for sensory neurons using identical solutions.

2.6. Drugs

Tetramethrin, DM, and PM were obtained from Sigma-Aldrich (Madrid, Spain) and initially diluted in DMSO at 100 mM. For in vitro and in vivo experiments, the stock solution prepared in a glass container was subsequently diluted in the appropriate medium and used at the stated concentrations (0.2 to 100 μM). The final concentration of DMSO was 0.1% or below. Pyrethroids were added to the recording chamber with a pipette to avoid possible sticking of these highly lipophilic compounds to the plastic tubing. Menthol (100 μM), allyl isothiocyanate (AITC; 100 μM) and capsaicin (1 μM), tetraethylammonium (TEA; 1 mM), 4-aminopyridine (4-AP; 1 mM), CdCl_2 (100 μM) and CsCl (1 mM), ibuprofen (50 nM), arachidonic acid (10 μM), cloxyquin (100 μM), thapsigargin (1 μM), and A-803467 (1 μM) were also purchased from Sigma (Madrid, Spain). Tetrodotoxin (1 or 7 μM) was obtained from Alomone Labs (Jerusalem, Israel).

2.7. Data analysis

Data are presented as mean $\pm$ SEM. Statistical differences between different sets of data were assessed by performing paired or unpaired Student *t* tests or Wilcoxon matched pairs test, as indicated, using Prism 5 (GraphPad Software, Inc) data analysis software. The significance level was set at $P < 0.05$ in all statistical analyses.

3. Results

3.1. Tetramethrin-activated sensory neurons are mainly nociceptors

Sensory alterations produced by pyrethroids require the activation of sensory nerve endings in the skin or viscera. To study the response of sensory neurons to pyrethroid exposure, we used intracellular calcium imaging in DRG sensory neurons labeled with Fura-2 calcium dye that permits the analysis of larger cell populations. Tetramethrin (20 μM) application increased intracellular Ca^{2+} in 124 DRG neurons of 265 (46.8% response; **Figs. 1A–C**) with a mean Ca^{2+} increase of 1.60 ± 0.04 -fold (ratio F340/F380). Interestingly, in most responding neurons (64.5%), the Ca^{2+} response followed an oscillatory pattern (**Figs. 1A and B**), whereas in 35.5% of the cells, TM produced a single Ca^{2+} peak. This oscillatory pattern was never observed when the same cells were stimulated with well-known agonists of different TRP channels present in cold (menthol, a TRPM8 agonist) or nociceptive sensory neurons (AITC and Capsaicin, agonists of TRPA1 and TRPV1, respectively). As shown in **Figure 1C**, TM-responding neurons had a significantly smaller diameter (18.1 ± 0.4 μm ; range 8.3–34.8; **Fig. 1C**; $n = 124$) than nonresponding cells (19.9 ± 0.6 μm ; range 8.3–44.0; $P < 0.05$). In addition, a high percentage of TM-responding cells were also positive for TRPA1 (62.9% also responded to AITC) or for TRPV1 (45.2% also

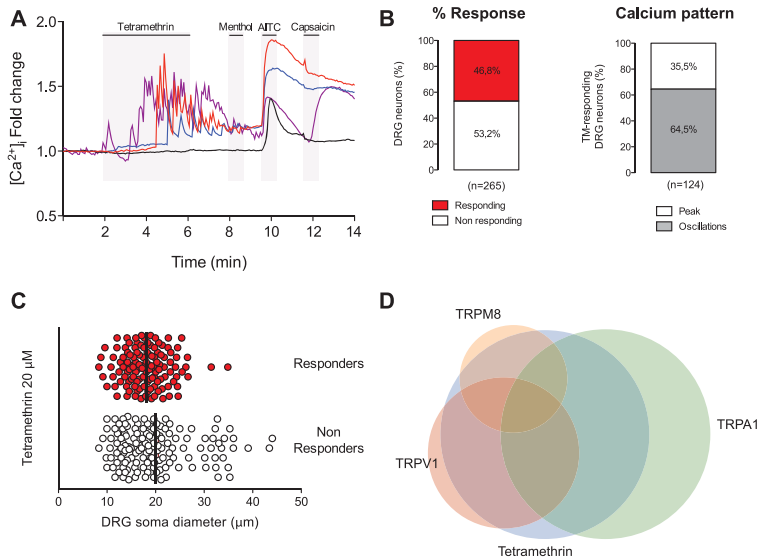


Figure 1. Tetramethrin (TM) excites a subset of sensory neurons with characteristics of nociceptors. (A) Wild-type mouse DRG sensory neurons were exposed to TM (20 μ M), the TRPM8 agonist menthol (100 μ M), the TRPA1 agonist allyl isothiocyanate (AITC, 100 μ M), and the TRPV1 agonist capsaicin (1 μ M), and intracellular calcium was measured by Fura-2 ratiometric imaging. A series of representative traces are shown. Tetramethrin induced a characteristic oscillatory calcium pattern in a large percentage of cells (red, purple, and blue traces). Calcium responses show that some cells are only activated by TM, whereas others respond also to agonists of TRPA1 or TRPV1. A non-TM responsive neuron that responds to AITC is shown in the black trace. (B) Left: quantification of the percentage of neurons responding to TM. Right: percentage of TM-responding cells showing an oscillatory calcium pattern or responding with a single calcium peak. (C) Soma diameter distribution of DRG neurons sensitive and insensitive to TM. Black bars show the mean soma value in each group. (D) Venn diagram showing the relative size and overlap between the population of TM-activated neurons and those activated by menthol, AITC, or capsaicin. DRG, dorsal root ganglion.

responded to capsaicin), whereas TRPM8-positive TM responders formed a minor population (12.1% responded to menthol but not to AITC) (Fig. 1D). All together, these results (presence of TRPA1/V1 and small cell size) indicate that many of the cells stimulated by pyrethroids were nociceptors. Because the Ca^{2+} increase triggered by pyrethroids can be due to an entry of extracellular Ca^{2+} , a release from intracellular stores or both, we performed experiments in the absence of extracellular Ca^{2+} (nominally Ca^{2+} -free solution) to study the origin of the Ca^{2+} peak. In Ca^{2+} -free conditions, none of 126 neurons studied produced any response after application of TM (20 μ M; supplemental Fig. 1A, available at <http://links.lww.com/PAIN/A485>). Reintroduction of extracellular Ca^{2+} produced an increase in cytosolic Ca^{2+} in 70 of 126 neurons (55.6%), indicating that an entry of extracellular Ca^{2+} is mediating the effects of pyrethroids. Also, in the presence of thapsigargin (1 μ M) to empty the intracellular calcium stores, the effect of TM was still present in a similar percentage of neurons (50.8%; supplemental Fig. 1B, C, available at <http://links.lww.com/PAIN/A485>).

The effects of pyrethroids on sensory neuron excitability are usually attributed to effects on voltage-gated Na^+ (Na_v) channels (increased action potential duration and depolarizing after potentials), but less is known about their effects on other conductances that control resting membrane potential and neuronal excitability. For this matter, we tested whether small- and medium-sized DRG neurons in culture (which are mainly nociceptors) were depolarized and fired action potentials when challenged with TM, a type I pyrethroid. Twenty-two DRG neurons with a mean capacitance of 22.7 ± 2.6 pF and a resting membrane potential of -54.9 ± 1.1 mV were recorded using the

current clamp mode of the patch-clamp technique. Twelve of these neurons ($\sim 54.5\%$ response) were depolarized by 15.7 ± 4.2 mV and fired action potentials (34.7 ± 12.7 spikes) in response to TM (20 μ M, Figs. 2A and B), whereas 10 neurons showed a small depolarization (3.7 ± 1.4 mV) that did not reach action potential threshold (Fig. 2B). To examine the possible involvement of K^+ currents active at the subthreshold range of membrane potentials, we recorded the effect of TM on membrane current in the presence of a general Na^+ , K^+ , and Ca^{2+} channel blocker cocktail (TTX, TEA, 4-AP, Cs^+ , and Cd^{2+}) at a slightly depolarized holding potential (-30 mV). Under these conditions, TTX-sensitive Na_v , K_v , K_{Ca} , and Ca_v channels should make a minimal contribution and noninactivating K^+ currents will be more activated, allowing assessment of the contribution of other channels (including K_{2P} channels) to the TM response. After TM application, a negative shift in the holding current was observed ($\Delta I_{\text{hold}} = -29.9 \pm 11.1$ pA; $n = 6$), suggesting a possible inhibition of active resting conductances (Fig. 2C). Tetramethrin also significantly reduced whole-cell currents elicited by depolarizing ramps in the presence of the channel blocker cocktail (Figs. 2C–E). The reversal potential of the depolarization-activated current was -45.9 ± 5.1 mV, indicating that K^+ is not the only contributor to this current and TTX-resistant persistent Na^+ current and/or Cl^- currents may make some contribution. In another set of experiments using the same experimental protocol, to exclude possible contributions of Na_v channel to the effect produced by TM, we included a high concentration of TTX (7 μ M) and the specific $\text{Na}_v 1.8$ blocker A-803467 (1 μ M) to minimize sodium currents. In these conditions, similar inhibitory effects were found after TM application (-76.5% ; $n = 5$; $P < 0.001$; not shown).

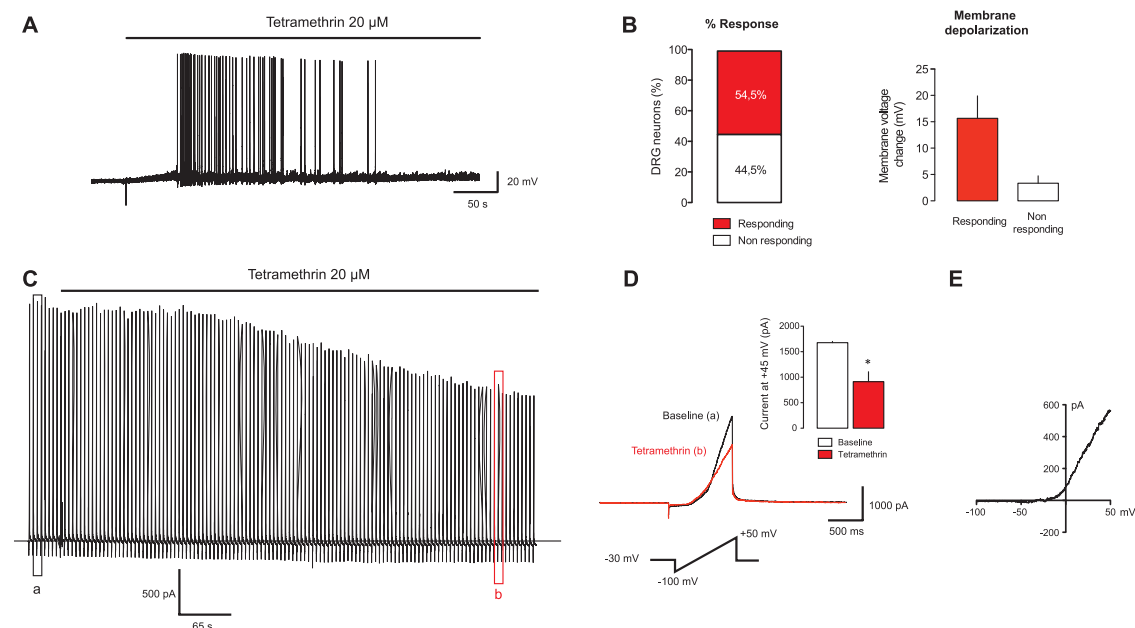


Figure 2. Tetramethrin (TM) activates sensory neurons. (A) Current-clamp recording on a DRG neuron activated by TM, a type I pyrethroid. Drug application depolarizes the cell sufficiently to trigger firing. (B) Quantification of the percentage of response ($n = 22$) and the amount of membrane depolarization induced by TM in neurons that fired action potentials (responding) or nonresponders. (C) Example of a membrane current recording in the presence of Na^+ , K^+ , and Ca^{2+} channel blockers (TTX, TEA, 4-AP, Cs^+ , and Cd^{2+}) at a holding voltage of -30 mV. Depolarizing ramps from -100 to $+50$ mV were applied to monitor the effects of TM in the whole cell current. (D) Left: detail of depolarizing ramps before (a) and during (b) TM application as indicated on the recording shown in (C). Inset: quantification of the current inhibited by TM application ($n = 6$). Data are expressed as mean $\pm$ SEM. $^*P < 0.05$; Student t test vs baseline. (E) Mean current subtracted (baseline current – current with TM) obtained from experiments as in (D). DRG, dorsal root ganglion.

on the total current elicited by a depolarizing ramp and measured at $+45$ mV (as in Fig. 2D).

3.2. Pyrethroids inhibit K_{2P} channels

Of the different ion channels that are thought to participate in setting the membrane potential and regulating it in the sub-threshold range, 4-AP-sensitive K_v , slowly-activating M channels (K_v7 and KCNQ), the $\text{Na}_v1.9$, and “leak” K_{2P} channels have been described as having the most significant influence.¹⁶ Within the K_{2P} family, TREK-1, TREK-2, TRAAK, and TRESK are proposed to play a significant role in sensory neurons, especially those of small and medium size.^{15,22,45} To further analyze the effects of pyrethroids in the K_{2P} channels expressed in native neurons, we searched for small/medium-sized neurons, where arachidonic acid (a known activator of TREK-1, TREK-2, and TRAAK^{19,26,35}) or cloxyquin (a TRESK-activating compound^{24,49}) increased the current measured at hyperpolarized voltages in high extracellular K^+ solution. In these conditions, background potassium channels activated by each compound should carry the majority of the current measured at hyperpolarized potentials. Voltage ramps from -100 to $+50$ mV were first recorded in physiological solution and then the extracellular bath was replaced by high K^+ solution (near symmetrical K^+ concentration). Under these conditions, voltage ramps had a reversal potential near 0 mV, indicating that K^+ was the major carrier of the current. As depicted in Figure 3A, addition of $10\text{-}\mu\text{M}$ arachidonic acid produced a 43% increase in the current measured at -95 mV in 5 of 9 neurons recorded (responding neurons: from -11.5 ± 3.6 to

-16.1 ± 4.8 pA/pF; $P < 0.05$). When $20\text{-}\mu\text{M}$ TM was added (in the presence of arachidonic acid), a significant inhibition was observed ($39 \pm 6\%$; from -16.1 ± 4.8 to -10.7 ± 3.9 pA/pF; $P < 0.05$). In parallel experiments, $100\text{-}\mu\text{M}$ cloxyquin produced a significant current increase of 414% at -95 mV in 6 of 8 neurons recorded (from -12.2 ± 3.5 to -38.1 ± 6.6 pA/pF; $P < 0.01$; Fig. 3B). Again, addition of TM (in the presence of cloxyquin) significantly inhibited the current by 42% (from -38.1 ± 6.6 to -20.1 ± 4.6 pA/pF; $P < 0.05$). These results indicate that native currents mediated by background K^+ channels were significantly affected by pyrethroid exposure.

To corroborate the inhibitory effect found on native currents, and to further characterize the specific effects of pyrethroids on K_{2P} channels, we studied them after expression in a heterologous system. Whole-cell currents from HEK293 cells transiently transfected with human TRESK (hTRESK) were recorded and responses to pyrethroids were measured. Treatment with $20\text{-}\mu\text{M}$ TM, DM, or PM had an inhibitory effect on hTRESK, decreasing its current by 33%, 30%, and 27%, respectively (Figs. 4A–C, G). Partial recovery of the current was seen after washout of the compounds. In a similar fashion, TM was also tested on rat and mouse TRESK orthologs, with similar inhibitory effects (Figs. 4G and H). The dose–response curve rendered a half-maximal inhibitory concentration (IC_{50}) of $10.7\text{ }\mu\text{M}$ for human and $34.9\text{ }\mu\text{M}$ for mouse TRESK (Fig. 4H). To determine whether TM was acting directly on the channel or near it (through the plasma membrane), and to discard an action through intracellular messengers or interaction with soluble intracellular proteins, we performed recordings in outside-out patches from HEK293 cells transfected with mTRESK

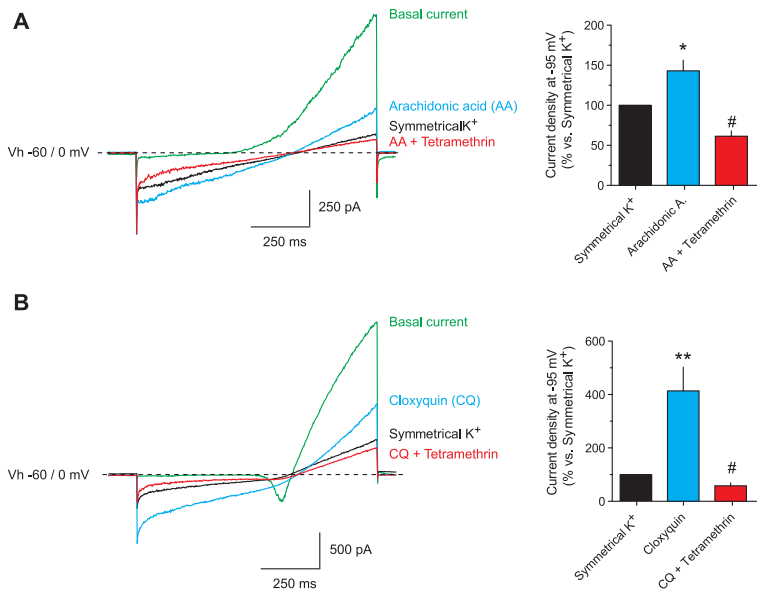


Figure 3. Pyrethroids inhibit native background currents in sensory neurons. (A and B) Representative recordings of whole-cell currents from small/medium-sized DRG sensory neurons. Depolarizing ramps from -100 to $+50$ mV were recorded from a holding voltage of -60 mV in physiological extracellular solution containing TEA (1 mM), 4-AP (1 mM), CdCl₂ (100 μ M), CsCl (1 mM), iberitoxin (50 nM), and TTX (1 μ M). Then, the extracellular solution was exchanged by a high K⁺ solution (135 mM) containing the same blockers. In near symmetrical K⁺ concentration, the current measured at hyperpolarized potentials should be mainly carried by background K⁺ channels. A known activator of TREK-1, TREK-2, and TRAAK (arachidonic acid, AA; 10 μ M; A) or TRESK (cloxyquin, CQ; 100 μ M; B) was used to specifically increase the current mediated by these channels. The effect of tetramethrin (TM, 20 μ M) was then evaluated. Bar graphs show the quantification of the currents at -95 mV expressed as % of current recorded in symmetrical K⁺ conditions. Data are expressed as mean $\pm$ SEM of 5 to 6 neurons recorded in each condition. * $P < 0.05$, ** $P < 0.01$ paired Student t test vs symmetrical K⁺. # $P < 0.05$; paired Student t test vs current activated by arachidonic acid or cloxyquin. DRG, dorsal root ganglion.

(supplemental Fig. 2, available at <http://links.lww.com/PAIN/A485>). In isolated membrane patches, an even greater inhibition of mTRESK current was observed after the application of TM 20 μ M (75.6%; $n = 7$ independent patches) compared with whole-cell effects.

Similarly, human TREK-2 (hTREK-2) and mouse TRAAK (mTRAAK) currents were inhibited by TM application, with IC₅₀ values of 75.6 and 12.6 μ M, respectively (Figs. 4E–H). The effect of TM on TREK-1 was slightly different to its fellow family members and the dose–response relationship showed a biphasic curve: at the lower range of concentrations tested (2–20 μ M), a stimulatory effect was observed while at a higher concentration (100 μ M), the effect was an inhibition (Figs. 4D, G, and H). Nevertheless, the inhibitory effect on TREK-1 was much smaller than those observed on the other K_{2P} channels tested. Altogether, the results obtained show that pyrethroids exert an inhibitory effect on the principal K_{2P} channels expressed in small- and medium-diameter sensory neurons, suggesting that the effects of pyrethroids on these channels could contribute to depolarize and activate these neurons.

3.3. Tetramethrin actions on peripheral terminals of sensory nerve fibers

Using the saphenous nerve-skin preparation, we studied whether pyrethroids could alter sensory transduction in peripheral nerve terminals and, if so, which subsets of somatosensory nerve fibers were affected by exposure to TM. A total of 50 fibers recorded from 29 skin flaps were examined (Table 1). Among those, 27 were C-units (mean CV: 0.51 ± 0.03 m/s), 16 were A δ (mean CV:

5.52 ± 1.0 m/s), and the remaining 7 were fast-conducting A β -fibers (mean CV: 20.2 ± 2.3 m/s) according to the classification provided by Zimmerman et al.⁵²

3.4. Tetramethrin effects on C- and A δ -nociceptors

Our analysis revealed that TM had excitatory effects in a proportion of C- and A δ -nociceptors; however, the type of effect differed between both groups. In addition, the mechanical responsiveness of both groups was differently altered in the presence of TM. At low concentrations (25–50 μ M), TM had an excitatory effect in 6/20 C-mechanosensitive units (mean von Frey threshold 28.5 ± 7.6 mN), whereas vehicle used to dilute TM did not produce significant effects (not shown). Besides, 5 of 7 further C-mechanoinsensitive units were also excited. Within seconds after application started to fire at low frequencies (mean discharge 0.32 ± 0.13 Hz, range 0.04–1 Hz) for ~ 15 minutes (an example of the activation of a C-mechanoinsensitive fiber is shown in Fig. 5; fiber 1). Higher concentrations (100–200 μ M) did not further increase firing rates, but mechanical responses were significantly inhibited in 3 of the mechanosensitive units. In the remaining 14 C-mechanosensitive units unresponsive to TM, a similar inhibition of mechanical responsiveness was observed in 3 units (fiber 2, Fig. 5). In summary, at low concentrations (25–50 μ M), TM evoked ongoing firing in about a third of C-nociceptors. At higher concentrations, TM had a clear desensitizing effect in the mechanical responsiveness of 50% of the mechanosensitive C-nociceptors (from 32 ± 7 to 7 ± 3 spikes, $n = 7$, $P < 0.05$ Wilcoxon matched pair test).

A similar proportion of A δ -nociceptors were excited by TM (6/16) at both low and medium-high concentrations in

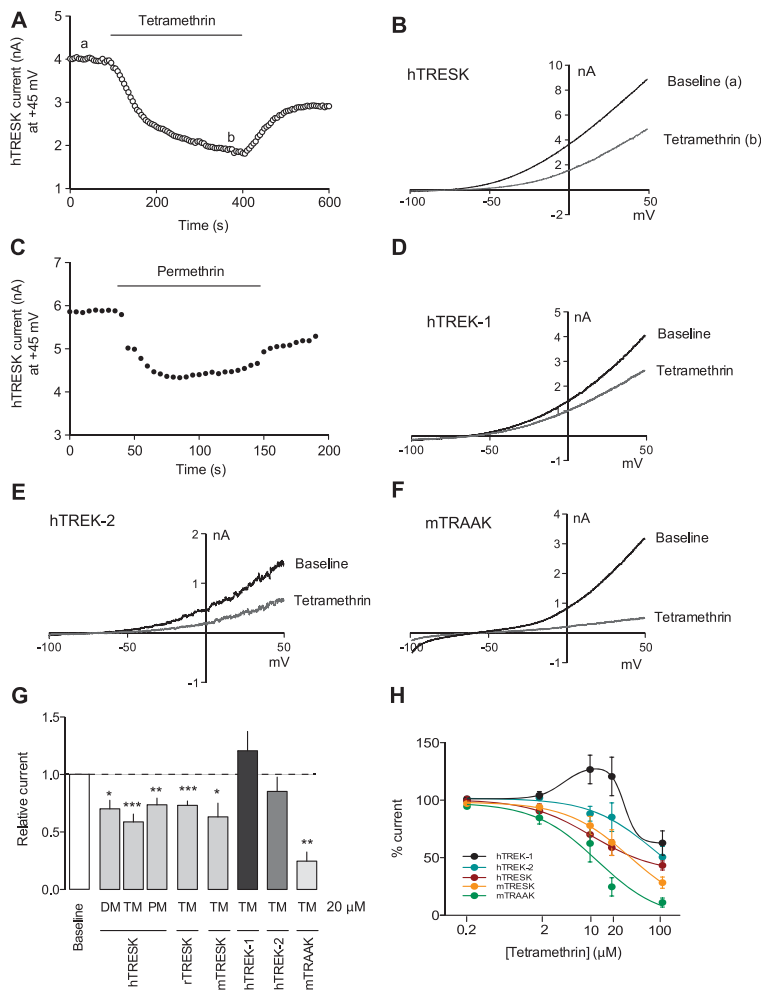


Figure 4. Pyrethroids inhibit K₂P channels expressed in heterologous systems. (A) Whole-cell current in a HEK293 cell transfected with human TRESK. Depolarizing ramps from -100 to +50 mV every 5 seconds from a holding voltage of -60 mV were applied. Current measured at +45 mV in each ramp is plotted against time. Tetramethrin application (TM, 20 μM) produced a significant decrease in the current. Washout of the drug partially recovered current values. (B) Voltage ramps recorded in baseline (a) and during TM application (b) from the experiment shown in (A). (C) Permethrin (PM, 20 μM), a widely used drug as topical treatment for scabies and head lice, also inhibits hTRESK. TRESK current was recorded as in (A). (D-F) Effect of TM (100 μM) on human TREK-1 (D), human TREK-2 (20 μM, E), and mouse TRAAK (20 μM, F) expressed in HEK293 cells. (G) Quantification of inhibitory effects of pyrethroids in K₂P channels. Currents were recorded as described for the experiment shown in (A). Tetramethrin, deltamethrin (DM), and PM were assayed at 20 μM. In each group, 6 to 10 cells were recorded per condition. Data are expressed as mean ± SEM. **P* < 0.05; ***P* < 0.01; and ****P* < 0.001 Student *t* test vs baseline. (H) Dose-response relationships of hTRESK, mTRESK, hTREK-1, hTREK-2, and mTRAAK.

a concentration-dependent manner (25-100 μM; mean discharge 8.55 ± 7.5 Hz, range 0.26-46 Hz). Most of them (5/6) showed a transient or sustained activity in the form of bursts

Table 1			
Tetramethrin actions on sensory nerve fibers.			
Fibres	C	Aδ	Aβ
Conduction velocity, m/s	0.51 ± 0.03	5.52 ± 1.00	20.2 ± 2.3
Tetramethrin effects			
No. of excited fibres (%)	9 (33)	6 (37)	5 (71)
Response to vFrey	Desensitized	Sensitized	Sensitized
Total number of fibres	27	16	7

characterized by apparent intraburst frequencies above 200 Hz (see fiber 1 in Fig. 6A and B for a representative example). The effect of TM on mechanical responses was evaluated in 13 of the 16 mechanosensitive units (mean von Frey threshold 9.27 ± 2.1 mN). In 10 of the units, the characteristic tonic discharge changed to a series of bursts of high frequency (up to 750-Hz intraburst frequency) as shown in Figure 6C. A desensitizing effect was only observed in one other Aδ-unit in which a single application of 200 μM inhibited evoked discharges from the exposed RF.

3.5. Tetramethrin effects on Aβ-fibers

Tetramethrin excited 5/7 fibers at all concentrations assayed, within seconds of application the fibers started to fire at low

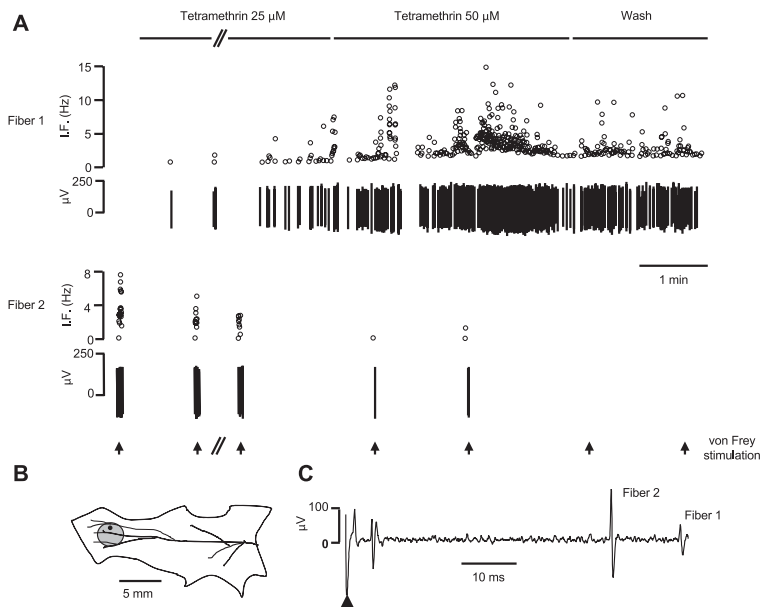


Figure 5. Tetramethrin (TM) activates cutaneous somatosensory C-fibers. Original recording of 2 single C-fibers recorded from the same nerve filament using the saphenous nerve-skin preparation. (A) The upper panel shows the firing of fiber 1 as instantaneous frequency (IF). This unit, which was mechanoinensitive, exhibited an ongoing, dose-dependent, transient response to TM application. Washout started as soon as the response began to decline. The lower panel shows the mechanical responses of fiber 2 to a 16-mN vFrey filament which decreased and vanished during and after TM superfusion (>20 minutes after washout). Arrows under the traces mark application of vFrey filament for 5 seconds. The gap (//) represents a 5-minute interval. (B) Representation of the preparation with the mechanical receptive field of fiber 2 indicated by the gray circle. The black dot marks the site of electrical stimulation. (C) Electrically evoked responses of the fibers studied. According to latency and the recording distance (24 mm), the calculated conduction velocity of fibers 1 and 2 were 0.48 and 0.4 m/s, respectively. The arrowhead indicates the stimulation artifact.

frequency and in a concentration-dependent manner (mean discharge 5.4 ± 1.9 Hz, range 0.59–10.65 Hz). Furthermore, the characteristic tonic discharge evoked by mechanical stimulation changed to a series of bursts of very high frequency in the 4 fibers tested (up to 1000-Hz intraburst frequency).

3.6. Behavioral effects of pyrethroids

Because our data show that pyrethroids activate a subset of small- and medium-sized sensory neurons as well as nerve fibers that are thought to be involved in nociception, we examined the possible analgesic effects of TM by measuring mouse somatosensory behaviors. Injection of TM (10 μ L, 100 μ M) into the hind paw produced a significant increase in nocifensive behaviors (13.3 ± 2.4 flinches/lickings; $n = 12$; $P < 0.001$) compared with vehicle injection (0.3 ± 0.2 ; $n = 10$; **Fig. 7A**). Moreover, TM decreased the mechanical threshold for paw withdrawal measured using von Frey filaments 15 minutes after injection, compared with the value obtained 30 minutes before injection (**Fig. 7B**). Vehicle injection did not produce significant effects on mechanical threshold. By contrast, noxious thermal sensitivity was not significantly altered by TM exposure in the radiant heat paw withdrawal test (**Fig. 7C**). Commercial products for the treatment of head lice in humans or insecticides usually contain pyrethroid concentrations ranging between 0.25% and 1.5% (between 8 and 40 mM depending on the compound). To assess whether a relevant concentration used in commercial applications significantly affect nociceptor activity, we applied TM 1 mM to the corneal surface, which is similar to what occurs when

a head lice shampoo gets to the eye. Blinking response, as a surrogate of nocifensive behavior, was largely increased on TM application compared with control vehicle (43.3 ± 9.5 vs 12.2 ± 1.6 blinks per 2 minutes; $n = 5$ each; $P < 0.01$). These results indicate that pyrethroid exposure produces painful responses and increases basal sensitivity to mechanical stimuli.

3.7. Lack of TRESK expression enhances pyrethroids effects

As mentioned earlier, K_{2P} channels play a significant role in preventing sensory neuron depolarization. Therefore, inhibition of K_{2P} channels by pyrethroids may contribute to increased neuronal excitability. Alternatively, although pyrethroids have inhibitory effects on K_{2P} channels, the remaining K^+ background current may still be acting as a “brake” to prevent excessive depolarization of sensory neurons. Our results (**Figs. 3 and 4**) demonstrated that one of the major inhibitory effects of pyrethroids is on TRESK current and, therefore, we examined whether a total lack of this channel further enhances or ameliorates pyrethroid effects. Using DRG neurons from TRESK KO animals, we examined the responses to TM in current-clamp and calcium imaging experiments similar to the analysis performed in WT animals. Twenty-four DRG neurons from TRESK KO mice were recorded in current-clamp mode with a mean capacitance of 20.5 ± 1.6 pF and a resting membrane potential of -58.0 ± 1.5 mV, which were not different from WT neurons. Twelve of these neurons were significantly depolarized (19.5 ± 4.4 mV) and fired action potentials in response to TM (50% response to 20 μ M TM, data not shown). The remaining 12

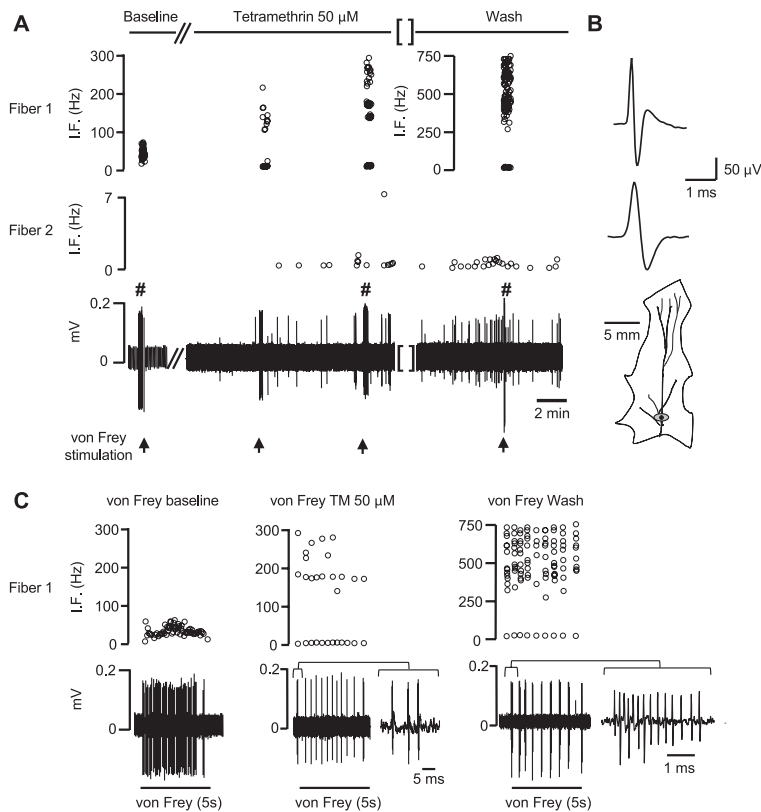


Figure 6. Pyrethroid exposure alters the firing pattern of cutaneous somatosensory fibers. Electrophysiological responses of 2 fibers recorded within the same filament before, during, and after application of TM. (A) Left panel: an A δ -fiber (fiber 1) responded with an ongoing discharge when stimulated with a gravity-driven von Frey filament (11.4 mN). Fiber 2 was a C-type mechanosensitive fiber. Central panel: the basal activity of fiber 1 was unaffected but the responses to the 11.4 mN von Frey (arrow) consisted in a high-frequency burst, which built with time. Fiber 2 becomes activated after application of 50 μ M TM. Right panel: the effects of TM on both fibers were irreversible and lasted >30 minutes after wash. (B) Averaged waveforms (>20 spikes) of fiber 1 and 2 in an expanded time base. The mechanical receptive field of fiber 1 (marked in gray) is shown below. The black dot indicates the site for electrical stimulation. Gap (/) represent a 5-minute interval and gap [] a 30-minute interval. (C) Expanded responses to von Frey application before (baseline), during application of 50 μ M TM, and after washout in fiber 1 (marked with # in the original recording shown in (A)). The responses are presented as the original recording and as instantaneous frequency (IF). A burst in each condition has been further expanded to emphasize the presence of bursts with very short interspike intervals. TM, tetramethrin.

neurons only showed a small depolarization (3.0 ± 2.6 mV) that was insufficient to trigger an action potential. When the effect of TM was assayed in calcium imaging experiments, 259 of 475 neurons elicited a significant intracellular calcium increase (54.5% response; **Figs. 8A and B**), which was similar to the value obtained in WT animals. Interestingly, the number of neurons showing oscillations in intracellular calcium levels was markedly decreased (30.2% compared with 64.5% in WT neurons; $P < 0.001$; **Figs. 8A and B**), and most neurons elicited a single and wider calcium peak in response to TM with a mean fold Ca^{2+} increase of 1.83 ± 0.04 , which was significantly greater compared with WT neurons ($P < 0.001$). Similarly to WT animals, the neurons activated by TM in KOs were again smaller in size than nonactivated neurons (**Fig. 8C**). A significant percentage of neurons responding to TM were positive for TRPA1 (22.0%) and TRPV1 (66.7%; **Fig. 8D**), whereas a small proportion was positive for TRPM8 (4%; menthol positive, AITC negative). In summary, our electrophysiological and calcium imaging data showed similar overall effects of TM on primary sensory neurons from WT and KO animals. To further evaluate the contribution of

TRESK, in WT animals, we pretreated neurons with cloxyquin (50 μ M) to enhance TRESK currents. In these conditions, the percentage of neurons activated by TM application in Ca^{2+} imaging experiments was significantly decreased (supplemental Fig. 3, available at <http://links.lww.com/PAIN/A485>) as well as the number of neurons showing an oscillatory calcium pattern. These results suggest that TRESK function is important to prevent sensory neuron activation and that inhibition of the channel plays a significant role in the activation of these neurons by TM.

We next tested whether deleting TRESK had any effects on TM responses at the behavioral level. Tetramethrin injection in the hind paw produced a significant increase in nocifensive behaviors compared with vehicle injection (TM: 29.5 ± 4.9 ; $n = 10$; vehicle: 0.5 ± 0.3 flinches/lickings; $n = 8$; $P < 0.001$; **Fig. 7A**). Comparison with WT responses revealed a significant increase in TM-induced nocifensive behaviors in KO animals ($P < 0.01$ vs TM in WT).

When mechanical sensitivity was assessed with von Frey filaments in KO animals under baseline conditions, the withdrawal threshold was found to be greatly reduced compared with WT

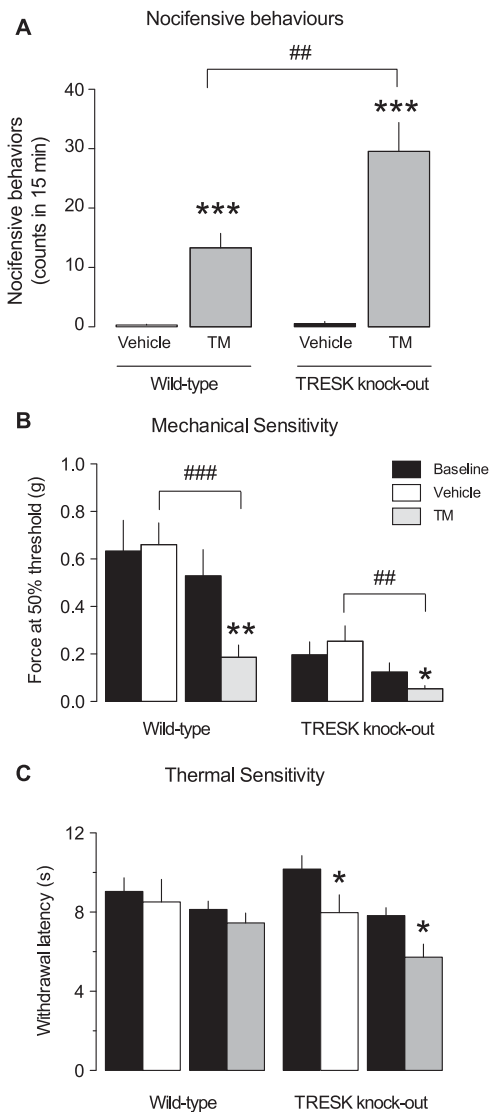


Figure 7. Pyrethroids produce pain-associated behavioral responses and decrease the threshold for mechanical stimuli. (A) Quantification of the number of flinching and licking behaviors after intradermal injection of tetramethrin (TM, 5 μ L, 100 μ M) or vehicle in the hind paw for wild-type and TRESK knockout mice. The mean number of counts over a period of 15 minutes is shown ($n = 6$ –10 animals per group). *** $P < 0.001$ Student t test vs vehicle injection. ## $P < 0.01$ Student t test WT vs KO mice. (B) Mechanical sensitivity: von Frey response thresholds before (baseline) and 15 minutes after vehicle or TM injection (100 μ M) for wild-type and TRESK knockout animals. * $P < 0.05$; ** $P < 0.01$ Student t test vs baseline. ### $P < 0.01$; #### $P < 0.001$ Student t test vehicle vs TM. (C) Thermal sensitivity: latency of response to radiant heat stimuli applied to the hind paw of wild-type and TRESK knockout mice before (baseline) and after vehicle or TM injection (100 μ M). * $P < 0.05$; Student t test vs baseline.

animals (WT: 0.58 ± 0.08 vs KO: 0.15 ± 0.03 g; $P < 0.001$; $n = 22$ and 18 respectively), indicating that TRESK might play a role in the neuronal pathways detecting mechanical stimuli. The withdrawal threshold was further decreased after TM injection

compared with vehicle in TRESK-deficient animals ($P < 0.01$; Fig. 7B). The mechanical threshold was also significantly smaller after TM application ($P < 0.05$) compared with the result obtained in WT animals. When thermal sensitivity was evaluated, no differences were found in baseline latencies between WT and KO animals. TM injection in the KO animals produced a small but significant decrease in latency compared with baseline levels, but this effect was also observed after vehicle injection, which could be related to an effect of the injection procedure specific to KO animals.

4. Discussion

Dermal exposure to pyrethroids causes paraesthesias (tingling, itching, burning, and stinging sensations) due to their ability to activate sensory neurons. Besides their well-known effects on Na_v channels, we explored whether other conductances present in the action potential subthreshold region can also be affected, in particular, the K_{2P} family of background channels. We report that pyrethroids inhibit K_{2P} channels and activate a large percentage of DRG neurons, many of them positive for distinct nociceptive markers (TRPV1 and TRPA1). This correlates well with activation of A δ - and C-fibers in response to TM as well as the nociceptive behaviors observed in mice.

Depolarization-induced neuronal firing observed after pyrethroid application is likely the result of either activation of a depolarizing conductance, inhibition of a K^+ current, or both. Recordings of small sensory neurons in the presence of a cocktail of ion channel blockers that should minimize the contribution of Na_v , K_v , K_{Ca} , and Ca_v channels, but that do not block K_{2P} s indicated that the TM-inhibited current had the kinetic characteristics of a K^+ current. Nevertheless, any TTX-resistant Na_v channels present could still be activated. In a different set of experiments, background currents were enhanced by arachidonic acid, a known activator of TREK-1/TREK-2/TRAAK, or by cloxyquin, that activates TRESK, allowing us to test the effects of TM in native cells. In both groups, TM produced a significant inhibition of the currents mediated by these channels. Similar inhibitory effects were corroborated in HEK293 cells expressing each type of channel. Taken together, our data indicate that pyrethroids inhibit K_{2P} channels.

Acute and chronic effects of pyrethroids on $\text{Na}_v1.8$ and $\text{Na}_v1.6$ are well characterized,^{7,21,33} but these effects are unlikely to participate in a subthreshold depolarization because they are not active at rest. We and others found that responses to pyrethroids are still present in the presence of TTX (Fig. 2),²¹ thus the TTX-sensitive $\text{Na}_v1.7$ does not seem to be necessary for pyrethroid effect. A possible effect on the TTX-resistant, noninactivating $\text{Na}_v1.9$ channel (expressed in nonpeptidergic C-fibers) might be more relevant. Acute exposure to PM increased $\text{Na}_v1.9$ current in muscle and vascular nociceptors, whereas chronic exposure produced an increased current without changing its voltage dependence.³² These acute and especially chronic effects have been implicated in the etiology of Gulf War Illness, a chronic pain syndrome linked to soldiers' exposure to pesticides and repellants, which contained significant amounts of PM.⁵ In view of our data, the participation of K_{2P} s should also be considered in the pathophysiological mechanism.

Currents mediated by K_v7 channels were initially upregulated and later downregulated on chronic exposure to pyrethroids.^{33,34} Initial upregulation was attributed to compensatory effects on nociceptor activation and the subsequent current decrease to persistent toxicity. Significant downregulation of delayed rectifier K^+ currents has also been reported after chronic exposure,

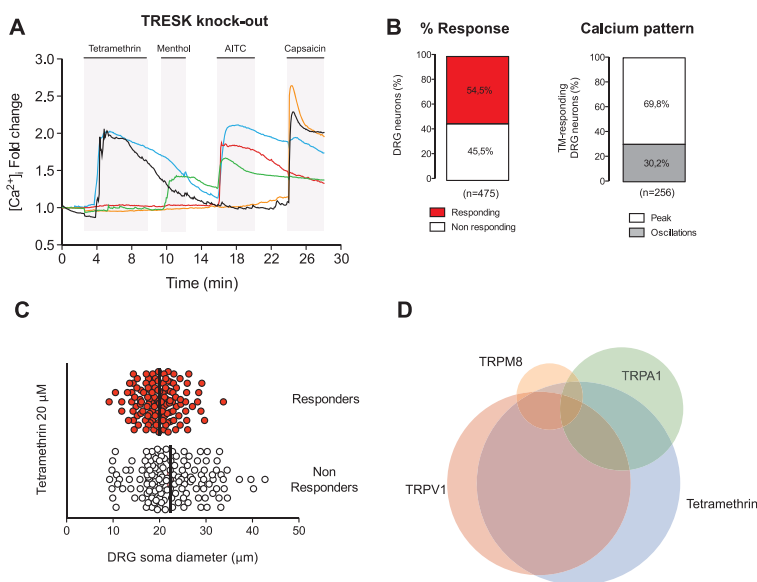


Figure 8. Tetramethrin (TM) effects on TRESK knockout sensory neurons. (A) Representative recordings of intracellular calcium in TRESK knockout mouse DRG sensory neurons. Tetramethrin (20 μ M), menthol (100 μ M), allyl isothiocyanate (AITC, 100 μ M), and capsaicin (1 μ M) were assayed. Tetramethrin activated a similar percentage of cells compared with wild-type neurons but a greater percentage of cells showed a single, larger calcium peak rather than an oscillatory pattern. (B) Left: quantification of the percentage of response to TM. Right: percentage of TM-responding cells showing an oscillatory calcium pattern or responding with a single calcium peak. (C) Soma diameter distribution of TRESK knockout DRG neurons displaying sensitivity and insensitivity to TM. Black bars show the mean soma value in each group. (D) Venn diagram showing the relative size and overlap between the population of TM-activated neurons and these activated by menthol, AITC, or capsaicin in the knockout mice. DRG, dorsal root ganglion.

coincident with the presentation of pain behaviors in rats.³⁴ These data suggest that the combined action of pyrethroids on different channels leads to neuronal activation and enhanced nociceptor excitability because of Na^+ currents enhancement, decreased K^+ currents, and possibly increased Ca^{2+} entry because of action potential broadening.

In heterologous systems and sensory neurons, pyrethroids significantly inhibited K_{2P} currents, similar to hydroxy- α -sanshool, a compound extracted from Szechuan peppercorns, which inhibits TRESK, TASK-1, and TASK-3 and activates sensory neurons.⁴ Sanshool also inhibits $Na_v1.3$, $Na_v1.7$, and $Na_v1.8$,⁴⁴ and the subsequent blocking effect on action potential firing is thought to mediate its analgesic effects by silencing A δ -nociceptors.⁴⁴ The synthetic alkylamide isobutylalkenyl amide, a sanshool derivative, also inhibits TRESK, activates sensory neurons, and produces tingling, numbing, and burning sensations in humans and painful behaviors in rodents.^{2,40,45} Another alkylamide insecticide, BTG502, structurally related to sanshool, inhibits Na_v channels by binding to a site that overlaps with the pyrethroid-binding site.¹⁷ These different compounds exhibit characteristic somatosensory effects via their actions on both Na^+ and K^+ channels expressed in sensory neurons and resulting sensations might depend on the particular neuronal subtypes affected. Effects on channels such as TRPs cannot be completely discarded, but these seem unlikely because we could find neurons not activated by TRPV1, TRPA1, or TRPM8 agonists that were still activated by TM (Fig. 1).

In peripheral nerve fibers, pyrethroids at low concentrations (20–50 μ M), activated A β -, A δ -, and C-fibers or potentiated their activation by other stimuli (eg, mechanical). Higher concentrations (100 to 200 μ M) usually produced desensitization and

inhibition of any previous mechanical response. Similar activating effects were reported for sanshool in A β -, A δ -, and C-fibers which were associated with modulation of TRESK, TASK-1, or TASK-3 channels.²⁵ Later, in fibers where sanshool did not evoke excitation, analgesic effects were linked to suppression of mechanical responses of somatosensory A β -mechanoreceptors and A δ -mechanonociceptors, through inhibition of Na^+ channels.⁴⁴ Pyrethroids might induce similar effects depending on the concentration and the nerve fiber targeted, thus at low concentrations, activation or sensitization of C-fibers would be prominent, likely due to a combination of effects on Na_v and K_{2P} channels. This is well correlated with the increase observed in nocifensive behaviors when TM is injected into the mouse hind paw, which decreases mechanical withdrawal threshold. By contrast, high concentrations appear to desensitize C-fibers by sustained depolarization, possibly due to combined effects on $Na_v1.9$ and K_{2P} inhibition. This persistent depolarization will inactivate Na_v channels and decrease mechanical responsiveness of nociceptive fibers. In addition, TM sensitized A β - and A δ -fibers to mechanical stimulation, even in fibers not directly activated by the compound. Often, bursting activity in response to mechanical stimulation was observed, which has also been reported after sanshool application and attributed to K^+ channel inhibition.²⁵

TREK-1, TREK-2, and TRAAK are involved in the transduction of mechanical, noxious, and non-noxious thermal stimuli by sensory neurons.^{3,31,36} Despite TM inhibition of these channels, we did not find significant effects on thermal sensitivity in mice, although a more detailed characterization would be required. By contrast, as in nerve fibers, mechanical sensitivity was enhanced, providing strong evidence that the effects of pyrethroids are

prominent in mechanoreceptive neurons. TRESK might be involved in the transduction of mechanical stimuli, not as a direct sensor but by regulating the excitability of sensory neurons involved in mechanodetection. TRESK is thought to prevent neuronal depolarization,¹⁸ and decreases in its expression after nerve injury or inflammation could contribute to neuronal hyperexcitability.^{15,28,45} In support of this role, we found an increased sensitivity to mechanical stimulation in TRESK KO animals, which is then further enhanced by TM exposure. In previous studies, knocking down TRESK using an siRNA increased mechanical sensitivity,⁴⁵ which is consistent with a lack of this channel enhancing the peripheral and/or central excitability of the nociceptive pathway. Interestingly, TM still enhanced mechanical sensitivity in TRESK KO mice, suggesting that TRESK is not the only target of TM and that the absence of TRESK further potentiates the effects on other channels rather than preventing TM effects. We could hypothesize that the pharmacological inhibition of TRESK by TM in WT animals might contribute to the increased mechanical sensitivity because knocking down the channel produces a similar effect. A role for other channels, including K_{2P} s is also possible.

According to recent reports, TRESK expression is restricted to some subtypes of sensory neurons,^{12,46} thus it is possible that despite the lack of this channel, remaining effects on other channels/neuronal subtypes are still prominent. Our analysis of the response of populations of primary sensory neurons did not reveal significant differences in the percentage of neurons activated by TM between WT and KO animals. However, calcium oscillations were seen in a large percentage of cells from WT animals while most cells in KO animals showed a single, longer calcium peak (Figs. 1A, B, 8A, and B). This larger calcium increase can produce a bigger neuronal activation or increased neurotransmitter release at the spinal synapse, thus producing greater pathway activation. In agreement, behavioral effects to TM injection were enhanced in KO mice. TRESK deletion might also increase the excitability of dorsal horn neurons because the channel was initially identified in the spinal cord, where it is highly expressed,³⁹ and recent reports indicate that TRESK might play a role in the modulation of nociceptive information at this level.^{10,20} We did not explore further the change in the calcium pattern, but it is tempting to speculate that the TRESK activation by Ca^{2+} /calcieneurin^{13,14} might limit depolarization and Ca^{2+} influx, thus preventing excessive neuronal activation. In the absence of the channel, the loss of this Ca^{2+} /calcieneurin-activated K^{+} current will result in a more prolonged depolarization and a larger and longer Ca^{2+} influx. The results presented here provide the first demonstration that pyrethroids can inhibit K_{2P} channels contributing to activation of pain pathways, where low concentrations will promote burning, itching, and stinging sensations, whereas desensitizing effects produced at higher concentrations can mediate tingling or numbing sensations.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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A. Castellanos, A. Andres, G. Callejo, and X. Gasull performed electrophysiological recordings in neurons and cell lines and calcium imaging. A. Castellanos performed behavioral experiments. A. Castellanos, A. Andres, N. Comes, and J. P. Giblin performed cellular cultures, plasmid generation, and transfection. L. Bernal and C. Roza performed skin-nerve recordings. A. Castellanos, A. Andres, A. Gual, N. Comes, and X. Gasull participated in the design of the study and performed the statistical analysis. X. Gasull conceived the study, oversaw the research, and prepared the manuscript with help from all others. All authors read and approved the final manuscript.

Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at <http://links.lww.com/PAIN/A485>.

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Anionic Phospholipids Bind to and Modulate the Activity of Human TRESK Background K⁺ Channel

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Abstract

The background K⁺ channel TRESK regulates sensory neuron excitability, and changes in its function/expression contribute to neuronal hyperexcitability after injury/inflammation, making it an attractive therapeutic target for pain-related disorders. Factors that change lipid bilayer composition/properties (including volatile anesthetics, chloroform, chlorpromazine, shear stress, and cell swelling/shrinkage) modify TRESK current, but despite the importance of anionic phospholipids (e.g., PIP₂) in the regulation of many ion channels, it remains unknown if membrane lipids affect TRESK function. We describe that both human and rat TRESK contain potential anionic phospholipid binding sites (apbs) in the large cytoplasmic loop, but only the human channel is able to bind to multilamellar vesicles (MLVs), enriched with anionic phospholipids, suggesting an electrostatically mediated interaction. We mapped the apbs to a short stretch of 14 amino acids in the loop, located at the membrane-cytosol interface. Disruption of electrostatic lipid-TRESK interactions inhibited hTRESK currents, while subsequent application of Folch Fraction MLVs or a PIP₂ analog activated hTRESK, an effect that was absent in the rat ortholog. Strikingly, channel activation by anionic phospholipids was conferred to rTRESK by replacing the equivalent rat sequence with the human apbs. Finally, in the presence of a calcineurin inhibitor, stimulation of a G_{q/11}-linked GPCR reduced hTRESK current, revealing a likely inhibitory effect of membrane lipid hydrolysis on hTRESK activity. This novel regulation of hTRESK by anionic phospholipids is a characteristic of the human channel that is not present in rodent orthologs. This must be considered when extrapolating results from animal models and may open the door to the development of novel channel modulators as analgesics.

Keywords K_{2P} channels · KCNK · Membrane phospholipids · Nociception · Neuronal excitability

Introduction

TWIK-related spinal cord K⁺ channel (TRESK) or K_{2P}18.1 (encoded by the *KCNK18* gene) is a member of the two-pore domain potassium channel (K_{2P}) family, which contains 15 members with a shared molecular architecture. K_{2P} channel proteins are comprised of four transmembrane domains and two pore-loop forming domains with intracellular N- and C-

termini [1]. Four pore-loops are required to form a functional K⁺ selectivity filter and therefore K_{2P} channels function as dimers, unlike the other K⁺ channel subfamilies (voltage-gated, calcium-activated, and inwardly rectifying K⁺ channels), which function as tetramers [2, 3]. K_{2P} channels play an important role in the maintenance and stabilization of the resting membrane potential, and in excitable cells such as neurons and cardiac myocytes, they modulate the shape and frequency of action potentials. They have been implicated in a wide range of physiological processes including nociception, somatosensation, nutrient and chemo-sensing, hormone secretion, sleep, and anesthesia [4–7].

The coding sequence of the human *KCNK18* gene was originally identified by a homology search of the draft human genome using the amino acid sequence of a previously identified K_{2P} channel, K_{2P}1.1 (also known as TWIK1, tandem of pore domains in a weak inwardly rectifying K⁺ channel), which led to its cloning from human spinal cord mRNA [8]. TRESK is selectively expressed in a subpopulation of sensory neurons of the dorsal root (DRG) and trigeminal ganglia (TG), which

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innervate peripheral regions of the body and are responsible for the detection of both innocuous (e.g., touch) and noxious chemical, thermal, and mechanical stimuli [9–14]. Studies on primary cultures demonstrated that TRESK accounts for a significant proportion of the “resting” or background K^+ conductance in DRG neurons from mouse and rat [11, 15]. The first evidence of a role for TRESK in the regulation of sensory neuron excitability came when DRG neurons from a mutagenized mouse strain carrying a loss-of-function mutation in the *Kcnk18* gene were compared with wild-type DRG neurons, revealing that the TRESK-mutant neurons were easier to excite by depolarizing stimuli [11]. Subsequent studies have demonstrated that pharmacological inhibition of TRESK by alkylamides, such as hydroxy- α -sanshool and isobutylalkylamide (IBA) or pyrethroids, produces sensory neuron activation and contributes to associated somatosensory and nocifensive behaviors [9, 10, 16, 17]. A dominant negative loss-of-function mutation in the *KCNK18* gene is linked to familial migraine with aura, and over-expression of mouse TRESK with the equivalent mutation in trigeminal neurons was shown to cause hyperexcitability, suggesting that TRESK is required for correct regulation of trigeminal neuron excitability [18, 19]. An alteration of TRESK functional expression may also be a contributing factor to the changes in sensory neuron excitability observed during inflammation and neuropathic pain since *Kcnk18* mRNA and protein expression are decreased in rat models of neuropathic pain [10, 20] and TRESK activity is inhibited in the presence of inflammatory mediators such as arachidonic acid [8, 21]. Increased TRESK activity as a consequence of over-expression was shown to dampen the excitability of TG and DRG neurons and could attenuate nerve-injury-induced allodynia, leading to the suggestion that augmenting TRESK activity may be a viable therapeutic strategy for the treatment of pain-related conditions [20, 22–24]. A better understanding of the underlying mechanisms that influence TRESK activity is essential for the rational design of novel channel modulators.

The activity of TRESK can be modulated by stimuli that produce changes in the composition and/or properties of the plasma membrane. In a recent study from our laboratory, we demonstrated that TRESK activity can be modulated by experimentally applied shear stress, changes in osmotic pressure, and by compounds that affect membrane bilayer properties, such as chloroform and chlorpromazine [21]. Volatile (e.g., isoflurane) and local (e.g., bupivacaine) anesthetics are potent enhancers and inhibitors of TRESK currents, respectively [25]. Although the precise molecular mechanisms of action are unclear, volatile anesthetics appear to modify ion channel activity via their incorporation into lipid bilayers and by binding to amphiphilic sites on channel proteins [26].

TRESK currents are strongly potentiated by increases in intracellular Ca^{2+} -concentration via Ca^{2+} -dependent binding of the activated phosphatase calcineurin to the channel, which mediates dephosphorylation of inhibitory phosphorylation

sites [27, 28]. This mechanism underlies the enhancement of TRESK activity observed upon $G_{q/11}$ -coupled receptor stimulation [27, 29, 30]. However, G_q -coupled receptor activation can also produce large decreases in the plasma membrane concentration of the phosphoinositide phosphatidyl-inositol-4,5-bisphosphate (PIP_2) via the stimulation of phospholipase C (PLC) activity, which hydrolyses PIP_2 to produce diacylglycerol (DAG) and IP_3 [31–33]. It is also of note that large influxes of Ca^{2+} via TRP channels that co-express with TRESK in sensory neurons have also been shown to have significant effects on global PIP_2 levels via activation of Ca^{2+} -activated PLC isoforms, for example, the PLC δ s, which are expressed in sensory neurons [34, 35]. Given the importance of PIP_2 in the regulation of the activity of a wide variety of ion channels and transporters [36] and the evidence for the role of PIP_2 in the regulation of other K_2P channels [37–39], it is feasible that changes in plasma membrane PIP_2 concentration may also contribute to physiological modulation of TRESK activity.

It is clear that the membrane environment and changes in lipid composition play an important role in the modulation of TRESK. However, there is no information to date regarding interactions between TRESK and membrane phospholipids and the consequences for channel function. In this study, we have investigated whether TRESK contains binding sites for membrane phospholipids and whether these interactions play a role in the modulation of channel activity.

Materials and Methods

In Silico Search for Putative Anionic Phospholipid Binding Sites

The primary sequences of human (GenBank accession number NP_862823.1) and rat (GenBank accession number AAS68516.1) TRESK were analyzed using the BH search program (available at <http://helixweb.nih.gov/bhsearch>). The threshold BH value for residues forming part of a potential membrane binding site was 0.6, and the search was performed with a window size of 10 to obtain scores for N- and C-terminal residues [40]. A search for the most common putative lipid-binding domains (the PH, PKC C1, PKC C2, PX, FYVE, GLA, GRAM, F-BAR, and ENTH domains) in human and rat TRESK was undertaken using the SMART program [41, 42].

Molecular Biology

Rat TRESK in the pcDNA3.1(+) vector (kindly provided by Dr. S. Yost, University of California-San Francisco, USA) was subcloned into the pEGFP-C3 vector (Clontech) using a *BamHI/XbaI* digest and used for transient transfection of cell

lines as previously described [21]. Human TRESK pcDNA3.1(+) vector was kindly provided by Dr. Y. Sano (Astellas Pharma Inc., Ibaraki, Japan) and subcloned into pEGFP-C2 (Clontech) vector with an *EcoRI/SmaI* digest [21]. pEGFP-mTRESK-1 was a kind gift from Dr. G. Sandoz (CNRS-Universite de Nice-Sophia Antipolis, France). pRK5-HA-mGluR5 vector was kindly provided by Dr. F. Ciruela (University of Barcelona, Spain). pNice-CiVSP was kindly provided by Dr. S.C. Kohout (Montana State University, MT, USA). hTRPA1-GFP was a kind gift from Dr. F. Viana (Instituto de Neurociencias-CSIC, Alicante, Spain).

GST-Fusion Proteins PCR fragments of the relevant portions of human or rat TRESK were produced, with addition of a 5' *BamHI* and 3' *XhoI* sites using primer 5' overhangs before subcloning into the pGEX-5X-3 vector (GE Healthcare) with a *BamHI/XhoI* digest. The in-frame stop codon present in the pGEX-5X-3 polylinker was used in all fusion proteins except for GST-hTRESK_(356–384), where a stop codon was added on the 5' overhang of the reverse primer. The sequences of the oligonucleotide primers used to generate each fusion protein are provided in [supplementary methods](#).

Human-Rat TRESK Chimeras Human-rat TRESK chimeras were constructed using a splicing by overlap extension PCR strategy. A vertebrate Kozak sequence was added to the 5' overhang of the forward flanking primer (GCCGCCACC) before the start codon to optimize protein expression of the chimeric constructs. For hTRESK_(1–163)-rTRESK_(185–405) (hTRESK-rat-loop-rTRESK) and rTRESK_{hTRESK(163–177)} (rTRESK-h-apbs), spliced PCR products were subcloned into pcDNA3.1(+) (ThermoFisher Scientific) with *BamHI/EcoRI* digests. The rTRESK_(1–184)-hTRESK_(163–384) (rTRESK-human-loop-hTRESK) and hTRESK_{rTRESK(184–197)} (hTRESK-r-apbs) constructs were subcloned into pcDNA3.1(+) with *BamHI/XhoI* digests. All restriction enzyme sites were added using primer 5' overhangs. The sequences of the oligonucleotide primers used to generate each chimera are provided in [supplementary methods](#).

Purification of GST Fusion Proteins

Growth of Bacterial Cultures Plasmids encoding the relevant fusion proteins were transformed into *Escherichia coli* (strain BL21, Novagen). Cultures were grown at 37 °C in minimal medium (0.4% (w/v) glucose, 1% (w/v) tryptone) supplemented with M9 salts (48 mM Na₂HPO₄, 22 mM KH₂PO₄, 8.6 mM NaCl, 18.7 mM NH₄Cl), 2 mM MgSO₄, 0.1 mM CaCl₂, and 100 mg/ml ampicillin until the OD₆₀₀ reached 0.5–0.7. After reaching the desired OD₆₀₀, expression of the fusion proteins was induced by addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM followed by incubation at 25 °C for 3 h. Note that

GST alone was induced with 1 mM IPTG. Bacteria were harvested by centrifugation at 3000g for 30 min at 4 °C, and pellets were stored at –20 °C until use in the purification procedure.

Purification Procedure Frozen pellets were resuspended in purification buffer supplemented with a protease inhibitor cocktail (phosphate-buffered saline [containing CaCl₂ and MgCl₂, Sigma-Aldrich, Spain], 0.5% (v/v) Tween-20, 0.5 mM EDTA, 0.5 mM PMSF, 1 μg/ml Aprotinin, 1 μg/ml Pepstatin, 1 μg/ml Leupeptin, and 1 μg/ml Antipain). Bacteria were subsequently lysed using a probe sonicator, and insoluble material was removed by centrifugation at 20,000g for 30 min at 4 °C. The supernatant was applied to a column containing Glutathione Sepharose (GE Healthcare, Spain), which had been pre-equilibrated with purification buffer, and allowed to flow through under gravity. The column was washed three times with 10 column volumes of purification buffer followed by two washes with 10 column volumes of purification buffer (without Tween-20). After washing, fusion proteins were eluted with 50 mM TrisHCl, pH 8 containing 10 mM glutathione. Glycerol was subsequently added to a final concentration of 10% (v/v), and proteins were stored at –20 °C until use in liposome binding assays.

Preparation of Liposomes for Liposome Binding Assay

Preparation of Folch Multilamellar Vesicles (MLVs) MLVs composed of Folch fraction I lipids were prepared from a 100 mg/ml stock solution in chloroform obtained from Sigma-Aldrich (Spain; Ref. B1502). Chloroform from an aliquot of stock lipids was evaporated under a stream of nitrogen gas and the residue further dried under a vacuum for 1 h (SpeedVac). Dried lipids were then resuspended in liposome binding buffer (20 mM HEPES KOH pH 7.4, 100 mM KCl, 1 mM EDTA) at a concentration of 1 mg/ml and incubated at 37 °C for 1 h with occasional vortexing. The resuspended lipids were stored at 4 °C and used within 24 h in the liposome-binding assay.

Preparation of MLVs of Defined Phospholipid Composition

MLVs of defined phospholipid composition were prepared at a final concentration of 1 mM by mixing together different molar percentages of chloroform stock solutions of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (PC, Catalogue number 850375P, Avanti Polar Lipids, Alabama, USA), 1,2-dioleoyl-*sn*-glycero-[phospho-L-serine] (PS, Catalogue number 840035P, Avanti Polar Lipids), and 1,2-dioleoyl-*sn*-glycero-3-[phosphoinositol-4,5-bisphosphate] (PIP₂, Catalogue number 850155P, Avanti Polar Lipids) as indicated in the text and figure legends. Dried lipids were then rehydrated by treatment in the same manner as the Folch fraction lipids.

Liposome Binding Assay All steps were performed at room temperature. Five micrograms of purified GST fusion protein was incubated with 100 μ g liposomes in a total volume of 150 μ l for 30 min followed by centrifugation at 21,000g for 1 h. After centrifugation, the supernatant was removed and 150 μ l 2 $\times$ Laemmli loading buffer (250 mM Tris HCl pH 6.8, 40% (v/v) glycerol, 8% (w/v) SDS, 0.008% Bromophenol blue, 20% (v/v) β -mercaptoethanol) was added. The pellet was resuspended in 150 μ l liposome binding buffer followed by addition of 150 μ l 2 $\times$ Laemmli loading buffer. Samples were incubated at 95 $^{\circ}$ C for 3 min before equivalent amounts of supernatant and pellet were analyzed by SDS-PAGE followed by Coomassie staining.

HEK293 Cell Line Culture and Transfection

HEK293T cells were cultured in DMEM with 10% FBS, 1% penicillin/streptomycin, and 1% glutamine, maintained at 37 $^{\circ}$ C and 5% CO₂ and seeded in 12-mm dishes 24 h before transfection. Cells were transiently transfected with EGFP-hTRESK, EGFP-rTRESK, and EGFP-mTREK-1 vectors, or cotransfected with pRK5-HA-mGluR5, hTRPA1-GFP or pNice-CiVSP, and EGFP-hTRESK vectors using XtremeGENE 9 transfection reagent (Roche, Mannheim, Germany), according to the manufacturer's instructions. Cells were used for patch-clamp experiments 24–48 h after transfection.

Electrophysiological Recordings

Electrophysiological recordings were performed as previously described [17, 43]. Briefly, recordings were performed with a patch-clamp amplifier (Axopatch 200B, Molecular Devices, Union City, CA) in transfected HEK293T cells. Patch electrodes were fabricated in a Flaming/Brown micropipette puller P-97 (Sutter instruments, Novato, CA). Membrane currents were recorded in excised patches of membrane using the inside-out configuration of the patch clamp technique, filtered at 2 kHz, digitized at 10 kHz, and acquired with pClamp 10 software. Electrodes had a resistance between 2 and 4 M Ω when filled with extracellular solution (in mM) 145 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES, and 5 glucose at pH 7.4; and bath solution (in mM) 140 KCl, 2.1 CaCl₂, 2.5 MgCl₂, 5 EGTA, 10 HEPES, and 2 ATP at pH 7.3. In these conditions, the K⁺ gradient across the patch is equivalent to that found in physiological conditions. One second depolarizing ramps from –100 to +100 mV every 10 s were used to record TRESK- or TREK-1-mediated currents. A holding voltage of –80 mV was used. In whole-cell experiments, which were used to study the effect of mGluR5 activation on hTRESK current, the pipette solution was (in mM) 155 KCl, 5 EGTA, 3MgCl₂, and 10 HEPES. FK-506 (1 μ M; Sigma-Aldrich, Madrid) was included in the pipette to block

calcineurin activity. The same extracellular solution (bath) was used. In cell-attached experiments in cells cotransfected with mGluR5 and hTRESK, the pipette contained the extracellular solution used in the bath. Data was analyzed with Clampfit 10 (Molecular Devices) and Prism 5 (GraphPad Software, Inc., La Jolla, CA). Series resistance was always kept below 15 M Ω and compensated at 70–80%. All recordings were done at room temperature (22–23 $^{\circ}$ C), 24–48 h after transfection.

Statistical Analysis

Data are presented as mean $\pm$ SEM. Statistical differences between different sets of data were assessed by performing paired or unpaired Student's *t* test as indicated. Statistical significance was set at **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

Results

Identification of Putative Binding Sites for Anionic Phospholipids in TRESK Using an In Silico Approach

In general, protein regions that associate with biological membranes do so via binding to anionic/acidic phospholipids, displaying a wide variation in binding mechanisms [44]. In initial studies, we used an in silico approach to search for putative lipid-binding domains in both the human and rat isoforms of TRESK. A search for the most common putative lipid-binding domains (the PH, PKC C1, PKC C2, PX, FYVE, GLA, GRAM, F-BAR, and ENTH domains) in TRESK using the SMART program [41, 42] yielded no positive results, indicating that it is unlikely that TRESK contains lipid-binding domains with a well-defined tertiary structure. However, there are numerous examples of K⁺ channels lacking these structurally defined lipid-binding domains that can still bind to acidic/anionic phospholipids via clusters of basic and hydrophobic amino acids [37, 45–48]. With this in mind, we analyzed the sequences of human and rat TRESK using BH search, a program that identifies putative membrane-binding sites on the basis of basic and hydrophobic amino acid content (available at <http://helixweb.nih.gov/bhsearch>) [40]. The results of this analysis are shown in Fig. 1. It identified two putative membrane-binding sites in human TRESK, one in the large cytoplasmic loop at the membrane-cytosol interface (amino acids 163–191) and the other in the C-terminus (amino acids 371–380, Fig. 1a, c). A similar analysis of rat TRESK also identified a shorter putative membrane-binding site in the large cytoplasmic loop, which aligned with that of the human channel (amino acids 198–207, Fig. 1b, c).

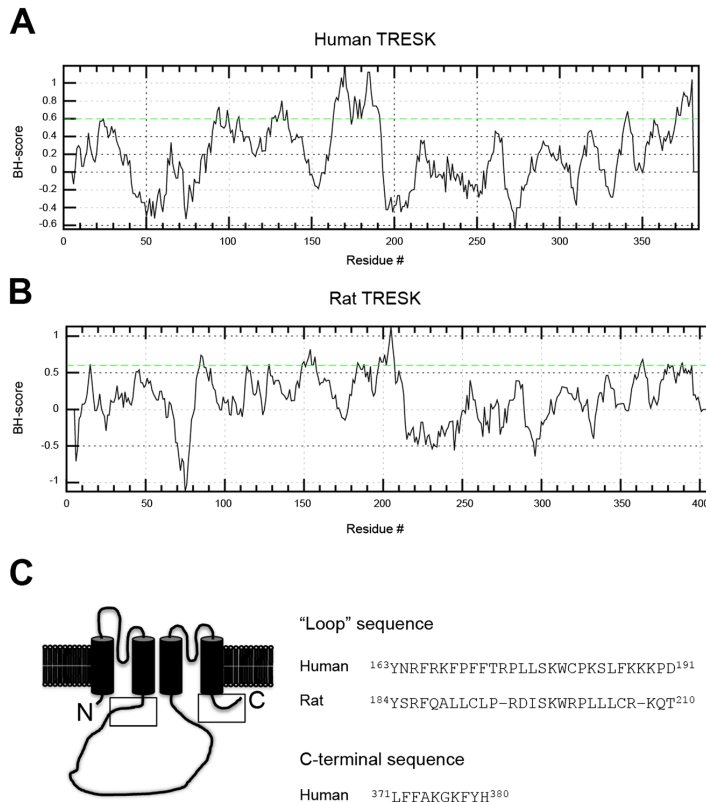


Fig. 1 In silico search for putative phospholipid binding sites in human and rat TRESK (K_{2p}18.1). The primary sequences of human (GenBank accession number NP_862823.1) and rat (GenBank accession number AAS68516.1) TRESK were analyzed using the BH search program. The threshold BH value for residues forming part of a potential membrane binding site was 0.6, and the search was performed with a window size of 10 to obtain scores for N- and C-terminal residues (Brzeska et al. 2010) [40]. **a** Results of the BH search analysis of

human TRESK revealed two potential membrane binding sites between amino acids 163–191 and 371–380. **b** Results of the BH search analysis of rat TRESK, revealing one potential binding site between amino acids 198–207. **c** Schematic representation of TRESK with boxes indicating the positions of the identified lipid-binding sites. The amino acid sequences of the putative binding sites are shown alongside. In the case of the “loop” sequence, the human and rat sequences are aligned

The Intracellular Loop of Human TRESK Contains a Site for Binding to Anionic Phospholipids

To analyze whether the anionic phospholipid binding sites identified in our in silico analysis could mediate TRESK interaction with membrane lipids, we examined whether regions of human and rat TRESK containing these putative sites could bind to multilamellar vesicles (MLVs) composed of Folch fraction I lipids, an organic extract of bovine brain enriched in phosphoinositides and phosphatidylserine [49]. Stretches of the large intracellular loops of human (amino acids 163–244) and rat (amino acids 185–267) TRESK, as well as the short C-terminus of human TRESK (amino acids 356–384), were expressed in *E. coli* as fusions to glutathione-S-transferase (GST) (GST-hTRESK_(163–244), GST-rTRESK_(185–267), and GST-hTRESK_(356–384), Fig. 2a). The purified GST fusion

proteins were used in a liposome-binding assay based on pelleting of liposome-associated proteins, as shown in Fig. 2b. Under the conditions of our assay, appreciable pelleting of GST alone was not observed in the presence or absence of Folch Fraction multilamellar vesicles (Folch MLVs; Fig. 2c–e, lanes 1–4). The GST fusion proteins of the human and rat loops also did not show significant pelleting in the absence of Folch MLVs (Fig. 2c, d, lanes 5–6). Strikingly, in the presence of Folch MLVs, strong pelleting of GST-hTRESK_(163–244) was observed (Fig. 2c, lanes 7–8) but not GST-rTRESK_(185–267) (Fig. 2d, lanes 7–8). The GST fusion protein of the C-terminal portion of human TRESK, GST-hTRESK_(356–384), showed significant pelleting in the absence and presence of Folch MLVs and was therefore not analyzed further (Fig. 2e, lanes 5–8). As shown in Fig. 2f, quantitative analysis of several experiments confirmed significantly

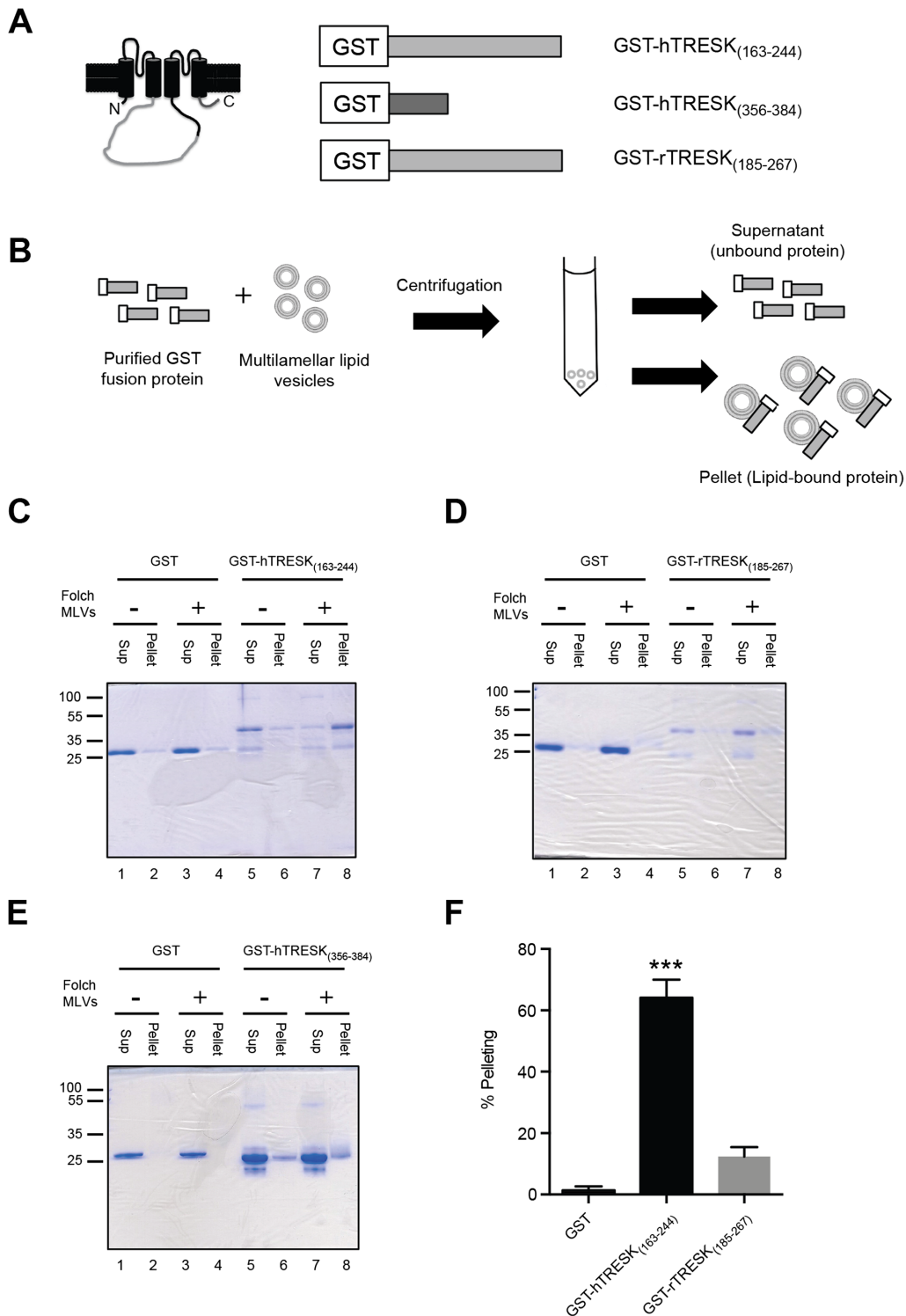


Fig. 2 Intracellular loop of human TRESK strongly binds to Folch Fraction Multilamellar Vesicles. **a** Schematic of the GST fusion proteins corresponding to the indicated regions of human and rat TRESK (K_{2p} 18.1). The large intracellular loop and the C-terminus are shown in light and dark gray, respectively. **b** Summary of liposome binding assay methodology. Representative Coomassie-stained gels of liposome binding assays using purified GST-hTRESK_(163–244) (**c**), GST-rTRESK_(185–267) (**d**), and GST-hTRESK_(356–384) (**e**). The positions of molecular weight standards (in kilodaltons) are indicated to the left of each gel. Lane numbers are indicated underneath the corresponding gels. **f** Quantification of the liposome binding assays showing the percentage of pelleting (mean $\pm$ SEM) after correction for the amount of pelleting in the absence of Folch MLVs. *** $p < 0.001$, Student's unpaired t test between GST-hTRESK_(163–244) and GST-rTRESK_(185–267)

stronger pelleting of GST-hTRESK_(163–244) ($64.14 \pm 5.84\%$, $n = 7$) compared to GST-rTRESK_(185–267) ($12.38 \pm 3.06\%$, $n = 4$; $p < 0.001$, unpaired two-tailed t test). It should also be noted that both fusion proteins displayed significantly more pelleting than GST alone (GST pelleting = $1.4 \pm 1.2\%$, $n = 9$; GST vs. GST-hTRESK $p < 0.0001$; GST vs. GST-rTRESK $p = 0.0017$, unpaired two tailed t tests). These in vitro observations demonstrate that the intracellular loop domain of human TRESK can interact with anionic phospholipids.

Folch Multilamellar Vesicles Activate Human TRESK Currents

After identification of the large intracellular loop of TRESK as a potential mediator of the interaction between the channel and anionic phospholipids, we went on to examine the functional consequences of disrupting electrostatic interactions between the channel and anionic phospholipids in the lipid bilayer. Cationic molecules, such as poly-lysine, interfere with electrostatic protein-lipid bilayer interactions by binding to and therefore “masking” negatively charged phospholipids [50]. Treatment with poly-cationic molecules produces inhibition of K^+ channels containing anionic phospholipid-binding domains [37, 51–53]. In HEK293 cells transfected with GFP-hTRESK and GFP-rTRESK, an initial application of poly-lysine ($1 \mu\text{g}/\text{ml}$) to the cytosolic side of inside-out patches produced significant current decreases of $35.8 \pm 5.4\%$ for hTRESK ($n = 7$; $p < 0.05$; Fig. 3d) and $36.9 \pm 4.9\%$ for rTRESK ($n = 4$; $p < 0.05$; Fig. 3d). The subsequent application of Folch Fraction Multilamellar Vesicles (MLVs) ($10 \mu\text{g}/\text{ml}$) to the cytosolic side of poly-lysine treated inside-out patches containing hTRESK produced a significant current activation of $125.5 \pm 51.7\%$ ($p < 0.001$; Fig. 3d). In contrast, almost no effect was found in rTRESK ($12.1 \pm 9.5\%$; $p = 0.101$; Fig. 3d). As a positive control, we assayed another K_{2p} channel previously shown to be modulated by anionic phospholipids, mouse TREK-1 (K_{2p} 2.1) [37, 38]. mTREK-1 displayed similar behavior to hTRESK, displaying sensitivity to poly-lysine (% decrease $33.6 \pm 4.8\%$; $n = 5$; $p < 0.01$; Fig. 3c, d) followed by subsequent activation by Folch Fraction MLVs (% increase $76.1 \pm 22.3\%$; $p < 0.01$). As

observed for hTRESK, a second application of poly-lysine produced a significant decrease in mTREK-1 current, reverting the previous lipid-mediated increase (Fig. 3b). It has been reported that membrane phospholipid binding of TREK-1 can modulate several properties of the channel, including voltage, stretch, and pH sensitivity [37]. We tested in inside-out patches whether hTRESK intracellular pH sensitivity was modified by the interaction between hTRESK and membrane phospholipids. As previously reported [8], acidic pH (from pH 7.2 to 5.5) decreased hTRESK current ($45.1 \pm 6.3\%$; $n = 5$; $p < 0.05$). Treatment with poly-lysine did not significantly modify the pH sensitivity of the channel ($32.2 \pm 10.4\%$; $n = 5$; $p = 0.335$). A similar result on pH sensitivity was obtained before and after treatment with Folch fraction (MLVs).

Our electrophysiological data demonstrated that the activity of TRESK is sensitive to poly-lysine treatment, suggesting that the disruption of electrostatic protein-bilayer interactions affects TRESK activity. However, when comparing the responses of rat and human TRESK to the application of Folch MLVs after poly-lysine treatment, important differences were observed. With rat TRESK, application of Folch MLVs restored poly-L-lysine inhibited currents to basal levels, presumably by sequestering the poly-L-lysine. However, with human TRESK, in addition to a sequestration effect, Folch MLVs potentiated channel activity above basal levels, which is likely to be due to an increase in the concentration of anionic phospholipids in the patch due to the incorporation of the Folch MLVs. The differential responses of human and rat TRESK to Folch MLVs are consistent with the presence of a stimulatory binding site for anionic phospholipids in human but not rat TRESK. The data from the liposome binding experiments shown in Fig. 2 suggest that this site may reside in the loop of human TRESK.

Mapping the Anionic Phospholipid Binding Site in the Cytoplasmic Loop of Human TRESK

The in silico analysis of human TRESK predicted that the site responsible for binding to anionic phospholipids resides between residues 163 and 191 (Fig. 1). Consistent with this, a GST-fusion protein containing this site showed significant interaction with Folch MLVs (Fig. 2c). On closer examination of the amino acid sequence of the putative anionic phospholipid-binding site, two separate clusters of positively charged amino acids could be distinguished. One of these clusters lies between amino acids 163 and 177 ($^{163}\text{YNRFKFPFFTRPLL}^{177}$, denoted “Cluster 1” in Fig. 4a) and the other between amino acids 177 and 191 ($^{177}\text{LSKWCPKSLFKKKPD}^{191}$, denoted “Cluster 2” in Fig. 4a). We therefore designed GST fusion proteins to test whether one or both of these stretches were necessary for interaction with Folch MLVs. As previously shown (Fig. 2c), GST-hTRESK_(163–244) pelleted strongly in the presence of Folch MLVs (Fig. 4b, left panel, lane 8 and Fig. 4c). Our analysis revealed that the fusion protein

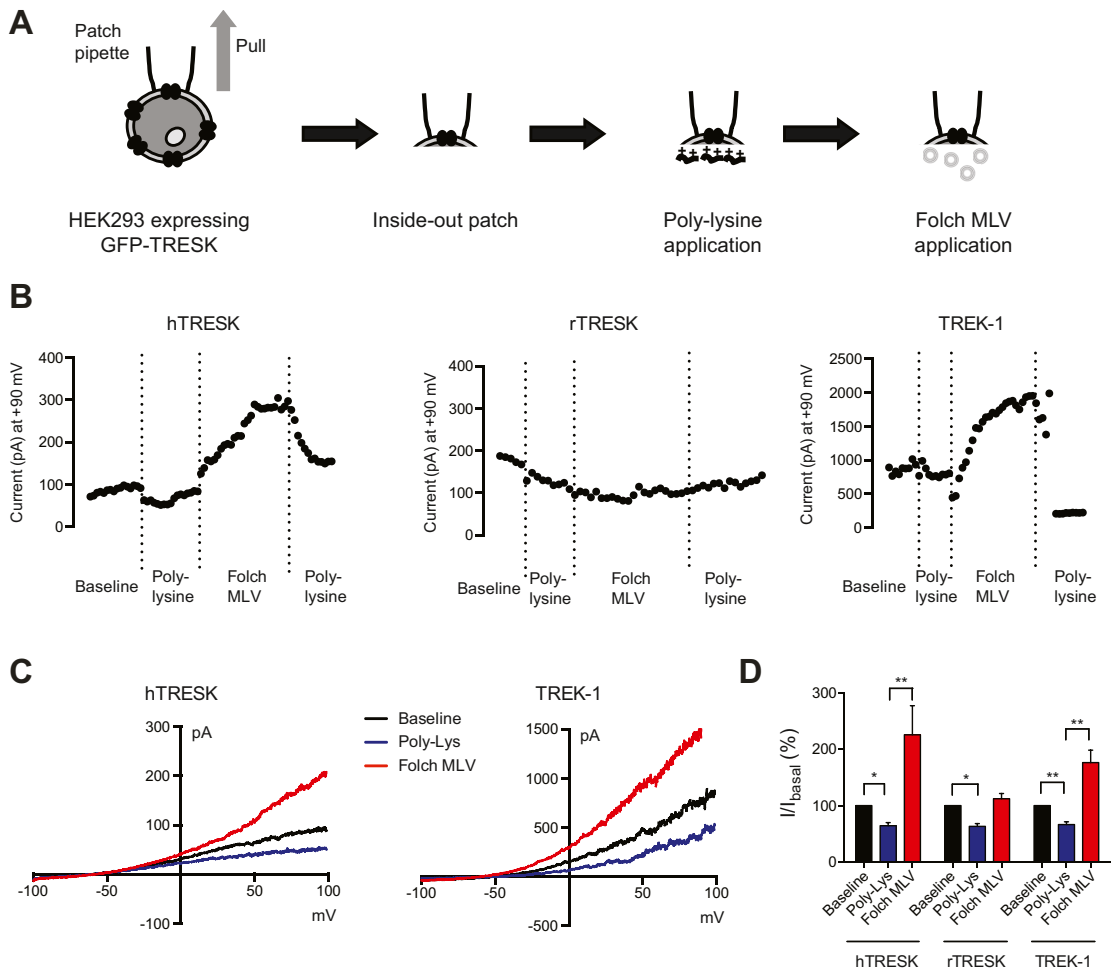


Fig. 3 Folch Fraction Multilamellar vesicles enhance human TRESK-mediated currents. **a** Schematic representation of the experimental protocol. **b** Representative time courses of human TRESK, rat TRESK, and mouse TREK-1 currents measured at +90 mV from consecutive depolarizing ramps. From a holding voltage of -80 mV, depolarizing ramps from -100 to +100 mV were recorded every 10 s on excised membrane patches in the inside-out configuration of the patch clamp technique. A physiological K⁺ gradient was used (bath solution 140 mM K⁺; pipette 5 mM K⁺). Transfected HEK293 cells expressing

GFP-hTRESK, GFP-rTRESK, and mTREK-1 were used. Poly-lysine and Folch MLVs were prepared in bath solution and applied at final concentrations of 1 and 10 µg/ml, respectively. **c** Representative currents elicited by depolarizing voltage ramps from -100 to +100 mV prior to (baseline) and during poly-lysine and Folch MLV application are shown. **d** Quantification of the experiments shown in panels b and c. GFP-hTRESK (*n* = 6), GFP-rTRESK (*n* = 4), and mTREK-1 (*n* = 5). **p* < 0.05; ***p* < 0.01, Student's paired *t* test

containing “Cluster 1,” GST-hTRESK_(163–207), pelleted significantly with Folch MLVs (Fig. 4b, right panel, lane 4 and Fig. 4c; % pelleting = 64.6 ± 5.5%, *n* = 3). In contrast, the fusion proteins containing either only “Cluster 2” (GST-hTRESK_(177–244)) or neither cluster (GST-hTRESK_(187–244)) showed little or no pelleting with Folch MLVs (Fig. 4b, center panel and Fig. 4c; % pelleting = 9.2 ± 5.2%, *n* = 4 for GST-hTRESK_(187–244) and 2.2 ± 2.2%, *n* = 4 for GST-hTRESK_(177–244)). These observations indicated that a stretch of 14 amino

acids between residues 163 and 177 in human TRESK is necessary for interaction with Folch MLVs.

Testing the Role of the Phospholipid-Binding Cluster in the Cytoplasmic Loop of Human TRESK Using Human-Rat TRESK Chimeric Constructs

The liposome binding data shown in Fig. 4 strongly suggested that the stretch of amino acids between residues 163 and 177

of human TRESK is essential to mediate a physical interaction with anionic phospholipids. We then attempted to investigate the role of this stretch of amino acids in the modulation of TRESK activity by designing a series of chimeras between human and rat TRESK. It is interesting to note that alignment of the anionic phospholipid binding stretch we have identified in human TRESK with the corresponding sequence in rat TRESK revealed significant differences (Fig. 5a). These differences may explain why the loop of human TRESK displays a much stronger binding to Folch MLVs compared to the rat loop (see Fig. 2). Of the four positively charged residues in the human stretch, only two are conserved in the rat sequence (R186 and R195). Furthermore, there is an aspartate residue in the rat sequence (D196), which at physiological intracellular pH would be negatively charged and therefore likely to interfere with anionic phospholipid-based interactions. As shown in Fig. 5b, two chimeras between human and rat TRESK were initially constructed. In the first chimera, the large intracellular loop, the third and fourth transmembrane domains of hTRESK were replaced with the corresponding sequence from rTRESK (hTRESK-rat-loop-rTRESK). A complementary second chimera was constructed in the same way (rTRESK-human-loop-hTRESK). The effects of poly-L-lysine followed by Folch MLV application to inside-out patches of HEK293 cells expressing each chimera were tested. In both cases, no significant effects of either poly-L-lysine or Folch MLVs on channel currents were observed (Fig. 5c).

A second set of chimeras was also tested where only the anionic phospholipid binding site (apbs) from human TRESK replaced the equivalent sequence in rat TRESK (rTRESK-h-apbs) or vice versa (hTRESK-r-apbs). The effects of poly-L-lysine followed by Folch MLV application to inside-out patches of HEK293 cells expressing each chimera were tested. As expected from liposome binding data in Fig. 2, replacement of the apbs in hTRESK by the rat sequence produced a chimeric channel unresponsive to poly-lysine ($-6.0 \pm 7.2\%$, $n = 7$) or Folch MLV application ($5.7 \pm 5.2\%$, $n = 7$; Fig. 5d, e). In agreement, a GST-r-apbs-hloop construct showed significantly less binding to Folch MLVs (21% pelleting; data not shown) compared to the normal human loop sequence (Fig. 2), providing further evidence for the importance of the human apbs in mediating interaction with anionic phospholipids. In contrast, when the apbs in rTRESK was replaced by the human one, the channel acquired responsiveness to poly-lysine ($-10.0 \pm 8.9\%$, $n = 11$; $p < 0.05$) and was potentiated by Folch MLV application ($128.7 \pm 45.2\%$, $n = 11$; $p < 0.05$; Fig. 5d, e), similarly to what was observed in the wild-type human channel.

The results of this analysis suggest that the apbs of hTRESK is sufficient to confer modulation by anionic phospholipids on rTRESK. However, in those chimeras where larger portions of each ortholog were switched (the rTRESK-human-loop-hTRESK and hTRESK-rat-loop-rTRESK chimeras), modulation by poly-lysine and Folch MLVs was lost. This suggests

that these chimeras had an altered channel tertiary structure resulting in disrupted channel gating and/or there are other elements in the human TRESK structure that can interfere with the stimulatory function of the apbs.

The Intracellular Loop of Human TRESK Binds to Liposomes Enriched with Phosphatidylserine and Phosphatidylinositol-4,5-Bisphosphate (PIP₂)

To determine whether the human TRESK intracellular loop displayed preferential binding for a particular type of anionic phospholipid and to further analyze potential differences between the lipid binding characteristics of the rat and human loops, we performed pelleting experiments using multilamellar liposomes of defined composition. GST-hTRESK_(163–244) pelleted strongly in the presence of liposomes enriched with anionic phospholipids, either 50% phosphatidylcholine (PC)/50% phosphatidylserine (PS) (Fig. 6a, lane 6) or 70% phosphatidylcholine/25% phosphatidylserine/5% phosphatidylinositol-4,5-bisphosphate (PIP₂; Fig. 6a, lane 8) but did not show significant pelleting with liposomes composed solely of the neutral lipid PC (Fig. 6a, lane 4). Quantification of pelleting from three independent experiments showed significant differences between PC-only liposomes and those enriched with anionic phospholipids (PS and PIP₂). However, no difference in pelleting was observed between the two types of liposomes enriched with anionic phospholipids (PS vs PIP₂) (Fig. 6c). In contrast, GST-rTRESK_(185–267) did not show specific pelleting with any of the liposomes tested (Fig. 6b, lanes 4, 6, and 8). We attempted to further characterize the lipid specificity of the human TRESK loop using commercially available strips spotted with various types of lipid (PIP strips from Thermo Fisher Scientific). However, no firm conclusions could be drawn due to a high level of GST binding to lipid spots (data not shown). The results of these liposome-pelleting experiments indicate that the intracellular loop of human TRESK contains a binding site for anionic phospholipids that does not display a preference for a specific lipid type.

PIP₂ Enhances Human TRESK Currents

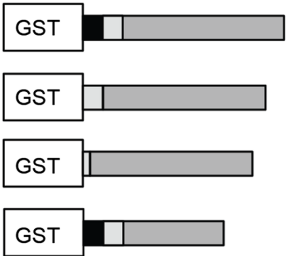
It is likely that the anionic phospholipid-binding site in the large cytoplasmic loop of human TRESK will bind preferentially to the phosphoinositide PI(4,5)P₂ under physiological conditions in native plasma membranes, on the basis of its trivalency at pH 7 and its concentration in the plasma membrane (approximately 1% of total lipid). In addition, physiologically relevant changes in plasma membrane PI(4,5)P₂ concentration can occur during G_{q/11}-coupled receptor stimulation by agonists such as hormones and neurotransmitters [54]. Therefore, we tested the effect of adding a water-soluble form of PI(4,5)P₂ (diC8:0 PI(4,5)P₂) to inside-out patches taken from HEK293 cells

A

¹⁶³YNRFRKFPFFTRPLL SKWCPKSLFKKKPD¹⁹¹

■ “Cluster 1”

■ “Cluster 2”

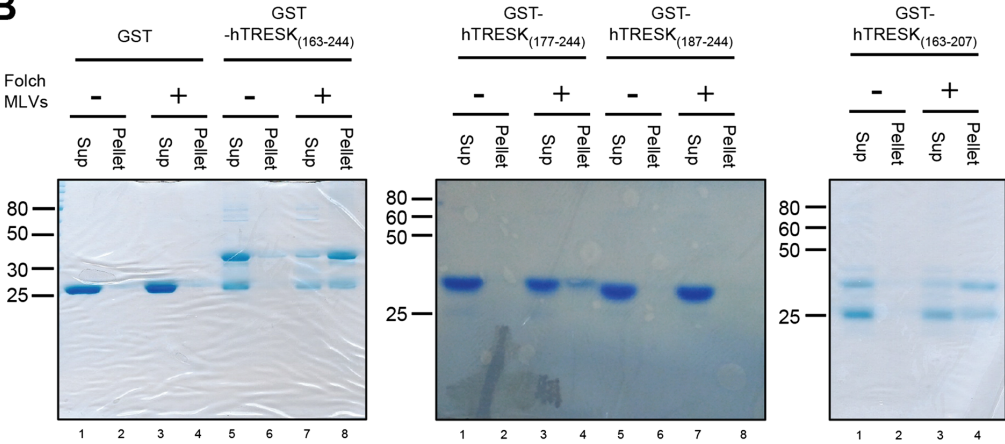


GST-hTRESK₍₁₆₃₋₂₄₄₎
GST-hTRESK₍₁₇₇₋₂₄₄₎
GST-hTRESK₍₁₈₇₋₂₄₄₎
GST-hTRESK₍₁₆₃₋₂₀₇₎

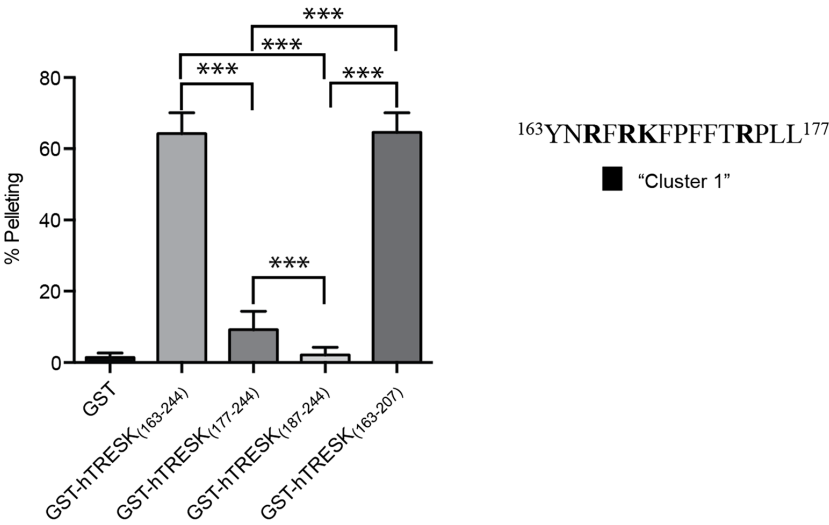
Interaction with Folch
MLVs?

Yes
No
No
Yes

B



C



¹⁶³YNRFRKFPFFTRPLL¹⁷⁷

■ “Cluster 1”

Fig. 4 A short stretch of amino acids in the cytoplasmic loop of human TRESK determines the anionic phospholipid binding. **a** Schematic representation of the GST fusion proteins corresponding to different portions of the large cytoplasmic loop of human TRESK. The amino acid sequences of the two “clusters” containing groups of positively charged amino acids are also indicated, with the numbers denoting the position in the primary sequence of human TRESK. “Cluster 1” (amino acids 163 and 177) and “Cluster 2” (amino acids 178–191) are indicated by the black and gray squares, respectively, on the fusion protein schematics. **b** Representative Coomassie-stained polyacrylamide gels of liposome binding assays. Left panel, results of a control experiment (shown for comparison) performed with GST and GST-hTRESK_(163–244) similar to the one shown in Fig. 2c. Centre panel, results of experiments with the fusion proteins GST-hTRESK_(177–244) and GST-hTRESK_(187–244). Right panel, results of a representative experiment performed with the GST-hTRESK_(163–207) fusion protein. **c** Quantitative summary (% pelleting; mean $\pm$ SEM; $n = 3$ –4 independent experiments per group) of the liposome binding assays showing percentage of pelleting after correction for the amount of pelleting in the absence of Folch MLVs. *** $p < 0.001$, Student's unpaired t test

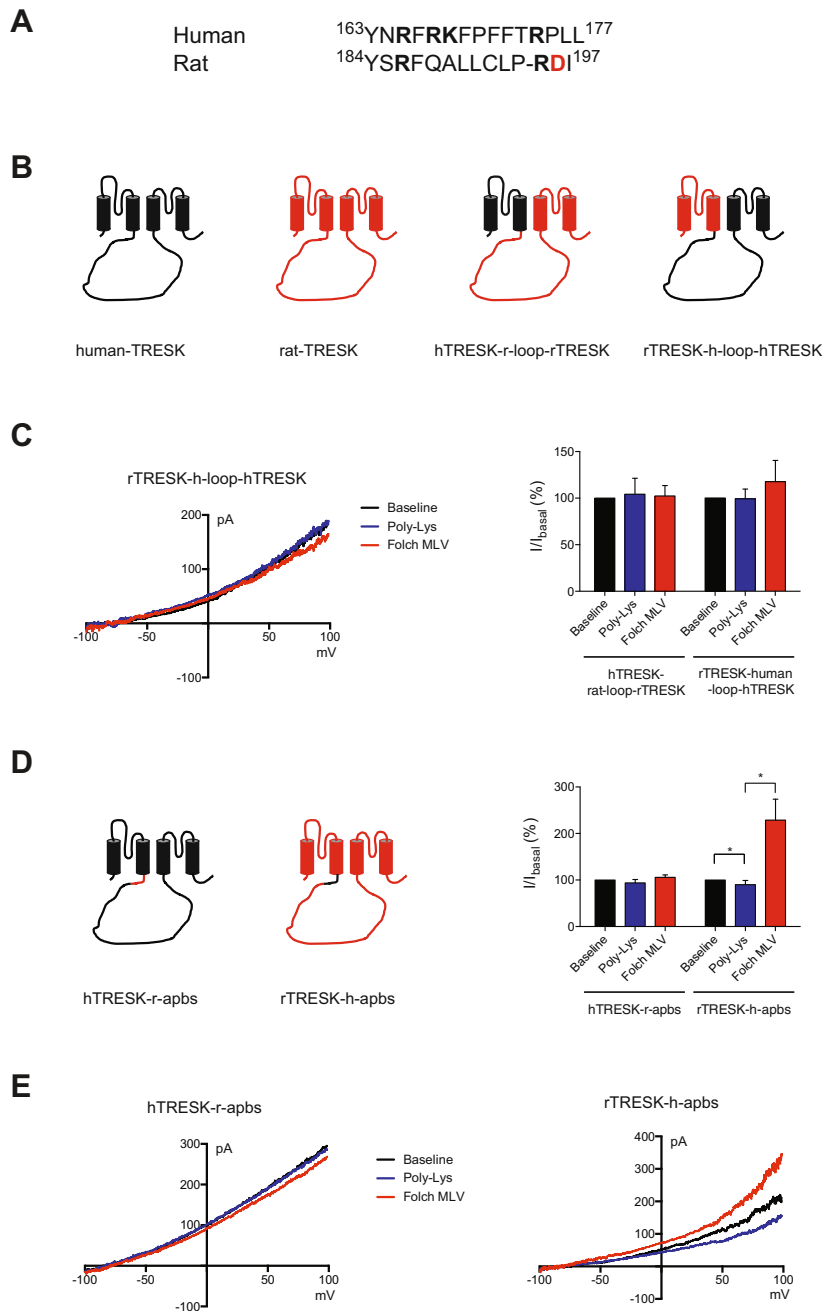
transiently expressing hTRESK. As displayed in Fig. 7a, b, in a similar fashion to Folch MLVs, diC8:0 PI(4,5)P₂ (5 μ M) produced a significant activation of hTRESK previously inhibited by 1 μ g/ml poly-L-lysine in seven out of nine patches tested (% increase $99.9 \pm 39.8\%$, $n = 7$; $p < 0.05$; Fig. 7c), while no effect was found in two additional patches. To assess if the membrane lipid binding is specific for PI(4,5)P₂ or just for anionic lipids, we tested the effect of PI(4,5)P₂ hydrolysis by the *Ciona intestinalis* voltage sensing phosphatase (CiVSP) as previously assayed for TREK-1 [55, 56]. Whole cell recordings in HEK293 cells expressing hTRESK with or without CiVSP did not show significant differences in the current activated by voltage ramps (-100 to $+50$ mV) before and after a prepulse ($+150$ mV, 200 ms) to activate the phosphatase (% inhibition: hTRESK $0.77 \pm 5.0\%$, $n = 4$; hTRESK+CiVSP $12.6 \pm 12.8\%$, $n = 5$; $p = 0.326$).

As previously described, changes in membrane PI(4,5)P₂ concentration after activation of GPCRs modulate the activity of several ion channels and membrane proteins [31, 32, 57–59]. It is well-known that TRESK is modulated by changes in intracellular Ca²⁺ through dephosphorylation by calcineurin [27]. In view of our results, we explored the possibility that a dual regulation of hTRESK by PIP₂ and Ca²⁺/calcineurin might exist. To address this, we cotransfected hTRESK and mGluR5 (a metabotropic glutamate receptor that can activate phospholipase C) in HEK293 cells and recorded hTRESK current in whole-cell experiments. To dissociate the possible effect of PI(4,5)P₂ concentration changes from the Ca²⁺-mediated modulation of hTRESK current, we included EGTA to chelate any free Ca²⁺ and FK-506 to block calcineurin activity. Under these conditions, stimulation of mGluR5 with 50 μ M glutamate produced a significant decrease in hTRESK current ($-38.2 \pm 7.9\%$, $n = 8$; $p < 0.001$; Fig. 7d, f) likely due to PIP₂ hydrolysis. In contrast, HEK293 cells transfected only with hTRESK did not show significant changes in whole-cell current after stimulation with

glutamate (control, Fig. 7f). Finally, to evaluate the relative contributions of the lipid- and Ca²⁺-modulatory effects on hTRESK, we performed experiments in HEK293 cells co-expressing mGluR5 and hTRESK in the cell-attached configuration of the patch-clamp technique, thus preserving native cytoplasmatic conditions. Glutamate application produced an overall increase in hTRESK current ($38.9 \pm 12.2\%$, $n = 12$; $p < 0.05$; Fig. 7e, left and 7f), consistent with previous reports [29, 60]. This indicates that, at least after stimulation of mGluR5 receptors, the activating effect of Ca²⁺/calcineurin is more important than the lipid-mediated inhibition. Interestingly, in 50% of the recordings (6 of 12), a biphasic effect was observed: an initial and transient increase in TRESK current followed by a sustained decrease ($-27.5 \pm 6.6\%$, $n = 6$), which could reflect an initial stimulatory effect of Ca²⁺/calcineurin on hTRESK followed by hydrolysis of PIP₂ at the plasma membrane (Fig. 7e, right). Nevertheless, some variability existed on the current increase/decrease observed that would reflect a competition between opposing modulatory mechanisms. An additional experiment in the same configuration (cell-attached) was performed in HEK293 cells co-transfected with hTRESK and hTRPA1 in order to determine the effect on hTRESK resulting from activation of a receptor/channel not linked to G-protein-mediated phospholipase C activation and membrane lipid hydrolysis but that its activation increases intracellular Ca²⁺. In these conditions, allyl isothiocyanate (AITC; 100 μ M), a well-known activator of TRPA1, produced a significant increase in hTRESK current ($170.6 \pm 29.3\%$, $n = 6$; $p < 0.01$; Fig. 7f, right), but no decrease in the current was observed. As a control, in HEK293 cells transfected only with hTRESK, AITC did not produce any significant change on the recorded current ($-13.1 \pm 6.4\%$, $n = 4$).

Discussion

In this study, we present evidence that argues for a species-specific modulation of TRESK by anionic phospholipids, requiring a juxtamembrane region in the large intracellular loop of the channel. It is well established that other members of the K_{2P} family (TREK-1, -2, TASK-1, -3) are activated by anionic phospholipids, with changes in local PIP₂ concentration appearing to be particularly important modulating factor [37–39, 61, 62]. In the case of TREK-1 (K_{2P}2.1), a short sequence of amino acids acts as a type of “phospholipid sensor” that links channel activity to PIP₂ concentration changes [37]. We used an in silico approach to identify regions in TRESK that could fulfill a similar role (Fig. 1). After identifying potential interacting regions, we used a liposome-binding assay to test their ability to bind to multilamellar vesicles enriched with anionic phospholipids (Folch fraction liposomes). These experiments revealed that, somewhat surprisingly, there appeared to be a difference in the ability of equivalent portions of the large cytoplasmic loop domain from



different TRESK orthologues to bind to anionic phospholipids. The region identified in the loop domain of hTRESK exhibits a much stronger binding to anionic phospholipids compared to the equivalent region in rat TRESK, probably

due to the presence of a cluster of four positively charged residues in the human stretch, while only two are conserved in the rat sequence (R186 and R195, see Fig. 5a). Additionally, the identified region in the rat contains a

Fig. 5 The anionic phospholipid binding site of human TRESK can confer stimulation by Folch MLVs on rat TRESK. **a** Comparison of the anionic phospholipid binding site (apbs) identified in human TRESK and the equivalent sequence in rat TRESK. **b** Representation of the human, rat, and human/rat chimeric TRESK channels tested. **c** Left: representative currents elicited by depolarizing voltage ramps from -100 to $+100$ mV prior to (baseline) and during poly-lysine ($1 \mu\text{g/ml}$) and Folch MLV ($10 \mu\text{g/ml}$) application are shown in the rTRESK-h-loop-hTRESK chimeric channel. Recordings were performed in inside-out membrane patches in a physiological K^+ gradient (bath solution 140 mM K^+ ; pipette 5 mM K^+). Right: quantification of the experiments in hTRESK-rat-loop-rTRESK ($n = 5$) and rTRESK-human-loop-hTRESK ($n = 6$) chimeric channels. Currents were measured at $+90$ mV from depolarizing ramps such as the example shown on the left. **d** Left: chimeric channels where the anionic phospholipid binding site (apbs) in human TRESK was replaced by the equivalent sequence from rat (hTRESK-r-apbs) or vice-versa (rTRESK-h-apbs). Right: quantification of the experiments in hTRESK-r-apbs ($n = 7$) and rTRESK-h-apbs ($n = 11$) chimeric channels. Currents were measured at $+90$ mV from depolarizing ramps, examples of which are shown in panel **e**. **e** Representative currents elicited by depolarizing voltage ramps from -100 to $+100$ mV prior to (baseline) and during poly-lysine and Folch MLV application are shown for the hTRESK-r-apbs and rTRESK-h-apbs chimeric channels. Recordings were performed in inside-out membrane patches in a physiological K^+ gradient. $*p < 0.05$; Student's paired t test

negatively charged aspartate residue (D196), which would be likely to interfere with any interaction with anionic phospholipids. We did not test the mouse TRESK orthologue, but analysis of its sequence shows that only one positive residue is present in the equivalent domain and it also contains the same negative residue present in rat, which probably makes unlikely an interaction with membrane lipids. In contrast to TREK-1 where the lipid-binding protein sequence resides in the C-terminal domain of the channel, evaluation of another possible lipid-binding sequence in the C-terminal domain of human TRESK did not show significant specific binding to Folch fraction liposomes. This further underlies the important regulatory role of the large intracellular loop of TRESK between transmembrane domains 2 and 3. For instance, several protein-protein interaction sites and phosphorylation motifs are present in this domain [27, 28, 60, 63–65], which exerts a similar role to the C-terminal domain in TREK-1. Changes in this lipid-binding domain such as the reduction in the number of positively charged amino acid residues and the presence of negatively charged residues (as occurs in the rat channel) appear to be critical for membrane lipid binding. This physical interaction does not appear to be important for the control of channel gating since human and rodent channels show similar activation profiles. However, it does seem to be important for “fine-tuning” of channel regulation.

Binding assays using liposomes with defined lipid compositions revealed that the loop domain of hTRESK bound to liposomes enriched with anionic phospholipids such as phosphatidylserine and PIP_2 but not to liposomes composed solely of the neutral phospholipid, phosphatidylcholine. Several ion channels and, in particular, potassium channels

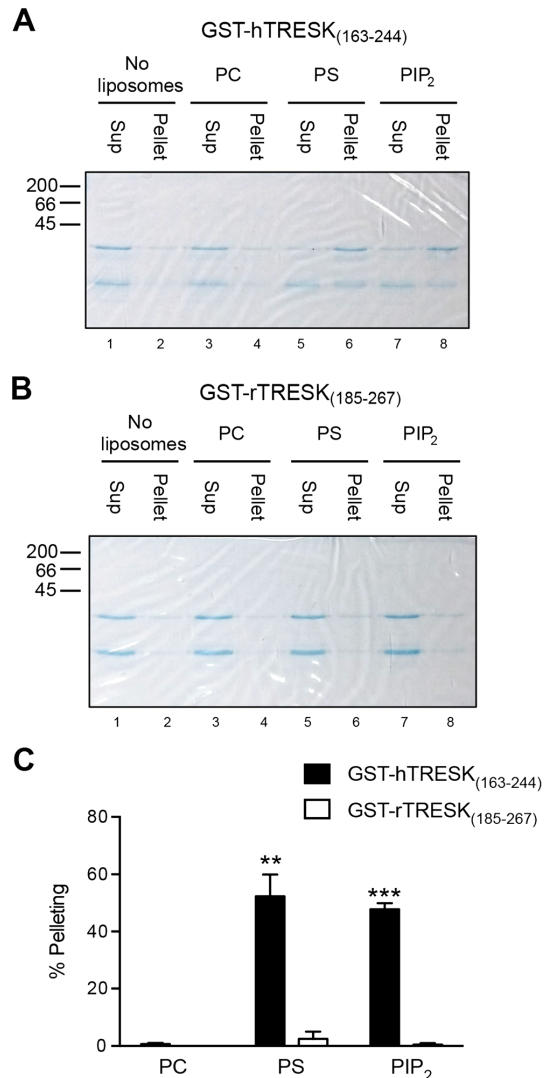


Fig. 6 Differential binding of the intracellular loops of human and rat TRESK to liposomes enriched with phosphatidylserine and phosphatidylinositol-4,5-bisphosphate (PIP_2). Coomassie-stained gels of representative liposome-binding assays with purified GST fusion proteins corresponding to equivalent portions of the large intracellular loop of human TRESK (amino acids 163–244) (**a**) and rat TRESK (amino acids 185–267) (**b**). The liposome compositions were (mole % in brackets) as follows: PC = phosphatidylcholine (100%); PS = phosphatidylcholine (50%)/phosphatidylserine (50%); PIP_2 = phosphatidylcholine (70%)/phosphatidylserine (25%)/phosphatidylinositol-4,5-bisphosphate (5%). **c** Quantitative summary (% pelleting; mean $\pm$ SEM) of three independent experiments for GST-hTRESK₍₁₆₃₋₂₄₄₎ and GST-rTRESK₍₁₈₅₋₂₆₇₎ showing the percentage of pelleting after correction for the amount of pelleting in the absence of liposomes. $**p < 0.01$; $***p < 0.001$, Student's unpaired t test vs. pelleting in the presence of PC-only containing liposomes

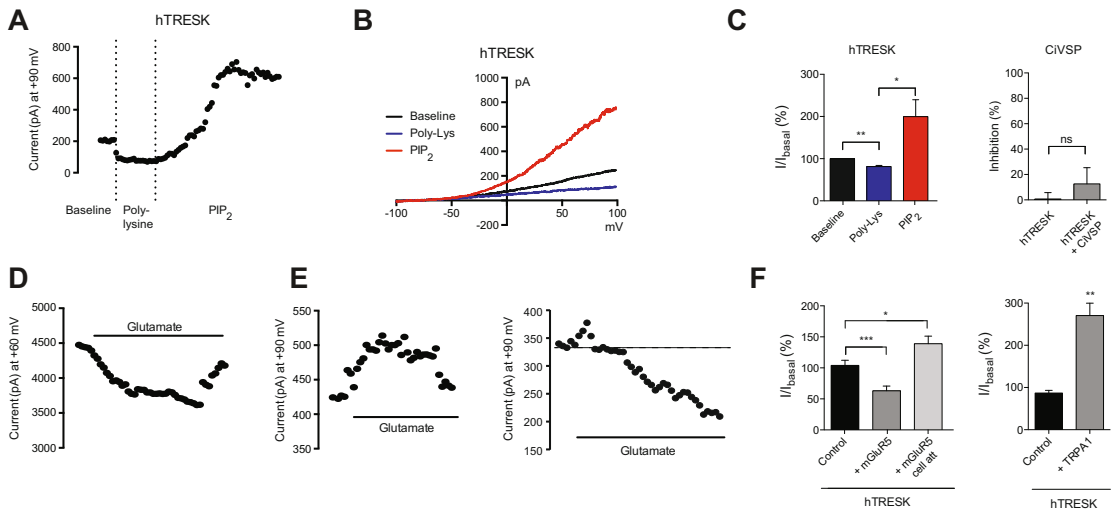


Fig. 7 PIP₂ enhances human TRESK-mediated currents. **a** Representative time course of human TRESK currents measured at +90 mV from consecutive depolarizing ramps. In transfected HEK293 cells expressing GFP-hTRESK, depolarizing ramps from −100 to +100 mV were recorded every 10 s (holding voltage −80 mV) on excised membrane patches in the inside-out configuration of the patch clamp technique. A physiological K⁺ gradient was used (bath solution 140 mM K⁺; pipette 5 mM K⁺). The effects of adding poly-lysine (1 μg/ml) and a water-soluble form of PI(4,5)P₂ (diC8:0 PI(4,5)P₂) (5 μM) to the bath solution (cytosolic side of the patch) on TRESK currents were measured. **b** Representative currents elicited by depolarizing voltage ramps from −100 to +100 mV prior to (baseline), during poly-lysine and PIP₂ application are shown. **c** Left: quantification of the experiments shown in panels **a** and **b** GFP-hTRESK (*n* = 7). Right: quantification of the effect on hTRESK current of PIP₂ hydrolysis by activation of CivVSP phosphatase. hTRESK (*n* = 4); hTRESK+CivVSP (*n* = 5). **p* < 0.05; ***p* < 0.01, Student's paired *t* test. **d** Representative time course of human TRESK currents in transfected HEK293 cells expressing GFP-hTRESK and mGluR5. Current was measured at +60 mV from consecutive depolarizing ramps from −100 to +60 mV recorded

every 10 s (holding voltage −60 mV) in the whole-cell configuration of the patch clamp technique. A physiological K⁺ gradient was used. The pipette solution contained EGTA and the calcineurin blocker FK-506. Glutamate (50 μM) was added to the bath as indicated. **e** Left: representative time course human TRESK currents in transfected HEK293 cells expressing GFP-hTRESK and mGluR5. Current was measured at +90 mV from consecutive depolarizing ramps from −100 to +100 mV recorded every 10 s (holding voltage 0 mV) in the cell-attached configuration of the patch clamp technique. Both the bath and the pipette contained a physiological solution. Glutamate (50 μM) was added in the bath as indicated. Right: example of a recording showing increasing and decreasing effects on hTRESK current. **f** Left: quantification of the experiments shown in panels **d** and **e**. Cells transfected only with GFP-hTRESK (control; *n* = 7); whole-cell recordings in GFP-hTRESK+mGluR5 cells (*n* = 8); cell-attached recordings in GFP-hTRESK+mGluR5 cells (*n* = 12). Right: quantification of the effect of TRPA1 activation (AITC 100 μM) on hTRESK current in HEK293 cells transfected only with hTRESK (*n* = 4) or with hTRESK+hTRPA1 (*n* = 6). **p* < 0.05; ***p* < 0.01; ****p* < 0.001, Student's paired *t* test

including K_{ir}, K_v, KCNQ, and K_{Ca} are regulated by phosphoinositides, including PIP₂ (for review see [66]). In fact, other channels of the K_{2P} family are regulated by different phospholipids as well as by PIP₂ [37–39, 67–69]. Here, we found that exogenous PIP₂ increased hTRESK activity in isolated inside-out patches. A closer inspection of the putative anionic phospholipid-binding region in hTRESK revealed the presence of two “clusters” of positively charged amino acids, which may be responsible for mediating binding to anionic phospholipids. We tested the role of these clusters in phospholipid binding using the liposome binding assay and determined that only one of these clusters is necessary to mediate binding to anionic phospholipids (Fig. 4). However, there is a possible smaller contribution from cluster 2 as the GST-hTRESK_(177–244) shows significant binding above GST and significantly more than the fusion protein lacking both clusters, GST-hTRESK_(187–244). Rat TRESK seems to have cluster

2, which may explain the residual binding activity observed in the pelleting assay (Fig. 2f).

In our experiments, hTRESK is able to bind to PIP₂ to increase the channel current. In TREK-1, it has been described that when endogenous phospholipids are “quenched” by poly-cationic poly-lysine, mechanosensitivity is significantly reduced [37]. Also, PIP₂ exerts a dual regulation of TREK-1 activation by intracellular pH (pHi) or mechanical stimuli. In the presence of PIP₂, mechanosensitivity or activation by pHi is reduced, but in the presence of poly-lysine, the effects are reverted and PIP₂ can promote activation of the channel [37–39]. TRESK is not directly activated but modulated by membrane stretch or by changing membrane curvature [21], in contrast to TREK-1 or -2 that can be directly activated by mechanical stimuli. It remains to be explored if stretch modulation of hTRESK current is enhanced or diminished by the binding to membrane phospholipids. Another possibility is

that changes in the intracellular pH (pHi) might produce protonation of some residues to modify the interaction with membrane lipids. In TREK-1 channels at acidic pHi, exogenous phospholipids transform the mechano-gated channel into a K⁺-selective leak conductance [37]. Also, blocking the interaction between the C-terminal domain and membrane lipids by addition of poly-lysine abolishes activation by acidic pHi [37, 38]. In hTRESK, acidic pHi produces the opposite effect and decreases the current, while alkalinization increases it [8]. We tested whether hTRESK intracellular pH sensitivity was modified by the interaction with membrane lipids, but no significant differences were found, indicating that at least intracellular pH sensitivity is not dependent on this interaction, in contrast to what occurs for TREK-1. Whether other biophysical properties of the channel are affected by the binding to membrane lipids remains to be studied.

Other K_{2P} channels are modulated by hormones and neurotransmitters through activation of different GPCRs linked to G_s-cAMP-PKA phosphorylation, G_q-PLC-DAG-PKC phosphorylation, or PIP₂ membrane depletion (for review see [3]). Interestingly, TRESK is the only member of the family regulated by Ca²⁺/calcineurin dephosphorylation, which increases the channel current [27]. Therefore, by increasing the cytosolic Ca²⁺ through membrane ion channels or after stimulation of G_{q/11}-coupled receptors and Ca²⁺ release from internal stores results in TRESK activation [27, 29, 30]. However, the net stimulation is likely to be the sum of two consequences of receptor activation: a Ca²⁺-dependent activation of TRESK by calcineurin and an inhibition due to PIP₂ hydrolysis in the vicinity of the channel. Here, we show that activation of a membrane GPCR linked to G_{q/11} activation and Ca²⁺ release (mGluR5) produces a significant decrease in channel current that is likely due to PIP₂ hydrolysis when the Ca²⁺-mediated effects are blocked (calcium-chelating buffer+inhibitor of calcineurin). In contrast, when both modulatory mechanisms can be activated, the overall effect in the majority of the cells is an increase of hTRESK current, indicating that the Ca²⁺/calcineurin effect predominates, although in some cells, a biphasic effect is observed. In this sense, stimulation of TRPA1, a receptor/channel that produces an intracellular Ca²⁺ increase but is not linked to G-protein activation and phospholipid hydrolysis, produced a bigger effect on hTRESK since it is only activating one of the modulatory mechanisms.

During inflammation or nerve injury, a mix of chemicals is released producing the sensitization of nociceptive sensory neurons and contributing to chronic pain [70]. These chemicals, in many cases, activate pronociceptive GPCRs [70, 71]. Because many pronociceptive receptors signal through PLC (and require PIP₂ for signaling), the modulation of hTRESK by membrane lipids might be relevant. Enhancing effects of Ca²⁺ on TRESK current have been described after stimulation with several neurotransmitters or inflammatory mediators such as bradykinin, 5-HT, glutamate,

lysophosphatidic acid (LPA), histamine, or muscarinic agonists [29, 30, 60]. All these studies used rodent (mostly mouse) TRESK orthologs where the Ca²⁺-calcineurin-mediated TRESK activation would be more prominent due to the lack of lipid binding and regulation. In contrast, in the human channel, a combination of both effects will be present. Depending on the receptor activated and the PIP₂ hydrolysis in restricted membrane microdomains, the excitatory or inhibitory effects on TRESK could prevail. In fact, several differences have been reported between the rat/mouse and human TRESK channels, including the sensitivity to anesthetics (higher enhancing efficacy to isoflurane and less blocking effect of lidocaine in hTRESK) [72], the docking of calcineurin to the channel, and their sensitivity to Ca²⁺ regulation [73] or the sensitivity to Zn²⁺ ions and pH [72, 74]. The fact that human and rodent coding sequences share only about 65% identity and 71% overall amino acid similarity is likely to be the reason for this different modulatory effect, including the lipid regulation described here. These species differences must be taken into consideration when extrapolating pharmacological results from rodent experimental models to human physiology or during drug development.

It has been proposed that in sensory neurons, TRESK counteracts membrane depolarization induced by external stimuli or diverse chemical substances in order to prevent excessive neuronal activation [3, 10, 11, 17, 30]. A recent report shows combined enhancement of TRESK and TRPV1 currents by LPA, an inflammatory mediator, with potentiation of hyperpolarizing TRESK currents counteracting to some extent the depolarizing effects derived from TRPV1 [30]. In fact, TRESK seems to be modulated by different stimuli, including Ca²⁺, membrane curvature, or arachidonic acid [8, 21, 27]. As an example, during inflammation, both LPA, arachidonic acid, and hypertonic conditions can be present, the first one enhancing TRESK current while the latter two decrease TRESK current [21, 30]. It is worth considering that, at least for the human channel, while activation of GPCRs will produce a modulation of TRESK current through membrane lipids and by Ca²⁺/calcineurin, activation of TRESK via Ca²⁺ influx through direct activation of membrane channels such as TRPV1 or TRPA1 will only produce a potentiation of the current, as shown in experiments where TRPA1 was activated. This would suggest that depending on the stimuli activating the sensory neuron, the role of TRESK counterbalancing the depolarizing effect of the stimulus might be different. Taken together, it appears that depending on the stimuli, TRESK can be modulated negatively or positively and the final amount of hyperpolarizing current counterbalancing depolarization might vary in different situations. Here, we show that membrane lipids also contribute to this regulation, at least in the human channel. In addition, regulation of TRESK expression is another factor to be added into the equation, since injury or inflammation produce a down-regulation of the channel

expression [10, 75]. Because TRESK represents a common regulation point by all these stimuli, it can be postulated as an interesting way to bypass the membrane receptor diversity and to target a point where different signaling pathways converge. In this way, analgesic drug development to enhance TRESK activity could be an interesting approach to treat pain.

In summary, here, we describe a novel regulation of human TRESK by anionic membrane lipids through interaction with a motif in the intracellular loop of the channel. This regulation is unique in the human channel and not present in rodent orthologs. This finding further expands the different properties of the human channel, which should be taken into account when extrapolating results from rodent models. Also, it opens the door to possible new drug developments that target this channel for the treatment of pain.

Authors' Contributions Authors JPG, AC, and AA carried out cellular cultures, plasmid generation, and transfection. JPG and IE performed construction of GST fusion proteins, chimeric constructs, and liposome binding assays. AC, AA, and XG performed electrophysiological recordings in cell lines. JPG and XG participated in the design of the study and performed the statistical analysis. JPG and XG conceived the study, oversaw the research, and prepared the manuscript with help from all others. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no competing financial interests

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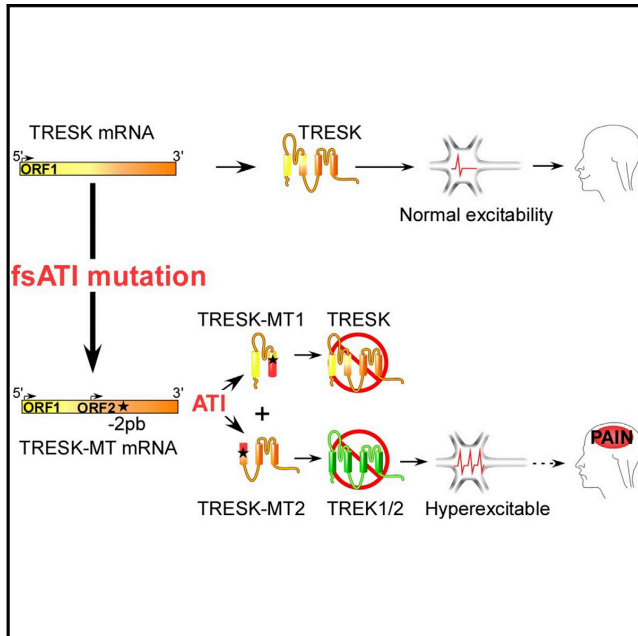
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Migraine-Associated TRESK Mutations Increase Neuronal Excitability through Alternative Translation Initiation and Inhibition of TREK

Graphical Abstract



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In Brief

Royal et al. demonstrate that migraine-associated frameshift mutations of TRESK, a two-pore-domain K⁺ channel, lead to the production of a second protein fragment, which carries the pathophysiological function by inhibiting TREK1 and 2, due to a mechanism called frameshift mutation-induced alternative translation initiation (fsATI).

Highlights

- TRESK, a K₂P channel implicated in migraine, heteromerizes with TREK1 and TREK2
- Migraine-associated TRESK-MT mutant induces formation of two proteins: MT1 and MT2
- MT2 is produced by frameshift mutation-induced alternative translation initiation
- MT2 inhibits TREK to increase neuronal excitability and induce a migraine phenotype



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Migraine-Associated TRESK Mutations Increase Neuronal Excitability through Alternative Translation Initiation and Inhibition of TREK

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SUMMARY

It is often unclear why some genetic mutations to a given gene contribute to neurological disorders and others do not. For instance, two mutations have previously been found to produce a dominant negative for TRESK, a two-pore-domain K⁺ channel implicated in migraine: TRESK-MT, a 2-bp frameshift mutation, and TRESK-C110R. Both mutants inhibit TRESK, but only TRESK-MT increases sensory neuron excitability and is linked to migraine. Here, we identify a new mechanism, termed frameshift mutation-induced alternative translation initiation (fsATI), that may explain why only TRESK-MT is associated with migraine. fsATI leads to the production of a second protein fragment, TRESK-MT2, which co-assembles with and inhibits TREK1 and TREK2, two other two-pore-domain K⁺ channels, to increase trigeminal sensory neuron excitability, leading to a migraine-like phenotype in rodents. These findings identify TREK1 and TREK2 as potential molecular targets in migraine and suggest that fsATI should be considered as a distinct class of mutations.

INTRODUCTION

Migraine is a common, disabling neurological disorder with a genetic, environmental, and hormonal component with an annual prevalence estimated at ~15%. It is characterized by attacks of severe, usually unilateral, and throbbing headache and can be accompanied by nausea, vomiting, and photophobia. Migraine is clinically divided into two main subtypes: migraine with aura (MA), when a migraine is preceded by transient neurological disturbances that are usually visual, and migraine without

aura (MO). Cortical spreading depression (CSD) underlies the aura, although its precise relationship to headache is unclear. Activation and sensitization of trigeminal neurons (TG) leading to the release of pro-inflammatory peptides is likely a key component in pain initiation and transmission in migraine (Nosedá and Burstein, 2013; Yan and Dussor, 2014).

Recent evidence points to a pivotal contribution of a variety of two-pore-domain potassium (K_{2P}) channels in chronic pain processing. The diverse K_{2P} channel family is made of 15 subtypes, which form 6 subfamilies. The activity of these channels drives the membrane potential toward the K⁺ equilibrium potential and therefore reduces cellular excitability. Expression of several K_{2P} channel subunits has been detected in nociceptive dorsal root ganglion and trigeminal neurons (Alloui et al., 2006; Bautista et al., 2008; Bliin et al., 2016; Morenilla-Palao et al., 2014; Noël et al., 2009; Yamamoto et al., 2009). One subtype of K_{2P} channels that is highly expressed in sensory neurons, TRESK, has been directly linked to MA via a causally linked 2-bp frameshift mutation (F139WfsX24) identified in the KCNK18 gene, which causes premature truncation of TRESK ("TRESK-MT"; Lafrenière et al., 2010; Wood, 2010). This mutation segregated perfectly with the MA phenotype in a large pedigree and was shown to produce a non-functional protein that can serve as a dominant negative that functionally downregulates the wild-type (WT) TRESK channel (Lafrenière et al., 2010; Wood, 2010). TRESK-MT has been shown to induce hyperexcitability of TG neurons (Guo et al., 2014; Liu et al., 2013), which likely underscores its role in migraine. However, in subsequent genetic screening studies, another missense TRESK variant, C110R, was identified (Andres-Enguix et al., 2012). TRESK-C110R, similar to TRESK-MT, exerts a dominant-negative effect on WT-TRESK in heterologous cells, but expression of this mutant was found to have no effect on TG excitability (Guo et al., 2014). This absence of effect explains why this mutant was found in both migraine patients and control subjects (Guo et al., 2014). Therefore, despite the fact that both mutations lead to the same apparent effect on TRESK function, only TRESK-MT is able to increase TG excitability and is linked to migraine pathophysiology.



Classically a eukaryotic mRNA is thought to contain one translation start codon, which allows the production of a single protein species. However, in some cases, eukaryotic ribosomes can recognize several alternative translation start sites to induce the formation of several different proteins from the same mRNA (Kochetov, 2008). This alternative translation initiation is a means of expanding the proteome (Kochetov, 2008) and has been shown to increase the functional diversity of K_{2P} channels (Thomas et al., 2008), raising the possibility that it plays a role in TRESK-mediated migraine pathophysiology.

In this study, we addressed why TRESK-MT (F139WfsX24), but not TRESK-C110R, is able to increase TG excitability and, potentially, play a role in migraines. Using single-molecule fluorescence and chemical optogenetic methods, we found that TRESK is able to heterodimerize with 2 distantly related K_{2P} channels from another subfamily, TREK1 and TREK2, and that TRESK-MT strongly inhibits TRESK, TREK1, and TREK2 currents. In stark contrast, we show that TRESK-C110R is only able to inhibit TRESK, but not TREK1 or TREK2. Furthermore, we show, using double knockout (KO) mice for TREK1 and TREK2, that TRESK-MT increases TG neuronal excitability by inhibiting TREK1 and TREK2. Consistent with a role for TREK1 and TREK2 in migraine induction, we found that double-KO mice for TREK1 and TREK2 present, at rest, a migraine-like allodynia phenotype. These results resolve the contradictory lack of effect of TRESK-C110R, which targets only TRESK and not TREK1 or TREK2. Strikingly, we next find that the 2-bp frameshift mutation of TRESK-MT puts an alternative start codon in frame, which leads to the translation of a second TRESK fragment, termed MT2, which specifically co-assembles with TREK1 and TREK2 to downregulate their function, leading to the TG excitability increase. Consistent with a role for MT2 in migraine induction, we found that MT2 expression within the trigeminal ganglia induced a migraine-like allodynia phenotype in rat. Finally, we find that other previously uncharacterized migraine-associated TRESK mutations also produce multiple fragments via alternative translation initiation (ATI) that can have distinct effects on TRESK and TREK channels. Together, these findings identify frameshift-induced ATI (fsATI) as a mechanism initiated by TRESK mutations, which leads to two protein fragments with dominant-negative effects on distinct channel targets to, ultimately, increase sensory neuron excitability, which may contribute to migraine induction.

RESULTS

TRESK Heteromerizes with TREK1 and TREK2

Despite the fact that K_{2P} channels share a similar architecture and global function, they share a low level of sequence identity, even between members of the same subfamily. Surprisingly, this low level of identity does not preclude heteromerization, as we and others recently showed within the TREK subfamily (Blin et al., 2016; Hwang et al., 2014; Lengyel et al., 2016; Levitz et al., 2016). Based on this and the fact that TG neurons express many K_{2P} channels (TREK1, TREK2, TRAAK, TASK1, and TASK3; Bautista et al., 2008; Yamamoto et al., 2009), we hypothesized that the difference between TRESK mutants is due to their differential ability to modify the function of other K_{2P} chan-

nels through heteromerization. To assess the ability of TRESK to heteromerize with other K_{2P} channels that are expressed in TG neurons, we used the single-molecule pull-down (“SiMPull”) assay (Jain et al., 2012) to visualize individual antibody-immobilized protein complexes on polyethylene-glycol-passivated glass coverslips (Figure 1A). We co-expressed GFP-TRESK with either hemagglutinin HA-TRESK, HA-TREK1, HA-TREK2, HA-TRAAK, HA-TASK1, or HA-TASK3 and assessed their ability to co-immunoprecipitate (coIP) GFP-TRESK via an anti-HA antibody. HA-TRESK, HA-TREK1, and HA-TREK2, were able to coIP many fluorescent GFP-TRESK spots (Figures 1B and 1C), whereas no GFP-TRESK spots were observed for HA-TRAAK, HA-TASK1, or HA-TASK3 (Figure 1C), indicating that TRESK co-assembly with other K_{2P} channels is specific for TREK1 and TREK2. Importantly, controls showed that identical results were observed in two different non-ionic detergents (Figure 1C), that similar expression levels were seen for GFP-TRESK when co-expressed with HA-TREK1 or HA-TRESK (Figure S1A), that all HA-tagged K_{2P} constructs were able to pull down themselves (Figures S1B and S1C), and that pull-down was dependent on the presence of the anti-HA antibody (Figure S1D) and confirm that TREK1, TREK2, and TRESK can be co-expressed in the same cultured TG neurons using immunofluorescence and single-cell RT-PCR (Figure S1F).

We next used photobleaching step analysis (Ulbrich and Isacoff, 2007) to determine the stoichiometry of TREK1 and TRESK complexes to test the hypothesis that they form heterodimers. First, we confirmed that HA-GFP-TREK1 form homodimers in our assay by observing ~70% 2-step bleaching for each when expressed and immunoprecipitated alone with anti-HA antibodies (Figure S2A), consistent with the formation of strict dimers with a GFP maturation rate of ~80%. Then, we counted the number of GFP-TRESK subunits within a HA-TREK1/GFP-TRESK complex by observing bleaching steps of GFP-TRESK coIP with immobilized HA-TREK1 (Figure S2C). The majority of fluorescence intensity trajectories showed one bleaching step (~70%; Figure S2C). This distribution is similar to the one observed for HA-TREK1/GFP-TREK1 complexes (Figure S2B) and agrees well with a 1:1 stoichiometry showing that TREK1-TRESK is primarily a heterodimer.

To test the functionality of the TREK1-TRESK heterodimer, we developed a heterodimerization assay based on an engineered “photoswitchable conditional subunit” (TREK1-PCS) of TREK1. The TREK1-PCS is a TREK1 subunit where the C terminus has been deleted to produce endoplasmic reticulum retention, which can be rescued through co-assembly with a full-length subunit (Sandoz et al., 2012). Following co-assembly and surface targeting, TREK1-PCS can then optically control the channel via a tethered photoswitchable blocker (“MAQ”), which attaches to a genetically engineered cysteine. Therefore, gain of photosensitivity of an identified co-expressed TREK-interacting subunit allows for the verification of a functional heteromer with TREK1. As expected, expression of TREK1-PCS alone did not generate a photoswitchable current (Figure 1D), but co-expression with either TREK1 or TRESK induced a robust photoswitchable current (Figures 1E and 1F), indicating that the TRESK subunit is able to co-assemble with TREK1-PCS. Consistent with SiMPull data, no photocurrent was observed when TASK1 (Figure 1G)

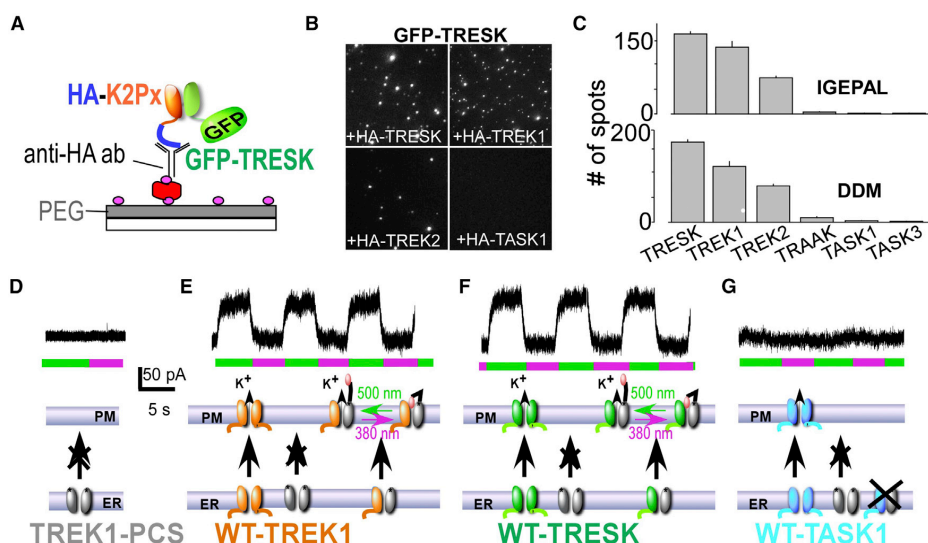


Figure 1. TRESK Heteromerizes Physically and Functionally with TREK1 and TREK2

(A) Schematic of single-molecule pull-down (SiMPull) of GFP-TRESK. HEK293T cells expressing GFP-TRESK and an HA-tagged K2P channel ("HA-K2Px") were lysed and then immobilized on a polyethylene glycol (PEG)-passivated coverslip conjugated to a biotinylated anti-HA antibody. (B and C) Representative images (B) (IGEPAL) and summary bar graphs (C) with 2 different detergents, IGEPAL and DDM, showing pull-down of GFP-TRESK by HA-TRESK, HA-TREK1, or HA-TREK2, but not by HA-TASK1, HA-TASK3, or HA-TRAAK. Data are represented as mean $\pm$ SEM. (D–G) Co-expression of TREK1-PCS with WT-TREK1 produces a heteromeric channel that traffics from the endoplasmic reticulum (ER) to the plasma membrane (PM) and which can be light gated due to attachment of a photoswitchable blocker to the TREK1-PCS. TREK1-PCS expression alone does not produce any photoswitchable current (D), but co-expression of TREK1 (E) or TRESK (F), but not TASK1 (G), leads to a photoswitchable current indicating that TREK1-TRESK form functional heteromers, but there is no functional assembly of TREK1 and TASK1. See also Figures S1, S2, and S3.

or TASK3 (Figure S1E) were co-expressed with TREK1-PCS. Furthermore, the bleaching step distribution of GFP-TREK1-PCS spots colPeped with immobilized HA-TREK1 is similar to HA-TREK1/GFP-TREK1; the majority of fluorescence spots showed one bleaching step ($\sim 70\%$; Figure S2D), supporting the conclusion that the light-gated TREK1-PCS/TRESK current is carried by a TREK1-PCS/TRESK heterodimer with a common pore.

Next, to test the functional properties of the TREK1-TRESK heteromer, we constructed a linked, tandem dimer to have a uniform representation at the surface of the cell of the TREK1-TRESK heteromer (Figure S3). This heteromeric channel displayed properties that are a mix of those from TREK1 and TRESK homodimers. Notably, TRESK is insensitive to arachidonic acid and TREK1 is sensitive (Figures S3A–S3C) and TRESK-TREK1 tandems show an intermediate sensitivity to arachidonic acid (Figure S3C). Furthermore, similar to TRESK, but not TREK1, TRESK-TREK1 tandems showed sensitivity to intracellular calcium, as tested with ionomycin application (Figures S3D and S3F). Consistent with this, the light-gated TREK1-PCS/TRESK current is also calcium sensitive (Figures S3E and S3F), confirming that the light-gated TREK1-PCS/TRESK current is carried by a TREK1-TRESK heteromer with a common pore.

Having found that TREK can physically and functionally heteromerize with TRESK and that all three channel subtypes are

co-expressed in sensory neurons, we next investigated the ability of TRESK mutants to modify TREK1 and TREK2 currents.

TRESK-MT, but Not TRESK-C110R, Acts as a Dominant Negative on TREK1 and TREK2 Channels

As previously shown (Andres-Enguix et al., 2012), both TRESK-MT and TRESK-C110R exert a dominant-negative effect on whole-cell TRESK currents (Figures 2A–2C). Because TREK1 can co-assemble with TRESK (Figure 1), we addressed the impact of the MT and C110R variants on TREK1 current. We found that TRESK-C110R co-expression did not modify TREK1 current, whereas TRESK-MT co-expression induced a near-complete inhibition of TREK1 current (Figures 2D–2F). Similar to TREK1, TRESK-MT, but not TRESK-C110R, strongly inhibited TREK2 current (Figures 2G–2I). This dominant-negative effect is specific and likely dependent on co-assembly because TASK1, TASK3, and TRAAK, which do not colP with TRESK (Figure 1), were not sensitive to TRESK-MT co-expression (Figure S4). To address why TRESK-C110R does not modify TREK1 or TREK2 current, we used the SiMPull assay to test the ability of TRESK-C110R to physically interact with TREK1. We co-expressed HA-TREK1 with either GFP-TRESK or GFP-TRESK-C110R and tested their ability to be colPeped with HA-TREK1 via an anti-HA antibody. Whereas GFP-TRESK was able to be colPeped with HA-TREK1, leading to many fluorescent spots,

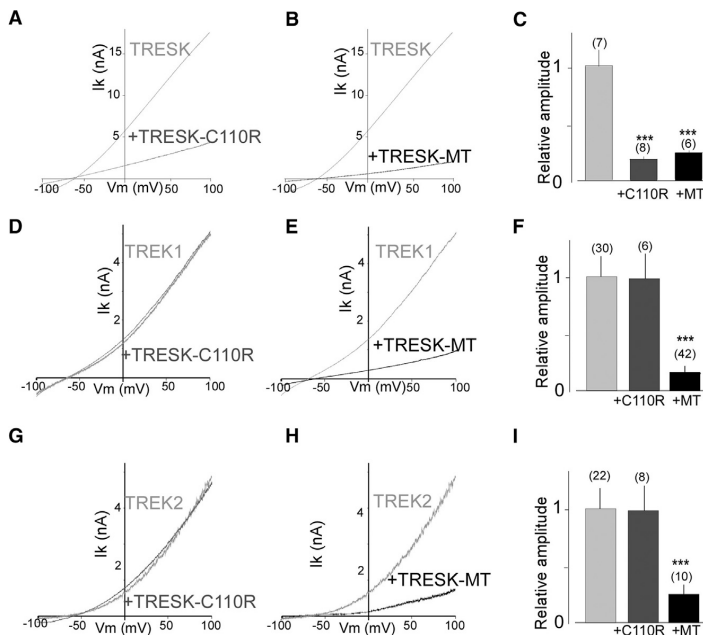


Figure 2. TRESK-MT, but Not TRESK-C110R, Acts as a Dominant Negative on TREK1 and TREK2 Channels

(A and B) Representative traces showing the effect of TRESK-C110R (A) and TRESK-MT (B) co-expression on TRESK current in HEK293T cells. Currents were elicited by voltage ramps (from -100 to 100 mV; 1 s duration).

(C) Bar graph summarizing the relative TRESK current amplitude at 0 mV for TRESK when TRESK-C110R and TRESK-MT are or not co-expressed. Data are represented as mean $\pm$ SEM.

(D–F) Same as (A)–(C) for TREK1.

(G–I) same as (A)–(C) for TREK2. The numbers of cells tested are indicated in parentheses on the graphs. Student's t test (** $p < 0.001$).

See also Figures S4 and S5.

very few spots were observed for GFP-TRESK-C110R (Figure S5A). This indicates that TREK1 can co-assemble with TRESK and that the C110R mutation leads to a drastic reduction of this association, explaining why TRESK-C110R has no effect on TREK1 current. Together, these data show that TRESK-MT can inhibit TRESK, TREK1, and TREK2, whereas TRESK-C110R is only able to inhibit TRESK. Based on the fact that TRESK-MT, but not TRESK-C110R, is able to induce TG neuron hyperexcitability (Guo et al., 2014; Liu et al., 2013), we hypothesized that TRESK-MT induces sensory neuron hyperexcitability primarily by acting on TREK1 and TREK2, not TRESK.

TRESK-MT Increases Neuronal Excitability through the Inhibition of TREK1 and TREK2

To investigate the role of TREK1 and TREK2 in the induction of TG hyperexcitability by TRESK-MT, we tested whether overexpression of GFP-TRESK-MT alters the passive and active electrophysiological properties of small-diameter (<25 μ m) TG neurons from wild-type or TREK1/TREK2 double-knockout ($TREK1^{-/-}/TREK2^{-/-}$) mice. As previously shown, TRESK-MT expression in WT TG neurons led to a decrease of the lamotrigine current (Figure S6), leading to an increase in excitability (Figure 3C), which included a decrease in the rheobase (74 ± 11 pA versus 47 ± 5 pA; $p < 0.05$ for TG neurons expressing GFP or TRESK-MT, respectively) and an increase in the number of action potentials (APs) evoked by suprathreshold current injections compared to control (Figures 3A and 3C). As shown in Figure 3B, neurons from $TREK1^{-/-}/TREK2^{-/-}$ mice were more excitable than WT TG neurons. These neurons have a smaller lamotrigine current

(Figure S6), smaller rheobase (55 ± 6 pA; $p < 0.05$) and a significant increase in the number of APs evoked by suprathreshold current injections compared to WT TG neurons. Consistent with a role for TREK1 and 2 in mediating the effects of TRESK-MT, TRESK-MT overexpression did not alter the excitability of $TREK1^{-/-}/TREK2^{-/-}$ mice (Figure 3D). $TREK1^{-/-}/TREK2^{-/-}$ TG neurons showed no increase in the number of evoked APs or a reduction in rheobase

(55 ± 6 pA versus 53 ± 5 pA for TG neuron expressing GFP or TRESK-MT, respectively; $p > 0.5$). Together, these data strongly support a major role for TREK1 and TREK2 in the control of TG neuron excitability and support the idea that TRESK-MT differs functionally from TRESK-C110R in its ability to target TREK1 and TREK2 to increase the excitability of TG neurons, which is likely a crucial step in the induction of migraines.

$TREK1^{-/-}/TREK2^{-/-}$ Mice Show a Mechanical Allodynia, which Is Not Increased by NO Donors

Having found that expression of the TRESK-MT mutant increases TG excitability through TREK1-TREK2 inhibition, we hypothesized that $TREK1^{-/-}/TREK2^{-/-}$ mice would show an increased susceptibility to a migraine-related phenotype. Migraine is associated with an increase of the sensitivity to all sensory modalities, and cutaneous allodynia can be used as a quantifiable marker of migraine disorder (Bates et al., 2010; Verkest et al., 2018). One approach to model acute and chronic migraine is the quantification of this increase in response to known migraine triggers, such as nitric oxide (NO) donors (Bates et al., 2010), including isosorbide dinitrate (ISDN) (Verkest et al., 2018). We quantified ISDN-evoked mechanical allodynia in $TREK1^{-/-}/TREK2^{-/-}$ and wild-type controls in acute and chronic conditions. In a first experiment, paw mechanical nociception thresholds were determined with a dynamic von Frey aesthesiometer before and during a 3-hr period after intraperitoneal injection of ISDN (10 mg/kg; Figure 3E; Bates et al., 2010; Verkest et al., 2018). In a second experiment, we assessed mechanical nociception thresholds in both $TREK1^{-/-}/TREK2^{-/-}$ and wild-type controls

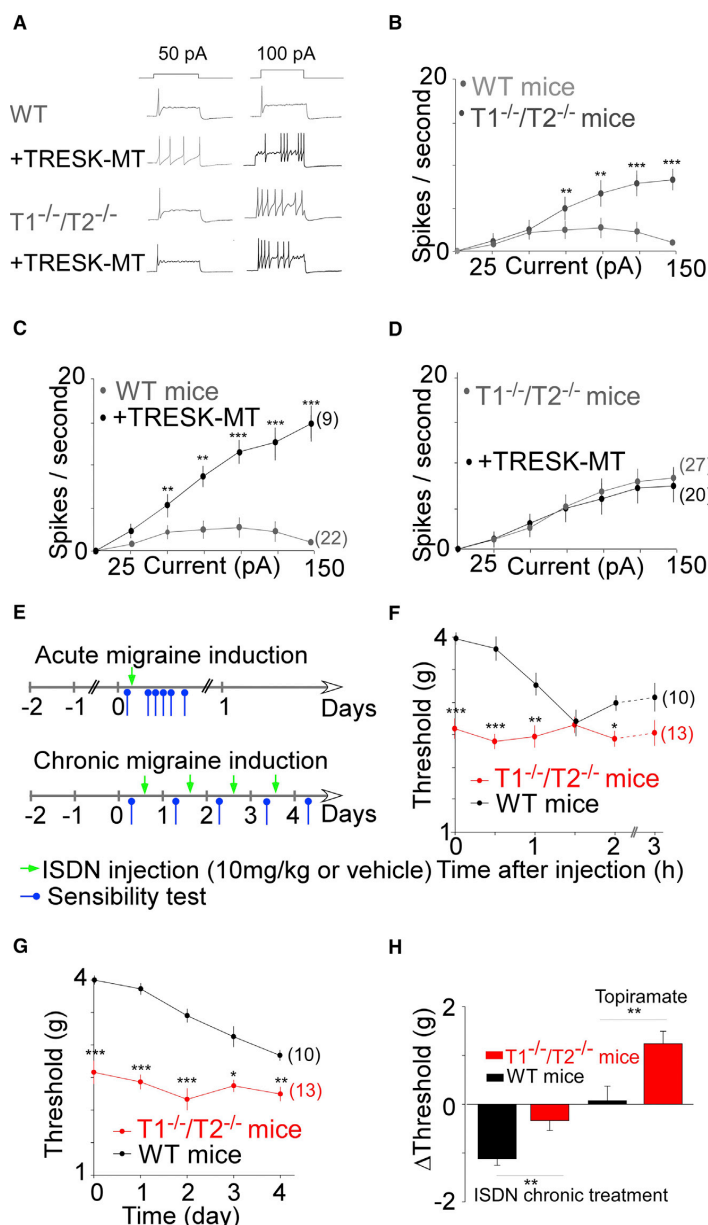


Figure 3. TREK1 and TREK2 Invalidation Increases Sensory Neuron Excitability and Mechanical Pain Perception

(A–D) *TREK1*^{-/-}/*TREK2*^{-/-} TG neurons are more excitable than WT TG neurons and are not sensitive to TRESK-MT overexpression.

(A) Representative traces of action potentials (spikes) generated by incremental depolarizing current injections in small-diameter TG neurons.

(B) Input-output plots of spike frequency in response to 1-s depolarizing current injection in untransfected, small TG neurons from WT and *TREK1*^{-/-}/*TREK2*^{-/-} double-KO mice.

(C and D) TRESK-MT acts as a dominant negative on TREK1 and TREK2 channels to increase excitability of TG neurons. Input-output plots of the spike frequency in response to 1-s depolarizing current injection in transfected small TG neurons from WT (C) and *TREK1*^{-/-}/*TREK2*^{-/-} double-KO mice (D) show that an increase in excitability elicited by TRESK-MT is observed in WT, but not *T1*^{-/-}/*T2*^{-/-} neurons.

The numbers of tested cells are indicated in parentheses on the plots and come from at least 4 different animals. Student's *t* test (**p* < 0.05; ***p* < 0.01; ****p* < 0.001).

(E–H) *TREK1*^{-/-}/*TREK2*^{-/-} double-knockout animals present a migraine-like hypersensitivity to mechanical stimuli.

(E) Schematic of experimental behavioral paradigms. Green arrows represent the injection of ISDN, a known migraine trigger. Blue arrows represent the measurement of mechanical sensitivity.

(F) Paw withdrawal mechanical threshold, assessed after the first ISDN injection, were significantly decreased in double-knockout animals and remained less than WT for the first 1.5 hr following ISDN injection.

(G) Mechanical responses, assessed prior to and after chronic ISDN injections, were significantly decreased in double-knockout animals.

(H) Variation (ΔThreshold (g)) of the paw withdrawal mechanical threshold induced by ISDN chronic treatment or by topiramate injection. Mechanical responses were assessed before and after ISDN chronic (4 days) treatment (left bars) and before and 2 hr after topiramate injection (right bars) in WT and *TREK1*^{-/-}/*TREK2*^{-/-} double-knockout ISDN-nontreated mice. Numbers of mice tested are indicated in parentheses on the plots; Student's *t* test to compare WT versus *TREK1*^{-/-}/*TREK2*^{-/-} mice (**p* < 0.05; ***p* < 0.01; ****p* < 0.001). Data are represented as mean ± SEM. See also Figure S6.

by intraperitoneally injecting ISDN every day for four days as a model of chronic migraine-associated pain (Figure 3E). We found that, at rest, *TREK1*^{-/-}/*TREK2*^{-/-} mice showed a decreased mechanical threshold compared to WT mice (2.6 ± 0.1 g versus 3.9 ± 0.1 g; *p* < 0.001). Notably, the basal mechanical threshold of

the injection of ISDN in *TREK1*^{-/-}/*TREK2*^{-/-} mice does not induce any modification of the mechanical threshold, which remained significantly lower than in wild-type controls during the first 1.5 hr following ISDN injection (Figure 3F; *p* < 0.001 after 30 min and *p* < 0.01 after 1 hr with a linear mixed-effects model).

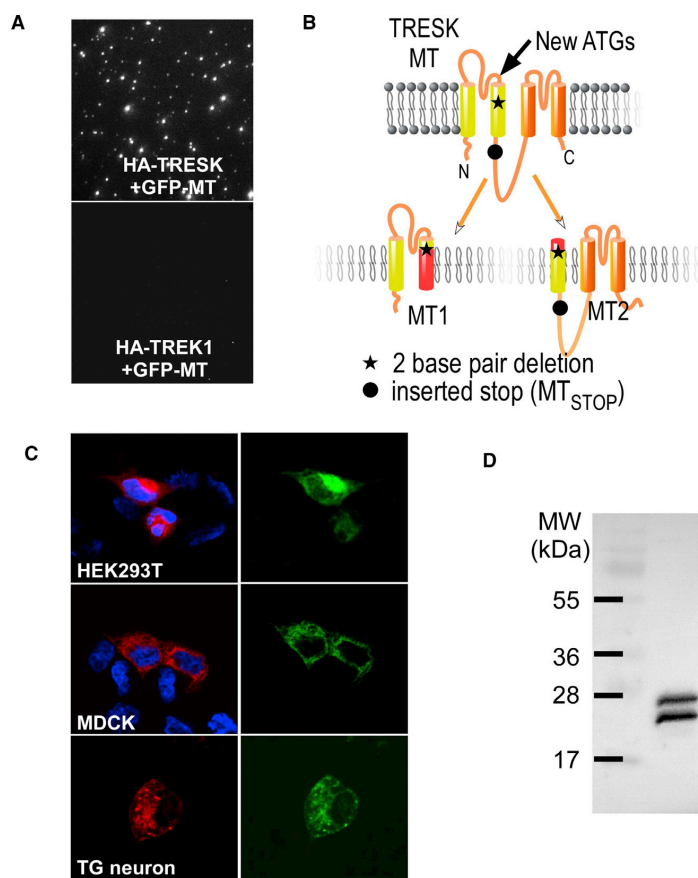


Figure 4. TRESK-MT Induces the Translation of a Second Protein, MT2

(A) Representative images from SIMPull experiments showing that GFP-TRESK-MT can be pulled down by HA-TRESK, but not by HA-TREK1.

(B) Cartoon showing the membrane topology of TRESK and the expected products induced by ATI in the TRESK-MT mutation. The region corresponding to aberrant sequences is shown in red.

(C) Co-synthesis of mCherry-MT1 and MT2-GFP products from the mCherry-TRESK-MT-GFP cDNA in HEK293T cells (top), MDCK (middle), and TG neurons (bottom). DAPI nuclear stain is shown in blue.

(D) Western blot against HA-TRESK-MT-HA probed with anti-HA antibodies from HEK293T cells lysate. See also Figure S10.

In the chronic migraine-associated pain assay, the mechanical thresholds remained significantly lower for *TREK1*^{-/-}/*TREK2*^{-/-} mice compared to wild-type controls, but the difference was strongly reduced (Δ mechanical threshold induced by 4 days ISDN treatment 0.33 ± 0.2 g in *TREK1*^{-/-}/*TREK2*^{-/-} versus 1.12 ± 0.1 g in WT mice; Figure 3G).

Having found that *TREK1*^{-/-}/*TREK2*^{-/-} mice show basal mechanical allodynia that cannot be further increased by an ISDN injection, we wondered whether a treatment used in prophylaxis in migraine patient, topiramate, could reverse this observed migraine-like phenotype as was observed in models of nitroglycerin (NO) donor-induced migraine (Pradhan et al., 2014) and in a model of ISDN-induced migraine in rats (Verkest et al., 2018). We assessed the mechanical nociception threshold in *TREK1*^{-/-}/*TREK2*^{-/-} mice before and 2 hr following the intraperitoneal injection of 30 mg/kg of topiramate. Treatment with topiramate reversed partially the chronic basal allodynia seen in *TREK1*^{-/-}/*TREK2*^{-/-} mice (Δ mechanical threshold of 1.2 ± 0.2 g; Figure 3H), as was previously observed for a nitroglycerin-evoked form of al-

lodynia (Pradhan et al., 2014). As a control, we tested non-treated ISDN WT mice and did not observe any significant shift of the mechanical threshold following topiramate treatment (Figure 3H).

These data demonstrate that, at rest, the *TREK1*^{-/-}/*TREK2*^{-/-} mice present an allodynia phenotype, which is similar to the phenotype observed in ISDN-treated WT animals. This is consistent with a role of TREK1/TREK2 in trigeminal sensory neuron hypersensitivity that is relevant to migraine, which is also supported by the reversion induced by topiramate, a drug used in the clinic to treat chronic migraine.

TRESK-MT Mutation Induces the Translation of a Second Protein, MT2

We next explored how TRESK-MT exerts its effects on TREK channels at the molecular level. The F139WfsX24 frameshift mutation

of TRESK-MT results in the premature truncation of the human TRESK protein from 384 amino acids (aas) to 162 aas. The truncated TRESK includes the first 138 aas of wild-type TRESK followed by a 24-aa aberrant sequence. The corresponding mutation has very similar effects on the mouse TRESK gene, generating a truncated protein with the first 149 aas of wild-type TRESK and followed by a 50-aa aberrant sequence at the C terminus (Figure 4B). We fused a GFP tag to the N terminus of the mouse TRESK-MT and tested its ability to be immobilized by HA-TRESK, HA-TREK1, or HA-TREK2 in the SIMPull assay. Surprisingly, only HA-TRESK was able to coIP GFP-TRESK-MT via an anti-HA antibody (Figure 4A). This result confirms that TRESK-MT associates with TRESK to induce its dominant-negative effect but raises the question of how TRESK-MT is able to inhibit TREK1 and TREK2 without direct association.

It has been hypothesized that ATI of eukaryotic mRNAs, including those that encode K_{TP} channels (Thomas et al., 2008), may provide a method to expand the proteome (Kochetov, 2008). A close examination of the nucleotide sequence of

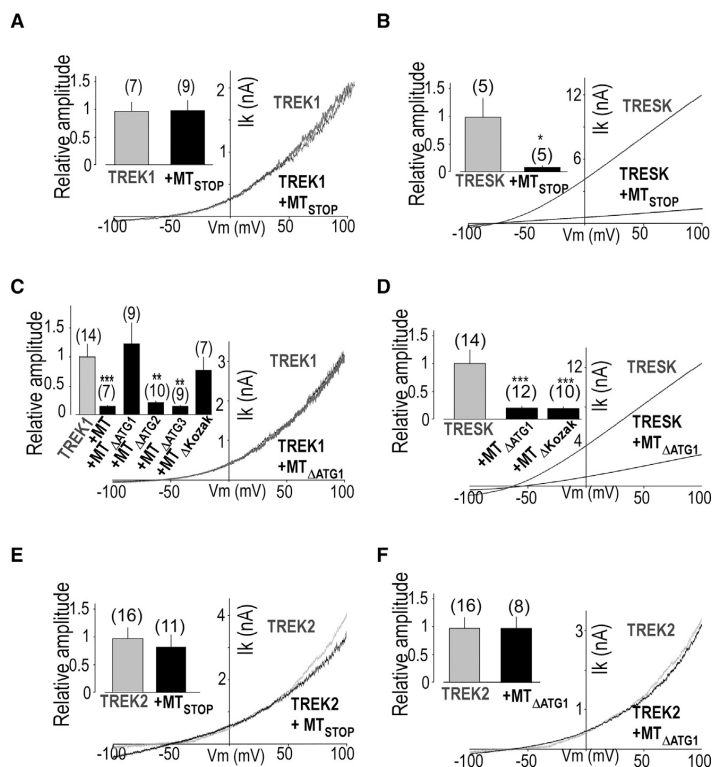


Figure 5. MT2 Mediates TREK1 Inhibition

(A) Representative traces showing the effect of introduction of a STOP codon at the beginning of the MT2 ORF within the 2-3 loop (TREK1-MT_{STOP}) on TREK1 current in HEK293T cells. Inset shows a summary of TREK1 relative current densities when TREK1-MT_{STOP} is co-expressed.

(B) Representative traces showing the effect of TREK1-MT_{STOP} on TREK1 current. (Inset) TREK1 relative current densities when TREK1-MT_{STOP} is co-expressed are shown.

(C) Representative traces showing the effect of introduction of a mutation of ATG at position +356 (ΔATG1) on TREK1 current and summary bar graph showing the effect of mutation of candidate alternative start codons (ΔATG1, ΔATG2, or ΔATG3) and mutation of the Kozak sequence surrounding ATG1 (ΔKozak) in TREK1-MT.

Currents were elicited by voltage ramps (from -100 to 100 mV; 1 s duration). (D) Representative traces showing the effect of TREK1-MT_{ΔATG1} on TREK1 current. (Inset) TREK1 relative current densities when TREK1-MT_{ΔATG1} is co-expressed are shown.

(E and F) same as (A) and (C) for TREK2. The numbers of cells tested are indicated in parentheses on the graphs. Cells come from at least two experimental days. Student's t test (*p < 0.05, **p < 0.01, and ***p < 0.001) shows the difference between TREK1, TREK2, or TREK2 and TREK1, TREK2, or TREK2 when co-expressed with different TREK1-MT constructs. Data are represented as mean ± SEM.

See also Figures S7 and S11.

TREK-MT revealed that the F139WfsX24 frameshift mutation places two new ATG codons in frame with the reference open reading frame of TREK (ATGs at position +356 and +407 for the human TREK cDNA and +389 and +490 for the mouse cDNA). We hypothesized that one of these codons may serve as an ATG site that can lead to the formation of a second truncated TREK protein, termed "MT2," that would include a short (either 2 or 19 aas) N-terminal aberrant sequence followed by the C-terminal part of MT2, including the 2-3 intracellular loop, TM3, P2 loop, TM4, and the C-terminal domains (Figure 4B). To test whether MT2 is co-translated with MT1, we introduced an N-terminal mCherry tag in frame with MT1 and a C-terminal GFP in frame with MT2 within the mouse TREK-MT cDNA ("mCherry-TREK-MT-GFP"). Expression of this construct led to HEK293T cells with both mCherry and GFP fluorescence, showing the co-translation of mCherry-MT1 and MT2-GFP (Figure 4C). This co-translation was observed in other cell lines, including MDCK cells (Figure 4C), as well as in primary TG neurons (Figure 4C). Next, we introduced an N-terminal HA tag in frame with MT1 and another one in frame with MT2 within the mouse TREK-MT cDNA ("HA-TREK-MT-HA"). Lysate from cells transfected with HA-TREK-MT-HA was probed in a western blot with anti-HA antibodies, and 2 bands, with a similar intensity, corresponding to the expected molecular weights for

MT1 (~23 kDa) and MT2 (~29 kDa) were detected (Figure 4D). Together, these data clearly show that TREK-MT leads to the production of two distinct fragments of TREK.

To probe the function of MT2, we introduced a stop codon into the MT2 open reading frame (ORF) of TREK-MT at the beginning of the 2-3 loop (Figure 4B), inducing the loss of expression of MT2 (Figure S7A). As shown in Figure 5, whereas the introduction of the stop codon in the MT2 ORF did not change the ability of TREK-MT to inhibit TREK current (Figure 5B), this stop codon abolished the ability of TREK-MT to produce a dominant-negative functional effect on TREK1 (Figure 5A). We next confirmed the importance of this second ORF by mutating, in TREK-MT, the putative ATG start codons one by one. Mutation of the first ATG abolished the ability of TREK-MT to inhibit TREK1 (Figure 5C), but not TREK (Figure 5D), whereas mutation of the second ATG did not alter the ability of TREK-MT to inhibit TREK1 current (Figures 5C and S7B). These data indicate that the ATG site is the first internal ATG. As a control, we mutated a third ATG, which is also present in the WT-TREK sequence, and found that it did not change the ability of TREK-MT to inhibit both TREK and TREK1 currents (Figures 5C and S7C). Similar to TREK1, introduction of a stop codon into the MT2 ORF or mutation of the first ATG also abolished the inhibition of TREK2 by TREK-MT (Figures 5E and 5F). To further

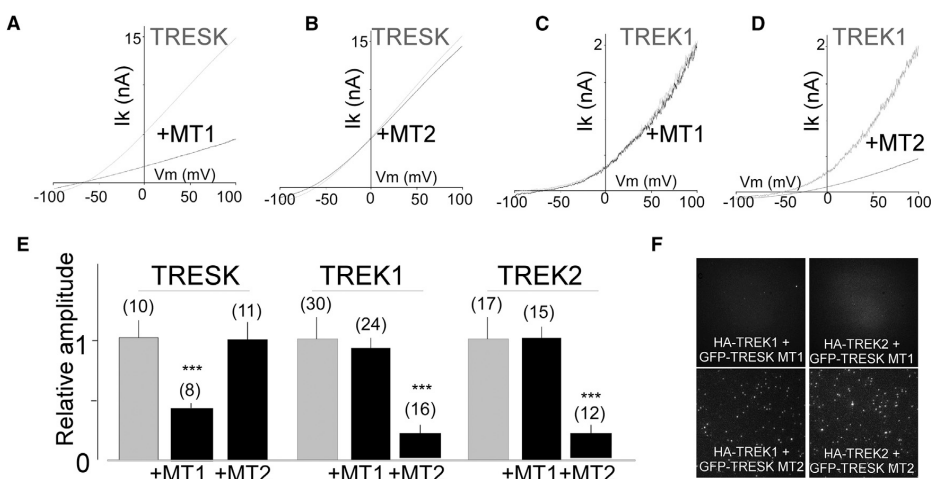


Figure 6. MT1 Acts as Dominant Negative on TRESK, whereas MT2 Acts as a Dominant Negative on TREK1 and TREK2 Channels

(A–D) Representative traces showing the effect of TRESK-MT1 (A and C) or TRESK-MT2 (B and D) co-expression on TRESK (A and B) or TREK1 (C and D) currents in HEK293T cells. Currents were elicited by voltage ramps (from -100 to 100 mV; 1 s duration).

(E) Bar graph summarizing the relative TRESK, TREK1, and TREK2 current amplitudes at 0 mV when MT1 or MT2 are co-expressed. The numbers of cells tested are indicated in parentheses on the graphs. Cells come from at least two experimental days. Student's *t* test ($***p < 0.001$). Data are represented as mean $\pm$ SEM. (F) Representative images showing that GFP-MT2, but not GFP-MT1, can be pulled down by HA-TREK1 and HA-TREK2 via an anti-HA antibody in the SIMPull assay with HEK293T cells.

See also Figures S7, S8, and S11.

demonstrate that the first ATG serves as a start codon, we mutated the potential Kozak sequence GCTATGG to CCTATGC and found that alteration of the Kozak sequence strongly reduced the inhibitory effect of TRESK-MT on TREK1 current (Figures 5C and S7D) without affecting the effect on TRESK (Figures 5D and S7E).

MT2, but Not MT1, by Acting as a Dominant Negative on TREK1 and TREK2 Channels, Increases Neuronal Excitability of WT Small TG Neurons, Leading to Facial Allodynia

To independently express MT1 and MT2 for functional characterization, we sub-cloned both ORFs into separate mammalian expression vectors. Co-expression of MT2 with TRESK did not modify TRESK current (Figures 6B and 6E), and MT1 co-expression induced a ~ 3 -fold decrease of the current, which was similar to what was observed for the co-expression of the full TRESK-MT construct (Figures 6A and 6E). On the contrary, co-expression of MT1 did not modify TREK1 current (Figures 6C and 6E), but co-expression of MT2 induced a ~ 4 -fold decrease of the current, similar to what was observed with co-expression of the full TRESK-MT construct (Figures 6D and 6E). Similar results were obtained for TREK2 (Figures 6E and S8). Consistent with the functional data, we found that GFP-MT2 is colPed with HA-TREK1 or HA-TREK2 in the SIMPull assay (Figure 6F).

To validate the physiological role of interaction between TREK1, TREK2, and MT2, we tested the functional effect of MT2 in TG neurons. Whereas MT1 expression did not alter the excitability of WT TG neurons (Figures 7A and 7B), MT2

increased it significantly (Figures 7A and 7C). In fact, MT1 did not modify the rheobase (74 ± 11 pA versus 79 ± 5 pA; $p > 0.5$ for TG neurons expressing GFP or MT1, respectively) and did not modify the number of APs evoked by suprathreshold current injections compared to control. Conversely, MT2 expression in WT TG neurons led to an increase in excitability (Figure 7), which included a decrease in the rheobase (74 ± 11 pA versus 55 ± 5 pA; $p < 0.05$ for TG neurons expressing GFP or MT2, respectively; $p < 0.05$) and an increase in the number of APs evoked by suprathreshold current injections compared to control (Figures 7A and 7C). We confirmed that this effect is linked to TREK1 and TREK2 because MT2 overexpression failed to increase the excitability of *TREK1*^{-/-}/*TREK2*^{-/-} TG neurons (Figure 7D).

Having found that MT2, by inhibiting TREK1 and TREK2, is sufficient to increase TG excitability, we asked whether MT2 expression in TG ganglia would induce a migraine-related phenotype. We conducted behavioral experiments in rats in which MT2 was virally overexpressed within the trigeminal ganglia. Rats allowed to test the mechanical pain threshold on the face, which is directly linked to TG excitability, constituting a relatively direct, reliable, and quantifiable marker of migraine disorder in clinical contexts as well as in NO-induced migraine (Pradhan et al., 2014; Kopruszinski et al., 2017; Harris et al., 2017). As shown in Figure 7F, MT2 expression in TG ganglia (Figure S9) significantly increased the facial mechanical threshold (3.3 ± 0.4 g versus 7.7 ± 0.4 g; $p < 0.001$). Furthermore, as was seen for the *TREK1*^{-/-}/*TREK2*^{-/-} mice, the basal mechanical facial threshold of MT2-expressing rats is similar to the threshold

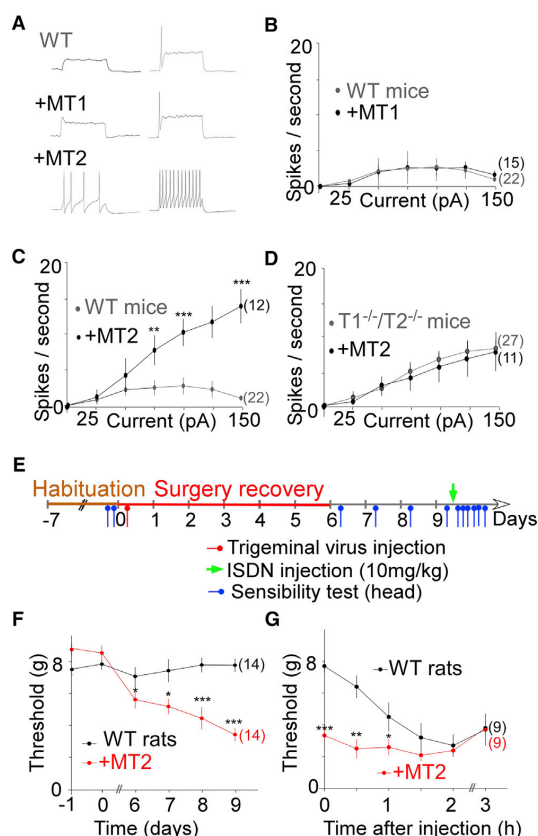


Figure 7. MT2, but Not MT1, Increases Neuronal Excitability of WT Small TG Neurons through TREK1 and TREK2 Inhibition

(A) Representative traces showing spikes generated by incremental depolarizing current injections (+50 pA and +100 pA) in small-diameter TG neurons. (B and C) Input-output plots of spike frequency in response to 1-s depolarizing current injections in WT small-diameter TG neurons transfected with either GFP ("WT"), the GFP-tagged MT1 subunit ("MT1"; B), or the GFP-tagged MT2 subunit ("MT2"; C). (D) Input-output plots of spike frequency show a lack of effect of GFP-MT2 expression on TG neurons from TREK1/TREK2 double-KO mice ($T1^{-/-}/T2^{-/-}$). (E-G) MT2 overexpression in TG leads to facial mechanical allodynia in rats. (E) Schematic of experimental behavioral paradigms. After a week of habituation, rats were injected with 10 μ L of AAV2 encoding for either MT2 + GFP or GFP. (F) Face withdrawal mechanical threshold assessed after trigeminal virus infection encoding either GFP (WT rat condition) or MT2. (G) Face withdrawal mechanical threshold, assessed after the first ISDN injection. The threshold for MT2-expressing rats was significantly decreased before the injection and remained less than WT for the first 1.5 hr following ISDN injection. The numbers of tested cells and rats are indicated in parentheses on the plots. Mice neurons come from at least 5 different animals. Student's *t* test (**p* < 0.05, ***p* < 0.01, and ****p* < 0.001). Data are represented as mean $\pm$ SEM. See also Figure S9.

See also Figure S9.

observed 1.5, 2, and 3 hr after acute ISDN injection in WT rats. Having found that MT2 overexpression induced allodynia, we quantified ISDN-evoked mechanical allodynia and found that MT2 overexpression in TG neurons prevents any further effect of ISDN on facial allodynia. This loss of effect of NO donors may be due to the fact that the "ceiling" level of allodynia has already been reached.

Together, these data demonstrate that, at rest, the overexpression of MT2 leads to an increase in TG excitability and a chronic cutaneous allodynia to a similar level to what is observed following NO-donor injection. This is also similar to the allodynia observed in $TREK1^{-/-}/TREK2^{-/-}$ double-KO mice (Figure 3).

MT2-Producing Alternative Translation Initiation Is Found in Other Migraine-Associated TRESK Mutants

Having found that MT2 is responsible for the migraine-associated increase in TG excitability and induction of a migraine-like phenotype through the inhibition of TREK1 and TREK2, we anticipated that other frameshift mutations may exist that place the ATG at position +356 in frame with the reference open reading frame of TRESK. Such mutations would lead to the formation of MT2. This mutation could be either a 2-bp deletion or 1-bp insertion in the region between the ATG at position +356 and the ATG at position +427 (Figure S10). We used the Exome Aggregation Consortium (ExAC) database (Lek et al., 2016) and found one variant (Y121LfsX44) with a T duplication (+1 pb; c.361dupT) that places the ATG site in frame with the TRESK ORF (Figure S9). We introduced this insertion into the mCherry-TRESK-GFP (mCherry-TRESK-c.361dupT-GFP) sequence and found, similar to mCherry-TRESK-MT-GFP (Figure 4C), that this construct led to HEK293T cells with both mCherry and GFP fluorescence (Figure 8A) due to the co-translation of both MT1 and MT2 proteins. Similar to TRESK-MT, this mutant is able to inhibit TRESK, TREK1, and TREK2 (Figure 8). As was seen for TRESK-MT (Figure 5), introduction of a stop codon into the MT2 ORF (TRESK c.361dupT_{STOP}) of this mutant abolished its ability to inhibit TREK1 and TREK2 (Figure 8), but not TRESK. Because this Y121LfsX44 mutation leads to the same molecular effects as TRESK-MT on TREK function, we hypothesized that it may be associated with a migraine phenotype. To address this, we looked in the ClinVar database (Landrum et al., 2016) and found that this mutant has been correlated with a migraine phenotype (RCV000490385.1).

DISCUSSION

Although initial findings of migraine-associated mutations of TRESK represented a major breakthrough (Lafrenière et al., 2010), a direct relationship between TRESK channel disruption and migraine has been challenged based on the discovery of a TRESK mutation (C110R) that produces a dominant-negative form of TRESK but is found in a control cohort population (Andres-Enguix et al., 2012). The presence of this mutation in control individuals indicates that a single non-functional TRESK variant alone may not be sufficient to cause migraines, consistent with the genetic complexity of this disorder. In this study, we have addressed this controversy and found that the migraine-associated

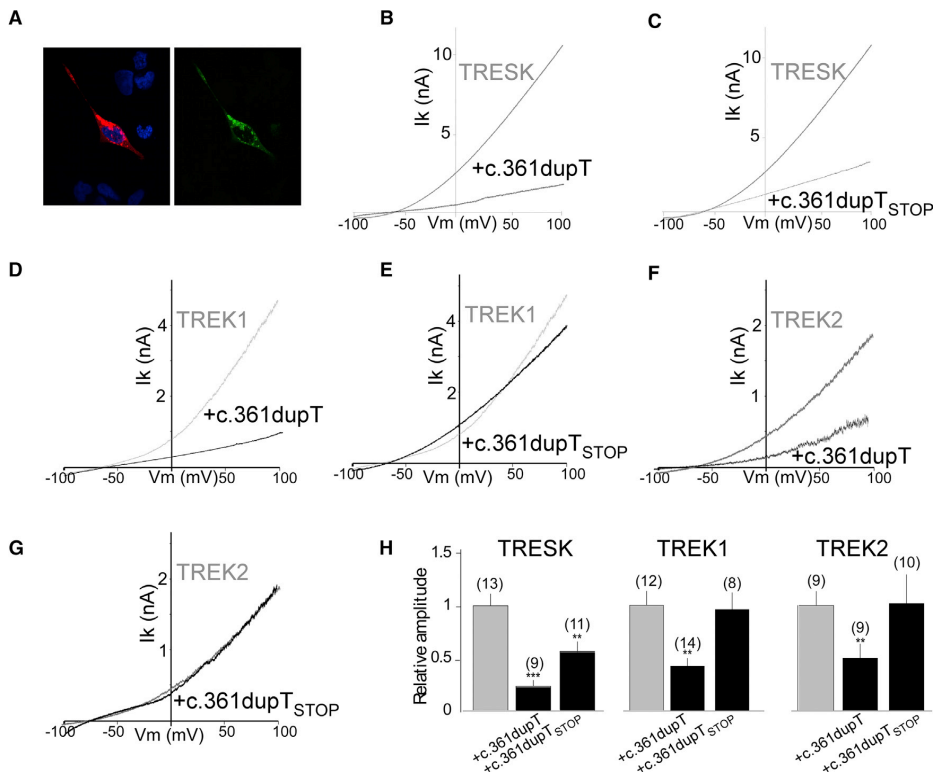


Figure 8. TRESK-c.361dupT (Y121LfsX44) Acts as a Dominant Negative to Reduce Both TRESK and TREK1 Current

(A) Co-synthesis of mCherry-MT1 and MT2-GFP products from the mCherry-TRESK-c.361dupT-GFP cDNA in HEK293T cells. (B and C) Representative traces showing the effect of TRESK c.361dupT (B) and TRESK c.361dupT_{STOP} (C) co-expression on TRESK current. Currents were elicited by voltage ramps (from -100 to 100 mV; 1 s duration). (D and E) Same as (B) and (C) for TREK1. (F and G) same as (A) and (B) for TREK2. (H) Bar graph summarizing the relative TRESK, TREK1, and TREK2 current amplitudes at 0 mV for TRESK, TREK1, and TREK2 when TRESK c.361dupT and TRESK c.361dupT_{STOP} are co-expressed. The numbers of cells tested are indicated in parentheses on the graphs. Cells come from at least two experimental days. Student's *t* test (** $p < 0.01$ and *** $p < 0.001$). Data are represented as mean $\pm$ SEM. See also Figure S10.

mutation of TRESK exerts its effects on sensory neurons by associating and serving as a dominant negative not only for TRESK but also for TREK1 and TREK2 channels. In stark contrast, TRESK-C110R is not able to regulate TREK channels. Consistent with a function of TREK1 and TREK2 in TRESK-MT-induced migraine, the *TREK1*^{-/-}/*TREK2*^{-/-} mice show a migraine-like hypersensitivity to mechanical stimuli. Surprisingly, we find that migraine-associated frameshift mutations of TRESK induce alternative translation initiation that allows the formation of a second product, MT2, which mediates the dominant-negative action on TREK1 and TREK2 when overexpressed. This dominant-negative action on TREK1 and TREK2, ultimately, leads to an increase in TG neuron excitability and a migraine-like hypersensitivity to facial mechanical stimuli. Supporting

this phenomenon as a general mechanism, we found another migraine-associated frameshift mutation in TRESK that produces a similar ORF shift, which also leads to the formation of MT2. Together, these findings support a role of fsAT1 and for TREK potassium channels as a key part of sensory neuron excitability and the underlying cellular mechanism of migraine.

We and others (Blin et al., 2016; Hwang et al., 2014; Lengyel et al., 2016; Levitz et al., 2016) have recently shown that K_{2P} channels are able to form both homodimeric and heterodimeric potassium channels. In this study, we show that TREK and TRESK readily assemble as heteromers. To co-assemble natively, TRESK and TREK must be expressed in the same cells. We and others have found that TG neurons from mice can co-express TREK1, TREK2, and TRESK (Bautista et al., 2008), and it

has been confirmed that TREK1 and TRESK are also co-expressed in human TG neurons (Flegel et al., 2015). Recent single-cell RNA sequencing data on dorsal root ganglion (DRG) sensory neurons from mice have also shown that co-expression of TRESK with TREK-2 and/or TREK-1 occurs in some subtypes of sensory neurons involved in pain signaling, including peptidergic and non-peptidergic subtypes (Usoskin et al., 2015; Zeisel et al., 2018). Single-channel recordings also identified these channels in medium- and small-sized sensory neurons of the DRG, which are likely involved in nociception (Kang et al., 2005). In this study, we have supported these previous findings by using immunohistochemistry and single-cell RT-PCR (Figure S1), as well as pharmacology (Figure S6), to confirm the co-expression of TRESK and TREK channels in sensory neurons. Nevertheless, the relative expression levels of TREK and TRESK in TG neurons are, as is the case for the DRG, likely variable, and therefore, we expect a differential population of homo and heteromers from cell to cell. Expression studies with single-cell precision are ultimately needed to precisely determine which subpopulations co-express different relative amounts of each channel. We show that this TRESK-TREK heteromer is a functional dimer because one TRESK is able to co-assemble with one TREK subunit to form a heterodimeric channel with a common pore using a photoswitchable conditional TREK1 (Sandoz et al., 2012). This is quite surprising given that TREK1/2 and TRESK are in different K_{2P} channel subfamilies and only show low sequence identity of ~19.7% (Sano et al., 2003). We previously found that all members of the TREK channel subfamily (TREK1, TREK2, and TRAAK) can co-assemble but that TREK was unable to interact with TASK channels. Similar to the other reported heteromers, TREK1-TRESK heterodimers show unique biophysical behavior that blends the properties of the parent subunits. Notably, TREK1-TRESK is both arachidonic-acid- and calcium-sensitive. In this study, we also show that TRESK does not heteromerize with three other K_{2P} channels, one from the TREK subfamily, TRAAK, and two from the TASK subfamily, TASK1 and TASK3. Together, this indicates that not all pairs of K_{2P} channel subunits are able to interact and that there are indeed rules of interaction that remain to be deciphered. Future work will be needed to determine the molecular mechanisms and structural interfaces that mediate specific K_{2P} heteromer assembly and the associated functional consequences of heteromerization.

TRESK channels are expressed in the DRG and show their highest expression levels in TG. In DRG and TG, TRESK is most abundant in the small- and medium-size sensory neurons (Dobler et al., 2007; Lafrenière et al., 2010). In TG neurons, introduction of TRESK-MT has been shown to reduce the lamotrigine-sensitive K^+ current, leading to an increase in excitability (Guo et al., 2014; Liu et al., 2013). This increase of TG excitability cannot be explained by TRESK inhibition because TRESK-C110R, which also strongly inhibits wild-type TRESK, does not inhibit the lamotrigine-sensitive K^+ current and does not increase TG excitability, explaining its lack of involvement in migraine (Guo et al., 2014). TREK1 and TREK2 are also expressed in DRG and show high expression levels in TG (Blin et al., 2016; Yamamoto et al., 2009). Furthermore, TREK1 and TREK2 have recently been shown to also be lamotrigine sensi-

tive (Walsh et al., 2016; Figure S6). We found that TRESK-MT inhibits TREK1 and TREK2 to increase TG excitability, showing that TREK1 and TREK2 control TG neuron excitability. To address the impact of this sensory neuron excitability increase, linked to TREK1 and TREK2 inhibition, we tested *TREK1^{-/-}/TREK2^{-/-}* mice for their susceptibility to a migraine-like phenotype. Migraine is associated with increased sensitivity to all sensory modalities, and it appears that cutaneous allodynia can be used as a quantifiable marker of migraine disorder (Bates et al., 2010). Increase of basal mechanical hyperalgesia induced by TREK1 and TREK2 invalidation, to a similar level compared to WT animals after ISDN injection, is in agreement with a KO-induced increase of sensory neuron excitability that is relevant to migraine (Brennan et al., 2013). Furthermore, trigeminal overexpression of MT2 in rats also induced a chronic facial allodynia. This facial allodynia can be a facial hypersensitivity without migraine; however, orbitofacial allodynia is clearly accepted as a reflection of the activity of TG neurons and therefore relevant to migraine (Pradhan et al., 2014; Koprussinski et al., 2017; Harris et al., 2017). Nevertheless, inhibition of TREK1/TREK2 by TRESK-MT2 fragments will depend on its physiological expression and remains to be demonstrated without overexpression. Topiramate was previously shown to inhibit NO-donor-induced acute or basal chronic allodynia (Pradhan et al., 2014). We found that topiramate was able to partially reverse TREK1-TREK2 invalidation-induced allodynia, which is consistent with its action as a prophylactic migraine therapy (Pradhan et al., 2014). Therefore, these results seem to indicate that the dysfunction of TREK1 and TREK2, and not of TRESK alone, contributes to the increase of TG excitability, which may lead to an alteration of pain processing like during migraine, suggesting that TREK1, TREK2, and TREK-TRESK heterodimers should be considered as new targets for migraine treatment.

An important remaining question is: how can TREK1 and TREK2 dysfunction lead to migraine phenotypes? Importantly, the TRESK-MT mutant has been found in migraine with aura phenotype (Lafrenière et al., 2010). Aura has been linked to CSD, which precedes the activation of TG neurons (Noseda and Burstein, 2013). TREK1 and TREK2 channel activity, by reducing TG excitability, may serve as a brake to prevent the pathological activation of TG neurons during the early stages of CSD. In patients expressing TRESK-MT, this mechanism may be reduced or eliminated, enhancing the activation of TG neurons, thus leading to migraines. Fitting the model in which an increase in TG excitability is the primary underlying cause of headaches, the TRESK-MT proband described in the original Lafrenière paper (Lafrenière et al., 2010; OMIM no. 613656) also showed migraine headaches in isolation without a preceding aura.

Most importantly, this study led us to uncover an undescribed mechanism involving alternative translation initiation. We provide evidence that the 2-bp deletion observed for TRESK-MT introduces an in-frame start codon with the reference open reading frame of TRESK, allowing the formation of MT2, the TRESK fragment responsible for the increase in TG excitability. Translation initiation of most eukaryotic mRNAs follows a linear scanning mechanism where the 40S ribosome is recruited to the

5' cap structure of the mRNA followed by downstream movement until an initiation codon is encountered (Kozak, 1999). In these cases, the translation initiation site is the first cap-proximal start codon for methionine (AUG). In most eukaryotic mRNA, this first AUG is embedded into the Kozak consensus sequence [A/G]-XX-ATGG (Kozak, 1984a, 1984b; Jackson et al., 2010). However, if the first AUG is used inefficiently, some ribosomes read through the site without recognition; this leaky scanning can result in translation initiation at a downstream position (Thomas et al., 2008). This has been observed for TREK1, where the second strong ATI site allows the physiological formation of a TREK1 channel with a shorter N terminus that leads to altered TREK1 ion selectivity (Thomas et al., 2008). Similar to what was seen with TREK1, the TRESK ATG at position +356 is embedded into the Kozak consensus sequence (GCTATGG), which may explain why this ATG can serve as an ATI. To further determine why the ATG at position +356 is able to serve as an ATI, we submitted the TRESK-MT sequence to TIS Miner and the ATGpr algorithms (Nishikawa et al., 2000), and both algorithms predicted that the ATG at position +356 is a strong start codon and it is the second possible start codon after ATG at position +1. Furthermore, we found that Kozak sequence mutation significantly reduced the TRESK-MT effect on TREK1 current. This indicates that leaky scanning may explain the generation of TRESK-MT2.

At the physiological level, ATI is thought to increase protein functional diversity, as is also the case with RNA splicing. For example, it was recently shown in osteogenesis imperfecta (OI) disease, that a causative missense mutation of c.-14C > T of the cDNA encoding IFITM5 creates an upstream ATG (ACG at position -15 was mutated to give ATG) in the 5' UTR in frame with IFITM5, which can serve as an ATI site, resulting in addition of an N-terminal 15-aa sequence (Lazarus et al., 2014). Here, we find that an ATG embedded by a strong Kozak sequence, downstream of the ATG at position +1, can be put in frame by a frameshift mutation to induce the translation of a second truncated protein. In the present work, this second product was found to target TREK1 and TREK2 to increase TG neuron excitability and to produce mechanical allodynia, linking this mutant to migraine. This represents the first example where a frameshift mutation downstream of an ATG start codon at position +1 creates a new ORF, allowing the production of a second product that is at the origin of a physiological disturbance. To see whether this fsATI is a general phenomenon in TRESK, we predicted that any mutations before the stop codon ATG at position +427 that put the ATG at position +356 in frame would induce the formation of an MT2 that would lead to disease. Indeed, we demonstrate that another frameshift mutation, c.361dupT (Y121LfsX44), also leads to the formation of MT2 and is correlated to migraine (Clinvar, RCV000490385.1). It is not possible to make any causal statements from this observation, but it is worth noting that this is the only other TRESK mutation that has been linked to a migraine phenotype. Together, this work shows that different frameshift mutations downstream of the first start codon can lead to ATI to produce a second protein that can carry the physiological function, suggesting that this mechanism may be widespread in nature and therefore needs to be

considered when analyzing frameshift mutations linked to human disorders.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes eleven figures and can be found with this article online at <https://doi.org/10.1016/j.neuron.2018.11.039>.

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AUTHOR CONTRIBUTIONS

Conceptualization, P.R., X.G., J.L., and G.S.; Methodology, P.R., A.B., A.A.-B., C.V., F.L., J.L., X.G., and G.S.; Investigation, P.R., A.A.-B., P.A.P., C.V., B.W., J.L., and G.S.; Writing – Original Draft, J.L. and G.S.; Writing – Review and Editing, P.R., A.B., F.L., X.G., J.L., and G.S.; Funding Acquisition, G.S.; Project Administration, G.S.

DECLARATION OF INTERESTS

Patent applications have been filed on TREK1/2 as potential targets to treat migraine (application with Inserm/Transfert).

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-HA-7 (western)	Sigma	Cat# H9658
anti-TRESK	abcam	Cat# ab96868; RRID:AB_10863722
Atto 488	Sigma-Aldrich	Cat# 41051; RRID:AB_260092
Biotinylated anti-HA clone 16B12	BioLegend	Cat# 901501; RRID:AB_901501
Cy3	abcam	Cat# ab146452
Cy5	abcam	Cat# ab146454
TREK1	Blin et al., 2016	N/A
TREK2	Blin et al., 2016	N/A
Bacterial and Virus Strains		
MT2-IRES2EGFP in pAAV	This paper	Vectorology facility, PVM, Biocampus Montpellier
IRES2EGFP in pAAV	This paper	Vectorology facility, PVM, Biocampus Montpellier
Biological Samples		
Mice trigeminal ganglia	This paper	N/A
Rat trigeminal ganglia	This paper	N/A
Chemicals, Peptides, and Recombinant Proteins		
Biotin-PEG	Laysan Bio	Item# Biotin-PEG-SVA-3400
Collagenase type II	ThermoFisher	Cat#17101015
Dako Fluorescent Mounting medium	Dako Corporation	Code S3023
DMEM media	ThermoFisher	Cat# 10566-016
FBS	Sigma-Aldrich	Cat# F9665
ISDN Risordan®	Sanofi	N/A
Laminin	Sigma-Aldrich	Cat# L2020
Lipofectamine 2000	Invitrogen	Cat#11668027
Moloney murine leukemia virus reverse transcriptase	Invitrogen	Cat# 28025013
NeutrAvidin	ThermoFisher	Cat# 31000
PEG	Laysan Bio	Item# MPEG-SVA-5000
Polybrene	Sigma-Aldrich	Cat# TR-1003
Poly-L-lysine	Sigma-Aldrich	Cat#P4707
Protease inhibitors	Roche Diagnostics	Cat#4693116001
Random hexamers	ThermoFisher	Cat# N8080127
SuperScript II	ThermoFisher	Cat# 18064014
Topiramate	Sigma-Aldrich	Cat# T0575
Trypsin	Sigma-Aldrich	Cat# T1763
Critical Commercial Assays		
mMESSAGE mMACHINE T7 Transcription Kit	Invitrogen	Cat # AM1344
Nucleospin RNA Plus XS kit	MACHEREY-NAGEL GmbH & Co. KG	Cat# 740990.50
PowerUp SYBR Green Master Mix	ThermoFisher	Cat# A25742
Experimental Models: Cell Lines		
HEK293T	ATCC	Cat#CRL11268
Mouse primary neurons	This paper	N/A
<i>X. laevis</i> oocytes	This paper	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Organisms/Strains		
Mouse: C57BL/6J	Charles River Laboratories	Strain Code 027
Mouse: TREK1/TREK2 dKO	Guyon et al., 2009	N/A
Rat: Sprague Dawley	Janvier Labs	Strain RjHan:SD
Oligonucleotides		
GAPDH sens cctggagaaacctccaagtatga	This paper	N/A
GAPDH reverse tgctgttgaaagtcgcaggaga	This paper	N/A
TREK1 forward catcttcacatcgttggtgctg	This paper	N/A
TREK1 reverse atcatgctcagaacagctgc	This paper	N/A
TREK2 forward aacagtgggtgccatcttcg	This paper	N/A
TREK2 reverse ccagcaagaagaaggcact	This paper	N/A
TRESK forward ctgcttccttgctgcctg	This paper	N/A
TRESK reverse aagaagagagcgtcaggaa	This paper	N/A
Recombinant DNA		
pcDNA3.1-GFP-X	This paper	N/A
pCMV-HA-X	This paper	N/A
pIRES2eGFP	Clontech	Cat#6029-1
Software and Algorithms		
Fiji/ImageJ	NIH	https://imagej.net/Fiji/Downloads
pClamp	Molecular Devices	http://mdc.custhelp.com/app/answers/detail/a_id/20260/~/axon%E2%84%A2-pclamp%E2%84%A2-11-electrophysiology-data-acquisition-%26-analysis-software
SigmaPlot	Systat Software Inc.	https://systatsoftware.com/products/sigmaplot/
Other		
Axioplan 2 Imaging Microscope	Zeiss	https://www.micro-shop.zeiss.com/?s=16103145829fcb6&l=en&p=us&f=a&i=10027
Axopatch 200A amplifier	Molecular Devices	https://fr.moleculardevices.com/products/axon-patch-clamp-system/amplifiers/axon-instruments-patch-clamp-amplifiers#ref
Camera EMCCD iXon	Andor	https://andor.oxinst.com/products/ixon-emccd-cameras
Dagan TEV-200A	Cornerstone	http://www.dagan.com/tev-200a.htm
Dynamic plantar aesthesiometer	Ugo Basil	Cat#: 37450
von Frey filaments	Bioseb	Modèle: Bio-VF-M

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Guillaume Sandoz (sandoz@unice.fr).

EXPERIMENTAL MODEL AND SUBJECT DETAILS**Knock-out mice**

Mice lacking TREK1 and TREK2 were generated as described ([Guyon et al., 2009](#)). Null mutations were backcrossed against the C57BL/6J inbred strain for 10+ generations prior to establishing the breeding cages to generate subjects for this study. Age- and sex-matched C57BL/6J WT mice, aged 9-12 weeks, were obtained from Charles River Laboratories (Wilmington, MA).

HEK cells

HEK (human embryonic kidney) 293T cells were purchased from ATCC and maintained at 37°, in 5% CO₂ in high glucose DMEM containing 10% fetal bovine serum and used from passage 10 to 40. One splitting per week was made on 35 mm diameter dishes.

Xenopus oocytes

Xenopus laevis oocytes were collected from female *Xenopus laevis* (agreement # 35382015121816318324V7) and dissociated using collagenase type IV (Levitz et al., 2016). Oocytes were injected with 50 nL of a cRNA at a concentration of 1 µg/µL and maintained at 18°C in ND96 solution (96 mM NaCl, 2 mM KCl, 2 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, pH 7.4). Oocytes were used 1 to 3 days after injections.

Rats

Experiments were performed on male Sprague Dawley rats (Janvier Labs) weighing 250 to 400 g (mean weight: 337 ± 16 g, 6 to 9 weeks old), and on male knock-out mice for TREK1 and TREK2 (weighting 20–25 g 7- to 13-weeks-old). Animals were housed in a 12 hour light-dark cycle with food and water available *ad libitum*. Animal procedures were approved by the Institutional Local Ethical Committee and authorized by the French Ministry of Research and the Spanish Ministry of Research according to the European Union regulations and the Directive 2010/63/EU (Agreements C061525 and 01550.03). Animals were sacrificed at experimental end points by CO₂ euthanasia.

WT mice

All mouse experiments were conducted according to national and international guidelines and have been approved by the local ethical committee (CIEPAL NCE). The C57BL/6J breeders were maintained on a 12 h light/dark cycle with constant temperature (23–24°C), humidity (45%–50%), and food and water *ad libitum* at the animal facility of Valrose.

METHOD DETAILS

Behavioral experiments

Drug administration and virus injection

For the topiramate experiment, mice were injected intraperitoneally with topiramate once at a dose of 30 mg/kg and mechanical nociception thresholds were assessed every 30 minutes for two hours after the injection.

The procedures of virus trigeminal injections are described by Long et al. (Long et al., 2017). Briefly, following general anesthesia with a cocktail of ketamine and xylazine (100 mg/kg and 10 mg/kg respectively in i.p.), rats were shaved on the right side, which is the injected side, and placed on a warmed surgical plate. The site of injection was determined using a notch between the condylar process and the ipsilateral angular process. The depth of injection was 9 mm. Antibiotics were used 5 days following injections. Viral vector suspension (10 µL, 10¹¹ transduction unit) containing 6 µg/mL of Polybrene was injected slowly over 1 min. Rats in the experimental group (randomly selected, n = 14) received 10 µL of viral vector containing the MT2 protein sequence, while those in the control group (randomly selected, n = 14) received the same amount of EGFP viral vector. Injections were made in a blind way for the behavior experimenter. Epifluorescence imaging and qPCR were performed to verify successful transduction of trigeminal ganglia by viral vector.

Mechanical sensitivity measurements

The face mechanical sensitivity was measured using calibrated von Frey filaments (Bioseb, France). Unrestrained rats placed in individual plastic boxes on top of a wire surface were trained over one week to stimulation on the periorbital area, following a progressive protocol, starting with non-noxious filaments during the first 3 days of training. The face withdrawal force threshold (g) was determined by the filament evoking at least three responses over five trials, starting with lower force filaments. Basal values were determined 2 days before experiments. Animals showing an outlier threshold for the basal value were excluded from the study.

The hindpaw mechanical sensitivity was evaluated with a dynamic plantar aesthesiometer (Ugo Basile, Italy). Unrestrained mice were placed in 10 individual plastic boxes on top of a wire surface. The mouse hindpaw was submitted to a force ramp up to 7.5 g during 10 s, the paw withdrawal force threshold (g) was assessed in three consecutive trials with at least 3–5 min between the trials and averaged to select animals. Basal values were determined 2 days before experiments. Animals showing an outlier threshold for the basal value were excluded from the study.

Migraine rodent models

The rodent model of NO-induced migraine was induced by intraperitoneal (i.p.) injection of ISDN (Risordan®, Sanofi) at 10mg/kg, a long-lasting NO donor. The vehicle control used in these experiments was 0.9% saline. The acute mechanical allodynia induced by a single ISDN injection was followed on the hindpaw for mice and on the face for rats before (basal value) and for 3 hours after injection, every 30 minutes. Animals showing an outlier threshold for the basal value were excluded from the study. Chronic mechanical allodynia was induced by a single daily injection of ISDN during 4 days. The hindpaw extra-cephalic mechanical sensitivity was measured each day before the ISDN i.p. injection for mice. Topiramate was tested on chronic mechanical allodynia on the 5th day and effects were followed every 30 min during 2 hours after injection in mice. Experiments were made on two batches of animals in duplicate.

Electrophysiology

HEK293T cell electrophysiology was performed 24–72 h after Lipofectamine transfection (with 1 to 1.6 μ g DNA) in solution containing (in mM): 145 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 10 mM HEPES. For co-expression of K2P channels and mutant channels, a DNA ratio of 1:1 was used. Glass pipettes of resistance between 3 and 6 M Ω were filled with intracellular solution containing (in mM): 140 KCl, 10 HEPES, 5 EGTA, 3 $MgCl_2$, pH 7.4. Cells were patch clamped using an Axopatch 200A (Molecular Devices) amplifier in the whole cell mode. Currents were elicited by voltage-ramps (from -100 to 100 mV, 1 s in duration) and the current density was calculated at 0 mV.

Oocyte two-electrode voltage clamp electrophysiology was performed in a 0.3-mL perfusion chamber; a single oocyte was impaled with two standard microelectrodes (1–2.5 M Ω resistance) filled with 3 M KCl, and maintained under voltage clamp using a Dagan TEV 200 amplifier in standard ND96 solution [96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM HEPES pH 7.4 with NaOH]. For the high K^+ solution contained 80 mM K^+ , 78 mM NaCl was replaced by KCl. Stimulation of the preparation, data acquisition, and analysis were performed using pClamp software (Molecular Devices).

Neuronal excitability was studied in small-diameter TG neurons transfected with 1 μ g of the pIRES2EGFP vector containing the X insert in which there is no N-terminal tag on the insert and EGFP is co-translated as a transfection marker or the pIRES2EGFP control plasmid with Lipofectamine 2000. Extracellular solution contained (in mM): 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, 10 glucose, pH 7.4 with NaOH, 310 mOsm. The pipette solution contained the following (in mM): 140 K-gluconate, 10 NaCl, 2 $MgCl_2$, 5 EGTA, 10 HEPES, 2 ATP-Mg, 0.3 GTP-Na, 1 $CaCl_2$ pH 7.3 with KOH, 290 mOsm. Recording pipettes had < 4.5 M Ω resistance. Series resistance (< 20 M Ω) was not compensated. Signals were filtered at 10 kHz and digitized at 20 kHz. After establishing whole-cell access, membrane capacitance was determined with amplifier circuitry. The amplifier was then switched to current-clamp mode to measure resting membrane potential (V_{rest}). Neurons were excluded from analysis if the V_{rest} was higher than -40 mV or if the input resistance was smaller than 200 M Ω . To test neuronal excitability, neurons were held at V_{rest} and injected with 1 s depolarizing currents in 25 pA incremental steps until at least 1 action potential (AP) was elicited.

Immunocytochemistry

Transfected neurons on coverslips were fixed with PBS containing 4% paraformaldehyde for 15 minutes at room temperature (RT), then permeabilized with PBS and 0.1% Triton X-100 (PBST) and blocked for 1 h with 5% horse serum (HS) in PBST. Primary and secondary antibodies were diluted in PBST and 5% HS and incubated for 1 h at RT. Three 5-min washes with PBST were carried out between each incubation step and at the end of the procedure. Coverslips were mounted in Dako Fluorescent Mounting medium (Dako Corporation, Carpinteria, CA, USA). The following antibodies were used: rabbit anti-TREK1 and TREK2 (Blin et al., 2016), anti-TRESK (ab96868, abcam) conjugated with Cy3 (ab146452, abcam), Cy5 (ab146454, abcam) and Atto 488 (Sigma) respectively. Microscopy analysis and data acquisition were carried out with an Axioplan 2 Imaging Microscope (Zeiss®).

Molecular Biology, Cell Culture and Gene Expression

Channel DNA was used in the pIRES2eGFP, pcDNA3.1 and pCMV-HA vectors. HEK293T cells were maintained in DMEM with 5% FBS on poly-L-lysine-coated glass coverslips in 12 well plates. Cells were transiently co-transfected using Lipofectamine 2000 (Invitrogen) with a total of 1–1.6 μ g of DNA total per 35 mm dish. When two genes were co expressed, a ratio of 1:1 DNA was used.

PCR amplification and quantification

Semiquantitative PCR were performed to determine the relative levels of TREK1, TREK2, and TRESK after RNA extraction and reverse transcription using primers described below. qPCR (10 μ L) was performed using the aforementioned reverse-transcribed cDNA (4 μ L) and the primers for TREK1 (forward: catcttcacctcgtttggctg, reverse: atcatgctcagaacagctgc, 240 pb), TREK2 (forward: aacagtgttgccatcttcg, reverse: ccagcaagaagaaggcact, 276 pb), TRESK (forward: ctgcttcctttgctgcctg, reverse: aagaagagagcgcctcaggaa, 256 pb) and GAPDH (forward: cctggagaacctgccaagtatga, reverse: tgctgttgaagtcgcaggaga) as a reference. After initial denaturation at 95°C for 15 s, 40 cycles of amplification (95°C for 15 s and 60°C for 1 minute) were performed.

Quantitative PCR were performed to determine the levels of GFP after inoculation of adenovirus vectors containing GFP MT2 sequences into trigeminal ganglia using the same protocol with specific primers for the GFP (forward: aagctgacctgaagtcctcatctgc, reverse: cttgtagttgccgcgtcctcgaa).

Analysis were made using the GAPDH as the housekeeping gene along with the 2- $\Delta\Delta$ Ct Calculation Method.

Primary cultures of mouse TG neurons

Trigeminal ganglion tissues were collected from postnatal day 8 mice of either sex and treated with 2 mg/ml collagenase type II (Worthington) for ~2 hours, followed by 2.5 mg/ml trypsin for 15 min. Neurons were dissociated by triturating with fire-polished glass pipettes and seeded on polylysine/laminin coated coverslips. The DMEM-based culture medium contained 10% fetal bovine serum and 2mM GlutaMAX (Invitrogen). Neurons were transfected at 1 d *in vitro* (DIV) with 1 μ g of DNA using Lipofectamine 2000 (Invitrogen). Transfected neurons were identified by the green fluorescence and patch clamp recordings were performed between DIV 3 and 5.

Single Molecule Pulldown (SiMPull)

For SiMPull experiments, a DNA ratio of 1:1 was used, with a total quantity of 1 μ g. 24 hours after transfection, HEK 239T cells were harvested from coverslips by incubating with Ca^{2+} -free PBS buffer for 20–30 minutes followed by gentle pipetting. Cells were lysed in buffer containing (in mM): 150 NaCl, 10 Tris pH 7.5, 1 EDTA, protease inhibitor cocktail (Thermo Scientific) and 1.5% IGEPAL (Sigma) or 1% DDM (Sigma). After 30–60 minute incubation at 4°, lysate was centrifuged for 20 minutes at 12,500 g and the supernatant was collected. Coverslips passivated with PEG (~99%)/ biotin-PEG (~1%) and treated with NeutrAvidin (Pierce) were prepared as described (Jain et al., 2012). 15 nM biotinylated anti-HA antibody (clone 16B12, BioLegend) was applied for 20 minutes and then washed out. Antibody dilutions and washes were done in T50 buffer with BSA containing (in mM): 50 NaCl, 10 Tris pH 7.5, and 0.1 mg/mL BSA. Lysate, diluted in lysis buffer containing 0.04% IGEPAL, was then applied to the chamber and washed away following brief incubation (~2 minutes). Single molecules were imaged using a 488 nm Argon laser on a total internal reflection fluorescence microscope with a 60x objective (Olympus). We recorded the emission light after an additional 3x magnification and passage through a double dichroic mirror and an emission filter (525/50 for GFP) with a back-illuminated EMCCD camera (Andor iXon DV-897 BV). Recordings were made in blind for the experimenter before being analyzed.

Single cell reverse transcription

The procedures of single cell RT-PCR are adapted from Johansen et al. (Johansen et al., 1995). The content of each cell was aspirated into the patch pipette by applying negative pressure. The flow of the cytosol in the pipette as well as the aspiration of the nucleus was controlled under the microscope. Only cell samples which included the nucleus were investigated in the present study.

The pipette was then released from the holder and mounted on a syringe to expel its content into a test tube. To the ~6.5 μ L of the pipette-content expelled into the test tube was added 3.5 μ L of a solution containing random hexamers (Invitrogen, 5 μ M final concentration), dithiothreitol (DTT, final concentration 10 mM), the four deoxyribonucleotide triphosphates (dNTP, Thermo Fisher, final 0.5 mM each), 20 U ribonuclease inhibitor (Promega), and 100 U Moloney murine leukemia virus reverse transcriptase (Invitrogen).

The total 10 μ L reaction was incubated for 1 h at 35°C for synthesis of single stranded cDNA, and then kept on ice until PCR.

Trigeminal neurons RNA extraction and reverse transcription

Total RNA was isolated from trigeminal neurons in suspension using a Nucleospin RNA Plus XS kit (from MACHEREY-NAGEL GmbH & Co. KG) according to the manufacturer's protocols and 1 μ g of RNA was reverse transcribed (with 10 nM random hexamers for 5 min at 65°C, then with 10 mM DTT, 0.5 mM each dNTP, 100 U SuperScript II (Invitrogen) 42°C for 50 min). Subsequently, the cDNA was quantified by qPCR with PowerUp SYBR Green Master Mix (ThermoFisher).

Vector preparation

Adenovirus vector (DJ) encoding an IRES2EGFP or MT2-IRES2EGFP were used. Following linearization, this vector was recombined with the mouse version of the MT2 protein. The recombinant was amplified with PCR, and DNA sequencing was performed to verify the DNA sequence. Viral vectors were packaged and harvested by transfection of HEK293T cells, followed by quantification of the viral titer through quantitative PCR. In addition, as a control, an adenovirus vector containing only the IRES2EGFP sequence was used.

Western blot analysis

24 to 48 hours after transfection using 1 μ g DNA with Lipofectamine 2000, HEK293T cells were homogenized in PBS containing saponin (0.5% w/v), Triton X-100 (0.5% w/v) and protease inhibitors (Roche Diagnostics, Basel, Switzerland). Lysates were clarified by centrifugation at 20 000 g for 30 min. Proteins were separated on 10% SDS polyacrylamide gel and blotted onto nitrocellulose membrane (Hybond-C extra, Amersham Biosciences, Freiburg, Germany). Detection was carried out using mouse monoclonal antibody clone HA-7 against the HA epitope (Sigma-Aldrich).

QUANTIFICATION AND STATISTICAL ANALYSIS

Single Molecule Pulldown

Movies of 250–500 frames were acquired at frame rates of 10–30 Hz. The imaged area was $13 \times 13 \mu\text{m}^2$. At least 5 movies were recorded for each condition and data was analyzed using custom software. Multiple independent experiments (at least 3 times) were performed for each condition. Representative datasets are presented to quantitatively compare conditions tested on the same day.

Electrophysiology

The numbers of cells tested are indicated in parentheses on top of graphs in each figure. Cells come from at least 3 batches of experiment. Student's t test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) are performed between the different conditions tested (indicated in each figure's legend). For neurons, the cells come from at least 4 different animals. When the data did not follow a normal distribution, Mann-Whitney U test were assessed.

Animal experiment

Numbers of mice and rats tested are indicated in parentheses in each figure. Student's t test to compare WT versus *TREK1*^{-/-}/*TREK2*^{-/-} mice (*p < 0.05, **p < 0.01, ***p < 0.001).

Quantitative PCR

Analysis were made using the GAPDH as the housekeeping gene along with the 2- $\Delta\Delta C_t$ Calculation Method. n represents the number of single cells tested.



TRESK background K⁺ channel deletion selectively uncovers enhanced mechanical and cold sensitivity

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Key points

- TRESK background K⁺ channel is expressed in sensory neurons and acts as a brake to reduce neuronal activation.
- Deletion of the channel enhances the excitability of nociceptors.
- Skin nociceptive C-fibres show an enhanced activation by cold and mechanical stimulation in TRESK knockout animals.
- Channel deletion selectively enhances mechanical and cold sensitivity in mice, without altering sensitivity to heat.
- These results indicate that the channel regulates the excitability of specific neuronal subpopulations involved in mechanosensitivity and cold-sensing.

Abstract Background potassium-permeable ion channels play a critical role in tuning the excitability of nociceptors, yet the precise role played by different subsets of channels is not fully understood. Decreases in TRESK (TWIK-related spinal cord K⁺ channel) expression/function enhance excitability of sensory neurons, but its role in somatosensory perception and nociception is poorly understood. Here, we used a TRESK knockout (KO) mouse to address these questions. We show that TRESK regulates the sensitivity of sensory neurons in a modality-specific manner, contributing to mechanical and cold sensitivity but without any effect on heat sensitivity. Nociceptive neurons isolated from TRESK KO mice show a decreased threshold for activation and skin nociceptive C-fibres show an enhanced activation by cold and mechanical stimulation that was also observed in behavioural tests *in vivo*. TRESK is also involved in osmotic pain and in early

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phases of formalin-induced inflammatory pain, but not in the development of mechanical and heat hyperalgesia during chronic pain. In contrast, mice lacking TRESK present cold allodynia that is not further enhanced by oxaliplatin. In summary, genetic removal of TRESK uncovers enhanced mechanical and cold sensitivity, indicating that the channel regulates the excitability of specific neuronal subpopulations involved in mechanosensitivity and cold-sensing, acting as a brake to prevent activation by innocuous stimuli.

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Introduction

The combined activation of depolarizing and hyperpolarizing ion channels determines the likelihood of excitation and generation of action potentials (APs) in specific subtypes of sensory neurons such as thermoreceptors, mechanoreceptors or nociceptors. Each subtype presents a characteristic pattern of expression of ion channels that define their intrinsic physiological properties such as firing pattern and AP characteristics (Zheng *et al.* 2019). Two-pore domain potassium channels (K_{2P}) are expressed in different subpopulations of sensory neurons, including nociceptors where they carry most of the 'leak' or background hyperpolarizing current (Enyedi & Czirják, 2010). Their electrophysiological properties allow them to carry K^+ currents over a wide range of membrane potentials and hence they are key determinants of neuronal excitability, decreasing the probability of depolarizing stimuli to reach AP threshold, as well as shaping the neuron firing response (Enyedi & Czirják, 2010). TRESK (TWIK-related spinal cord K^+ channel), along with other K_{2P} family members TREK-1, TREK-2 and TRAAK channels, generate the major contribution to leak currents in trigeminal (TG) and dorsal root ganglion (DRG) neurons (Patel *et al.* 1998; Kang & Kim, 2006; Dobler *et al.* 2007; Yamamoto *et al.* 2009; Tulleuda *et al.* 2011). These channels have been implicated in pain perception induced by mechanical, thermal and chemical stimuli, as well as in neuropathic and inflammatory pain (Alloui *et al.* 2006; Noël *et al.* 2009; Tulleuda *et al.* 2011; Marsh *et al.* 2012; Acosta *et al.* 2014; Pereira *et al.* 2014). The modulatory effect that these channels will exert on nociceptors will depend on their expression pattern and their individual properties. Indeed, deletion of one of the K_{2P} channels enhances the specific sensitivity to certain external stimuli but not others, rather than exerting a general brake on neuronal excitability by decreasing the total K^+ current. In humans, rats and mice, TRESK shows a high expression in DRG and TG neurons and is particularly enriched in sensory ganglia compared to other neural and non-neural tissues (Kang & Kim, 2006; Dobler *et al.* 2007; Marsh *et al.* 2012; Manteniotti *et al.* 2013; Flegel *et al.* 2015; Usoskin *et al.* 2015; Ray *et al.* 2018; Zeisel *et al.* 2018).

Single-cell RNA sequencing data have provided further insight into the role of TRESK, showing that it is present in a subpopulation of low-threshold mechanoreceptors involved in touch sensation (expressing TRKB and Piezo2) and, predominantly, in non-peptidergic nociceptors (Usoskin *et al.* 2015; Zeisel *et al.* 2018; Zheng *et al.* 2019). Interestingly, TRESK expression is down-regulated in different pain conditions, comprising sciatic nerve axotomy (Tulleuda *et al.* 2011), spared nerve injury (Zhou *et al.* 2013) and chronic inflammation (Marsh *et al.* 2012), contributing, together with other changes, to enhance neuronal excitability. In contrast, increasing TRESK expression resulted in a decrease in neuronal excitability and amelioration of painful behaviours (Zhou *et al.* 2012, 2013; Guo & Cao, 2014). Similarly, mice with a loss-of-function mutation in TRESK [G339R] showed a significant reduction of outward K^+ current, increased excitability and reduced rheobase (Dobler *et al.* 2007). Furthermore, a frameshift mutation leading to the truncation of the channel has been associated with familial migraine with aura, thus involving TRESK in the enhanced activation of the trigeminovascular system and the release of inflammatory neuropeptides in the meninges and cerebral vessels triggering migraine pain (Lafrenière *et al.* 2010). This is supported by a recent study demonstrating that mutant TRESK can heteromerize with TREK-1/2 to decrease their function and trigger migraine pain (Royal *et al.* 2019).

Despite the fact that TRESK shares some functional properties with other K_{2P} channels, its high expression in sensory neurons points to a relevant and yet unexplored role in sensory perception and pain. We report that TRESK removal uncovers enhanced cold and mechanical sensitivity without affecting thermal sensitivity to warm or hot conditions.

Methods

Animals

All behavioural and experimental procedures were carried out in accordance with the recommendations of the

International Association for the Study of Pain (IASP) and were reviewed and approved by the Animal Care Committee of the University of Barcelona and by the Department of the Environment of the Generalitat de Catalunya, Catalonia, Spain (nos. 8466, 8468, 8548, 6869 and 9876). Female and male C57BL/6N mice between 8 and 15 weeks old were used in all experimental procedures [RNA extraction and quantitative PCR (qPCR), cell cultures, electrophysiology, calcium imaging, nerve fibre recording and behavior] unless differentially indicated. Mice were housed at 22°C with free access to food and water in an alternating 12 h light and dark cycle.

TRESK (*Kcnk18/K2P18.1*) knockout mice (KO) and wild-type (WT) littermates were obtained from the KOMP Repository (Mouse Biology Program, University of California, Davis, CA, USA). The TRESK KO mouse was generated by replacing the complete *Kcnk18* gene by a ZEN-UB1 cassette according to VelociGene's KOMP Definitive Null Allele Design. At 3 weeks of age, WT or KO newborn mice were weaned, separated and identified by ear punching. Genomic DNA was isolated from tail snip samples with a Maxwell Mouse Tail DNA Purification Kit (Promega, Madison, WI, USA). PCR was performed with primers to detect the *Kcnk18* gene: forward 5'-ACCAA CACCAAGCTGTCTTGTTC-3' and reverse 5'-AGAC AGATGGACGGACAGACATAGATG-3' or the inserted cassette in the KO mice: forward (REG-Neo-F) 5'-GCAG CCTCTGTTCCACATACACTTCA-3' and reverse (gene-specific) 5'-AGACTTCTCCAGGTAACAACCTCTGC-3'. The PCR mixture contained 1 µl DNA sample, 2.5 µl PCR buffer (10× concentration), 2 µl dNTP mixture (2.5 mM), 0.5 µl (20 µM) forward and reverse primers, 0.2 µl Taq DNA polymerase (5 U µl⁻¹), 1.7 µl MgCl₂ (25 mM), 6.5 µl betaine (5 M), 0.325 µl DMSO and 9.7 µl water (final volume of 25 µl). PCR amplifications were carried out with 31 cycles in a programmable thermal cycler (Eppendorf AG, Hamburg, Germany). The programme used was: 94°C for 5 min and cycles of 94°C for 15 s, 60°C for 30 s, 72°C for 40 s, with a final extension at 72°C for 5 min. PCR products were analysed by electrophoresis in 1% agarose gels. Once identified, genotyped animals were used as breeders for colony expansion and their offspring were used in all experimental procedures in which mice were required.

Behavioural studies

Female and male WT or TRESK KO mice between 8 and 15 weeks of age were used in all behavioural studies. To avoid stress-induced variability in the results, mice were habituated to the experimental room and the experimental setup prior to testing. Behavioural measurements were done in a quiet room, taking great care to minimize or avoid discomfort of the animals. All experimenters were blinded to the genotype or the drug/vehicle assayed.

Mouse mechanical sensitivity

Mechanical sensitivity of WT and TRESK KO mice was assessed using the 'up and down' method by the application of calibrated von Frey filaments (North Coast Medical, Inc., Morgan Hill, CA, USA) as previously described (Tulleuda *et al.* 2011; Castellanos *et al.* 2018). The von Frey filaments [size: 2.44, 2.83, 3.22, 3.61, 3.84, 4.08, 4.17, 4.31 and 4.56; equivalent to (in grams) 0.04, 0.07, 0.16, 0.4, 0.6, 1, 1.4, 2 and 4] were applied perpendicularly to the plantar surface of the hind paw and gently pushed to the bending point for 5 s. The 50% withdrawal threshold was determined using the up and down method (Chaplan *et al.* 1994). A brisk hind paw lift in response to von Frey filament stimulation was regarded as a withdrawal response. A dynamic plantar aesthesiometer (Ugo Basile, Gemonio, Italy) was also used to assess mechanical sensitivity. A von Frey-type 0.5 mm filament was applied with a 10 s ramp (0–7.5 g) and the hind paw withdrawal threshold of mice was recorded.

Mouse thermal sensitivity

Hot plate and cold plate test. After habituation, the cold/hot plate apparatus (Ugo Basile) was set to 2, 52 or 56°C and animals were individually placed in the centre of the plate. Latency time to elicit a nocifensive behaviour (a jump or a paw lick/lift) was counted with the apparatus stopwatch and the average of three separate trials was used as a measurement. Since the temperatures tested are in the noxious range, a cut-off time of 25 s was established to avoid tissue damage.

Dynamic hot plate test. In contrast to the conventional hot plate, the dynamic hot plate allows the testing of a wide range of temperatures. Before testing, animals were habituated and later placed individually within the hot/cold plate apparatus where the plate temperature increased from 30 to 50°C at 1°C min⁻¹. To determine the temperature that is perceived as noxious for mice and to quantify pain-related behaviours, the number of jumps at each temperature was scored.

Radiant heat test. The heat sensitivity of mice was assessed by measuring hind paw withdrawal latency from a radiant infrared source (Hargreaves' method) using the Ugo Basile Model 37370 Plantar test. Each measurement was the mean of three trials spaced 15 min apart. For all experiments, infrared intensity was set to 30% and a cut-off time of 20 s was established to avoid skin burn damage.

Thermal place preference test. The thermal place preference test is a test of better comfort temperature

rather than an indicator of temperature aversion. The hot/cold plate apparatus was placed side by side with a complementary plate and a small divider platform was situated between them to connect the two devices (Ugo Basile). The reference plate was always set at 30°C and the test plate was set at 50, 40, 30, 20, 15 or 10°C. Animals were habituated at the experience room for 30 min before testing and then they were allowed to investigate the testing setup for 10 min or until animals crossed consistently from one plate to the other. Mice were then placed individually onto the centre of the platform and once they crossed to a plate the cumulative time they spent on each plate for a total of 5 min was then counted. Only animals that performed properly when both plates were set to 30°C were used for the study (50% of the time at each plate and more than two crossings). To exclude the possibility that animals could be learning which was the reference plate, the position of the reference and the test plate was switched randomly between trials.

Cold plantar assay

To complement the cold plate test, we studied noxious cold sensitivity of TRESK KO mice using the cold plantar assay (Brenner *et al.* 2012). This assay produces an unambiguous nocifensive response that is easily identified when compared to the cold plate test. Animals were placed on top of a 1/8-inch thick glass plate and were enclosed in transparent boxes separated by opaque dividers to prevent animals seeing each other. The cold probe consisted of a modified 3 ml syringe filled with freshly powdered dry ice. This powder was then packed into a pellet and its surface was flattened. Using a mirror to target the mouse hind paw, we applied the dry ice pellet below the glass, making sure that the paw was completely in contact with it. This delivers a cooling ramp to the mice paw and a few seconds later withdrawal responses occur. The withdrawal latency time was measured with a stopwatch and the final withdrawal latency time for each animal was the average of three trials, which were tested at intervals of at least 15 min. A cut-off time of 20 s was used to prevent tissue damage.

Evaluation of nocifensive behaviour

Using a 30 g needle, 10 µl of a solution containing 100 µM allyl isothiocyanate (AITC; 10%), capsaicin (1 µg/10 µl) or their vehicle solutions were administered intradermally into the plantar surface of the hind paw. Mice behaviour was observed and the number of flinches and lickings of the paw were counted for a 5-min period starting immediately after the injection. On the day previous to testing, animals were habituated to the testing room and to the handling procedure. The flinching and licking test was also used

to examine the painful response of mice to different osmolality solutions, both in naive and in sensitized conditions. Then, 10 µl of the following solutions were administered in the hind paw to different groups of male mice: NaCl 33% (hypotonic, 100 mOsm kg⁻¹), NaCl 2% (hypertonic, 622 mOsm kg⁻¹), NaCl 10% (hypertonic, 3157 mOsm kg⁻¹) and PBS (isotonic, 298 mOsm kg⁻¹). A different group of animals was used to study osmotic pain under inflammatory conditions. A 5 µl injection of prostaglandin E₂ (PGE₂; 10 µM) was injected into the hind paw of each mouse and 30 later, 10 µl of NaCl 33% or NaCl 2% were injected into the sensitized paw. After the injection of different osmolality solutions, nocifensive behaviours of paw licking and shaking were manually counted for 5 min.

CFA model of inflammatory pain

After baseline measurements and under brief isoflurane anaesthesia, complete Freund's adjuvant (CFA, Sigma-Aldrich, St Louis, MO, USA; 20 µl; 1 mg ml⁻¹) was injected subcutaneously (glabrous skin) in the hind paw of mice to induce a local inflammation. Mechanical von Frey threshold and heat withdrawal latency (radiant heat test) was examined at different time points after the injection (1 h, 5 h, 1 day, 3 days, 7 days and 16 days).

Formalin test

After habituation, mouse hind paw was subcutaneously injected with formalin (10 µl of a 5% formaldehyde solution) and nocifensive behaviours were measured for 50 min after the injection. The cumulative time spent shaking and licking the injected paw was counted with a stopwatch in 5 min periods.

Oxaliplatin-induced cold hypersensitivity

Prior to oxaliplatin injection, naive thermal preference was measured with the thermal place preference test set at 20°C vs. 30°C. Thirty minutes later, paw withdrawal latency to cold stimuli was determined using the cold plantar assay. A single intraperitoneal injection of oxaliplatin (6 mg kg⁻¹ in PBS with 5% glucose) was delivered to mice. Cold preference and cold sensitivity were re-evaluated 90 h after oxaliplatin injection, a time point that is known to correlate with the peak of oxaliplatin-induced cold hyperalgesia (Descœur *et al.* 2011).

Cuff-induced neuropathic pain model

The sciatic nerve cuffing model was used to induce mechanical and thermal hyperalgesia in the hind paw of mice, as described by Yalcin *et al.* (2014). Mice were

housed individually to avoid stress derived from the surgery and to prevent injury. Cardboard rolls were placed into their home cages to provide shelter and additional stimulation after the surgery. All surgeries were done under aseptic conditions using intraperitoneal ketamine/xylazine anaesthesia. The left leg of mice was shaved from the hip to the knee and the surgical field was disinfected. A 0.5 cm incision parallel to the femur was made to expose the common branch of the sciatic nerve, which appeared after separating the muscles close to the femur. A drop of sterile physiological saline was applied to prevent the nerve from dehydrating and this concluded the procedure for the sham group. The 'cuff' consisted of a 2-mm-long piece of polyethylene tubing (PE-20) with an inner diameter of 0.38 mm and an outer diameter of 1.09 mm, opened by one of its sides. For the cuff group, the sciatic nerve was gently straightened with two sterile sticks and the 'cuff' was inserted around the main branch of the nerve and closed by applying moderate pressure with surgical forceps. To ensure that the 'cuff' was correctly positioned and closed, it was turned gently around the nerve. Both sham and cuff surgeries ended by suturing the incision with surgical knots. After the procedure, animals were placed in their respective home cages lying on their right side and were under constant monitoring until they were completely awakened. Mechanical and heat sensitivity of mice were determined with the von Frey and the radiant heat tests as previously described, before surgery and 5, 7, 14 and 21 days after surgery.

Skin-nerve preparation and single fibre recordings

The isolated skin-saphenous nerve preparation for single C-fibre recording was used as previously described (Noël *et al.* 2009). The hind paw skin of male mice 10–20 weeks of age was isolated with the saphenous nerve. The skin was pinned corium side up in a perfusion chamber with the nerve being pulled in a recording chamber filled with paraffin oil. The skin was perfused with warm (~ 30 – 31°C) synthetic interstitial fluid (SIF; in mM): 120 NaCl, 3.5 KCl, 5 NaHCO₃, 1.7 NaH₂PO₄, 2 CaCl₂, 0.7 MgSO₄, 9.5 sodium gluconate, 5.5 glucose, 7.5 sucrose and 10 Hepes, pH 7.4 adjusted with NaOH, saturated with O₂/CO₂ 95%/5%. Isolated nerve fibres were placed on a gold recording electrode connected to a DAM-80 AC differential amplifier (World Precision Instruments, Sarasota, FL, USA), Digidata 1322A (Molecular Devices, Union City, CA, USA) and Spike2 software (Cambridge Electronic Design, Cambridge, UK) to record extracellular potentials. The skin was probed with mechanical stimulation with a glass rod and calibrated von Frey filaments to characterize the mechanical sensitivity of nerve fibres. The C-fibres were classified according to their conduction velocity of less than 1.2 m s^{-1} measured by electrical stimulation (Stimulus Isolator A385, World Precision Instruments).

The skin receptive field of C-fibres was isolated from the surrounding bath chamber with a stainless steel ring 0.8 cm in diameter, an internal volume of 400 μl , and a hot or cold temperature-controlled SIF was infused into the ring through a bipolar temperature controller CL-100 (Warner Instruments, Hamden, CT, USA). Electrical recordings were amplified ($\times 10\,000$), band-pass filtered between 10 Hz and 10 kHz and stored on computer at 20 kHz. The APs were detected and analysed offline with the principal component analysis extension of the Spike2 software (Cambridge Electronic Design).

RNA extraction and quantitative real-time PCR

Mouse tissue samples were obtained from DRG, kept in RNAlater solution (Ambion, Austin, TX, USA) and stored at -80°C until use. Total RNA was isolated using the Nucleospin RNA (Macherey-Nagel, Düren, Germany) and first-strand cDNA was then transcribed using the SuperScript IV Reverse Transcriptase (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Quantitative real-time PCR was performed in an ABI Prism 7300 using the Fast SYBR Green Master mix (Applied Biosystems, Foster City, CA, USA) and primers (detailed below) obtained from Invitrogen (ThermoFisher Scientific). Amplification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts was used as a standard for normalization of all qPCR experiments and gene-fold expression was assessed using the ΔC_T method. All reactions were performed in triplicate. After amplification, melting curves were obtained and evaluated to confirm correct transcript amplification. Gene-specific primers used were: TRESK (forward 5'-CTCTCTTCTCCGCTGTCGAG-3'; reverse 5'-AAGAGAGCGCTCAGGAAGG-3'); TREK-1 (forward 5'-CACTGTGAGTTTTGCACATGG-3'; reverse 5'-GGGACTGGACTTTTTCTGAATC-3'); TREK-2 (forward 5'-GCAGCTTTCCCTTAGACCAG-3'; reverse 5'-CCAGGGACATTCATTTTGGA-3'); TRAAK (forward 5'-CATCCAAAGAGCCTTCCAGA-3'; reverse 5'-ATTTGGCAACCACTGGACTC-3'); TRPA1 (forward 5'-GCAGGTGGAACCTCATACCAACT-3'; reverse 5'-CACTTTGCGTAAGTACAGAGTGG-3'); TRPV1 (forward 5'-CCCATTGTGCAATTGAGCAT-3'; reverse 5'-TTCCTGCAGAAGAGCAAAGAGC-3'); and GAPDH (forward 5'-ATGTGTCCGTCGTGGATCTGA-3'; reverse 5'-GCTGTTGAAGTCGAGGAGAC-3').

Culture of DRG neurons

Mice were killed by decapitation under anaesthesia (isoflurane) and thoracic, lumbar and cervical DRG were removed for neuronal culture as previously described (Tulleuda *et al.* 2011; Callejo *et al.* 2013). Briefly, DRGs were collected and maintained in cold (4 – 5°C)

Ca^{2+} - and Mg^{2+} -free PBS supplemented with 10 mM glucose, 10 mM Hepes, 100 UI ml^{-1} penicillin and 100 $\mu\text{g ml}^{-1}$ streptomycin until dissociation. Subsequently, ganglia were incubated in 2 ml HAM F-12 with collagenase CLS I (1 mg ml^{-1} ; Biochrome AG, Berlin, Germany) and bovine serum albumin (BSA, 1 mg ml^{-1}) for 1 h 45 min at 37°C followed by 15 min trypsin treatment (0.25%). Ganglia were then resuspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (100 $\mu\text{g ml}^{-1}$) and L-glutamine (100 mg ml^{-1}) and mechanical dissociation was conducted with fire-polished glass Pasteur pipettes of decreasing diameters. Neurons were centrifuged at 100 g for 5 min and re-suspended in culture medium (DMEM + 10% FBS, 100 $\mu\text{g ml}^{-1}$ penicillin/streptomycin, 100 mg ml^{-1} L-glutamine). Cell suspensions were transferred to 12-mm-diameter glass coverslips pre-treated with poly-L-lysine/laminin and incubated at 37°C in a humidified 5% CO_2 atmosphere for up to 1 day, before being used for patch-clamp electrophysiological recordings or calcium imaging experiments. Nerve growth factor or other growth factors were not added.

Calcium imaging

Cultured DRG neurons from wild-type and TRESK KO mice were loaded with 5 μM fura-2/AM (Invitrogen) for 45–60 min at 37°C in culture medium. Coverslips with fura-2-loaded cells were transferred into an open flow chamber (0.5 ml) mounted on the stage of an inverted Olympus IX70 microscope equipped with a TILL monochromator as a source of illumination. Pictures were acquired with an attached cooled CCD camera (Orca II-ER, Hamamatsu Photonics, Shizuoka, Japan) and stored and analysed on a PC computer using Aquacosmos software (Hamamatsu Photonics). After a stabilization period, pairs of images were obtained every 4 s at excitation wavelengths of 340 nm (λ_1) or 380 nm (λ_2 ; 10 nm bandwidth filters) in order to excite the Ca^{2+} -bound or Ca^{2+} -free forms of the fura-2 dye, respectively. The emission wavelength was 510 nm (12-nm bandwidth filter). Typically, 20–40 cells were present in the microscope field. $[\text{Ca}^{2+}]_i$ values were calculated and analysed individually for each single cell from the 340- to 380-nm fluorescence ratios at each time point. Only neurons that produced a response >10% of the baseline value and that, at the end of the experiment, produced a Ca^{2+} response to KCl-induced depolarization (50 mM) were included in the analysis. Several experiments with cells from different primary cultures and different animals were used in all the groups assayed. The extracellular (bath) solution used was 140 mM NaCl, 4.3 mM KCl, 1.3 mM CaCl_2 , 1 mM MgCl_2 , 10 mM glucose and 10 mM Hepes (pH

7.4 with NaOH). Experiments were performed at room temperature.

Electrophysiological recording

Electrophysiological recordings in DRG sensory neurons were performed as previously described (Callejo *et al.* 2013, 2015; Castellanos *et al.* 2018). Briefly, recordings were performed with a patch-clamp amplifier (Axopatch 200B, Molecular Devices) and restricted to small DRG neurons (<30 μm soma diameter), which largely correspond to nociceptive neurons (Le Pichon & Chesler, 2014). Patch electrodes were fabricated in a Flaming/Brown micropipette puller P-97 (Sutter instruments, Novato, CA, USA). Electrodes had a resistance of 2–4 M Ω when filled with intracellular solution (in mM): 140 KCl, 2.1 CaCl_2 , 2.5 MgCl_2 , 5 EGTA, 10 Hepes and 2 ATP (pH 7.3). Bath solution was (in mM): 145 NaCl, 5 KCl, 2 CaCl_2 , 2 MgCl_2 , 10 Hepes and 5 glucose (pH 7.4). The osmolality of the isotonic solution was 310.6 ± 1.8 mOsm kg^{-1} . Membrane currents were recorded in the whole-cell patch-clamp configuration, filtered at 2 kHz, digitized at 10 kHz and acquired with pClamp 10 software. Data were analysed with Clampfit 10 (Molecular Devices) and Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). Series resistance was always kept below 15 M Ω and compensated at 70–80%. All recordings were made at room temperature (22–23°C), 18–24 h after dissociation. To study sensory neuron excitability, after achieving the whole-cell configuration in the patch clamp technique, the amplifier was switched to current-clamp bridge mode. Only neurons with a resting membrane voltage below –50 mV were considered for the study. To study neuronal excitability, we examined the resting membrane potential (RMP); AP rheobase (minimum current to elicit an AP; obtained with 400 ms depolarizing current pulses in 10 pA increments); whole-cell input resistance (R_{in}) was calculated on the basis of the steady-state I – V relationship during a series of 400 ms hyperpolarizing currents delivered in steps of 10 pA from –50 to –10 pA; AP amplitude was measured from RMP to AP peak, AP duration was measured at 50% of AP amplitude and hyperpolarizing afterpotential (HAP) was measured as the voltage difference between the firing level of the spike (beginning of the ascending phase of the AP) and the potential of the HAP. The interspike interval was measured from peak to peak of two succeeding APs. The average of the initial three interspike intervals from spikes elicited by a 1 s current ramp from 0 to 500 pA was used (Fig. 2C).

Drugs

All reagents and culture media were obtained from Sigma-Aldrich (Madrid, Spain) unless otherwise

indicated. Menthol (100 μM), AITC (100 μM) and capsaicin (1 μM) were also purchased from Sigma.

Data analysis

Data are presented as mean $\pm$ SD and, in some specific analyses, the median, coefficient of variation or geometric mean are also provided. Statistical differences between different sets of data were assessed by performing paired or unpaired Student's *t* tests, Mann–Whitney test, Wilcoxon matched pairs test, one-way or two-way ANOVA plus the Holm–Sidak correction for multiple comparisons, Chi-square test or Fisher's exact test, as indicated. The significance level was set at $P < 0.05$ in all statistical analyses. Data analysis was performed using GraphPad Prism 8 software and GraphPad QuickCalcs online tools (GraphPad Software).

Results

TRESK deletion reduces total background current and enhances neuronal excitability

TRESK channels have been detected in small and medium-sized sensory neurons, together with other members of the K_{2P} family of background K^+ channels (Kang & Kim, 2006; Usoskin *et al.* 2015; Zeisel *et al.* 2018). To confirm the loss of TRESK expression in homozygous knockout animals and to assess possible compensatory effects on the expression of other K_{2P} channels, we examined the mRNA expression in DRGs by real-time qPCR. TRESK mRNA was undetected in KO mice while a significant expression was found in WT animals (Fig. 1A). We next examined the expression of TREK-1, TREK-2 and TRAAK (the most well-expressed K_{2P} members in sensory neurons). Analogously to what has been reported in a previous study (Chae *et al.* 2010), mRNA for these channels were present in WT and KO mice at similar levels, thus excluding any compensation effect. Furthermore, the expression of TRPA1 and TRPV1, two channels highly expressed in nociceptors, was also unchanged.

To assess whether removal of TRESK modifies K^+ background currents in nociceptors, we recorded total K^+ current from small-diameter DRG neurons (soma size $<30 \mu\text{m}$; capacitance $<45 \text{ pF}$) that largely correspond to nociceptive neurons with C-fibre conduction velocities (Ma *et al.* 2003).

Putative nociceptors isolated from KO mice displayed a significantly lower K^+ current density than nociceptors of WT mice, both at -25 mV (15.3 ± 8.5 ; $n = 31$; vs. $9.7 \pm 7.8 \text{ pA pF}^{-1}$; $n = 50$; for WT and KO mice, respectively; unpaired *t* test $P = 0.004$), and at -135 mV ($-11.1 \pm 8.9 \text{ pA pF}^{-1}$ vs. $-7.3 \pm 5.6 \text{ pA pF}^{-1}$; for WT and KO mice, respectively; unpaired *t* test

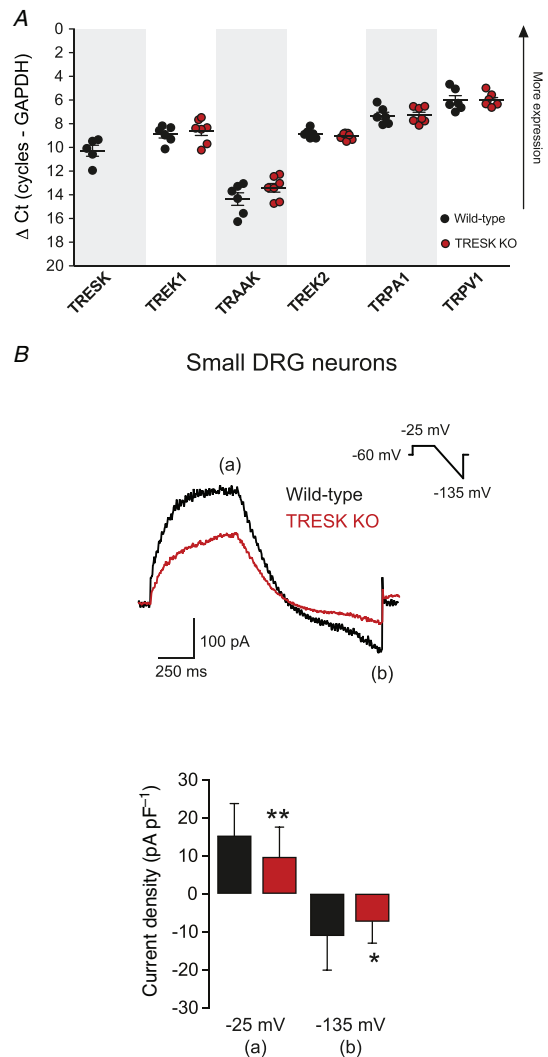


Figure 1. Nociceptive sensory neurons lacking TRESK have a decreased standing outward current

A, expression profile of K_{2P} channels, TRPA1 and TRPV1 in mouse sensory neurons from wild-type (WT) and TRESK knockout (KO) mice. mRNA expression obtained by quantitative PCR shows no significant differences between WT and KO animals after genetic deletion of TRESK. The y-axis shows the ΔCt (number of cycles target – number of cycles GAPDH) for the different mRNAs. Note that the y-axis has been inverted to visually show that lower ΔCt numbers indicate higher expression. Each dot represents a single animal (WT $n = 6$; TRESK KO $n = 7$). B, top: representative recordings of whole-cell currents from small DRG sensory neurons using a protocol to minimize activation of voltage-gated transient K^+ outward currents (holding voltage -60 mV). Bottom: quantification of currents at the end of the pulse (mean $\pm$ SD) at -25 mV (a) and at the end of the ramp (-135 mV ; b) showing significant differences among groups (* $P < 0.05$; ** $P < 0.01$ unpaired *t* test; WT $n = 31$; TRESK KO $n = 50$).

$P = 0.038$), indicating that the absence of TRESK has a significant functional consequence on the membrane current (Fig. 1B). In a second set of nociceptor recordings, we investigated the functional consequence of TRESK deletion on neuronal excitability by measuring their resting membrane potential and AP firing properties (Fig. 2A and B). WT and TRESK KO nociceptors did not present significant changes in RMP (58.3 ± 13.6 vs. -56.2 ± 7.7 mV, respectively; unpaired t test $P = 0.540$; Fig. 2B), suggesting that TRESK might have a minor role in setting the RMP and other K^+ channels are possibly more important in setting the RMP in the cell body. This is in agreement with previous data showing that RMP was

not significantly modified after TRESK down-regulation or deletion (Dobler *et al.* 2007; Tulleuda *et al.* 2011). Interestingly, current-clamp recordings indicated that rheobase (minimum current injected to elicit an AP) was significantly lower in TRESK KO nociceptors (Fig. 2B, $P = 0.0013$, unpaired t test) compared to nociceptors from WT littermates. This effect is probably a consequence of the increased membrane resistance found in TRESK KO neurons (1262.8 ± 655.3 M Ω) compared to controls (WT: 792.7 ± 661.7 M Ω ; $P = 0.045$, unpaired t test; Fig. 2B). As expected, AP amplitude was not significantly altered ($P = 0.406$, unpaired t test) because this parameter is more dependent on the activity of voltage-dependent sodium

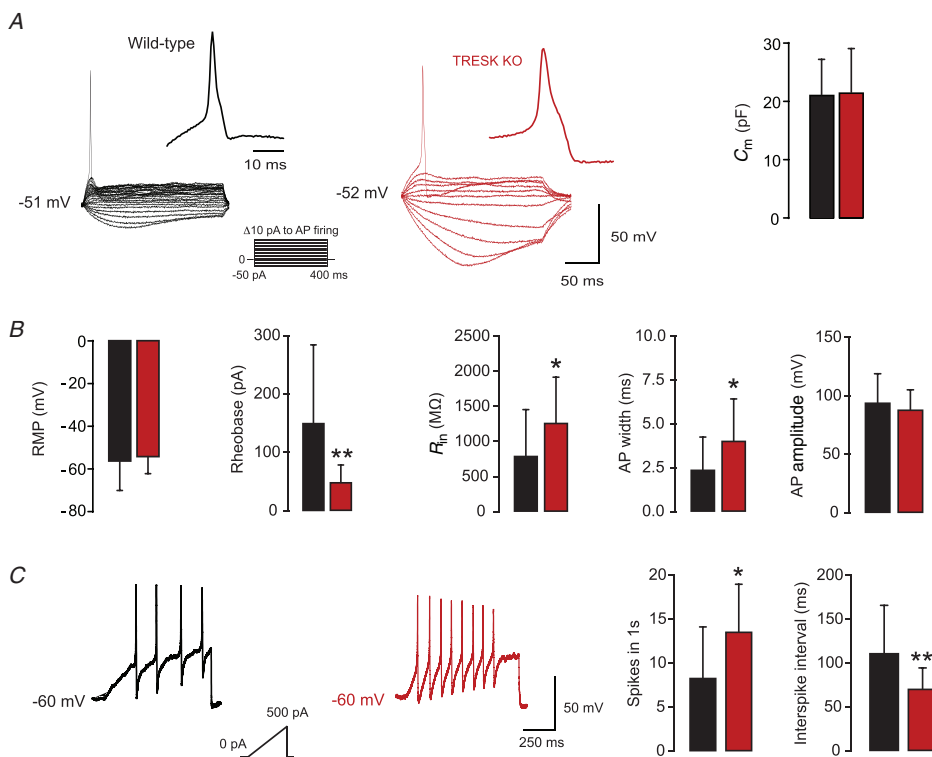


Figure 2. Nociceptive sensory neurons lacking TRESK present a higher excitability

A, representative whole-cell current-clamp recordings from wild-type (WT) and TRESK knockout (KO) nociceptive sensory neurons elicited by hyperpolarizing or depolarizing 400 ms current pulses in 10 pA increments from -50 pA. Right: mean membrane capacitance (C_m) from neurons studied. B, quantification of the electrophysiological parameters analysed in WT (black bars, $n = 13$) and TRESK KO (red bars, $n = 27$) sensory neurons. RMP: resting membrane potential. Rheobase was measured using 400 ms depolarizing current pulses in 10 pA increments; R_{in} : whole-cell input resistance was calculated on the basis of the steady-state I - V relationship during a series of 400 ms hyperpolarizing currents delivered in steps of 10 pA from -50 to -10 pA. Action potential (AP) amplitude was measured from the RMP to the AP peak. AP duration/width was measured at 50% of AP amplitude. Data are presented as mean $\pm$ SD. Statistical differences between groups are shown (* $P < 0.05$, ** $P < 0.01$ unpaired t test). C, examples and quantification of neuronal excitability as the number of APs fired in response to a depolarizing current ramp (0–500 pA, 1 s) from a holding voltage of -60 mV (black, WT $n = 10$; red, TRESK KO $n = 18$). The peak to peak interspike interval value between the four initial APs was measured and averaged. Statistical differences between groups are shown (* $P < 0.05$; ** $P < 0.01$ unpaired t test).

channels (Na_v). However, APs were significantly wider in nociceptors from TRESK KO animals, probably reflecting the consequence of the decrease in total K^+ current (width at 50% AP: TRESK KO 4.03 ± 2.4 ms; $n = 27$ neurons; WT: 2.38 ± 1.9 ms; $n = 13$; $P = 0.041$; Fig. 2B). In a similar fashion, HAP measured from the firing level of the spike was also reduced in KO neurons (TRESK KO 36.5 ± 7.7 mV; $n = 27$ neurons; WT: 46.4 ± 8.1 mV; $n = 13$; $P = 0.001$). To investigate if these changes affect the excitability of nociceptors, we injected a depolarizing current ramp (0–500 pA, 1 s) and counted the number of APs generated for each genotype. As shown in Fig. 2C, TRESK KO neurons fired on average more spikes (13.6 ± 5.4 spikes; $n = 18$) than WT neurons (8.3 ± 5.8 spikes; $n = 10$; $P = 0.023$), and showed a reduced inter-spike interval (KO: 70.6 ± 23.9 ms vs. WT: 111.2 ± 54.7 ms; $P = 0.006$), indicating that removal of TRESK increased the excitability of sensory neurons.

To further characterize the responsiveness of sensory neurons in TRESK KO mice, we measured the intracellular Ca^{2+} signals ($[\text{Ca}^{2+}]$) of cultured DRG neurons from WT and KO mice in response to capsaicin (1 μM ; a TRPV1 agonist) and AITC (100 μM ; a TRPA1 agonist), two markers of nociceptive neurons, and menthol (100 μM ; a TRPM8 agonist), a marker of cold sensory neurons. Among WT sensory neurons (total number of neurons analysed = 1124), 49.2% responded to capsaicin, 40.4% to AITC and 7.0% to menthol. Responses to both capsaicin and AITC were seen in 17.9% of the neurons (Fig. 3A and B). A percentage of neurons did not respond to any of the agonists tested (246 neurons, 21.9%). Responses to capsaicin (45.7%) and menthol (9.1%) were not different in neurons from TRESK KO mice ($n = 1228$), but the percentage of neurons activated by AITC was significantly lower (24.7%, $P = 0.009$). Neurons not responding to any agonists totalled 446 (36.3%). The diameters of sensory neurons responding to the different agonists between WT and KO animals were not different, indicating that they represent similar populations of DRG neurons (mean soma diameter WT: 20.2 ± 5.9 μm ; KO: 20.3 ± 5.6 μm). In parallel experiments, the response of cultured trigeminal neurons was assessed. These showed no significant differences for the response to the different agonists (data not shown) between genotypes. Therefore, the diminished response to AITC is restricted to DRG neurons.

We next assessed if the reduced AITC response of DRG neurons from TRESK KO mice had a behavioural correlate by injecting AITC (100 μM) into the mouse hind paw. In agreement with the decreased response to AITC obtained in calcium recordings, nocifensive behaviour measured as the time spent licking or shaking the paw significantly diminished in KO compared to WT mice (TRESK KO: 9.0 ± 7.3 s; WT: 18.1 ± 13.0 s; $P = 0.039$; $n = 13$ for each group; Fig. 3C). In contrast, painful behavioural responses to capsaicin injection did not differ between KO

and WT mice (40.0 ± 22.7 s vs. 28.8 ± 13.6 s, respectively; $P = 0.236$; Fig. 3C). Injection of vehicles for each compound did not produce significant behavioural effects (data not shown). To further investigate to what extent chemical nociception was altered by the absence of TRESK, we used the formalin test (Tjølsen *et al.* 1992; Abbott *et al.* 1995), which is characterized by an initial phase (phase I; 0–5 min) due to direct nociceptor activation (McNamara *et al.* 2007) and a second phase (phase II; 15–50 min) that is attributed to a combined nociceptive input together with central spinal sensitization (Tjølsen *et al.* 1992). The phase I nocifensive response has been directly linked to the activation of TRPA1 (McNamara *et al.* 2007), although high concentrations of formalin are still able to induce some pain in TRPA1 KO mice (Fischer *et al.* 2013). Interestingly, animals lacking TRESK showed a diminished response to a 5% formalin injection in phase I (30.8 ± 15.0 s; $P = 0.002$; Fig. 3D) compared to control WT animals (69.9 ± 17.6 s). The decreased response in phase I of TRESK KO animals is in agreement with the decreased nocifensive response observed after AITC injection (Fig. 3C) which corroborates the decreased fraction of AITC-sensitive DRG neurons from TRESK KO animals in culture. All these observations confirm that TRPA1 activation is decreased in TRESK KO animals. Phase II of the formalin test can be further split into phase IIa (15–25 min) and IIb (25–50 min), where IIa has a higher nociceptive input than IIb. TRESK KO animals showed a decreased licking time during phase IIa (107.7 ± 39.9 s) compared to WT animals (154.8 ± 42.2 s; $P = 0.026$); this probably reflects decreased nociceptor activation as found in phase I. In contrast, phase IIb of the formalin test did not show significant differences and licking behaviour was similar between groups ($P = 0.814$, Fig. 3D). Injection of hypertonic saline stimulates primary afferent nociceptors and produces pain in humans (Alessandri-Haber *et al.* 2003, 2005). This response can be further enhanced by sensitization of nociceptors with PGE_2 . Injection of hypotonic stimuli did not induce significant nocifensive behaviour in resting conditions ($P = 0.146$, Fig. 3E) but, as previously described (Alessandri-Haber *et al.* 2003; Alloui *et al.* 2006), nocifensive responses were enhanced after sensitization with PGE_2 in WT mice but not in TRESK KO mice ($P = 0.019$, unpaired *t* test WT vs. KO). Nocifensive responses to mild hypertonic saline were diminished in TRESK KO animals, both in resting conditions (2% or 10% NaCl, $P = 0.049$ and $P = 0.002$) and after sensitization with PGE_2 ($P = 0.002$; Fig. 3E). Again, these effects are similar to that reported after knocking out TREK-1 and TREK-2, but not in the single TRAAK KO mice (Alloui *et al.* 2006; Noël *et al.* 2009; Pereira *et al.* 2014), thus implying that TREK-1, TREK-2 and TRESK are involved in the sensitivity to hypertonic stimuli and their absence prevents sensitization by PGE_2 .

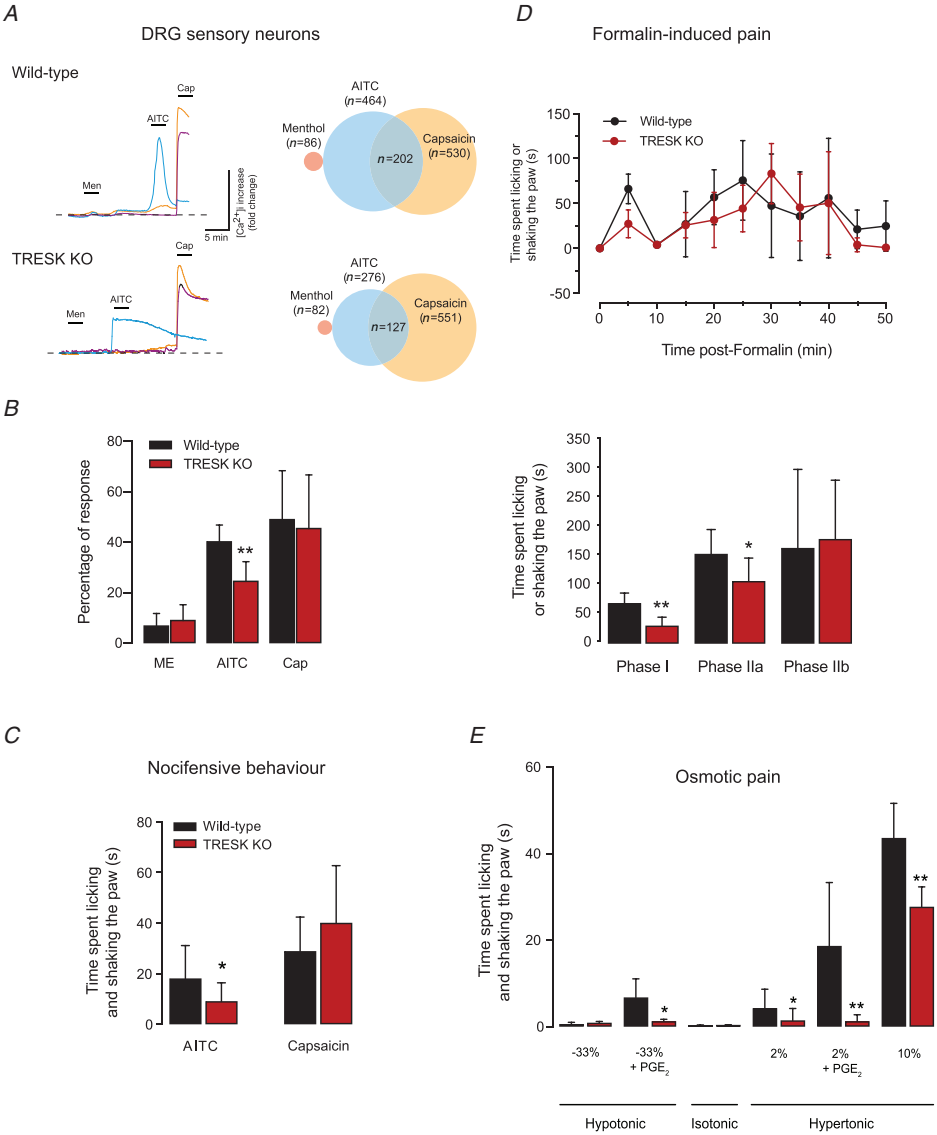


Figure 3. TRESK KO mice present diminished responses to osmotic stimuli and TRPA1 activation
A, representative recordings of intracellular calcium (Fura-2 ratiometric imaging) of wild-type (WT) or TRESK knockout (KO) DRG sensory neurons exposed to menthol (100 μ M), allyl isothiocyanate (AITC, 100 μ M) and capsaicin (1 μ M). Venn diagrams show the relative size and overlap between the populations of neurons activated by each agonist. The number of total cells analysed was 1124 (WT) and 1228 (KO). The number of responding cells in each subgroup is shown in parentheses. Non-responding neurons to any of the agonists assayed were 246 (WT) and 446 (KO). B, quantification of the percentage of neurons responding to each agonist in intracellular calcium recordings. Statistical differences between groups are shown (** $P < 0.01$ unpaired t test). C, nocifensive behaviour: quantification of the time the animals spent licking and shaking the paw after intradermal injection of AITC (10%) or capsaicin (1 μ g/10 μ l) in the hind paw for WT and TRESK KO mice. The mean time spent showing nocifensive behaviours over a period of 5 min is shown (WT $n = 13$; KO $n = 13$ animals). D, formalin-induced pain. Top: time course of licking/shaking behaviour directed to the formalin-injected hind paw. Bottom: quantification of cumulative time spent in phase I (0–10 min), IIa (11–30 min) and IIb (31–50 min); $n = 5$ –6 animals per group. E,

Mice lacking TRESK present mechanical allodynia and normal heat perception

Because TRESK KO enhances nociceptor excitability, we next analysed the sensitivity of nerve fibres from TRESK KO mice to different types of stimuli and whether the activation of sensory fibres was correlated to behavioural responses to these stimuli. Saphenous nerve C-fibres from WT ($n = 14$) and TRESK KO mice ($n = 22$) were recorded with the nerve-skin preparation (Zimmermann *et al.* 2009). Mechanical thresholds were determined with calibrated von Frey filaments applied on the receptive fields of C-fibres. TRESK KO mice presented an enhanced sensitivity to mechanical stimuli. The mean threshold value for the whole population was not significantly different in KO animals (mean $\pm$ SD: 27.6 ± 24.6 mN; median: 22 mN) compared to WT mice (mean $\pm$ SD: 33.2 ± 23.3 mN; median: 22 mN; $P = 0.268$, Mann–Whitney test), but threshold values from KO mice showed a wider distribution and a significant shift towards lower values (Fig. 4A). This wider spread might mean a subset of C-fibres have lost the inhibitory effect of TRESK, and can now activate with lower-intensity mechanical stimuli. To examine such a possibility, we compared the percentage of fibres that presented a lower threshold for activation by mechanical stimuli. Our results indicate that KO mice presented a significant percentage of fibres with lower thresholds (<12 mN; 40.9%) compared to fibres from WT animals (0%; $P = 0.006$; Fisher's exact test). This wider distribution of mechanical thresholds towards lower values in the fibres from KO animals can be also shown by the larger coefficient of variation (WT: 70.2% vs. KO: 89.1%) and by the geometric mean (WT: 27.8 mN vs. KO: 17.6 mN; Fig. 4A, blue lines), which normalizes the ranges being averaged, and thus no range dominates the weighting. This suggests that TRESK function prevents C-fibre activation by low-intensity mechanical stimuli and therefore deletion of the channel reveals mechanical sensitivity with low threshold values in a fraction of C-fibres (Fig. 4A). We then compared the mechanical sensitivity of TRESK KO to WT mice with the von Frey up and down method. Hind paw mechanical threshold was significantly lower in TRESK KO mice compared to WT mice ($P < 0.0001$ for males and females; Fig. 4B), indicating that the subset of C-fibres with lower activation thresholds by mechanical stimuli have an important functional consequence because

they precipitate mechanical allodynia in awake, freely moving mice. Measures of mechanical sensitivity with the dynamic plantar aesthesiometer also confirmed significant differences to pressure application between WT (5.06 ± 0.61 g; $n = 27$) and KO animals (4.66 ± 0.81 g; $n = 22$; t test $P = 0.027$; Fig. 4B).

Although other members of the K_{2P} family have been shown to be involved in thermosensation and pain perception in response to hot or cold stimuli (Alloui *et al.* 2006; Noël *et al.* 2009; Pereira *et al.* 2014), TRESK activity is not modulated by changes in temperature in the physiological range (Kang & Kim, 2006). Despite this lack of thermal sensitivity of the channel, it remains possible that the channel modulates the excitability of C-fibres involved in heat sensitivity. We addressed this question by recording responses of C-fibres activated by a heat-ramp applied on their receptive fields in the skin. TRESK KO and WT mice did not show significant differences in heat thresholds for the activation of C-fibres ($37.6 \pm 2.8^{\circ}\text{C}$, $n = 35$ C-fibres for WT; $38.1 \pm 3.9^{\circ}\text{C}$, $n = 37$ C-fibres for KO; $P = 0.502$ t test; Fig. 4C), nor the distribution of these thresholds over a range of temperatures, nor the number of spikes fired by heat-sensitive C-fibres during heat-ramps (32.6 ± 28.2 spikes WT; 34.8 ± 28.3 spikes TRESK KO; $P = 0.720$ t test; Fig. 4C), indicating that TRESK does not seem to have a major role in the detection of warm or hot conditions. We then tested heat sensitivity *in vivo* using the Hargreaves and hot plate tests. Heat sensitivity was unaltered in TRESK KO mice compared to WT in the radiant heat Hargreaves test ($P = 0.08$ males; $P = 0.603$ females, Fig. 4D), thus confirming the observations made in C-fibres recordings. To further evaluate a possible implication of TRESK in heat pain, we evaluated the sensitivity to more extreme temperatures in the hot plate test at 52 and 56°C , but no significant differences were detected (Fig. 4D). This indicates that the extreme heat sensitivity is conserved in TRESK KO mice. The dynamic hot plate with a ramp of temperature has been proposed as a valuable method to differentiate between thermal allodynia and hyperalgesia (Yalcin *et al.* 2009). The number of jumps of the mice on a hot plate when the plate temperature is increased from 39 to 50°C did not show any difference between TRESK KO and WT, neither for the total number of jumps nor the temperature at which animals displayed their first jump (Fig. 4E). These behaviour and skin-nerve preparation results indicate that there is no significant contribution of TRESK to heat sensitivity.

osmotic pain: quantification of the time spent licking and shaking the paw after intradermal injection of hypotonic (-33%) or hypertonic (2% and 10%) stimuli in the hind paw of WT and TRESK KO mice. The mean time spent showing nocifensive behaviours over a period of 5 min is shown. Six to 11 animals were evaluated in each group. When indicated, a 5 μl injection of prostaglandin E_2 (PGE_2) was injected in the hind paw of each mouse 30 min before the test to sensitize nociceptors. Statistical differences are shown as * $P < 0.05$, ** $P < 0.01$ (Student's unpaired t test) between WT and KO mice.

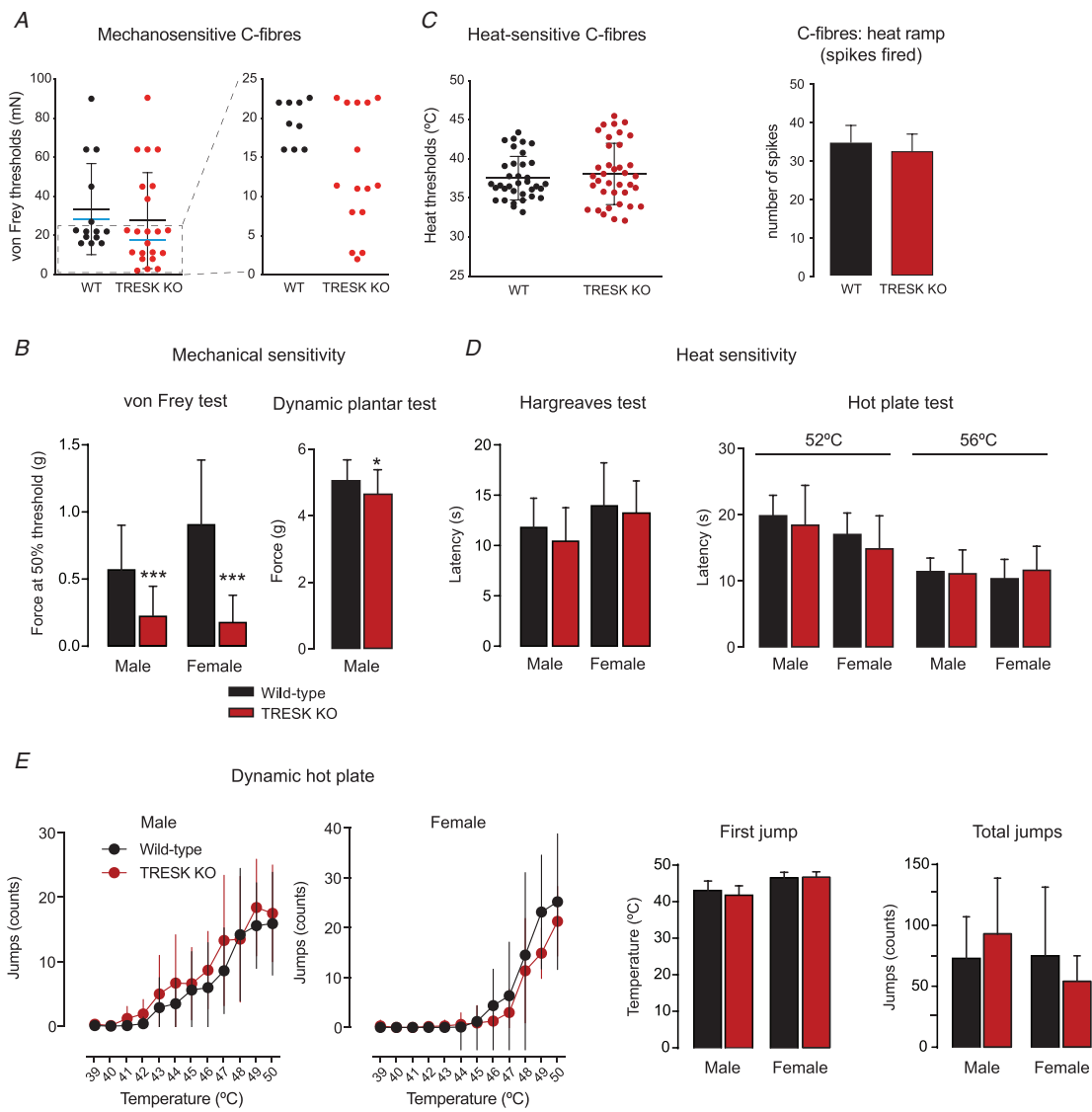


Figure 4. TRESK-lacking mice present mechanical allodynia and normal heat sensitivity
A, mechanical threshold obtained with von Frey hairs from saphenous nerve C-fibres. Left: distribution of von Frey thresholds. Mean $\pm$ SD is shown (black lines). The geometric mean is indicated as a blue line. Wild-type (WT) $n = 14$, knock-out (KO) $n = 22$. Right: magnification of 0–25 mN range of von Frey thresholds to highlight the larger distribution of fibres from KO mice to lower threshold values. B, mechanical sensitivity. Left: von Frey response thresholds obtained with the up and down method in male and female WT and TRESK KO animals (male WT $n = 58$, KO $n = 33$; female WT $n = 18$, KO $n = 19$). Right: latency to hind paw withdrawal in the dynamic plantar test (male WT $n = 27$; KO $n = 22$). Statistical differences are shown as $*P < 0.05$, $***P < 0.001$ (Student's unpaired t test) between WT and KO mice. C, left: distribution of heat thresholds from C-fibres recorded from WT ($n = 35$) and TRESK KO mice ($n = 37$), measured in skin-nerve experiments. Right: mean number of spikes fired by heat-sensitive C-fibres during a heat-ramp from 30 to 50°C. No significant differences were found. D, heat sensitivity. Left: radiant heat (Hargreaves test) in male (WT $n = 39$; KO $n = 38$) and female animals (WT $n = 18$; KO $n = 15$). Right: noxious heat sensitivity to 52 and 56°C (hot plate test) in male (WT $n = 8/9$; KO $n = 13/11$) and female animals (WT $n = 9/9$; KO $n = 12/8$). E, dynamic hot plate: in contrast to the conventional hot plate,

Elevated perception of low temperatures in the absence of TRESK channel

Thermosensitivity to low temperatures is governed by different mechanisms and by distinct neuronal sub-populations than warm/hot perception (Lolignier *et al.* 2016). We evaluated the role of TRESK in the cold sensitivity of C-fibres by recording fibre activity with the saphenous nerve preparation upon cooling their receptive fields in the skin from 30 to 10°C over 90 s (Fig. 5A–D). C-fibre recordings from TRESK KO mice showed that although the total number of cold-sensitive fibres did not differ significantly (58% in WT and 66% in KO mice), there were significant changes in the fractions of mechano-cold and mechano-heat and cold-sensitive polymodal C-fibres compared to WT mice. Specifically, we observed in KO mice an increase in the fraction of mechano-cold C-fibres compared to WT mice (37% vs. 20% C-MC, respectively; Fig. 5A). This was accompanied by a reduction of the percentage of mechano-heat-cold C-fibres (C-MHC) in KO mice (29%) compared to WT (38%; $P < 0.01$; chi-square test; Fig. 5A). The distribution of C-fibre activation thresholds by cold stimuli indicated a clear shift toward higher temperature in the KO (between 26 and 28°C) than in the WT mice (Fig. 5B and C). Despite the mean or median cold thresholds not being different ($P = 0.565$, Mann–Whitney test; Fig. 5C), we observed a tendency to have more fibres activated at temperatures above 25°C in KO animals (58%) compared to WT (42%), although this difference did not reach statistical significance (Fisher's exact test; $P = 0.099$). The total response of TRESK KO fibres to a 90 s cooling ramp (33.1 ± 29.5 spikes) was similar to that of WT fibres (34.1 ± 25.1 spikes; $P = 0.90$ unpaired t test). However, the distribution of the activity upon cooling was higher at temperatures between 25 and 19°C in TRESK KO fibres (Fig. 5D). The baseline activity between 30 and 31°C was also higher in TRESK KO than WT fibres ($0.3 \text{ spikes s}^{-1}$ for TRESK KO and $0.1 \text{ spikes s}^{-1}$ for WT; $n = 36$ and $n = 31$ respectively; $P < 0.001$, t test). These experiments indicate that the temperature for activation of cold-sensitive C-fibres was higher in TRESK KO mice, but also that the overall fibre activity in response to cooling did not depend drastically on the presence of TRESK.

In agreement with observations in C-fibres, behavioural responses to cold were also altered in animals lacking TRESK. Sensitivity to noxious cold (2°C; cold plate test)

was enhanced in knockout animals from both sexes, with shorter latency times to elicit a nociceptive behaviour ($P = 0.006$ males; $P = 0.041$ females, Fig. 5E). In addition, cold sensitivity to more moderate temperatures assessed with the cold plantar assay was significantly higher in TRESK KO mice ($P < 0.0001$ males, $P = 0.008$ females, Fig. 5F). Finally, we assessed whether changes in cold sensitivity modified the preference the mice displayed between the two different temperatures. TRESK KO animals spent more time on the reference plate (at 30°C) than on the experimental plate at 20°C ($P = 0.015$, one-way ANOVA plus Holm–Sidak correction for multiple comparisons, Fig. 5G) compared to WT animals, further supporting previous evidence from cold-sensitive C-fibre recordings and cold sensitivity assays. In contrast, place preference assays at other temperatures (10, 15, 30, 40, 50°C) did not show significant alterations between mice genotypes, corroborating the non-involvement of TRESK in the perception of cool, warm or hot conditions (Fig. 4).

Mice lacking TRESK show selective changes in persistent inflammatory and neuropathic pain

To assess the contribution of TRESK in chronic pain conditions, the behaviour of TRESK KO and WT mice was measured after intraplantar CFA injection, a model of persistent inflammatory pain. Both genotypes developed mechanical and thermal hypersensitivity in the injected hind paw beginning 1 h after injection and lasting at least 16 days (Fig. 6A). Despite the initial difference in basal mechanical thresholds ($P = 0.047$), the extent of mechanical allodynia was similar between WT and KO animals 1 h after CFA injection and during the entire 16-day observation period ($P > 0.05$; two-way ANOVA plus Holm–Sidak correction for multiple comparisons). The development of thermal hyperalgesia was similar between the two genotypes, thus showing that TRESK does not contribute significantly to peripheral sensitization of nociceptors due to inflammation. The sciatic nerve cuff-model was used to evaluate TRESK contribution in persistent neuropathic pain. The mechanical hypersensitivity developed in KO animals was significantly higher to begin with ($P = 0.0006$; Fig. 6B) but no significant differences were found after sciatic nerve cuffing compared to WT animals. Sham surgery did not show significant effects on this parameter in any

the dynamic hot plate allows the testing of a wide range of temperatures. Plate temperature was ramped from 30 to 50°C at 1°C min^{-1} . To determine the temperature that is perceived as noxious for mice and to quantify pain-related behaviours, the number of jumps at each temperature was scored. Left: number of jumps elicited at each temperature in male and female WT and TRESK KO mice ($n = 11$ – 15 animals per group). No significant differences were obtained between WT and TRESK animals (one-way ANOVA with Holm–Sidak correction). Right: mean temperature at which animals made the first jump (threshold) and total number of jumps for male and female mice in the whole range of temperatures. Values for temperatures between 30 and 38°C are not shown in the plots because they were probably detected as non-noxious and did not produce any observable response.

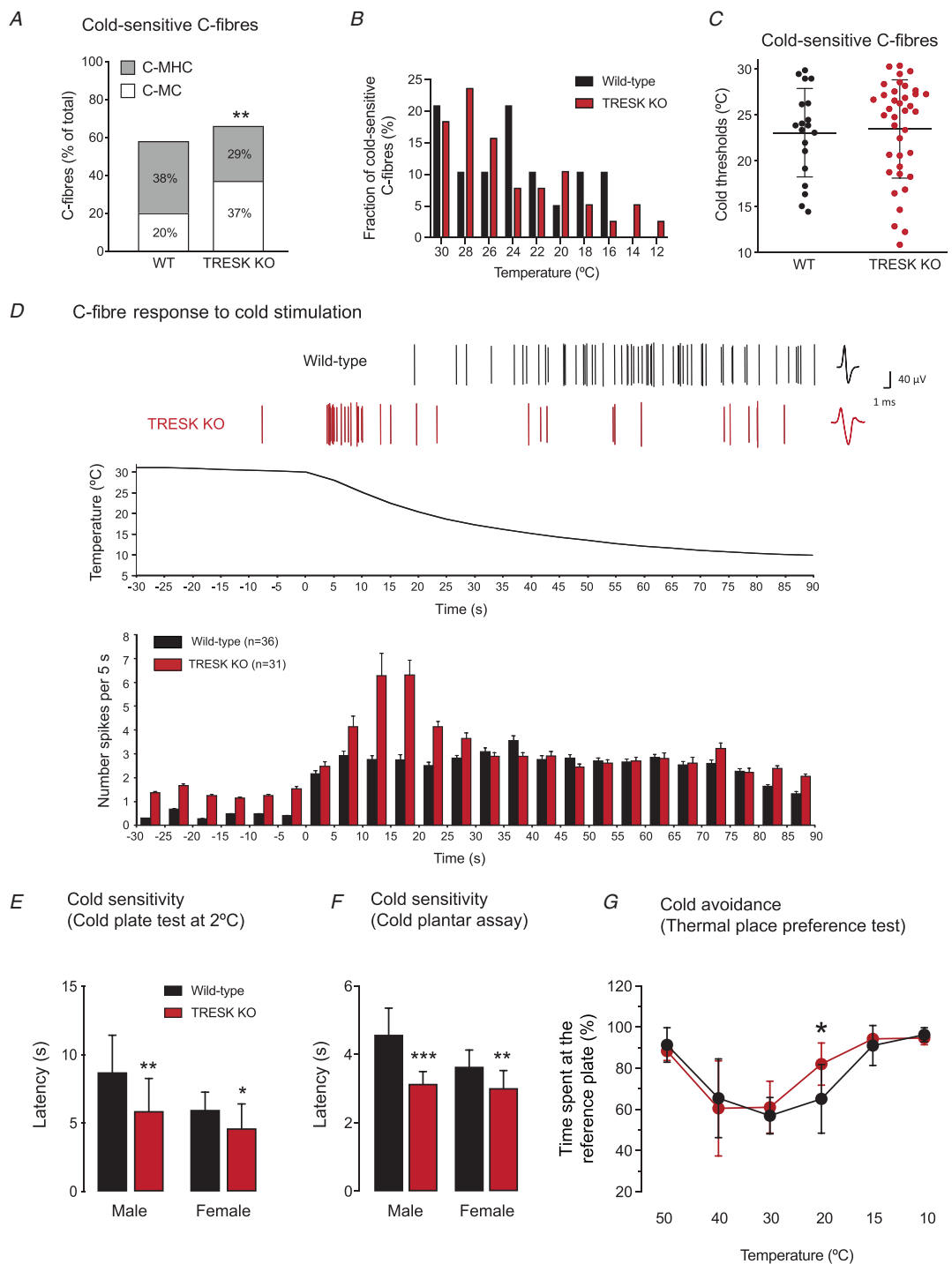


Figure 5. Cold allodynia in TRESK-deleted mice

genotype compared to baseline values (Fig. 6B). Also, when the percentage change in threshold (difference vs. basal value) was compared between WT and KO animals, this showed similar values (at 5 days, KO: $-45.1 \pm 42.7\%$; WT: $-37.9 \pm 29.6\%$; $P = 0.742$; at 7 days, KO: $-47.3 \pm 41.5\%$; WT: $-45.0 \pm 47.3\%$; $P = 0.931$), indicating that nerve injury exerted a similar effect in both genotypes but, because KO had a lower mechanical threshold to begin with, animals reached lower mechanical thresholds on days 5 and 7. Mechanical hypersensitivity was undistinguishable between groups at later stages, at 14 and 21 days after injury. Heat sensitivity showed a similar level of hyperalgesia after neuropathy, akin to the lack of implication of TRESK in thermal perception. Surprisingly, withdrawal latencies from KO animals recovered to baseline values faster than in the WT group after 21 days, which could indicate an excitotoxic death of some of the primary afferents due to excessive hyperactivity. Finally, we evaluated chemotherapy-induced neuropathic pain with the anti-cancer drug oxaliplatin, which induces cold allodynia in a majority of patients. We assessed whether the development of cold hyperalgesia after oxaliplatin treatment was modified in TRESK KO. As expected, WT mice showed an enhanced cold sensitivity 90 h after the oxaliplatin injection. Both the cold plantar assay ($P = 0.0002$; paired *t* test) and thermal place preference test ($30/20^\circ\text{C}$; $P = 0.0045$; unpaired *t* test) showed significant differences compared to pre-injection values, indicating a higher sensitivity to cold stimuli in neuropathic mice (Fig. 6C). In contrast, when TRESK KO animals were tested in the cold plantar assay, we did not observe any further decrease in paw withdrawal latency compared to the baseline value ($P = 0.912$), which was already lower than that of WT animals. Again, thermal place preference between 20 and 30°C did not show significant differences after oxaliplatin treatment ($P = 0.770$), suggesting that the enhanced cold sensitivity due to TRESK removal cannot be further increased by neuropathy induced by oxaliplatin.

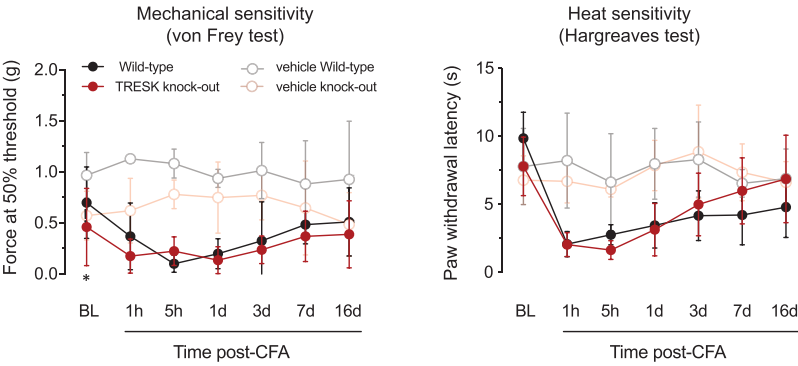
Discussion

TRESK is highly expressed in sensory ganglia (Kang & Kim, 2006; Dobler *et al.* 2007; Bautista *et al.* 2008; Tulleuda *et al.* 2011; Manteniotis *et al.* 2013; Nguyen *et al.* 2017; LaPaglia *et al.* 2018; Ray *et al.* 2018) within specific subtypes of sensory neurons, mainly nociceptors and especially in non-peptidergic neurons (Chiu *et al.* 2014; Usoskin *et al.* 2015; Li *et al.* 2016; Nguyen *et al.* 2017; Zheng *et al.* 2019). TRESK's main role has been attributed to preventing neuronal depolarization (Enyedi & Czirják, 2010), and reduction of its expression after nerve injury or inflammation contribute to neuronal hyperexcitability (Dobler *et al.* 2007; Tulleuda *et al.* 2011; Marsh *et al.* 2012). Mutations in this channel have been linked to the enhanced nociceptor excitability that occurs during familial migraine with aura (Lafrenière *et al.* 2010; Royal *et al.* 2019). Here, we propose that TRESK balances the effects of depolarizing stimuli, acting as a brake to prevent the activation of specific subpopulations of sensory neurons.

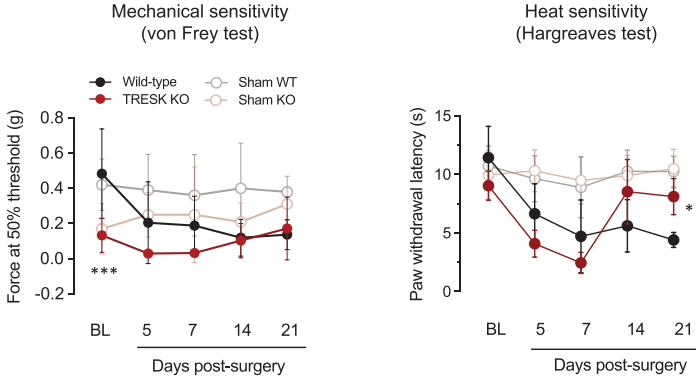
Nociceptors lacking TRESK presented electrophysiological changes and an enhanced excitability consistent with its putative role to prevent neuronal depolarization (Kang & Kim, 2006). RMP was similar between TRESK KO and WT neurons, indicating that TRESK does not contribute significantly in setting this parameter, as previously suggested (Dobler *et al.* 2007). Indeed, other K_{2P} members and other K^+ channel families have been proposed to control membrane polarization at rest (Du *et al.* 2018). We found that TRESK, TREK-1 and TREK-2 were similarly expressed in DRGs, while the expression of TRAAK was lower. Others have found a slightly higher expression of TRESK over TREK-1 or TREK-2 while the expression of TRAAK varies in different studies but in general appears less than for TRESK (Dobler *et al.* 2007; Marsh *et al.* 2012). Despite unchanged mRNA expression of other leak channels (TREK-1/2, TRAAK) in TRESK KO mice, it is possible that post-translational

A, fractions of cold-sensitive C-fibres in wild-type (WT) (total number of C-fibres = 35) and TRESK knockout (KO) ($n = 37$ C-fibres) mice, measured in skin-nerve experiments. Numbers in bars are the percentage of mechano-heat-cold (C-MHC) and mechano-cold (C-MC) fibres. A significant difference in the distribution of cold fibres is shown (** $P < 0.01$, chi-square test). B, distribution of the C-fibres activated by cold at different temperatures between WT and KO animals. C, distribution of cold thresholds from fibres recorded (WT $n = 19$; KO $n = 38$). Mean and SD are shown. D, representative experiments of C-fibres activated by a cold ramp. Top: action potentials (Spikes) fired in response to a temperature decrease are shown for WT and TRESK KO cold C-fibres. The average action potential is presented on the right. A representative cold ramp from 30 to 10°C is shown below. Bottom: histogram of mean responses to cooling, 5-s bin, of C-fibres from WT and TRESK KO mice. E and F, cold sensitivity measured with the cold plate (2°C) and cold plantar assays in male and female WT and TRESK KO animals ($n = 11$ –19 animals per group). G, cold avoidance measured in the thermal place preference test. The percentage of time spent at the reference plate (30°C) at each experimental temperature is shown ($n = 8$ –14 male animals per group). Statistical differences are shown as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ with Student's unpaired *t* test (E, F) or one-way ANOVA plus Holm–Sidak correction for multiple comparisons (G) between WT and KO mice.

A CFA-induced inflammatory pain



B Cuff-induced neuropathic pain



C Oxaliplatin-induced cold allodynia

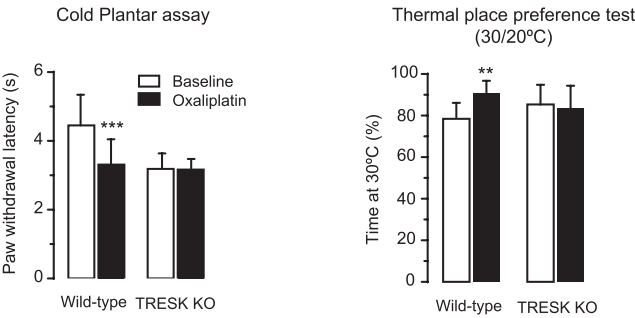


Figure 6. Changes in chronic pain in TRESK-deleted mice

A, mechanical and thermal sensitivity in the complete Freund's adjuvant (CFA)-induced inflammatory pain model. Mechanical sensitivity was measured with von Frey filaments (up and down method) and thermal sensitivity was measured with the Hargreaves test ($n = 7$ animals CFA groups; $n = 5$ animals vehicle groups). B, mechanical and thermal sensitivity in the cuff-induced neuropathic pain model. Mechanical sensitivity was measured with

modifications of these proteins in TRESK KO mice exist, thus enhancing the functional contribution of these other K^+ currents in the absence of TRESK to maintain the membrane potential within the physiological range. Also, a compensatory effect by other channels (e.g. HCN, KCNQ) cannot be discarded. TRESK might have a major impact on the range between the RMP and the AP threshold, and thus effects observed in excitability appear to be mainly attributable to the specific removal of TRESK. Still, these effects could be underestimated by the fact that TRESK shows different levels of expression in different types of nociceptors (Usoskin *et al.* 2015), so studying specific subpopulations of genetically labelled nociceptors (mainly non-peptidergic) might produce larger effects in excitability after TRESK deletion. In fact, a recently published characterization in TG neurons shows that TRESK is present in 73% of IB4⁺ non-peptidergic neurons, while only 29% of CGRP⁺ neurons (mainly peptidergic nociceptors) express TRESK (Weir *et al.* 2019). In agreement, IB4⁺ nociceptive TG neurons showed an enhanced excitability compared to IB4[−] neurons. Another study reported similar results in TG neurons but, surprisingly, lumbar DRG neurons from the same TRESK KO mice were not found to be hyperexcitable (Guo *et al.* 2019). Although we did not differentiate between IB4⁺ and IB4[−] neurons in this study, our data support previous findings of enhanced excitability of nociceptive sensory neurons (small DRGs) either after TRESK deletion or after a significant decrease in its expression (Dobler *et al.* 2007; Tulleuda *et al.* 2011; Yang *et al.* 2018; Weir *et al.* 2019). These effects have been also found after a decreased TRESK expression in a functional TRESK[G339R] knockout mice (Dobler *et al.* 2007; Kollert *et al.* 2015), after sciatic nerve axotomy (Tulleuda *et al.* 2011) or in a model of cancer-associated pain (Yang *et al.* 2018). This is in agreement with the neuronal activation observed by compounds blocking TRESK (Bautista *et al.* 2008; Tulleuda *et al.* 2011; Castellanos *et al.* 2018) or with the decreased excitability when over-expressing the channel (Guo & Cao, 2014; Yang *et al.* 2018), indicating that the regulation of TRESK expression is an important factor to control sensory neuron excitability.

Here, we found that instead of producing a general increase in excitability, removal of TRESK has a selective effect on certain types of fibres/sensory neuron subtypes

affecting specific sensory modalities. In a similar fashion, genetic deletion of TREK-1 modifies mechanical, heat and cold pain perception (Alloui *et al.* 2006) but when TRAAK is deleted, cold sensitivity remains unaffected and animals only present an enhanced heat sensitivity. Interestingly, deleting both channels (TREK-1/TRAAK double KO mice) potentiates the effects on cold sensitivity compared to TREK-1 alone (Noël *et al.* 2009). Besides, deletion of TREK-2 only enhances thermal sensitivity to non-aversive warm conditions but not to cold stimuli (Pereira *et al.* 2014). Finally, eliminating the BK_{Ca} channel does not modify acute nociceptive or neuropathic pain, but enhances persistent inflammatory pain (Lu *et al.* 2013), highlighting its specific participation during inflammation-induced hyperalgesia. Our data suggest that the lack of TRESK enhances cold sensitivity by increasing the percentage of C-fibres activated at moderate cold, which is translated at the behavioural level by an enhanced sensitivity to cold stimuli. An increase in cold sensitivity has also been reported in TRESK KO mice using the acetone test (Guo *et al.* 2019). In our study, the fraction of C-fibres activated by cold (C-MHC and C-MC; 58% in WT mice) was higher than other reports (35–45%), which could be due to the smaller number of fibres sampled compared to other studies (Bautista *et al.* 2007; Noël *et al.* 2009; Pereira *et al.* 2014; Winter *et al.* 2017) but, as occurred after deletion of other K_{2P} channels, the fraction of cold-sensitive C-fibres was enhanced in TRESK KO mice compared to WT animals. Interestingly, the larger fraction of cold-sensitive C-MC fibres (37%) in KO animals compared to WT animals (20%) suggests that, in physiological conditions, TRESK silences a population of neurons that would be normally activated at lower temperatures. This is consistent with a possible role of TRESK in preventing cold allodynia, acting as a brake to avoid C-fibre activation at moderate cool conditions. In fact, knocking out TRESK mimics cold allodynia and oxaliplatin injection is not able to further increase cold sensitivity. Oxaliplatin-induced cold allodynia has been attributed to a combined remodelling of ion channels, including down-regulation of TREK-1, TREK-2, TRAAK, K_v1.1 and K_v1.2, coupled with up-regulation of TRPA1, Na_v1.8 and HCN1, while other channels (TRPM8) are not significantly modified (Descœur *et al.* 2011; Pereira *et al.* 2014). It has been proposed that a reduced activity of K_{2P} channels when temperature decreases is responsible for

von Frey filaments (up and down method) and thermal sensitivity was measured with the Hargreaves test ($n = 6$ animals in each group). C, oxaliplatin-induced cold sensitization model. Left: paw withdrawal latency to the cold plantar assay before (baseline) and 90 h after oxaliplatin injection in WT and TRESK KO animals ($n = 9$ and 10 animals per group). Right: cold avoidance measured in the thermal place preference test. The percentage of time spent at the reference plate (30°C) versus the experimental temperature (20°C) is shown ($n = 6$ and 8 animals per group). Statistical differences between WT and KO mice are shown as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Two-way ANOVA plus Holm–Sidak correction for multiple comparisons (data in A and B) or Student's paired t test (C; baseline vs. oxaliplatin) were used.

releasing the excitability brake exerted by these channels and, combined with depolarizing thermo-TRP channel activation, triggers neuronal activation (Kang & Kim, 2006; Noël *et al.* 2009; Pereira *et al.* 2014). This seems not to be the case for TRESK, as its activity (channel open probability) is not significantly modified by temperature between 20 and 37°C (Kang & Kim, 2006). The possible modulation of the channel at lower temperatures it is unknown and remains to be tested.

TRPA1 was initially proposed to be involved in noxious cold sensing (Story *et al.* 2003) but recent reports have questioned its role, hypothesizing that it might be regulating cold sensitivity indirectly, rather than acting as a cold-sensing receptor (Lolignier *et al.* 2016; Yarmolinsky *et al.* 2016; Winter *et al.* 2017). If TRESK and TRPA1 are partially co-expressed in some neurons, one would expect an increase in the responses triggered by TRPA1 activation but, to our surprise, we observed the opposite effect despite TRPA1 mRNA expression being unchanged. It is possible that some other type of functional regulation is present, but we did not explore this possibility further. By contrast, it seems unlikely that enhanced cold sensitivity in TRESK KO animals is related to the observed decrease in TRPA1 activity. K_v channels can blunt cold responses by opposing depolarizing stimuli in TRPA1⁺ and TRPM8⁺ neurons (Madrid *et al.* 2009; Memon *et al.* 2017). It is possible that TRESK might exert a similar effect, hence silencing cold responses in normal conditions. Therefore, releasing this excitability brake by knocking out or down-regulating TRESK expression enhances cold sensation. We did not find differences in the cellular responses to menthol, which activates TRPM8, a well-known sensor of low temperatures (Bautista *et al.* 2007). Also, TRESK and TRPM8 seem to be expressed in different subpopulations of sensory neurons (Usoskin *et al.* 2015; Nguyen *et al.* 2017) and the transcriptome of TRPM8⁺ sensory neurons does not show an appreciable TRESK expression (Morenilla-Palao *et al.* 2014). Therefore, the enhanced cold sensitivity in TRESK KO mice does not seem related to activation of TRPM8⁺ fibres. Nevertheless, TRPM8⁺/IB4⁻ trigeminal neurons have been reported to be more excitable in the TRESK KO compared with WT neurons (Guo *et al.* 2019), and thus an increase in activation of TRPM8⁺ fibres after TRESK deletion cannot be completely discarded. Recently, a new cold sensor (GLR-3) has been identified in *Caenorhabditis elegans* and the kainate receptor GluK2 (mouse GLR-3 homolog) has been proposed to mediate cold sensitivity in mouse DRGs (Gong *et al.* 2019). Whether this cold sensor is expressed in the same neurons that contain TRESK it is as yet unknown. Even if this is the case, it remains to be examined in greater depth.

Mechanical sensitivity is also enhanced in TRESK KO animals, which present a higher number of C-fibres with lower mechanical thresholds and an increased response

to application of von Frey hairs. Similar results were previously reported after knocking down TRESK expression or after injecting TRESK blockers, where animals showed mechanical allodynia (Tulleuda *et al.* 2011; Castellanos *et al.* 2018; Yang *et al.* 2018). Likewise, trigeminal expression of a mutated form of TRESK linked to migraine results in facial mechanical allodynia (Royal *et al.* 2019). Other studies have also reported an increase in innocuous touch sensitivity in mice after TRESK deletion, although they did not find a significant difference in punctate mechanical sensitivity (Weir *et al.* 2019). A decrease in the withdrawal threshold to application of von Frey filaments in the forehead skin (trigeminal area) has also been reported but, surprisingly, not in the hindpaw (Guo *et al.* 2019). In our study, mechanical sensitivity is enhanced by shifting the mechanical threshold toward lower values in a population of sensory fibres, and therefore, a higher number of fibres is activated by low-intensity mechanical stimulation. The Piezo2 channel is involved in the detection of both innocuous touch and high-threshold noxious mechanical stimuli due to its expression in low-threshold mechanoreceptors (LTMRs) and nociceptors (Ranade *et al.* 2014; Dhandapani *et al.* 2018; Murthy *et al.* 2018; Szczot *et al.* 2018), and has been proposed to play a crucial role in the generation of mechanical allodynia during inflammation and neuropathic pain (Murthy *et al.* 2018). TRESK is also highly expressed in these populations of sensory neurons involved in mechanical hypersensitivity (Usoskin *et al.* 2015). We can hypothesize that when these neuronal populations lack TRESK, they are more readily activated by mechanically activated currents coming through Piezo2 or other mechanosensitive channels such as the recently identified TACAN (Beaulieu-Laroche *et al.* 2018), which would depolarize sensory neurons more effectively. We have not evaluated whether LTMRs lacking TRESK are more excitable and whether other touch modalities such as light touch (brush stroke) are also enhanced, but mechanical allodynia present in these animals suggests that they might also be affected. In agreement, a recent report found an enhanced response to innocuous touch stimuli (Weir *et al.* 2019).

TRESK is notably expressed in MrgprD⁺ neurons (Usoskin *et al.* 2015; Zheng *et al.* 2019) and deletion of this subpopulation of neurons abolishes mechanical but not thermal pain (Cavanaugh *et al.* 2009). Here, the mechanical hypersensitivity found after TRESK deletion might suggest an enhanced excitability of MrgprD⁺ neurons. In contrast, mechanical sensitization that occurs after inflammation or injury is not further enhanced by the absence of TRESK. Our data suggest that the molecular effect of injury, down-regulating TRESK expression (Tulleuda *et al.* 2011), has already been achieved by genetic removal of TRESK, and thus any further decrease in mechanical threshold observed is likely to be due to

other mechanisms sensitizing nociceptors or derived from central effects.

TRESK KO mice did not display relevant differences in heat sensitivity. Only the thermal place preference between 20 and 30°C showed a difference that seems to reflect the enhanced cold sensitivity rather than any effect on warm/heat sensitivity. In agreement, TRESK KO C-fibres did not show any specific effects upon temperature increase compared to WT. No significant alterations on heat sensitivity either observed were after CFA-induced inflammation or in the neuropathic pain model. In contrast to other K_{2P} and TRP channels (Alloui *et al.* 2006; Noël *et al.* 2009; Julius, 2013; Pereira *et al.* 2014; Vriens *et al.* 2014; Yarmolinsky *et al.* 2016), the role of TRESK in neurons detecting warm or hot stimuli seems not to be highly relevant. Chemical sensitivity was altered for some stimuli (AITC, osmosensitivity) but not for others (capsaicin, menthol). Whether TRESK is directly involved in detecting osmotic stimuli is unlikely, although we previously described that hypertonic and hypotonic stimuli modulate TRESK currents (Callejo *et al.* 2013). Similar results in osmotic pain were reported in animals lacking TREK or TRPV4 channels (Alessandri-Haber *et al.* 2005; Alloui *et al.* 2006; Noël *et al.* 2009; Pereira *et al.* 2014). Similarly to TREK-1/2 KO mice (but not TRAAK KO), PGE₂ was unable to sensitize nociceptors to osmotic stimuli in TRESK KO animals. It has been proposed that the negative regulation of TREK-1/2 channels by cAMP/PKA downstream of the G-coupled PGE₂ receptor might be involved in the lack of PGE₂-mediated nociceptor sensitization. PKA is known to phosphorylate TRESK and keep it in a resting state where the channel is less active (Czirják & Enyedi, 2010) but a direct link between PKA activation, TRESK activity and osmotic sensing is still unknown. In fact, neurons activated by radial stretch and hypotonic stimuli seem to express TRESK, as they are sensitive to hydroxy- α -sanshool, a TRESK blocker (Bhattacharya *et al.* 2008). These neurons were initially classified as LTMRs or non-peptidergic nociceptors, which is in agreement with the TRESK expression pattern obtained from single-cell mRNA sequencing (Usoskin *et al.* 2015).

In summary, we describe that genetic removal of TRESK specifically enhances mechanical and cold sensitivity in mice, not affecting the sensitivity to other stimuli. The development of specific TRESK openers/activators would be worthwhile to improve mechanical hypersensitivity and cold allodynia during chronic pain conditions.

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Additional information

Competing interests

The authors have no conflicts of interest to declare.

Author contributions

A.C., A.A.B. and G.C. performed electrophysiological recordings in neurons and calcium imaging. A.C., A.P.C. and A.A.B. performed behavioural experiments. A.C. and A.A.B. carried out primary cell cultures. A.C., G.C., A.P.C. and N.C. performed qPCR experiments. A.N. and J.N. performed skin-nerve recordings. A.C., D.S., J.N., N.C. and X.G. participated in the design of the study and performed the statistical analysis. X.G. conceived the study, oversaw the research and prepared the manuscript with help from all others. All authors read and approved the final manuscript.

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Keywords

cold, ion channels, mechanosensitivity, neuronal excitability, pain, potassium channel, sensory neurons

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Statistical Summary Document



Review

The Background K⁺ Channel TRESK in Sensory Physiology and Pain

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Abstract: TRESK belongs to the K_{2P} family of potassium channels, also known as background or leak potassium channels due to their biophysical properties and their role regulating membrane potential of cells. Several studies to date have highlighted the role of TRESK in regulating the excitability of specific subtypes of sensory neurons. These findings suggest TRESK could be involved in pain sensitivity. Here, we review the different evidence available that involves the channel in pain and sensory perception, from studies knocking out the channel or overexpressing it to identified mutations that link the channel to migraine pain. In addition, the therapeutic possibilities are discussed, as targeting the channel seems an interesting therapeutic approach to reduce nociceptor activation and to decrease pain.

Keywords: potassium channel; pain; sensory neuron; somatosensation; migraine; hyperexcitability

1. Introduction

Potassium channels integrate the largest family of ion channels, with about 80 genes encoding for alpha subunits [1]. Four large families of K⁺ channels have been identified in mammals according to their structural and functional characteristics: voltage-gated K⁺ (K_v) channels, Ca²⁺-activated K⁺ (K_{Ca}) channels, inwardly rectifying K⁺ (K_{ir}) channels, and two-pore domain background K⁺ (K_{2P}) channels. They participate in numerous cellular functions in almost all cell types of the body, including K⁺ homeostasis, cell volume regulation, cellular excitability, and proliferation. In particular, the polarity of the plasma membrane depends on the function of specific K⁺ channels that allow the K⁺ efflux through the membrane according to its electrochemical gradient, driving the membrane potential of the cell to negative values (near −80 mV). This value is close to the equilibrium potential for K⁺ due to the high concentration of K⁺ inside the cells (about 140 mM) compared to extracellular medium (4 mM). In addition to their role in setting the resting membrane potential, K⁺ channels regulate cellular excitability. This is especially important in neurons, where potassium currents oppose the depolarization caused by other excitatory Na⁺, Ca²⁺ or nonselective cation channels, thus contributing to regulate neuronal firing and shaping the action potential.

Among the different K⁺ channel families, the family of K_{2P} K⁺ channels was the last one identified and described, which, to date, has 15 members to date, grouped into six subfamilies (TWIK, TREK, TASK, TALK, THIK, and TRESK) based on sequence and functional similarities [1,2].

The first K_{2P} channel identified was TWIK1, for Tandem of pore domains in a Weak Inward-rectifying K⁺ channel [3]. Now, this subfamily also contains TWIK2 and KCNK7 (K_{2P}7.1).

The TREK (TWIK-Related K⁺ channel) subfamily contains TREK1, TREK2, and TRAAK (TWIK-Related Arachidonic acid Activated K⁺) channels. Members of this subfamily are activated by arachidonic acid, polyunsaturated fatty acids (PUFAs), volatile anesthetics, and pain-related stimuli. The TASK (TWIK-related Acid-Sensitive K⁺ channel) subfamily contains TASK1, TASK3, and TASK5 (K_{2p}15.1, KCNK15), and these channels have the common property of being inhibited by extracellular acidification. The TALK (TWIK-related ALkaline pH-activated channel) subfamily includes TALK1, TALK2 (K_{2p}17.1, KCNK17) and TASK2 that have an important sensitivity to extracellular alkaline pH. The THIK (Tandem pore domain Halothane-Inhibited K⁺) channel subfamily is composed by THIK1 and THIK2, and both channels are inhibited by halothane. Finally, the TRESK (TWIK-Related Spinal cord K⁺) subfamily has only one member, TRESK (K_{2p}18, KCNK18), which has the lowest structural and functional similarity to other K_{2p} channels, and it is the only K_{2p} regulated by intracellular Ca²⁺ concentration through calcineurin-mediated dephosphorylation.

In contrast to other K⁺ channel families that have one pore-forming domain for each subunit, K_{2p} channels have two pore domains and four transmembrane domains. Consequently, functional K_{2p} channels are dimers and not tetramers as most of the other K⁺ channels are. K_{2p} channel activity produces constitutive leak currents, which are mostly independent of the membrane potential, with some minor exceptions. As mentioned earlier, their main role in most cell types is attributed to the regulation of membrane potential, as they constitute the leak of potassium through the plasma membrane (accordingly they are commonly referred as leak or background potassium channels) that equilibrates with the function of the Na⁺/K⁺ pump and help to set the resting membrane potential. For this reason, they influence neuronal excitability over a wide range of membrane potentials, especially between resting and action potential threshold. Thus, K_{2p} channels are one of the main sustained K⁺ conductance that establish the resting membrane potential in neurons, where they can also shape the duration, frequency, and amplitude of the action potential.

Basic biophysical properties of this family of channels, regulation, and interaction with other proteins are reviewed elsewhere, including some comprehensive and extensive reviews [2,4–7]. This review is intended to highlight the role of the last described K_{2p} member, TRESK, in somatosensory perception and pain. The role of other K⁺ channels in pain, including K_{2ps}, has been nicely reviewed in several articles [8–11].

2. TRESK Identification and Expression

TRESK was initially identified and cloned from human spinal cord in 2003 [12]. Later and despite its low homology (65%), the mouse ortholog (initially identified as TRESK-2) was cloned from cerebellum and testis by other groups [13,14]. Initially, TRESK was only reported to be expressed in spinal cord in humans, but, in mice and rats, it was also detected in spleen, thymus, pancreas, kidney and testis [14], although other studies have not found a significant expression on some of these organs [15]. In the rodent nervous system, TRESK expression has been detected in cortex, cerebellum, brainstem, and spinal cord, but, especially, a high expression of the channel was found in sensory neurons of the dorsal root (DRG) and trigeminal ganglia (TG), as well as in neurons of the superior cervical ganglion and nodose ganglion [15–21]. Within sensory ganglia, TRESK seems predominantly expressed in small and medium diameter sensory neurons that mainly correspond to nociceptive neurons [16,22–25].

Recent studies of massive RNA sequencing, both bulk or at a single-cell level, have identified TRESK expression in selected subpopulations of mouse and human DRG sensory neurons [26–30]. In addition, trigeminal sensory neurons show a high expression of TRESK with a similar distribution pattern [31–34]. In particular, TRESK shows a predominant expression in non-peptidergic IB4⁺ sensory neurons (NP), which confirms previous immunohistochemistry data. NP1 and NP2 subgroups of non-peptidergic neurons show an important expression, while peptidergic sensory neurons (PEP2) show little expression [27]. The characteristic gene expression pattern of these subgroups (NP1, 2) suggests their involvement in neuropathic pain and itch. In addition, a significant expression of TRESK

was detected in a subpopulation of low-threshold mechanoreceptors (NF1) expressing high levels of TrkB and likely involved in the detection of touch and proprioception [26,27]. A similar TRESK expression pattern has been reported in subsets of sensory neurons from the trigeminal ganglia [32]. Recent studies using labeled subpopulations of DRG neurons from genetically modified mice have also identified TRESK expression in MrgprD⁺ non-peptidergic nociceptors, in CGRP⁺ peptidergic nociceptors and in A δ -low threshold mechanoreceptors (TrkB⁺) [35]. At a functional level, native single channel currents with electrophysiological and pharmacological properties of TRESK have been reported in mouse and rat sensory neurons, corroborating expression studies [16,19,36,37]. TRESK, together with TREK-2, seems to constitute the major component of K⁺ background current in medium- and small-sized sensory neurons [16].

3. Involvement of TRESK in Pain

From its initial identification and by analogy to other channels of the family, such as TREK-1/2 or TRAAK, a putative involvement of TRESK in pain sensitivity was suggested. As mentioned earlier, TRESK gene expression is elevated in sensory neurons, specifically in small and medium-sized neurons that mainly correspond to nociceptors. In addition, when compared to other channels of the K_{2P} family, TRESK presents the highest expression in rodent sensory neurons, followed by TRAAK, TREK2, TREK1, TWIK1, and TWIK2, although relative expression may vary between studies [19,21,25]. In humans, the most expressed channels in DRG and TG are THIK-2, TASK1, and TWIK1, followed by TREK1, TASK2, and TRESK [33], but other studies found TRESK as the most expressed channel in human TG [20,31,38].

3.1. TRESK Expression and Sensory Neuron Excitability

Studies using mice with a total or a functional knockout of the channel have shown that TRESK ablation enhances the excitability of nociceptive sensory neurons [19,23,25,39], confirming that the channel regulates neuronal excitability. This is accompanied by a decrease in total K⁺ current and an increase in membrane input resistance. All this data indicates that a decrease in channel expression or function facilitates neuronal depolarization and activation; thus, in physiological conditions, the channel acts as a brake to prevent neuronal activation in response to depolarizing stimuli.

In this sense, a reduction of TRESK expression has been reported in different models characterized by hyperexcitability of sensory neurons. In an initial report, Tulleuda et al. described a marked decrease in TRESK expression in rat lumbar DRGs three weeks after sciatic nerve axotomy [18]. In addition, a reduction of TRESK expression by siRNA injection (thus mimicking the effects of axotomy) resulted in an enhanced sensitivity to mechanical stimuli but not to radiant heat stimulation. The study showed that, similarly to other ion channels and particularly some K⁺ channels [10], peripheral injury or axotomy reduces the expression of TRESK, consequently enhancing sensory neuron excitability. Other studies reported similar effects using different models of chronic neuropathic or inflammatory pain [21,40,41]. Interestingly and despite the fact that sensory neuron primary culture is a common model used in many studies, ganglia dissociation produces an inevitably axotomy where neurons become injured. Consequently, they show an enhanced expression of known injury markers (e.g., ATF3 or Cacna2d1) and quickly become hyperexcitable. Similar to what occurs after axotomy, a decrease in TRESK expression was observed after culturing DRGs [18]. In fact, a recent report that has compared the transcriptome of native versus cultured DRGs shows that the latter display an increased expression of several genes associated with nerve injury or inflammation (BDNF, MMP9, ATF3, or GAL) which indicates an injury-like phenotype of cultured DRGs [42]. Again, TRESK was downregulated in cultured neurons compared to its native expression in the ganglion. Whether expression of the channel recovers its normal levels after injury or if reinnervation occurs it is still unknown.

Regardless of possible effects of cell culture, studies comparing sensory neurons from WT or TRESK KO mice have found significant differences in their excitability. Weir et al. have shown that deletion of TRESK enhances the excitability of non-peptidergic (IB4⁺) trigeminal sensory neurons while the excitability of peptidergic neurons was not significantly modified compared to WT cells [23],

consistent with the higher expression of the channel in the non-peptidergic neuron population. We reported a similar effect on DRG nociceptive neurons from a different TRESK KO mice, although in this case, no distinction was made between peptidergic and non-peptidergic neurons [25]. A third study reported similar results in TG neurons but, surprisingly, lumbar DRG neurons from the same TRESK KO mice were not found to be hyperexcitable [39]. In a functional TRESK[G339R] knockout mice, sensory neurons also showed a reduced current threshold to fire an action potential and an enhanced excitability [19,24]. Surprisingly, none of these studies found significant differences in resting membrane potential after deleting TRESK, suggesting that either TRESK removal is compensated by the concurrence of other channels or that the channel has a minor role on setting the resting membrane potential. Besides, all these studies indicate that TRESK have a major impact on the range between the resting membrane potential and the action potential threshold to modulate the excitability of sensory neurons (in particular, non-peptidergic ones). In agreement, overexpression of the channel using viral vectors decreased the excitability of sensory neurons and diminished pain sensitivity in animal models [41,43,44] (Figure 1).

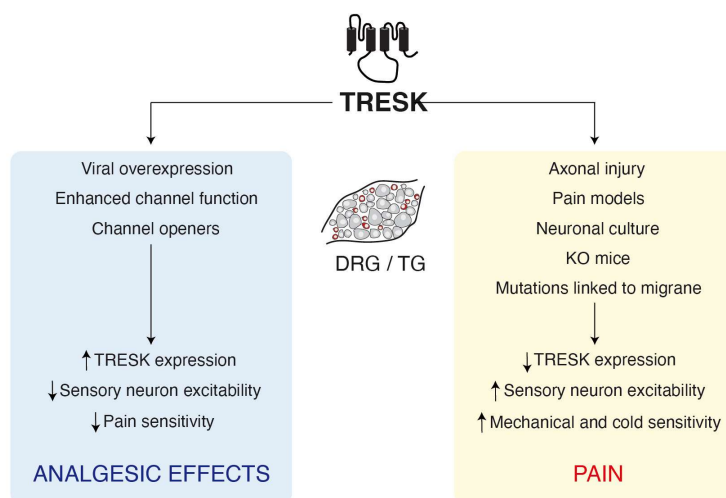


Figure 1. TRESK regulation is linked to pain or analgesic effects. Axonal and neuronal injuries or TRESK mutations conduct to a decrease in channel function that leads to an increase in pain sensitivity. Enhancement of TRESK expression or channel openers decreases the excitability of sensory neurons producing analgesic effects.

Removal of TRESK, rather than producing a general increase in excitability, has a selective effect on certain types of fibers/sensory neuron subtypes, thus affecting specific sensory modalities. Similar modulation of pain sensitivity to distinct stimuli has been encountered for other channels of the family. For instance, genetic ablation of TREK-1 modifies mechanical, heat and cold pain perception [45] but TRAAK deletion only enhances heat and mechanical sensitivity, while cold sensitivity remains unaffected [46]. Interestingly, if both channels are eliminated (TREK-1/TRAAK double KO mice), mice show an enhanced cold sensitivity compared to TREK-1^{-/-}. Besides, deletion of TREK-2 only enhances thermal sensitivity to non-aversive warm conditions without affecting cold perception [47]. Knocking out TRESK produces a significant enhancement of mechanical and cold sensitivity, while sensitivity to heat remains largely unaffected or the effects are much smaller [23,25,39]. This seems to be related to a percentage of peripheral C-fibers activated by lower mechanical thresholds or in a warmer range of temperature, compared to fibers from WT mice [25]. These results suggest that in normal conditions, TRESK prevents the activation of nociceptive fibers by innocuous or low intensity stimuli, therefore only the high intensity ones can depolarize peripheral nerve endings sufficiently to produce neuronal

firing. This seems restricted to certain modalities, but not others, such as heat sensing fibers in which excitability is probably regulated by other ionic mechanisms.

In addition to the downregulation of TRESK after axotomy, similar effects have been found in other models of persistent pain. During cutaneous inflammatory pain, a decrease in TRESK mRNA has been reported although the effects were rather small and in a limited number of animals [21]. In TRESK KO mice, an inflammatory pain model produced by injection of Complete Freund's Adjuvant (CFA) in the hind paw did not show significant differences compared to WT mice, neither in the development of mechanical nor thermal hypersensitivity, except for the initially enhanced mechanical sensitivity present in KO animals [25]. Despite the data is not conclusive, it seems that the involvement of the channel in the development of inflammatory pain is rather limited. Models of neuropathic pain (complete axotomy, spared nerve injury or spinal nerve ligation) have shown a marked reduction of TRESK expression in the DRGs, which likely contributes to the enhanced mechanical and thermal hypersensitivity [18,40,44,48]. Recovery of TRESK expression levels by viral transfection partially reversed mechanical allodynia [40,44]. A similar TRESK downregulation in DRGs has been reported in a model of cancer-associated pain and overexpression of the channel suppresses tumor-induced neuronal hyperexcitability and pain hypersensitivity in this bone metastasis model [41]. In a nerve-cuffing induced neuropathic pain model, we did not find significant differences in the development of mechanical and thermal hypersensitivity between WT and TRESK KO animals, having a similar effect in both genotypes. Because KO mice had a lower mechanical threshold to begin with, animals reached lower mechanical thresholds 5 to 7 days post-injury, but mechanical hypersensitivity was undistinguishable between groups at later stages (14 and 21 days). Radiant heat sensitivity showed a similar level of hyperalgesia after neuropathy, in agreement with the lack of implication of TRESK in thermal perception [25]. Interestingly, TRESK KO mice presented cold allodynia, similar to that induced by oxaliplatin treatment in WT mice. In this sense, oxaliplatin treatment did not further enhance the cold hypersensitivity achieved by TRESK ablation [25]. Whether TRESK ablation produces other cellular changes driving to hypersensitivity of cold-sensing nociceptors remains to be elucidated.

3.2. Calcineurin Modulation of TRESK and Pain Sensitivity

The regulation of TRESK by calcineurin-mediated dephosphorylation [13], a unique feature among other K_{2P} channels, it is thought to contribute to TRESK function. Neurotransmitters or other compounds acting on membrane receptors that finally increase cytosolic calcium, specially $G_{\alpha q}$ -coupled receptors, will enhance TRESK current, thus opposing neuronal depolarization by these stimuli [13,36,49,50]. According to several studies, TRESK remains constitutively phosphorylated (at residues S252, S262, S264, and S267 in the human channel; Figure 2) in resting conditions and calcium binding to calcineurin phosphatase rapidly activates the channel by dephosphorylation. If phosphatase activity is discontinued by recovery of low calcium levels, the channel will be rephosphorylated by kinases and will return to the resting state. In this effect, protein kinase A (PKA) and microtubule affinity-regulating (MARK) kinases seem to play a significant role [13,50–52] (Figure 2). Besides, protein kinase C (PKC) indirectly activates the human channel, as treatment with phorbol 12-myristate-13-acetate (PMA) enhanced its current in *Xenopus laevis* oocytes [53]. In contrast, the mouse channel was reported to be insensitive to PMA [12]. Although the intermediate signaling pathway was not initially described, novel-type PKC isoforms (nPKC) seem to be involved (rather than conventional PKC isoforms). In any case, the effect was independent of direct PKC-mediated phosphorylation of the channel since mutation of putative PKC phosphorylation sites did not abolish the PMA effect [53]. Confirmation of this effect was later reported and the likely mechanism of action involves the nPKC-dependent inhibition of the kinase responsible for the rephosphorylation of the channel at S264 [54].

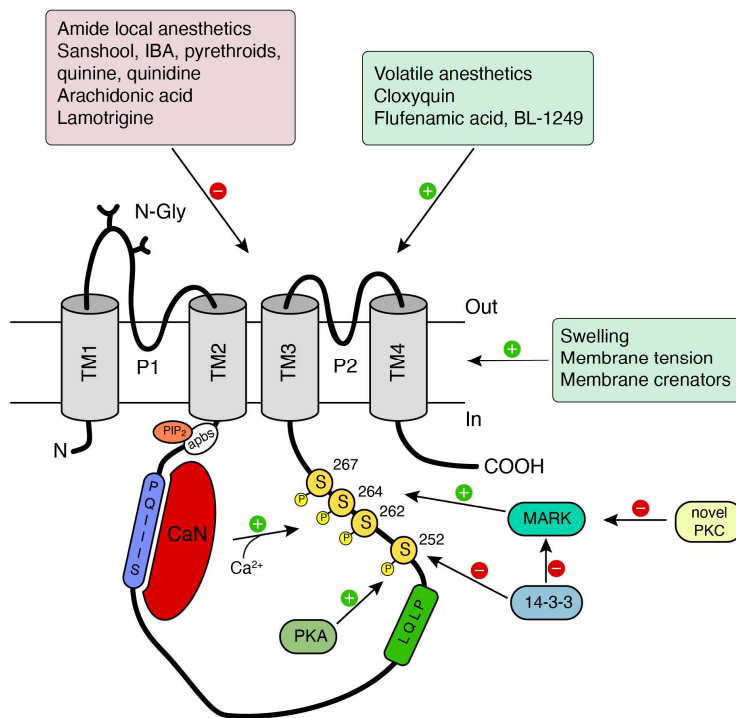


Figure 2. Regulation of TRESK by extracellular stimuli or by intracellular pathways and interacting proteins. Schematic model of the human TRESK channel in the resting state. Known activators and inhibitors/blockers of the channel are indicated on top, as well as stimuli that modulates channel function through the membrane. Regulatory and interaction sites with membrane lipids and PIP₂ (apbs) or calcineurin (PQIIS and LQLP) are shown. Calcineurin interacts with the LQLP motif upon calcium binding to dephosphorylate serines S262, S264, and S267. Other regulatory proteins (PKA, 14-3-3 and microtubule affinity-regulating kinases (MARK) and sites of action are indicated.

It has been proposed that calcineurin-inhibitor pain syndrome (CIPS) that occurs as a result of the phosphatase activity inhibition by immunosuppressive drugs, such as tacrolimus (FK506) or cyclosporin A, could be due to impaired modulation of TRESK current. This would increase neuronal excitability to enhance pain sensitivity [55]. This hypothesis has never been confirmed by detailed studies, but an interesting report has given some insights. Using a model of pain hypersensitivity in bone cancer-bearing rats, the authors show that intrathecal injection of calcineurin enhances TRESK mRNA and protein expression, as well as TRESK-mediated current [41]. These effects are correlated with both, a decrease in the excitability of DRG sensory neurons and an amelioration of mechanical and thermal hypersensitivity. Similar effects are found when doing a pretreatment with a NFAT inhibitor peptide, as NFAT (nuclear factor of activated T cells) is involved in the regulation of TRESK expression downstream of calcineurin activation. In contrast, exogenous application of tacrolimus or a calcineurin siRNA to cultured or in vivo DRGs increases TRESK phosphorylation and produced opposite effects (increase) on excitability and pain sensitivity. Similarly, interfering peptides that inhibit TRESK dephosphorylation render comparable effects on excitability. This data indicates that calcineurin inhibition deactivates TRESK channels through the suppression of TRESK dephosphorylation but also reduces TRESK protein and mRNA abundance. The study supports the hypothesis proposed for CIPS, although other effects of calcineurin inhibition apart from the regulation of TRESK should also be taken into consideration.

3.3. TRESK and Migraine

TRESK was involved in migraine pathophysiology in a study aimed to identify mutated brain-expressed ion channel genes in migraine patients [20]. A frameshift mutation in the KCNK18 gene (F139WfsX24) was present in several members of a family suffering from familial migraine with aura, while was absent in other family relatives that did not suffer from migraine pain. This mutation produces a truncated and non-functional TRESK channel (from 384 to 162 aa) with dominant-negative effects that can suppress wild-type channel function. The truncated channel includes the intact cytosolic N terminus, the first transmembrane region, the first extracellular loop and an aberrant sequence of 24 aa at the C terminus [20,56]. Expression of this mutated channel in HEK293 cells confirmed the dominant-negative effects observed in *Xenopus* oocytes and highlighted that not only the TRESK current is diminished (heterodimerization of wild-type and mutant TRESK subunits) but also the plasma membrane levels of the channel. When expressed in trigeminal sensory neurons, the mutated channel produced an increase in membrane resistance, a lower current threshold for action potential firing and an enhanced excitability of trigeminal sensory neurons.

Surprisingly, other mutations that inactivate TRESK were not correlated with migraine [57,58]. A number of KCNK18 missense variants (R10G, A34V, C110R, S231P, A233V) were also identified in unrelated sporadic migraine and control cohorts [20], as well as in Italian migraine patients [59]. Some variants (R10G, S231P, and A233V) did not appear to significantly affect TRESK current, [57] but, unexpectedly, the C110R variant, despite producing a complete loss of TRESK function, did not show an association with the disease, as it was expressed both in control and sporadic migraine cohorts. A similar outcome was reported for the A34V variant, although this mutant can still form functional channels. Furthermore, the C110R variant expressed in trigeminal neurons did not produce a significant increase in excitability [58]. These studies suggested that the presence of a single non-functional variant of TRESK alone is probably not sufficient to develop migraine.

These inconsistencies have been recently resolved in a study reporting that the TRESK mutation F139WfsX24 produces a nonfunctional channel (MT1) and a second protein fragment (MT2) that co-assembles with and inhibits TREK1 and TREK2 channels [60]. First, the study shows that TRESK can heteromerize with TREK1 or TREK2 subunits to form heteromeric channels. Second and more important, this mutation but not others (e.g., C110R), produces a second protein fragment due to the generation of a new translation initiation site (a mechanism termed frameshift mutation-induced alternative translation initiation). This second protein MT2 exerts a dominant negative effect on heteromeric channels, thus achieving a combined inhibition of TRESK, TREK1 and TREK2 channels. An increase in neuronal excitability is observed when MT2 is expressed in trigeminal neurons but not when only MT1 (truncated TRESK channel) is expressed. Finally, viral transfection of MT2 into the rat trigeminal ganglion produces a migraine-like phenotype, where an increase in facial mechanical sensitivity is observed over several days [60]. Interestingly, the facial mechanical allodynia elicited by injection of MT2, cannot be further enhanced by ISDN (isosorbide dinitrate) injection, a well-known model of migraine-like mechanical hypersensitivity. Database mining for other TRESK mutations found another described variant (Y121LfsX44) that again produces a second protein by the same mechanism. This mutation was also shown to produce a second protein that can interact with TREK channels. Importantly, this mutant has been associated with a migraine phenotype in the ClinVar database (www.ncbi.nlm.nih.gov/clinvar/).

In a recent study, human nociceptors with the F139WfsX24 mutation were generated from induced pluripotent stem cells from migraine patients [61]. Non-peptidergic nociceptors containing the mutation showed an increased excitability compared to these obtained from healthy control subjects. Correction of the mutation by CRISPR-Cas9 technology returned TRESK expression levels and neuronal excitability to normal values comparable to controls. Finally, the authors showed the therapeutic possibilities of enhancing TRESK activity. Cloxyquin, a TRESK activator that will be discussed in the next chapter, was able to reduce spontaneous firing in iPSC-derived nociceptors carrying the F1449V gain-of-function mutation in Nav1.7, that causes erythromelalgia [61]. In addition, in a rodent

model of migraine generated by administration of nitroglycerine, cloxyquin treatment was able to reduce mechanical and thermal hypersensitivity, highlighting the potential of TRESK as an analgesic drug target. Recently, a new missense mutation (W101R) was identified in a male individual having intellectual disability and migraine with brainstem aura [62]. This mutation markedly reduces TRESK current amplitude, although the channel retains its K^+ selectivity and the modulation by calcineurin. Interestingly, and in addition to the known association to migraine, the mutation links the channel to intellectual disability pathogenesis, a finding that remains to be confirmed by future studies.

3.4. TRESK and Mechanosensation

From the observation of tingling and numbing sensations produced by Szechuan peppercorns, Bautista et al. identified the natural compound hydroxy- α -sanshool as the responsible for activation of sensory neurons producing these effects [63]. Sanshool activates sensory neurons by depolarizing the cellular membrane through inhibition of the K_{2P} background channels TRESK, TASK1, and TASK3. It was proposed that the tactile component of these sensations might result from the activation of large-diameter, touch-sensitive fibers, whereas the pungent or irritant qualities may involve excitation of non-peptidergic, capsaicin-sensitive small-diameter nociceptive fibers. These studies suggest that tingling paresthesia elicited by sanshool is mediated by activation of mechanosensitive somatosensory neurons through inhibition of K_{2P} channels, in particular TRESK channels. In turn, numbing reported by sanshool might be mediated by desensitization of activated neurons. In fact, using the skin-nerve preparation, it has been shown that sanshool activates all ultrasensitive D-hair fibers and, to a lesser extent, some populations of pressure-sensitive $A\beta$ fibers and low conduction velocity C fibers [64]. Later, it was described that sanshool also inhibits $Na_v1.3$, $Na_v1.7$, and $Na_v1.8$, and the subsequent blocking effect on action potential firing is thought to mediate its analgesic effects by silencing $A\delta$ mechanonociceptors [65]. A sanshool synthetic derivative, isobutylalkenyl amide (IBA) also induces tingling sensations in humans [66]. In rats, IBA inhibits TRESK current, activates nociceptive C-type fibers and induces nocifensive behaviors and mechanical allodynia [18,67]. Similar to sanshool or IBA, dermal exposure to pyrethroid insecticides causes paresthesias (tingling, itching, burning, and stinging sensations) due to their ability to activate sensory neurons. As the former compounds, pyrethroid insecticides inhibit TRESK (as well as other K_{2P} channels) and activate peripheral $A\beta$ -, $A\delta$ -, and C-fibers, increasing mechanical sensitivity when injected subcutaneously [68].

Another evidence of TRESK's involvement in the function of mechanosensory neurons was obtained by stimulation of cultured DRG neurons with radial stretch, which activates nociceptors and mechanoreceptors [69]. Using hydroxy- α -sanshool and different TRP activators, two populations of stretch-sensitive neurons were described: low threshold mechanoreceptors/proprioceptors and non-peptidergic nociceptors. Both these populations of stretch-sensitive neurons express TRESK channels [69]. It has been shown that, in contrast to mechanically-gated channels, direct mechanical stimulation of the cell membrane does not activate or inhibit TRESK [70]. Nevertheless, changes in membrane tension modulates channel activity [37]. TRESK current is enhanced by cell swelling and inhibited by cell shrinkage, indicating that changes in membrane curvature/shape/tension modify channel activity/opening. The use of membrane crenators or cup-former substances (e.g., volatile anesthetics), which preferentially insert into the external or internal leaflet of the bilayer, respectively, and change membrane tension, corroborate these findings. In fact, the potent activation of TRESK by volatile anesthetics, such as isoflurane or halothane, could be mediated by modulation of the channel through membrane lipids.

In summary, a relationship between TRESK and mechanical sensitivity has been well demonstrated. The increase in mechanical sensitivity observed in different studies (knocking out TRESK in mice; mutations that knockdown the channel; pharmacological TRESK inhibition) suggests that TRESK is involved in the transduction of mechanical stimuli, not as a direct sensor but by regulating the excitability of sensory neurons involved in mechanodetection. Whether modulation of TRESK by

membrane tension also contributes to the detection of mechanical stimuli in peripheral nerve endings remains to be studied in detail.

4. TRESK Modulation and Pharmacology: Therapeutics

Basic biophysical properties, as well as modulation of TRESK activity by different intracellular kinases and phosphatases or interaction with several proteins, has been nicely reviewed [2,71,72]. As mentioned before, several compounds inhibiting the channel have been described, including sanshool, IBA, pyrethroids, quinine, quinidine, arachidonic acid, amide local anesthetics (e.g., bupivacaine) or different ions (Ba^{2+} , Hg^{2+}). For therapeutic purposes, TRESK activating compounds (openers) have more interest due to their action diminishing neuronal activation and producing analgesic effects. Besides the activating effects of volatile anesthetics on TRESK and other $\text{K}_{2\text{P}}$ channels, only a few compounds have been described as TRESK activators. Flufenamic acid, BL-1249, and its synthetic derivatives are TRESK openers that seem to act through a stabilization of an open state of the channel [73]. Using a baculovirus TRESK FluxOR cell-based assay, Wright et al. identified cloxyquin (5-chloroquinolin-8-ol), an old drug used to treat intestinal infections due to its anti-amoebic, anti-bacterial and anti-fungal properties [74]. Cloxyquin analogues generated accordingly to the structure-activity relationship also behaved as channel activators. Interestingly, the drug seems to possess some selectivity as it did not affect other potassium channels as ROMK (Kir1.1) or hERG (human Ether-à-go-go-Related Gene, Kv11.1) [74]. In addition, it is selective for TRESK among other $\text{K}_{2\text{P}}$ s [75]. Cloxyquin activates TRESK in its resting state, by a mechanism independent of Ca^{2+} /calcineurin-mediated dephosphorylation. In fact, cloxyquin did not further increase TRESK after activation of the channel by receptor-mediated calcium increase or in mutant channels mimicking the dephosphorylated state [75]. When tested in DRG sensory neurons, cloxyquin also increased the background K^{+} current in a subset of neurons, indicating that it is able to activate native channels [68,75]. As mentioned earlier, cloxyquin reduced spontaneous firing in iPSC-derived nociceptors carrying the F1449V gain-of-function mutation in Nav1.7, but, more importantly, this drug reduced mechanical and thermal hypersensitivity in a mouse model of migraine by administration of nitroglycerine [61]. The identification of cloxyquin and its in vitro and in vivo effects reinforce the therapeutic potential of targeting TRESK for analgesic purposes and opens the door to find new and selective compounds to activate the channel for the treatment of migraine or other pain-related diseases.

In the search and development of TRESK channel openers or inhibitors, species differences need to be taken into account. Despite the similarities between the human and rodent (mouse or rat) channels, some pharmacological differences have been described. TRESK inhibition by local anesthetics (bupivacaine and lidocaine) has about 10-fold greater potency on rodent TRESK than on the human ortholog. In contrast, activation by volatile anesthetics is more prominent on the human channel [15,76]. Differences also exist on the sensitivity to other stimuli, such as pH or Zn^{2+} , being human TRESK almost insensitive but not mouse and rat orthologs. Interestingly, cloxyquin similarly activates the human and rodent channel, thus not showing significant pharmacological differences.

Some differences have also been described regarding the intracellular regulation of the channel. Human TRESK interact with anionic membrane phospholipids and is regulated by PIP_2 generated by activation of GPCRs, whilst, in rodent orthologs of the channel, this regulation is not present [77]. Significant differences are also present in other species (e.g., zebrafish), where TRESK channels are not regulated by Ca^{2+} /calcineurin [78]. These species differences must be taken into consideration when extrapolating pharmacological results from rodent or other experimental animal models to human physiology, as well as during drug development.

5. Conclusions

As described, several studies to date have highlighted the role of TRESK in regulating the excitability of specific subtypes of sensory neurons. Targeting the channel seems an interesting therapeutic approach to reduce nociceptor activation and decrease pain. Some recent studies have pointed out the involvement of the channel in the regulation of the sensitivity to specific types of

stimuli but not others. In addition, the involvement of the channel seems more prominent in certain alterations of the sensitivity (mechanical or cold allodynia) rather than other painful conditions (inflammatory pain). The contribution of the channel to specific sensory modalities and on the pathophysiology of different pain-related diseases remains to be fully established, but it seems plausible that enhancing the channel function could ameliorate mechanical or cold allodynia experimented by many patients suffering from diverse medical conditions (e.g., migraine) or pharmacological treatments (e.g., oxaliplatin). The fact that the channel expression is restricted to certain types of neurons, mainly sensory neurons, makes it an attractive target for a selective therapeutic intervention. In addition, strategies to increase or recover channel expression after injury could be beneficial for certain neuropathic painful conditions. Development of specific activators of the channel is desired to expand the current available analgesic agents.

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C. ALTRES ARTICLES PUBLICATS

Bibliografia

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The LRRC8-mediated volume-regulated anion channel is altered in glaucoma

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Regulation of cellular volume is an essential process to balance volume changes during cell proliferation and migration or when intracellular osmolality increases due to transepithelial transport. We previously characterized the key role of volume-regulated anion channels (VRAC) in the modulation of the volume of trabecular meshwork (TM) cells and, in turn, the aqueous humour (AH) outflow from the eye. The balance between the secretion and the drainage of AH determines the intraocular pressure (IOP) that is the major casual risk factor for glaucoma. Glaucoma is an ocular disease that causes irreversible blindness due to the degeneration of retinal ganglion cells. The recent identification of Leucine-Rich Repeat-Containing 8 (LRRC8A-E) proteins as the molecular components of VRAC opens the field to elucidate their function in the physiology of TM and glaucoma. Human TM cells derived from non-glaucomatous donors and from open-angle glaucoma patients were used to determine the expression and the functional activity of LRRC8-mediated channels. Expression levels of LRRC8A-E subunits were decreased in HTM glaucomatous cells compared to normotensive HTM cells. Consequently, the activity of VRAC currents and volume regulation of TM cells were significantly affected. Impaired cell volume regulation will likely contribute to altered aqueous outflow and intraocular pressure.

Glaucoma is a chronic disease in which retinal ganglion cell degeneration leads to an optic nerve damage that results in visual field loss. This group of optic neuropathies represent a significant cause of blindness worldwide¹. Although the precise molecular mechanisms leading to glaucoma are poorly understood it is known that intraocular pressure (IOP) is the main risk factor for glaucoma development. IOP is maintained through a balance between the amount of aqueous humour (AH) produced in the ciliary processes and the AH drainage. In humans, the main outflow route of AH outflow consists of the trabecular meshwork (TM) tissue and Schlemm's canal (SC). TM cells actively regulate the drainage of AH, thereby maintaining a physiological intraocular pressure (IOP)². Although the bases for AH outflow regulation are still unknown, different cellular mechanisms have been associated to the trabecular meshwork physiology including composition and remodelling of TM extracellular matrix³, contraction / relaxation³ and volume regulation of trabecular cells^{4–6}, among others. When functionality of TM is impaired, an increased resistance to the eye fluid results in ocular hypertension and glaucoma⁷.

Cell volume regulation is crucial for cell division, migration and death⁸. Swollen cells recover their initial volume by the transport of solutes (especially K^+ and Cl^-), organic osmolytes and water through the plasma membrane (PM); this cellular mechanism is known as regulatory volume decrease (RVD)⁹. TM cells possess a RVD^{5,6} mediated at least by the Na^+/H^+ antiporter⁵, the $Na^+-K^+-2Cl^-$ co-transporter^{5,10}, the large-conductance calcium activated potassium channel (BKCa) and the volume-regulated anion channel (VRAC)^{5,6}. Volume of trabecular cells influence aqueous outflow since compounds that induce TM cell swelling reduce outflow facility and compounds known

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Individual	Age (years)	Race	Gender	Number
1	51	Caucasian	Male	Normotensive
2	58	African American	Male	Normotensive
3	45	African American	Male	Normotensive
4	48	Caucasian	Female	Secondary open-angle glaucoma
5	70	Caucasian	Male	Primary open-angle glaucoma
6	78	African	Male	Primary open-angle glaucoma
7	78	Caucasian	Female	Primary open-angle glaucoma
8	67	Caucasian	Male	Primary open-angle glaucoma

Table 1. Summary of Individual Parameters.

to shrink trabecular cells increase it^{4–6,11}. We and others have described how BKCa and VRAC ion channels can modulate aqueous outflow facility as a consequence of regulating the volume of trabecular cells^{5,6,12}. Besides volume regulation, VRAC participates in cellular proliferation, migration, apoptosis and release of glutamate¹³. It is widely known that VRAC mediates the ubiquitous swelling-activated chloride current (ICl_{swell})⁹. The well-described electrophysiological properties of VRAC are outwardly rectification, inactivation at large depolarized potentials and iodide over chloride selectivity¹³ while its molecular identity has been highly controversial for decades¹⁴.

Leucine-Rich Repeat-Containing 8A (LRRC8A) has been identified in a genome-wide loss of function screening^{15,16} as a protein indispensable for the VRAC activity. Specific knockdown of LRRC8A dramatically reduces swelling-activated iodide influx, release of taurine and glutamate^{15–17} and the ability to modulate cell volume^{15,16}. LRRC8A was cloned from a patient with congenital agammaglobulinemia, a disease characterized by a deficiency of circulating B lymphocytes¹⁸. LRRC8A is the first member of protein family that contains five different members (LRRC8A–LRRC8E). The traffic of the LRRC8B–LRRC8E subunits to the cell surface depends on the co-expression with LRRC8A¹⁶. LRRC8 proteins contain a leucine-rich repeat domain at the C-terminus¹⁹ and it has been proposed to have four transmembrane segments²⁰ and a similar topology to pannexins²¹. Because LRRC8A overexpression causes an unexpected suppression of endogenous VRAC currents^{16,22}, it has been speculated that a very specific stoichiometry of LRRC8 subunits is required to form functional VRAC. In this sense, VRAC appear to require a heteromeric composition with at least one main subunit LRRC8A and at least another LRRC8 family member^{15,16}. Recent reports suggest that functional channels may operate as hexamers^{21,23,24} containing at least three different LRRC8s²⁵. Notably, different combinations of LRRC8B–E plus LRRC8A yield VRAC currents with different inactivation kinetics, rectification and single-channel conductance²². As pointed by mutations in the essential subunit LRRC8A²², the composition of VRAC determines the channel permeability profile. In addition to transport neurotransmitters and neuromodulators²⁵, LRRC8A and LRRC8D subunits have been involved in anticancer drug uptake and LRRC8D is associated to blasticidin transport²⁶. In this context, and because VRAC is implicated in aqueous outflow^{5,6}, the recent attribution of this activity to LRRC8 proteins paves the way to determine their potential role in TM physiology and glaucoma.

Methods

Human trabecular meshwork cell lines. Human transformed TM cells from a non-glaucomatous donor (HTM-5) and from an individual affected by primary open-angle glaucoma (POAG) (HTM-3) were kindly obtained from Abbot Clark (University of North Texas - Health Science Center, TX, USA)²⁷. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 mg/mL L-glutamine, 100 U.I./mL penicillin and 100 µg/mL streptomycin. Cell passages were performed using Trypsin-EDTA. Reagents were obtained from Sigma-Aldrich. As an *in vitro* model of steroid-induced glaucoma, dexamethasone (DEX; 100 nM in DMEM) or vehicle (0.1% ethanol) was added to control HTM-5 cells every other day during 3 days or 7 days. At designated time points, cells were collected and analysed for mRNA (LRRC8A–LRRC8E and myocilin (MYOC)) and protein (LRRC8A and MYOC).

Human trabecular meshwork primary cells. Trabecular meshwork (TM) cultured cells from three normotensive non-glaucomatous human donors were kindly provided by D.W. Stamer (Duke University Eye Center, Durham, NC, USA)^{28,29} and T. Borrás (University of North Carolina, Chapel Hill, NC, USA). Cell cultures were maintained in low glucose DMEM supplemented with 10% FBS, 100 mg/mL L-glutamine, 100 U.I./mL penicillin and 100 µg/mL streptomycin in a humidified 5% CO₂ atmosphere at 37 °C. After confluence, TM cells were passaged using Trypsin-EDTA. Glaucomatous HTM cells from five patients were obtained using the explant method from trabeculectomies performed by Dr. M. I. Canut (Institut Universitari Barraquer, Barcelona, Spain) as described previously³⁰ (Table 1). Briefly, a small piece of TM was removed by a punch trabeculectomy and was kept in PBS at 4 °C for not more than 24 h. Human samples were handled in accordance with the principles expressed in the Declaration of Helsinki. TM tissue was carefully attached to the bottom of a 35-mm dish and covered with a drop of DMEM supplemented with 20% FBS, 100 mg/mL L-glutamine, 100 U.I./mL penicillin and 100 µg/mL streptomycin under a glass coverslip. Dishes were incubated in a humidified 5% CO₂ atmosphere at 37 °C and medium was changed every other day. Cells were allowed to grow from the explant for a period of 4 weeks. Once confluent, TM cells were passed to a T-25 flask, labelled as passage 1, and were maintained in the same medium with 10% FBS. Cells were used at passages 3 to 7 and changed into media containing 1% FBS for one week prior to experimentation. All reagents were obtained from Sigma (Madrid, Spain).

Gene		PCR primer sequence		Annealing (°C)	Product size (bp)
LRR8A	Forward	5'-GGGTTGAACCATGATTCCGGTGAC-3'		54	133
	Reverse	5'-GAAGACGGCAATCATCAGCATGAC-3'			
LRR8B	Forward	5'-ACCTGGATGGCCACAGTAATAG-3'		56	126
	Reverse	5'-ATGCTGGTCAACTGGAACCTCTGC-3'			
LRR8C	Forward	5'-ACAAGCCATGAGCAGCGAC-3'		52	132
	Reverse	5'-GGAATCATGTTTCTCCGGGC-3'			
LRR8D	Forward	5'-ATGGAGGAGTGAAGTCTCCTGTCG-3'		54	126
	Reverse	5'-CTTCCGCAAGGGTAAACATTCCTG-3'			
LRR8E	Forward	5'-ACCGTGGCCATGCTCATGATTG-3'		54	62
	Reverse	5'-ATCTTGTCTGTGTACCTGGAG-3'			
ELAM-1	Forward	5'-CCCATGGAACACAACCTGTACATT-3'		54	118
	Reverse	5'-AGCTTTACACGTTGGCTTCTCGTT-3'			
MGP	Forward	5'-CGCCTTAGCGGTAGTAACCTTGT-3'		48	371
	Reverse	5'-TTTCTTCCCTCAGTCTCATTG-3'			
MYOC	Forward	5'-AGCACGGGTGCTGTGGTGATC-3'		49	338
	Reverse	5'-AAGGTGCCACAGATGATGAA-3'			
KCNMA1	Forward	5'-TACTTCAATGACAATATCCTCACCT-3'		50	210
	Reverse	5'-TTAGGGGATGGTGGTTGTTATGGT-3'			
NKCC1	Forward	5'-CAAGACATACCGGCAGATCAG-3'		50	109
	Reverse	5'-CGAAAAGGTGCTGTGTCTAGT-3'			
18S	Forward	5'-GGCGCCCCCTCGATGCTCTTAG-3'		60	Internal reference for RT-PCR
	Reverse	5'-GCTCGGCCCTGCTTTGAACACTCT-3'			

Table 2. Primer sequences, annealing conditions and amplicon lengths for each amplified gene.

Patch clamp procedures. Patch clamp experiments were performed at 22–23 °C using the whole-cell configuration without any leak subtraction. Electrodes were fabricated in a micropipette puller P-97 (Sutter instruments) with a resistance of around 3 MΩ. A Ag/AgCl ground electrode mounted in a 3 M KCl agar bridge was employed. The setup consists of an inverted microscope (Axiovert 35 M Zeiss), an amplifier (Axopatch 200B, Molecular Devices) and the pClamp 10 software (Molecular Devices). Data analysis was performed with Clampfit 10 (Molecular Devices) and Prism 4 (GraphPad Software, Inc.). Series resistance was kept at <15 MΩ and compensated

at 70–80%. Tamoxifen was purchased from Sigma-Aldrich (Madrid). Swelling-activated Cl[−] currents were recorded by depolarizing pulses from −100 mV to +100 mV in 20 mV voltage steps applied every 5 seconds from a 0 mV holding potential, using an intracellular pipette solution containing (in mM): 150 NMDGCl, 1.2 MgCl₂, 1.0 EGTA, 2.0 ATP, 0.5 GTP, 10 Hepes; pH 7.35 adjusted with tris-base (303 mOsm/Kg). The isotonic bath solution contained (in mM): 150 NMDGCl, 0.5 MgCl₂, 1.3 CaCl₂, 10 Hepes and 20 D-Sorbitol; pH 7.35 adjusted with tris-base (301.5 ± 3 mOsm/Kg). The hyposmotic solution contained (in mM): 100 NMDGCl, 0.5 MgCl₂, 1.3 CaCl₂, 10 Hepes; pH 7.35 adjusted with tris-base (211 ± 3 mOsm/Kg, −30%). To evoke whole-cell outward K⁺ currents, cells were clamped at −60 mV and depolarizing pulses from −100 to +90 mV were applied in 10-mV steps. For clarity only pulses from 0 to +40 mV are shown. The pipette solution was (in mM): 140 KCl, 2.1 CaCl₂, 2.5 MgCl₂, 5 EGTA, and 10 HEPES, pH 7.3 with 0.5 M KOH. The bath solution was (in mM): 145 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES and 5 glucose, pH 7.4 with NaOH. Osmolalities were measured using a Vapro osmometer (Wescor) and adjusted with sorbitol.

RNA extraction and reverse transcription reaction. RNA extraction was conducted with the RNeasy® Mini Kit from QIAGEN (Valencia). Reverse transcription reactions were carried out with spectrophotometrically measured RNA (5 µg; Nanodrop, ThermoFisher Scientific) using the Cloned AMV First-Strand mRNA Synthesis Kit (Invitrogen, ThermoFisher Scientific) according to the manufacturer's recommendations³¹.

Gene primers. Primers sets for LRR8A-LRR8E subunits designed by T. Jentsch and collaborators were used¹⁶. Primers for glaucoma-related genes (MYOC, ELAM-1, MGP), Na⁺-K⁺-2Cl[−] cotransporter (NKCC1), BKCa channel (KCNMA1) as well as the ribosomal 18 S RNA were designed using the Primer Designing Tool of the National Center for Biotechnology Information (NCBI) and purchased from Invitrogen (ThermoFisher Scientific) (Table 2). Target specificity was obtained by performing Basic Local Alignment Search Tool (BLAST) comparisons against the entire nucleotide database (<http://www.ncbi.nlm.nih.gov>).

Standard PCR reaction and sequencing. PCRs were carried out in a DNA thermal cycler (Eppendorf) to verify the size of amplified products. The protocol was 95 °C for 30 seconds and cycles of 95 °C for 30 seconds, variable annealing temperature for 60 seconds, 68 °C for 60 seconds, and a final extension at 68 °C for 5 min. Amplification of ELAM-1, MGP and MYOC genes was verified by sequencing (Stab Vida) using the <http://www.ncbi.nlm.nih.gov> database.

Relative quantitative real-time PCR. Normalized mRNA quantification was performed by relative quantitative real-time PCR technology with Fast SYBRTM Green Master Mix using an ABI Prism[®] 7500 Fast Real-Time PCR System and the 7500 System SDS software (Applied Biosystems, ThermoFisher Scientific). Fold Changes were calculated by the formula $2^{-\Delta\Delta CT}$ ³¹. Melting curves were carried out to determine melting temperatures in order to check the specificity of the amplified products. All experiments were performed by triplicate.

Perfused human anterior segment organ cultures. Non-glaucomatous human eyes were obtained from national eye banks following signed consent of the patients' families. All procedures were in accordance with the Tenets of the Declaration of Helsinki. After dissection, pairs of ocular anterior segments were perfused at a constant flow and pressure (P) was monitored as described previously³¹. After a stable baseline of 24 h, a ΔP of 31.96 ± 3.25 mmHg was applied to one eye and the contralateral eye was maintained at physiological pressure (≈ 15 mmHg). After 7 d, anterior segments were immersed in RNAlater (Ambion) to accomplish dissection of TM tissue and RNA extraction³¹.

RNA microarrays. RNAs from perfused anterior segments were hybridized to Human Genome U95Av2 or U133Plus2.0 GeneChips (Affymetrix, ThermoFisher Scientific) at the University of North Carolina at Chapel Hill Functional Genomics Core Facility. Affymetrix GeneChip Microarray Suite 5.0 (Affymetrix, ThermoFisher Scientific) and 7.3.1 GeneSpring GX Expression Analysis software (Agilent Technologies) were used to analyse differential gene expression between high P and control P TM tissues³¹.

Total protein extraction and western blotting. Cultured HTM cells were lysed with a standard 1% Triton X-100 buffer supplemented with 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin and 1 mM phenylmethylsulfonyl fluoride (PMSF) as protease inhibitors. PierceTM BCA Protein Assay Kit (Thermo Scientific) was used to quantify total protein in order to analyse the same amount of HTM-5/HTM-3 paired extracts by western blot (WB). PVDF membranes were incubated with LRRC8A polyclonal antibody (1:1000, Bethyl Laboratories), β -actin polyclonal antibody (1:5000, Sigma-Aldrich) or MYOC monoclonal antibody (1:1000, Santa Cruz Biotechnology) and secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit (1:10000) or goat anti-mouse (1:2000) antibodies (Jackson ImmunoResearch Laboratories). Immunoreactive bands were captured using the WesternBrightTM Quantum detection kit for HRP (Advanta) and visualized by chemiluminescence on Carestream[®] Kodak BioMax MR films (Sigma-Aldrich) or with a LAS-3000 Mini gel Imaging System (Fujifilm). All experiments were done by triplicate.

Biotinylation of cell surface proteins. HTM cell-surface biotinylation was carried out using the EZ-Link[®] Sulfo-NHS-LC-Biotin (sulfosuccinimidyl-6-[biotin-amido]hexanoate) reagent (Thermo Scientific). *N*-Hydroxysuccinimide (NHS) esters of biotin react with primary amines of lysine aminoacidic residues exposed extracellularly. The cross-linking reaction was quenched with 0.1 M Glycine. Cells were scrapped with 1% Triton X-100 buffer with the same cocktail of protease inhibitors than for total protein extraction. When protein content was extracted from the supernatant, a fraction of 10% (in volume) was stored at -20°C as starting materials. To isolate the labelled proteins, cell lysates were purified on Neutravidin[®] Agarose Resins (Thermo Scientific) and eluted with 1X Laemmli SDS loading buffer. Starting materials and biotinylated fractions were analysed by western blotting to quantify the LRRC8A levels. Experiments were performed by triplicate.

Measurements of cell volume with calcein fluorescence. Fluorescence intensity of intracellular calcein was used to quantify changes in cell volume³². HTM cells were loaded with 0.5 μ M calcein-AM (Calbiochem, Merck KGaA) at 37°C for 2 min. After acute harvesting, coverslips were mounted on an inverted, epi-fluorescence microscope Olympus IX70 (Olympus). Using a xenon lamp and a monochromator Polychrome IV (Till Photonics), calcein was excited every 20 sec at 488 nm and light emitted at 520 nm was detected with a digital charge-coupled device CCD camera and the AquaCosmos 2.5 Software (Hamamatsu). Isotonic solution contained (in mM): 125 NaCl, 5.5 KCl, 1.3 CaCl_2 , 1.25 MgCl_2 , 20 Hepes, 10 dextrose, 20 BDM; pH 7.4 adjusted with NaOH (292 mOsm/Kg). Hypotonic solution was (in mM): 80 NaCl, 5.5 KCl, 1.3 CaCl_2 , 1.25 MgCl_2 , 20 Hepes, 10 dextrose, 20 BDM; pH 7.4 adjusted with NaOH (213 mOsm/Kg, 27%). Osmotic behaviour of each cell was analysed by applying 14% hypotonic (251 mOsm/Kg) / hypertonic (333 mOsm/Kg) before experiments. Only cells with an osmotic behaviour were analysed to determine the RVD mechanism¹².

Data analysis. Results are presented as mean $\pm$ SEM. Data were analysed with paired or unpaired Student's *t*-tests. Entire I-V curves of different experimental conditions were analysed with two-way analysis of variance (ANOVA) plus Bonferroni post-tests. Prism 4.0 software was used (GraphPad Software). $p < 0.05$ was considered statistically significant.

Results

Validation of both glaucomatous (HTM-3) and control (HTM-5) trabecular meshwork cells at a molecular level.

Trabecular meshwork cell lines from a non-glaucomatous donor (HTM-5) and from a primary open-angle glaucoma patient (POAG) (HTM-3) were used²⁷. Prior to undertaking experiments, cell types were validated by quantifying Myocilin (MYOC) mRNA/protein in control HTM-5 cells treated with Dexamethasone (DEX) and the expression levels of glaucoma-related genes in glaucomatous HTM-3 cells. Glucocorticoids induce ocular hypertension³³ and many studies have shown that induction of MYOC by the glucocorticoid DEX is specific to the TM tissue. HTM-5 cells were treated either with DEX (100 nM in 0.1% ethanol) or vehicle (0.1% ethanol) for 3 or 7 days. MYOC showed low expression in vehicle-treated HTM-5 cells but its expression was significantly increased after 3 or 7 days of DEX exposure (7.69 ± 2.15 -fold and 9.92 ± 2.68 -fold,

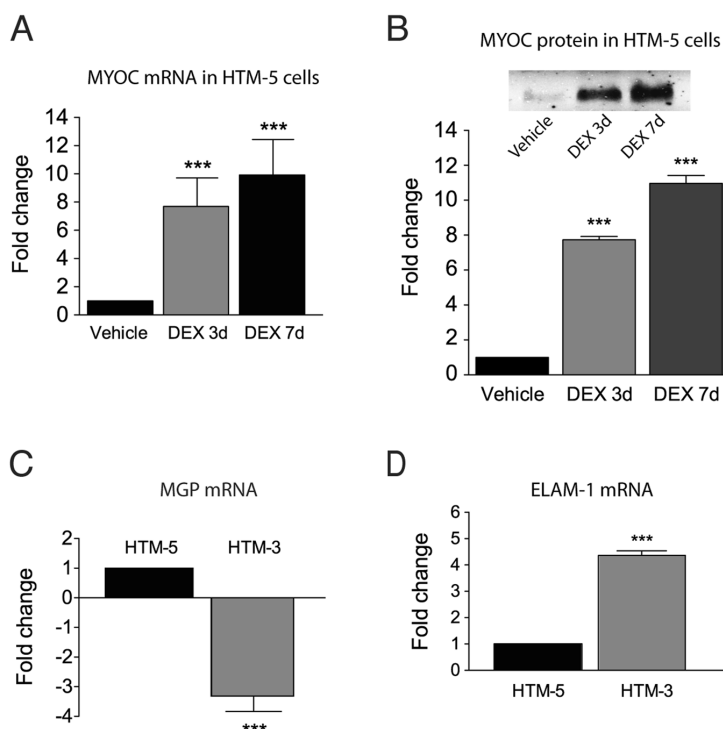


Figure 1. Validation of HTM-5 and HTM-3 cell lines. **(A)** Induction of Myocilin (MYOC) mRNA in HTM-5 cells treated with dexamethasone (DEX, 100 nM) at two different time points (3 days and 7 days) compared to HTM-5 cells treated with vehicle (0.1% ethanol). Values are the mean $\pm$ SEM of $n = 8$ cells (** $p < 0.001$ DEX-treated cells vs. vehicle, Student's t -test). **(B)** MYOC protein quantified in HTM-5 extracts treated with vehicle, DEX 3 days and DEX 7 days. MYOC was significantly increased by DEX. Values are mean $\pm$ SEM of $n = 8$ cells (** $p < 0.001$ DEX-treated cells vs vehicle, Student's t -test). *Inset*: Representative western blot showing MYOC in HTM-5 cells. A full-length blot is presented in Supplementary Fig. 1B. **(C)** mRNA expression of Matrix Gla Protein (MGP) and **(D)** Endothelial-Leukocyte Adhesion Molecule-1 (ELAM-1) in glaucomatous HTM-3 cells normalized to HTM-5 control cells. MGP was significantly down-regulated and ELAM-1 was significantly up-regulated in HTM-3 compared to HTM-5, as it has been described in glaucoma. Values are mean $\pm$ SEM of $n = 3$ cells (** $p < 0.001$ HTM-3 versus HTM-5, Student's t -test).

respectively, $n = 3$, $p < 0.001$) (Fig. 1A). Similarly, DEX increased MYOC protein by 7.74 ± 0.19 -fold (3 days) and 10.97 ± 0.45 -fold (7 days) in comparison to vehicle-treated cells ($n = 3$, $p < 0.001$) (Fig. 1B). To assess whether specific alterations described in glaucoma were present in the HTM-3 cells, we evaluated the expression of Matrix Gla Protein (MGP) and Endothelial-Leukocyte Adhesion Molecule 1 (ELAM-1). Glaucomatous cells present a lower expression of MGP, a gene encoding a protein that acts as an inhibitor of vascular mineralization^{30,34}. Accordingly, we found a decreased expression of MGP in HTM-3 compared to HTM-5 cells (-3.32 ± 0.52 -fold; $n = 3$, $p < 0.001$; Fig. 1C). ELAM-1, which encodes a secreted glycoprotein involved in cell adhesion, is specifically induced in glaucoma TM cells and has been considered the first molecular marker of this ocular disease³⁵. In this sense, ELAM-1 mRNA was significantly overexpressed in HTM-3 compared to HTM-5 cells (4.36 ± 0.18 -fold; $n = 3$, $p < 0.001$; Fig. 1D), further indicating that HTM-3 cells present characteristic changes found in glaucoma. Standard PCR was performed to verify the size of all amplified products and amplification of ELAM-1, MGP and MYOC glaucoma-related genes was checked by sequencing (data not shown).

Current density and functional activation of volume-regulated anion channels (VRAC) are altered in glaucomatous HTM-3 cells. We next studied whether VRAC activity was affected in glaucomatous TM cells compared to the normotensive ones. As shown in Fig. 2A–C, in control HTM-5 cells the current is inactive in isotonic conditions (3.13 ± 1.96 pA/pF at $+100$ mV) and rapidly activates after 4 min of hypotonicity, reaching a maximum at 10 min (22.61 ± 5.85 pA/pF) ($n = 5$, $p < 0.001$). As expected, VRAC presented a fast activation and then a slow time-dependent inactivation at highly depolarized voltages (Fig. 2A). Current-voltage (I - V) curves exhibited a typical outward rectification and a time-dependent decay ($+60$ to $+100$ mV) (Fig. 2B). Moreover, activation of VRAC was reversible when returning to isotonic conditions showing no significant

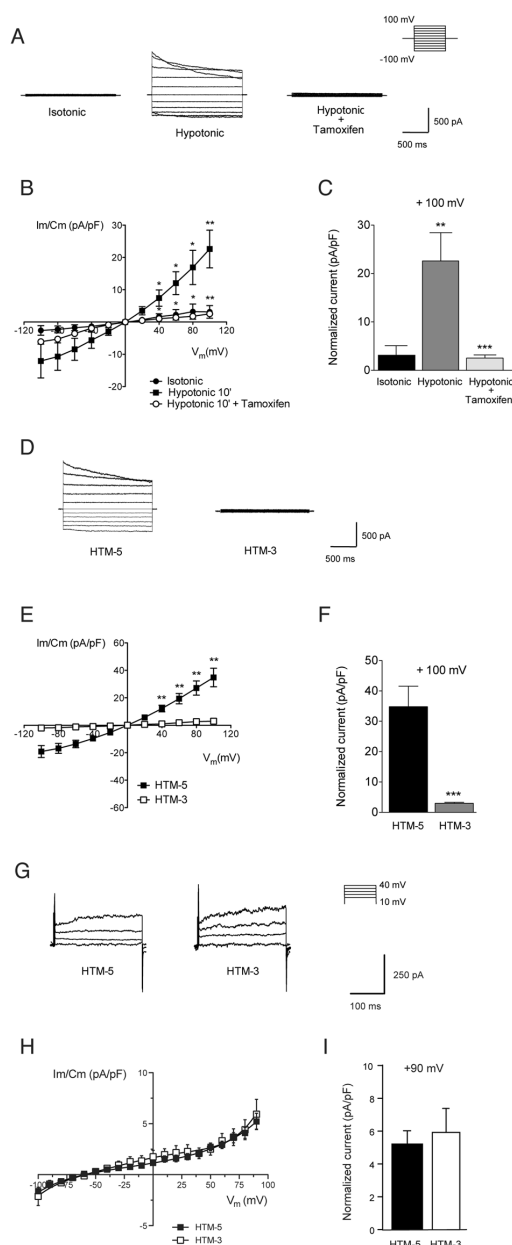


Figure 2. Activation of VRAC and BKCa currents in HTM cell lines. **(A)** Representative VRAC currents recorded in isotonic extracellular medium, and at 10 min in hypotonic extracellular medium (30%) before and after the addition of tamoxifen (100 μ M). **(B)** Current-voltage (I-V) curves showing VRAC currents in HTM-5 cells in (●) isotonic conditions, (■) hypotonic conditions, and (○) hypotonic conditions + tamoxifen. Values are the mean $\pm$ SEM of $n = 5$ independent cells. Current density is determined normalizing currents against cell capacitance (pA/pF). VRAC currents increased significantly after 10 min of hypotonicity ($p < 0.001$). $^{**}p < 0.01$, $^{*}p < 0.05$ isotonic vs. hypotonic, ANOVA *plus* Bonferroni post-tests. Tamoxifen blocked most of VRAC currents activated under hypotonic conditions ($p < 0.001$). $^{**}p < 0.01$, $^{*}p < 0.05$ hypotonic versus hypotonic + tamoxifen, ANOVA *plus* Bonferroni post-tests. **(C)** Peak currents measured at +100 mV in HTM-5 cells at each experimental condition ($^{**}p < 0.01$ isotonic vs. hypotonic, $^{***}p < 0.001$ hypotonic vs. hypotonic + tamoxifen, Student's *t*-test). **(D)** Representative experiment showing VRAC currents under hypotonic conditions in control HTM-5 cells and glaucomatous HTM-3 cells (recorded as described in A). **(E)**

Normalized I-V relationship activated in (■) HTM-5 and (□) HTM-3 at 10 min in hypotonic conditions. Values are mean $\pm$ SEM of $n = 10$ cells (HTM-5) and $n = 7$ cells (HTM-3). VRAC currents were significantly lower in HTM-3 compared to HTM-5 cells ($p < 0.001$). ** $p < 0.01$ HTM-3 vs. HTM-5, ANOVA plus Bonferroni post-tests. (F) Peak currents measured at +100 mV under hypotonicity (** $p < 0.001$ HTM-3 vs. HTM-5, Student's t-test). (G) Representative outward K^+ currents mediated by the high conductance Ca^{2+} -activated K^+ (BKCa) channel in control HTM-5 and glaucomatous HTM-3 cells. (H) Current-voltage curves for BKCa in (■) non-glaucomatous and (□) glaucomatous HTM cells. Results are the mean $\pm$ SEM ($n = 11$ HTM-5, $n = 10$ HTM-3). No significant differences were found in current densities between cell lines (Two-way ANOVA and Bonferroni post-tests). (I) Peak currents measured at +90 mV in control HTM-5 and glaucomatous HTM-3 cells (glaucoma vs. non-glaucoma, Student's t-test).

differences to those recorded in isotonic initial medium ($n = 5$, not shown). When the VRAC blocker tamoxifen was added to hypotonic medium, VRAC currents were significantly reduced to 2.54 ± 0.65 pA/pF ($p < 0.0001$; Fig. 2C) and outward and inward currents were blocked in a voltage-independent manner along the entire I-V curve (hypotonic vs hypotonic + tamoxifen, $p < 0.05$, $p < 0.01$). In HTM-5 cells, VRAC inactivated slowly ($\tau = 3821.03 \pm 262.31$ ms) at depolarized voltages (+100 mV) and showed a significant outward rectification (0.52 ± 0.02 , $n = 10$) measured as the ratio between inward/outward currents at -100 mV/+100 mV. In contrast, VRAC activity was significantly diminished in HTM-3 cells: while the current was absent in isotonic conditions (1.50 ± 0.36 pA/pF) (not shown), hypotonic medium only produced a small increase (2.62 ± 0.37 pA/pF; $n = 7$) compared to the effect found in HTM-5 cells (34.82 ± 6.75 pA/pF; $n = 10$) at 100 mV ($p < 0.0001$; Fig. 2D–F). In addition to the reduced current density, HTM-3 cells also showed a delayed activation of VRAC currents compared to those activated in HTM-5 cells at equivalent voltages. To assess whether other ionic conductances were also altered by glaucoma, we recorded total outward K^+ current in the same cell types. TM cells present a high-conductance Ca^{2+} -activated K^+ (BKCa) channel as previously characterized which contributes to cell volume regulation⁴⁶. All control HTM-5 and glaucomatous HTM-3 cells recorded displayed outward K^+ currents under isotonic conditions (Fig. 2G,H) and, unlike VRAC, K^+ currents were not significantly altered between control and glaucomatous HTM cells (HTM-5: 5.22 ± 0.80 pA/pF; HTM-3: 5.93 ± 1.46 pA/pF at +90 mV, Fig. 2I). These results suggest that glaucomatous cells showed a specific alteration of VRAC, while other currents involved in volume regulation are not equally altered.

Functional activity of the VRAC is diminished in primary glaucomatous HTM cells. To further establish a correlation with glaucoma progression and to corroborate the findings obtained in HTM cell lines, we examined the activation of VRAC currents in primary TM cells from five different patients with open-angle glaucoma and three non-glaucomatous human donors. Again, whole-cell currents were recorded in isotonic solution that was replaced by hypotonic medium to activate VRAC (Fig. 3A–C). Under isotonic conditions, non-glaucomatous cells showed small currents of 0.85 ± 0.12 pA/pF at +80 mV (not shown) but after 5 min in hypotonic medium, a significant activation of VRAC was observed (34.02 ± 3.10 pA/pF at +80 mV, Fig. 3B) ($p < 0.001$ hypotonic vs. isotonic, $n = 15$). Currents increased further between 5 and 10 min and decreased significantly when returning to isotonic solution (not shown). In glaucomatous cells 33 of 39 exhibited VRAC currents in hypotonic conditions, while no activation was found in the other 6 cells, in contrast to control cells that all showed activation of VRAC. Additionally, VRAC activation was smaller compared to control cells being 1.12 ± 0.14 pA/pF in isotonicity and 11.88 ± 1.10 pA/pF in hypotonicity at +80 mV (Fig. 3B) ($p < 0.001$ hypotonic vs. isotonic, $n = 33$). As seen in transformed cell lines, native glaucoma HTM cells showed a delayed activation of VRAC in comparison to control cells at equivalent voltages, in addition to the significant lower current densities. I/V curves showed significant differences between the VRAC current amplitude in open-angle glaucoma and normotensive control cells for the whole positive voltage range ($p < 0.001$, Fig. 3B). Current density at +80 mV was also represented as a scatter plot for each individual tested ($p < 0.001$, Fig. 3C).

Molecular expression of LRRC8A-LRRC8E subunits and the main LRRC8A protein that form VRAC are decreased in glaucomatous HTM-3 cells. To determine whether the decrease in VRAC currents in glaucomatous cells was due to a decreased expression of the mRNA expression of LRRC8 genes, we quantified their relative abundance by qPCR. All LRRC8s were found expressed in HTM-5 cells, being LRRC8A the most expressed one. For comparison purposes, the levels of LRRC8A mRNA were used to normalize the expression of the other subunits. Expression levels of the other subunits, expressed as percentage of LRRC8A (100%) were $18.13 \pm 2.29\%$ for LRRC8B, $16.05 \pm 2.01\%$ for LRRC8C, $56.24 \pm 9.50\%$ for LRRC8D and $6.57 \pm 0.85\%$ for LRRC8E ($n = 4$, Fig. 4A). When the expression profile of LRRC8A-LRRC8E was determined in glaucomatous HTM-3 cells, a significant decrease in all subunits was found compared to HTM-5 cells (in fold change): -3.02 ± 0.52 in LRRC8A, -69.83 ± 1.54 in LRRC8B, -5.34 ± 1.04 in LRRC8C, -76.42 ± 12.68 in LRRC8D and -28.67 ± 3.48 in LRRC8E ($n = 4$, Fig. 4B). Standard PCR was done to verify the size of amplified products (not shown). We also determined the mRNA expression of the BKCa channel and the $Na^+-K^+-2Cl^-$ cotransporter, two of the main proteins involved in the TM cell volume regulation together with VRAC. According to the channel activity, the expression of KCNMA1 (coding for the α -1 subunit of the BKCa) was not significantly altered in glaucomatous HTM-3 cells ($8.7 \pm 1.3\%$ from the expression in HTM-5). In contrast, NKCC1 (coding for the $Na^+-K^+-2Cl^-$) was significantly down-regulated in HTM-3 cells ($-30.2 \pm 0.9\%$ from HTM-5, Fig. 4C), similarly to what has been previously reported³⁷.

At the protein level, LRRC8A was also decreased by $52.91 \pm 2.34\%$ in HTM-3 compared to HTM-5 cells ($n = 7$ protein extractions, $p < 0.001$; Fig. 5A). Despite the decreased level of LRRC8A mRNA and total protein, it could

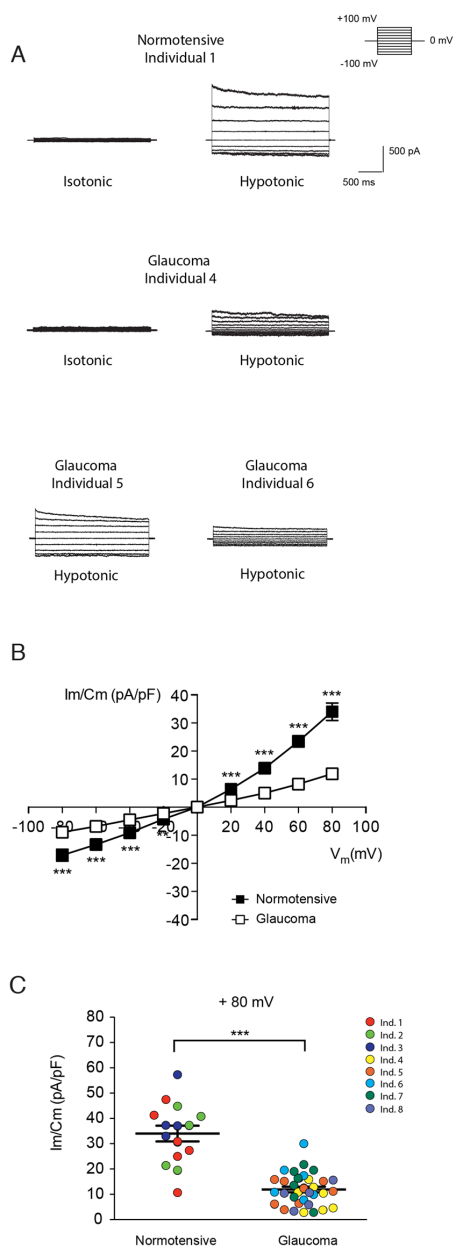


Figure 3. Activation of VRAC currents in primary HTM cells. **(A)** Representative whole-cell currents activated in primary non-glaucomatous (Ind. 1) and glaucomatous HTM cells (Ind. 4, 5 and 6) by depolarizing pulses, from -80 mV to $+80$ mV in 20 mV steps applied every 5 seconds from a 0 mV holding potential. VRAC currents were recorded in isotonic medium, and after 5 min in hypotonic medium ($\sim 30\%$) (For Ind. 5 and 6 only hypotonic conditions are shown). **(B)** Current-voltage (I-V) curves for VRAC in (■) non-glaucomatous and (□) glaucomatous HTM cells under hypotonic conditions for 5 min. Values are shown as the mean $\pm$ SEM of non-glaucomatous ($n = 15$) and glaucomatous ($n = 33$) primary HTM cells, from three and five individuals, respectively. The hypotonic-mediated increase in VRAC current was significantly smaller in glaucoma compared to control primary HTM cells ($p < 0.001$). $***p < 0.001$ glaucoma vs. non-glaucoma, ANOVA plus Bonferroni post-tests. **(C)** Peak currents measured at $+80$ mV in control and glaucoma primary HTM cells in hypotonic conditions ($***p < 0.001$ glaucoma vs. non-glaucoma, Student's t-test) represented as a dispersion (the colours indicate the eight individuals studied, Table 1).

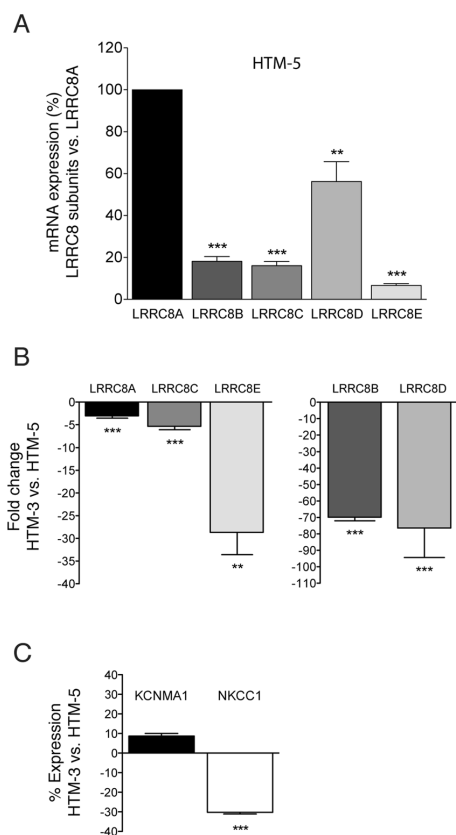


Figure 4. Expression of LRRC8A-LRRC8E, KCNMA1 and NKCC1 genes in HTM cells. **(A)** Relative abundance of LRRC8 mRNAs in HTM-5 cells. Results are shown as the percentage of expression of LRRC8B-LRRC8E compared to the expression of the main subunit LRRC8A normalized against 18S RNA. Values are mean $\pm$ SEM of 4 sets of independent experiments performed by triplicate. LRRC8 were expressed in HTM-5, being LRRC8A the most abundant subunit (** $p < 0.01$, *** $p < 0.001$, Student's t-tests). **(B)** LRRC8A-E expression was significantly down-regulated in glaucomatous HTM-3 cells vs. control HTM-5 cells (** $p < 0.01$, *** $p < 0.001$, Student's t-tests). Results are expressed as fold change normalized against 18S RNA. Values are mean $\pm$ SEM of 4 sets of independent experiments done by triplicate. **(C)** Gene expression of KCNMA1 (BKCa) and NKCC1 ($\text{Na}^+\text{-K}^+\text{-2Cl}^-$) in HTM-3 compared to HTM-5 cells determined by relative quantitative real-time PCR. KCNMA1 expression was not significantly altered in glaucomatous HTM-3 cells (Student's t-tests) while the expression of NKCC1 was significantly down-regulated in HTM-3 versus HTM-5 (*** $p < 0.001$, Student's t-tests). Results are expressed as fold change normalized against 18S RNA. Values are mean $\pm$ SEM of $n = 3$ (KCNMA1) and $n = 4$ (NKCC1) independent experiments done by triplicate.

still be possible that the amount of protein reaching the PM would be sufficient to produce large VRAC currents similar to those activated in normotensive cells. Hence, we quantified the protein levels of LRRC8A at the PM by biotinylation of cell-surface proteins. These studies revealed that while the proportion of LRRC8A on the PM in HTM-5 cells was $67.83 \pm 2.08\%$ of the total protein, this proportion falls to $34.36 \pm 2.25\%$ in HTM-3 cells ($n = 5$ independent biotinylation experiments, $p < 0.001$; Fig. 5B). Interestingly, LRRC8A protein was significantly diminished by $32.65 \pm 1.61\%$ in primary HTM cells derived from patients with open-angle glaucoma (Ind. 5–8) compared to native HTM cells from normotensive donors (Ind. 1–3) ($p < 0.001$; Fig. 5C). Altogether our data suggest that the decreased VRAC currents in glaucomatous cells is due to a decrease in LRRC8 mRNA expression combined with a reduction in the total and surface LRRC8A protein.

Cell volume regulation is impaired in glaucomatous HTM-3 cells. We measured the Regulatory Volume Decrease (RVD) activated in HTM cells upon hypotonic shock by means of calcein fluorescence. After 5 min in isotonic medium to establish the initial baseline, HTM cells were exposed to hypotonic medium (27%) to swell the cells and trigger a subsequent RVD. As predicted by the low VRAC currents, the RVD was significantly impaired in glaucomatous cells compared to that activated in control cells (Fig. 6A). Hence, HTM-5 exhibited an

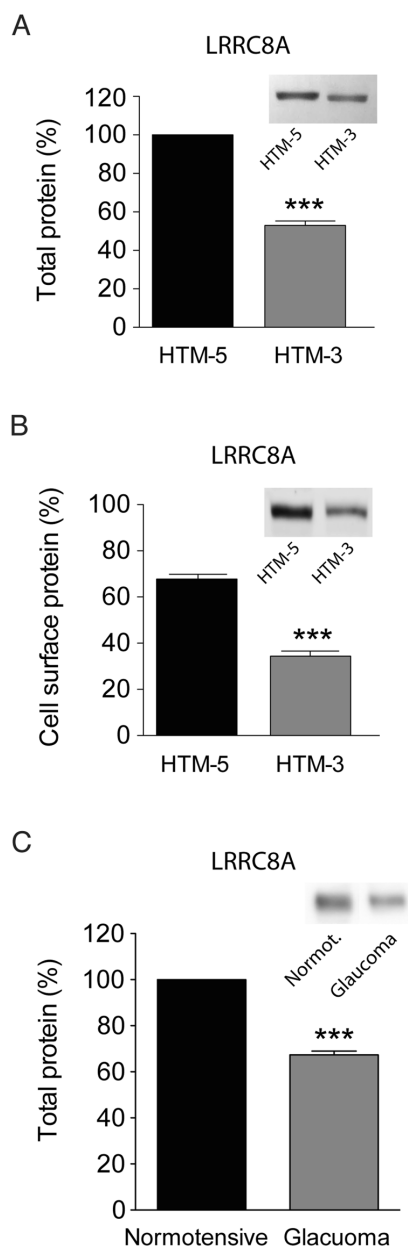


Figure 5. Quantification of LRRC8A protein at HTM cells. **(A)** LRRC8A was significantly lower in HTM-3 compared to HTM-5. Data are expressed as the percentage of LRRC8A in HTM-3 normalized to its amount in HTM-5. Values are mean $\pm$ SEM of 7 protein extractions ($***p < 0.001$, Student's t-tests). Total protein was previously quantified to load the same amount of protein of the two cell lines and the LRRC8A signal was normalized by β -actin. *Inset:* Representative western blot showing LRRC8A (95 KDa) in HTM-5 and HTM-3 cell extracts. Full-length blot is presented in Supplementary Fig. 5A. **(B)** Relative abundance of LRRC8A protein at cell surface in relation to total LRRC8A protein expressed as a percentage. Quantification shows a lower amount of LRRC8A protein at the PM of HTM-3 cells compared to that at HTM-5 cells. Values are mean $\pm$ SEM of 5 independent experiments ($***p < 0.001$, Student's t-tests). *Inset:* Representative WB of cell surface biotinylation showing LRRC8A. Full-length blot is shown in Suppl. Figure 5B. A set of experiments without biotin was done as a negative control (not shown). **(C)** LRRC8A protein was also significantly lower in primary TM cells from glaucoma patients in comparison to non-glaucomatous donors. Protein extracts were previously

quantified to load the same amount of each cell sample from Ind. 1–3 (control, normotensive) and Ind. 5–8 (glaucoma), and LRRC8A signal normalized by β -actin. Values are mean $\pm$ SEM of two different film intensities of two western blots from the same protein extracts (***) $p < 0.001$, Student's *t*-tests). *Inset*: Representative WB showing LRRC8A in control (Ind. 1) and glaucoma (Ind. 5) protein samples. Full-length blots are in Supplementary Fig. 5C.

RVD of $11.91 \pm 0.50\%$ (range 2.15–34.02%) after 30 min of hypotonicity ($n = 152$ cells) while in HTM-3 this value was of $4.99 \pm 0.40\%$ (range 0.04–24.19%; $n = 79$ cells; Fig. 6B). When isotonic solution was restored, HTM-5 volume decreased under baseline and slowly returned to initial resting values. This rebound effect was proportional to the activated RVD (Fig. 6A). Alteration of the regulatory mechanisms to restore TM cell volume in glaucoma may compromise the physiological maintenance of the AH outflow.

Down-regulation of LRRC8 genes in human anterior ocular segments perfused under elevated pressure conditions. To corroborate the relevance of our results from cellular models in the pathophysiology of glaucoma, we took advantage of previously performed microarray experiments in human TM tissue from ocular anterior segments perfused at normal/elevated pressure for 7 days. We reanalysed the microarray data looking for changes in the newly identified VRAC molecular components³¹. Analysis of DNA microarrays showed a significant down-regulation of LRRC8B, LRRC8C and LRRC8D genes in response to high pressure in four out of the five individuals tested. These results further reinforce the finding that ocular hypertension impairs VRAC expression and function in the TM tissue (Fig. 7).

The expression of LRRC8 genes and the LRRC8A protein is induced in a glaucoma model *in vitro*. It is well known that corticosteroids cause a specific form of glaucoma (steroid-induced open angle glaucoma)³⁸, characterized by expression of specific genes (e.g. Miocytin), extracellular matrix (ECM) remodelling and an important up-regulation of a large number of genes. To assess if VRAC was differentially affected in this type of glaucoma, we used a cellular model by incubating HTM-5 cells with 100 nM DEX (or vehicle) during 3 or 7 days. Real-time qPCRs showed that the five LRRC8A-E isoforms of VRAC were mostly induced by DEX. Fold changes were 1.88 ± 0.74 in LRRC8A, 3.35 ± 1.11 in LRRC8B ($p < 0.05$), 1.55 ± 0.50 in LRRC8C, 2.44 ± 0.24 in LRRC8D ($p < 0.001$) and 3.62 ± 1.97 in LRRC8E at 3 days of DEX and 2.68 ± 1.16 in LRRC8A ($p < 0.05$), 2.21 ± 0.19 in LRRC8B ($p < 0.001$), 1.15 ± 0.22 in LRRC8C, 5.76 ± 2.21 in LRRC8D ($p < 0.05$) and 2.39 ± 0.70 in LRRC8E ($p < 0.05$) after 7 days of DEX ($n = 3$ –5 experiments by triplicate, Student's *t*-tests; data not shown). Consequently, western blot of protein extracts from vehicle and DEX-treated HTM-5 cells were performed with LRRC8A and β -actin antibodies. After normalization, levels of LRRC8A protein were found induced by 33.38% ($p = 0.167$) at 3 days and 106.17% ($p = 0.014$) at 7 days of DEX compared to vehicle. Thus, LRRC8 mRNA and the LRRC8A protein were altered by DEX as it occurs in glaucomatous cells, but in the opposite direction. Because MYOC is induced by DEX specifically in the TM, its expression was used to validate the effect of DEX on HTM-5 cells. As expected, MYOC was induced by 4.65 ± 1.22 -fold ($p < 0.05$) and 8.26 ± 1.63 -fold ($p < 0.01$) at 3 and 7 days of DEX respectively, similarly to previously reported data³⁹.

Discussion

Volume-regulated anion channels are fundamental to maintain cellular volume in vertebrate cells⁴⁰. We and others, previously demonstrated the participation of this channel in volume regulation of TM cells and aqueous outflow^{5,6} but it was not until recently that LRRC8 proteins have been identified as the molecular components of VRAC^{15,16}. LRRC8A is the most important subunit and required with at least one of the other LRRC8A–LRRC8E subunits to form functional VRAC^{15,16,25,41}. This finding permitted us to characterize its expression and activity in the pathophysiology of glaucoma. Using established TM cell lines, allowed us to have enough starting material to perform different electrophysiological, biochemical, and imaging techniques. Because cell lines might undergo some changes due to the culture conditions, we validated them by quantifying the expression of different well-known markers present in glaucomatous TM cells. MYOC was discovered when its expression was induced by DEX in human TM. Importantly, DEX treatment causes ocular hypertension and today it is accepted that DEX-induced overexpression of MYOC is associated to steroid-induced glaucoma³⁹. As expected, a chronic treatment on normotensive HTM-5 cells with DEX produced the induction of MYOC mRNA and protein levels, which is specific of TM cells⁴². Then, we rationally selected MGP and ELAM-1 genes, known to be relevant to outflow function, in order to validate HTM-3 cells. MGP gene encodes an inhibitor of calcification that protects soft tissues like vascular smooth muscle cells from mineralization⁴³. MGP is highly abundant in adult TM but its expression decreases in glaucomatous TM cells³⁰, as we corroborated in HTM-3 cells. Also, the first molecular marker for glaucoma, ELAM-1³⁵, was found induced in HTM-3 glaucomatous cells compared to control cells, where it was almost absent. Altogether, our data show that HTM cells present characteristic features found in normal TM and several of the changes that occur during POAG.

VRAC was first recorded in human T lymphocytes⁴⁴ and since then, biophysical properties have been characterized in several cell types⁸. Similarly to previous data in primary bovine trabecular meshwork cells (BTM)^{6,12} and HTM cells⁵, the HTM-5 cell line present currents with characteristic electrophysiological properties of VRAC (outward rectification, voltage-dependent inactivation, and inhibition by tamoxifen). To date, mechanisms by which cell swelling activates VRAC are far from being understood. The debate is whether the channel itself senses the change in volume or whether changes in membrane tension or other factors are required for gating⁸. A decreased ionic strength⁴⁵ and other processes independent to cellular volume like shear stress⁴⁶, intracellular GTP- γ S⁴⁷, purinergic signalling⁴⁸ and ROS⁴⁹ also activate VRAC. Now, it is known that specific biophysical properties of VRAC depend on the type of cell, mainly due to the diversity generated by the combination of LRRC8

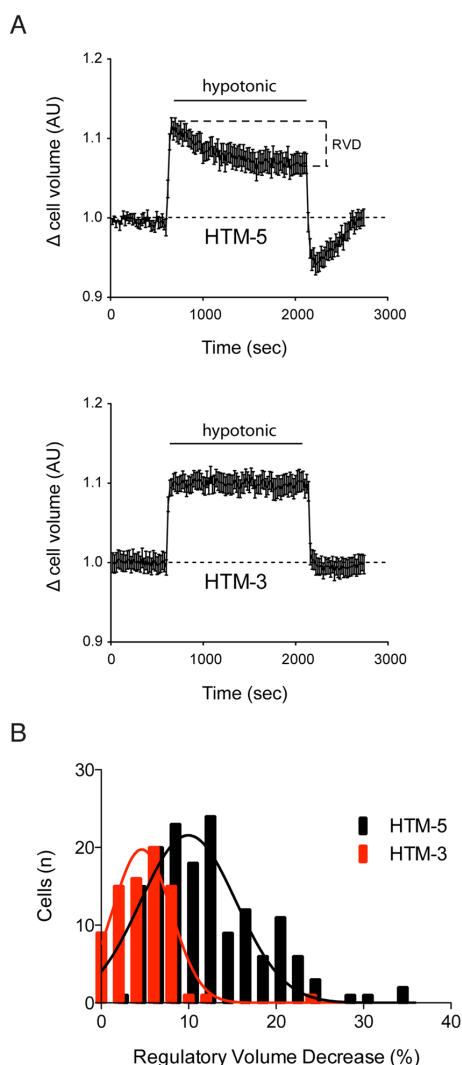


Figure 6. Volume regulation of HTM cells. (A) RVD activated in HTM-5 and HTM-3 cells was measured using calcein as an index of cellular volume (see Material and Methods for details). After baseline at isotonic conditions (10 min), swollen was induced by application of hypotonic bath solution (27% for 30 min) to activate the RVD. Glaucomatous HTM-3 cells showed an inappreciable RVD in comparison to the RVD activated in control HTM-5 cells. Results are plotted as mean $\pm$ SEM of a set of two representative experiments ($n = 17$ HTM-5 cells, $n = 15$ HTM-3 cells). (B) RVD is determined as the percentage of cell volume recovery during the hypotonic period being significantly lower in HTM-3 cells ($n = 79$) than in HTM-5 cells ($n = 153$). Values are represented versus the distribution of the number of cells in each group (** $p < 0.001$, Student's t -tests).

subunits, and that distinct LRRC8 proteins can coexist in a cell to form heterohexameric VRAC with specific intrinsic properties^{16,22,25,41}. Currents with stronger outward rectification have been recorded in HEK cells lacking LRRC8B, LRRC8C and LRRC8E in comparison to HEK cells without LRRC8B, LRRC8D, LRRC8E and LRRC8B LRRC8C LRRC8D. Moreover, and in contrast to LRRC8C-mediated currents, LRRC8D- and LRRC8E subunits generate currents that inactivate faster and at less positive potentials¹⁶. In control HTM-5 cells, VRAC currents show a significant outward rectification and a slow inactivation compared to previously published results^{16,22}. All the LRRC8A-LRRC8E mRNAs have been detected in control HTM-5 cells, where the obligatory subunit for VRAC, LRRC8A, is highly expressed. However, the particular stoichiometry of the VRAC complexes assembled in these cells would require a complete and detailed biophysical characterization.

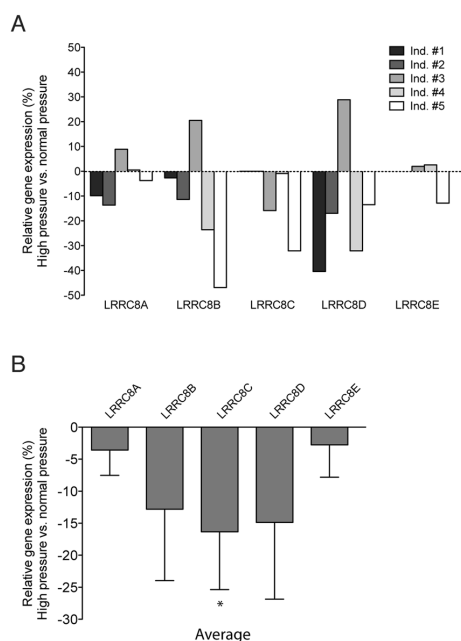


Figure 7. Differential expression of LRRC8A-LRRC8E genes under pressure in human TM. Paired ocular anterior segments from five donors were perfused at high P or physiological P during 7 d as in Material and Methods. For each individual, the relative expression of LRRC8 genes in human TM tissues was determined with Affymetrix GeneChips U95Av2 (individuals #1 and #2) or U133Plus2.0 (Ind. #3, #4, and #5). **(A)** Bars represent changes in the LRRC8 expression by high P treatment in each individual, plotted separately to assess individual molecular response to pressure. Because the two types of microarrays contain $\approx 12,625$ and $54,678$ probes respectively, LRRC8C and LRRC8E were not detected in the U95Av2 platform (Ind. #1 and #2). **(B)** Expression of LRRC8 genes as mean $\pm$ SEM shows their tendency to decrease by elevated pressure insults. LRRC8C down-regulation is the only statistically significant because of the differential molecular response to pressure of the individual #3. (* $p = 0.038$ by the Student's t-test).

In glaucomatous HTM-3 cells VRAC currents show a much lower density and a delayed activation compared to those activated in control cells. Notably, the activity of VRAC was also decreased in native TM cells from five patients with open-angle glaucoma (Ind. 4–8). Individual 4 was a case of secondary glaucoma due to idiopathic uveitis (IOP 35 mmHg pre-trabeculectomy and 14 mmHg at 3 months after surgery without pharmacological treatment). This patient suffered several outbreaks of uveitis in the past for which she received treatment with cortisone. It is not possible to discern whether ocular hypertension was caused by inflammation or corticosteroids. Because the maximum pharmacological treatment did not allow good control of the hypertension, surgery was necessary. At the time of trabeculectomy, she did not receive steroids for ten years. It is important to note that the other glaucomatous native HTM cell lines are derived from POAG patients (Ind. 5–8). All of them were treated with beta-blockers and carbonic anhydrase inhibitors, plus prostaglandins (Ind. 5–7) before surgery. In contrast to VRAC, outward K^+ currents mediated by BKCa were unaffected in HTM-3 cells. This indicates that the down-regulation of VRAC function in HTM-3 is specific and not a general effect on other proteins/channels. We previously found that RVD is markedly attenuated by tamoxifen (a VRAC blocker) in BTM cells¹². Accordingly, inhibition of Cl^- or K^+ channels with NPPB or TEA respectively, is sufficient to abolish RVD in HTM cells, while it is only partially reduced blocking the K^+-Cl^- -symport with DIOA⁵. Therefore, although the amount of LRRC8A protein could be acceptable, the decreased activity of VRAC may largely impair the regulation of cell volume. The expression of $Na^+-K^+-2Cl^-$, one of the main transporters of the RVD, is also decreased in HTM-3 cells by 29%, as described in TM cells from glaucoma patients³⁷. These proteins might contribute to the decreased ability to regulate the volume of glaucomatous cells. Interestingly, the same authors that found the $Na^+-K^+-2Cl^-$ diminished in glaucoma TM cells, described that its expression and activity was stimulated in HTM and BTM cells after DEX exposure⁵⁰. This proves once again that some genes altered in glaucoma are differentially affected by DEX, which induces its expression.

Approximately 40% of patients who are long-term treated with corticosteroids develop a pronounced rise of IOP and POAG and up to 90% of POAG cases are considered "steroid responders"⁵¹. Reorganization of the cytoskeleton⁵², an increased ECM deposition⁵³, and altered synthesis of specific proteins⁵⁴ in TM cells are some of the mechanisms proposed for steroid-induced glaucoma. Expression of MYOC have been found induced in TM in almost half of the POAG cases⁵⁵ and MYOC protein is enhanced in the TM by the steroid DEX³⁹. For all

this, the use of DEX has been used as a glaucoma model *in vitro*. In our study, DEX induced MYOC expression in HTM-5 cells, showing a consistent response of this control cell line to steroids. We also found an enhanced expression of LRRC8A-E genes, a response in the opposite direction to the found in cells from POAG. There is much evidence that gene expression is changed in TM cells in response to DEX^{42,56,57}. Borrás and coworkers reported expression changes in human TM tissues in response to DEX for 6–8 days by microarray analysis. Among the genes with expression levels than changed more than 20-fold by DEX, a higher number were up-regulated (40) than down-regulated (18). In addition, a significant set of the genes induced by DEX were located in chromosomal regions linked to glaucoma⁴². Rozsa and collaborators also identified a larger number of genes enhanced than decreased after 21 days of DEX treatment in the TM. In fact, exposure of HTM to steroids for weeks increases the synthesis of several proteins that may be relevant for trabecular function^{39,54,58}. Thus, corticoids tend to increase above all, the expression of specific proteins that could be associated to the development of steroid-induced glaucoma. Because a normal cytoskeleton is needed for the RVD mechanism⁵⁹, we used Phalloidin 555 to study F-actin on glaucomatous and control TM cell lines but we did not find evident changes (not shown). However, we can not discard that other mechanical properties such as stiffness of the TM, that have been shown to differ in POAG, could compromise the correct regulation of cell volume⁶⁰.

Grant and co-workers previously showed that VRAC currents were activated in hypotonic conditions but they did not found significant differences in VRAC between normotensive (NTM) and glaucomatous (GTM) TM cells, although a tendency might exist⁶¹. Surprisingly, they report VRAC activation in isotonic conditions, especially in NTM cells. In none of our studies with primary HTM, BTM or transformed TM cells VRAC was active at rest (isotonic) as it has been widely published⁶². These differences could be due to technical reasons and the use of non-selective chloride solutions. Solutions containing with NMDG-Cl⁻ (as used here) are a standard when recording this type of currents. Also, biophysical and pharmacological properties indicate that our chloride currents are mediated by VRAC^{6,12}. Our data indicate that the reduced VRAC currents in glaucomatous cells are the consequence of a combined decrease in mRNA of all LRRC8 subunits and LRRC8A protein. Importantly, microarray data from ref.³¹, revealed a slight but significant down-regulation of LRRC8B, LRRC8D and LRRC8E genes in TM tissues in response to pressure insults. Therefore, the expression pattern of LRRC8 under high-pressure conditions changed in the same direction than in glaucoma but in a lesser extent, regardless some individual differences. An altered composition of AH and/or an increased shear stress caused the aqueous outflow under pressure could contribute to the differential gene expression found in glaucoma.

The subunit composition of the functional complexes dictates the substrate selectivity, since LRRC8A/LRRC8D expression is associated to the release of taurine and LRRC8A, LRRC8C and LRRC8E heteromers favour anion permeation⁴¹. LRRC8D, but not LRRC8C or LRRC8E, is also required for the blasticidin uptake²⁶. Therefore, the called volume-sensitive organic osmolyte-anion channel (VSOAC) and VRAC would refer to differently composed LRRC8 heteromers⁴¹. Because *LRRC8D*^{-/-} HEK cells show diminished swelling-induced taurine efflux and a decreased RVD but normal anion currents, organic metabolites could participate in volume regulation¹⁶. In addition, a lower amount of LRRC8A has been associated to a lower capacity to modulate cell volume^{15–17}. In this regard, we have found a significant expression of LRRC8A and LRRC8D mRNAs in HTM-5 cells that show the capacity to restore part of cell volume in hypotonic conditions. Otherwise, Kunzelmann and collaborators probed that LRRC8A had little impact on volume regulation of HeLa cells. LRRC8A siRNAs and the channel inhibitor NS3728 slightly attenuate cell volume regulation but it was strongly delayed by the inhibition of the K⁺-Cl⁻ symport. In a similar way, RVD was detected in HCT116 cells lacking LRRC8A but not in the BHY cell line without this subunit⁶³. Therefore, participation of LRRC8A to cell volume regulation may depend on the cell type. Noteworthy, maximal VRAC currents were observed at 2.5 min of hypotonic medium (33%) and cell volume recovery was completed at 3.5 min in HeLa⁶³. The temperature at which the experiments were done (37 °C) could explain fast VRAC currents and the strong RVD since it is confirmed that both are attenuated at 20 °C. In this sense, BTM cells exhibit a RVD of 21% after 30 min of hypotonicity (40%) at 22–23 °C⁶ consistent to that measured in human TM cells⁵. Similarly, we recorded maximal VRAC currents at 15 min and a RVD of 11% after 30 min of 27% hypotonicity in HTM-5 cells. Although the molecular basis of cell volume regulation remains unknown the role of the temperature-sensitive PLA₂ has been suggested to be important for current density and time-dependent inactivation of VRAC and for RVD⁶³. Thus, VRAC is a key player of RVD activated in trabecular cells, that also implies activation of the BKCa, the K⁺-Cl⁻ symport, the Na⁺-K⁺-Cl⁻ cotransporter, and the Na⁺/H⁺ antiport^{5,6}. Taken all the data into account, VRAC is probably essential for volume regulation in many cell types including TM cells^{15,16,40,41} but not quite indispensable in others⁶³. Because BKCa function is not altered in HTM-3 cells, we propose that at least VRAC is required for TM cell volume regulation.

Volume of trabecular cells contribute to the aqueous outflow facility^{4–6,10,11}. As we determined previously, inhibition of VRAC and BKCa reduced the ability to recover outflow facility under hypotonic conditions while specific activation of BKCa showed the opposite effect⁶. Consequently, down-regulation of LRRC8 subunits in glaucomatous TM cells could explain the impaired modulation of cellular volume underlying an increased resistance to aqueous outflow. Aside from the volume regulation, LRRC8-mediated channels are associated to processes like transport of modulators, extracellular signal transduction and cell proliferation⁴⁰. The cellularity of the TM tissue decreases with age⁶⁴ and decreases further in eyes from POAG patients⁶⁵. In fact, laser trabeculoplasty stimulates the replication of TM cells as a part of its mechanism of action⁶⁶. A reduced number of functional VRAC in glaucomatous trabecular cells could be associated to a lower degree of proliferation.

In conclusion, glaucomatous TM cells shows a decreased VRAC activity due to a down-regulation of LRRC8 mRNA and protein, which impairs cell volume regulation. This might be associated to the altered AH outflow in open angle glaucoma patients. Finding activators of LRRC8-channels could be very useful in the trabecular physiology as they could be used to manage ocular hypertension and glaucoma.

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Author Contributions

Authors A.A.B., A.C., X.G. and N.C. performed electrophysiological recordings and calcein imaging. MIC was responsible of the trabeculectomies. A.A., A.C., and N.C. carried out cellular cultures. M.C. and N.C. did the mRNA expression studies. R.E. and N.C. performed LRRC8A protein quantification and biotinylation assays. MR did the T.M. cytoskeleton study. M.R. and N.C. were responsible of treatments and experiments with D.E.X. T.B. and N.C. performed the microarrays analysis. X.G. and N.C. designed the experiments, analysed data and performed the statistics. N.C. supervised the study and prepared the text with the help of the other authors who read and approved the final manuscript.

Additional Information

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