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Phenotyping of sigma-1 receptor knock-out rodents

Xavier Codony Soler

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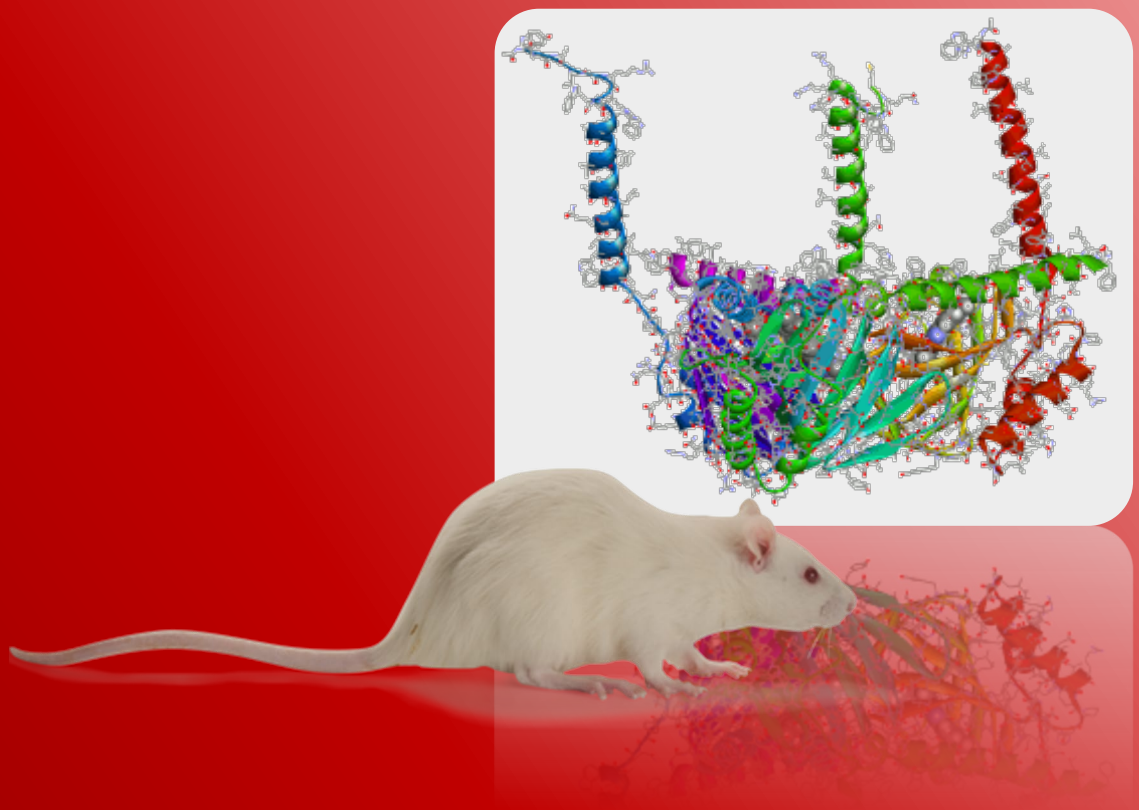

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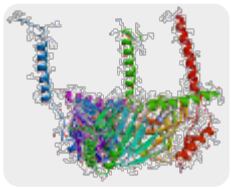
Xavier Codony Soler

PhD Thesis

Illustrations in the cover page:



Molecular representation of the Sigma-1 receptor structure.

Wistar outbred rat (Hsd:WI)

UNIVERSITAT DE BARCELONA

Facultat de Farmàcia
i Ciències de l'Alimentació

Programa de Doctorat en Biomedicina
Departament de Farmacologia, Toxicologia i Química Terapèutica

Aquesta tesi s'ha dut a terme als laboratoris de Welab Barcelona,
al Parc Científic de Barcelona.

Phenotyping of Sigma-1 receptor knock-out rodents

Memòria presentada per Xavier Codony Soler per optar al grau de doctor per la Universitat de Barcelona

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FACULTAT DE FARMÀCIA

Universitat de Barcelona

Welab BARCELONA

Als meu pare i la meva mare, per donar-me
la llibertat d'escollir.

A la Rosa Maria, la Maria, la Clàudia i en Martí,
el millor de la meva vida.

DON'T DREAM YOUR LIFE,

BUT LIVE YOUR DREAM

Mark Twain

AGRAÏMENTS

Abans de començar ni tan sols a pensar en aquest apartat ja sabia que seria el que em generaria més neguit. Voldria que tothom que ha tingut algun paper en l'elaboració d'aquesta tesis hi tingués la merescuda referència i no voldria que ningú se sentís menystingut o oblidat.

A més, he tingut el privilegi de treballar amb molta gent al llarg dels trenta-i-un anys que porto en el món de la farmacologia i molts companys i companyes han tingut un paper, més gran o més petit, en el camí que jo he fet en el món de la ciència.

Tenint en compte aquest dos condicionants, i assumint la impossibilitat de fer referència a tothom, començo amb un agraïment genèric a tots els que he conegut en la meva etapa prèvia a la meva entrada als Laboratoris del Dr. Esteve S.A. (ara Esteve Pharmaceuticals S.A.) i la llarga etapa al laboratori farmacèutic, on vaig fer la major part de la meva carrera professional.

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I ara, des de fa un parell d'anys, estem en aquesta nova aventura que és diu Welab Barcelona. Alguns ja hi eren el 1991. altres han anat arribant i d'altres han anat

marxant, quan els tocava... o massa aviat. Ja fa temps que són temps complicats per les empreses que es dediquen a descobrir nous fàrmacs.

En qualsevol cas, aquí estem, treballant amb el mateix objectiu de treure algun fàrmac que, poc o molt, ajudi a algú a viure millor.

I en aquest present, sense dubte he donar les gràcies al Dr. Manel Merlos per haver acceptar co-dirigir aquesta tesis, però sobretot agrair-li haver-me encoratjat a no defallir en els moments en els que, amb la venda del centre d'R+D a Leitat i la maleïda pandèmia, el més fàcil hauria estat deixar-ho córrer per tercer cop.

El meu agraïment al Dr. Joan Carles Laguna per haver acceptat la co-direcció de la tesis i donar-me la llibertat necessària de fer-la amb les limitacions d'estar treballant.

Gràcies José Miguel, tant pel teu suport en la realització d'aquesta tesis, com per animar-me a créixer professionalment.

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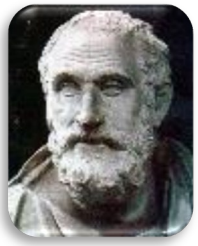
Un agraïment enorme a tots els companys de Welab Barcelona que fan possible, amb el seu esforç i tossuderia, que seguim aquí, lluitant per tirar endavant!

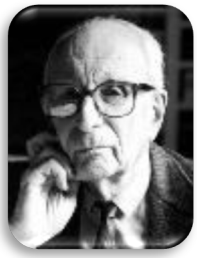
Finalment, gràcies Rosa Maria, per la paciència i el suport incondicional.

Mataró, 14 de Març del 2022



"Στην πραγματικότητα υπάρχουν δύο πράγματα, η επιστήμη και η γνώμη, το πρώτο γεννά γνώση, το δεύτερο την άγνοια."
Ἱπποκράτης
(~460 a.c. - ~370 a.c.)
(De fet hi ha dues coses, la ciència i l'opinió,
la primera engendra el coneixement, la segona la ignorància. Hipòcrates)

"In questioni di scienza, l'autorità di un migliaio di persone non vale tanto quanto l'umile ragionamento di un singolo individuo."
Galileo Galilei
(1564 – 1642)



"Le savant n'est pas celui qui fournit les vraies réponses,
c'est celui qui pose les vraies questions."
Claude Levi-Strauss
(1908-2009)

SUMMARY

The sigma-1 receptor is a chaperone that is primarily expressed in the mitochondria-associated endoplasmic reticulum (MAM). It was cloned years ago from different tissues of various species and its structure has been recently elucidated.

In 2003, our laboratory generated the first knock-out (KO) mouse for the sigma-1 receptor. These mice served to demonstrate the involvement of the sigma-1 receptor in acute and chronic pain. In addition to pain, the sigma-1 receptor has been implicated in other physiological processes and pathological conditions including depression and addiction. The sigma-1 receptor has also been shown to be associated with the regulation of other proteins, including dopamine (DAT) transporter.

In this work, the generation of a sigma-1 receptor KO rat model is reported. The behavioural response of sigma-1 KO mice in two models of depression has been characterized, and the physiological and behavioural phenotyping of sigma-1 KO rats has been carried out, with special attention to the possible effects of the absence of the receptor in models of depression and addiction.

By means of CRISPR / Cas9 technology, two KO strains for the sigma-1 receptor with deletions of 218bp and 7bp were obtained. Wild-type (WT) and the two KO strains showed no significant differences in the Irwin test, spontaneous locomotor activity, open field test, startle response, or pre-pulse inhibition. In contrast, the results obtained in response to mechanical or thermal stimulation led us to select the strain with a deletion of 218bp. Using this strain, WT (+/+), KO heterozygous (+/-) and KO homozygous (-/-) rats were phenotyped. No significant differences were found in growth or survival curves, nor in most of the assessed physiological or behavioural parameters. Regarding depression, no significant difference was found in the acute study after administration of fluoxetine (an antidepressant with sigma-1 affinity) or venlafaxine (without sigma-1 affinity) on the day of the test. This was an expected outcome, based on the literature and previous findings regarding the ineffectiveness of antidepressants in an acute treatment. In the sub-acute study, the tendency for lower immobility during training observed in mice was confirmed. On test days 1 and 7, neither fluvoxamine nor

venlafaxine showed any efficacy in reducing immobility. In contrast, at day 14, the two antidepressants significantly reduced immobility only in KO rats. Given that there is no sigma-1 receptor in these rats and that both antidepressants showed activity, regardless of whether they had sigma-1 affinity, it seems that the efficacy may be due to some change in their action on SERT.

Regarding locomotor activity, as a surrogate measure of addictive potential, the set of results seems to indicate a greater role of the sigma-1 receptor in the rearing activity, especially as stereotyped behaviour, rather than its merely exploratory activity, being the difference between the two genotypes mainly at high doses. There have been fewer differences in locomotor activity and, surprisingly, no differences after cocaine administration, that has a sigma-1 affinity. Differences between mobility and rearing can be assigned to the dopaminergic pathways involved, meso-limbic for mobility, and nigro-striatal for stereotypes.

In conclusion, in rats, deletion of the gene encoding the sigma-1 receptor generates a viable phenotype very similar to the WT strain under normal conditions. Behavioural response under conditions of environmental stimulation and / or pharmacological treatment, reveals some differences between WT and KO, in both mice and rats. The absence of the receptor seems to lead to the adaptation of other proteins. The results further support the concept that the sigma-1 receptor may be involved in the development of depression and addiction and drugs acting on sigma-1 receptors could be useful in such pathologies.

RESUM

El receptor sigma-1 és una xaperona que s'expressa principalment al reticle endoplasmàtic associat a mitocòndries (MAM, de l'anglès Mitochondria-Associated endoplasmic reticulum Membranes). Va ser clonat ja fa anys a partir de diferents teixits de diverses espècies i recentment se n'ha elucidat l'estructura.

L'any 2003 el nostre laboratori va generar el primer ratolí knock-out (KO) pel receptor sigma-1. Aquests ratolins van servir per demostrar la implicació del receptor sigma-1 en el dolor agut i com crònic. A banda del dolor, el receptor sigma-1 s'ha implicat en altres processos fisiològics i condicions patològiques incloent depressió i addicció. Així mateix, s'ha demostrat que el receptor sigma-1 està associat i participa en regulació d'altres proteïnes, entre elles el transportador de dopamina (DAT).

En aquest treball reportem la generació d'un model de rata KO pel receptor sigma-1. S'ha caracteritzat la resposta conductual dels ratolins KO sigma-1 en dos models de depressió i s'ha procedit a fer el fenotipat fisiològic i conductual de les rates KO sigma-1, amb especial atenció als possibles efectes de l'absència del receptor en models de depressió i addicció.

Mitjançant la tecnologia de CRISPR/Cas9 s'obtingueren dues soques KO pel receptor sigma-1 amb delecions de 218bp i 7bp. Cap de les dues soques mostraren diferències destacables en el test d'Irwin, activitat locomotora espontània, exploració en camp obert, *startle response* o *pre-pulse inhibition* amb respecte a la soca *wild-type* (WT). En canvi, els resultats obtinguts en resposta a l'estimulació mecànica o tèrmica ens portaren a seleccionar la soca amb una deleció de 218bp.

Amb aquesta soca es va procedir al fenotipat de rates WT (+/+), heterozigots KO (+/-) i homozigots KO (-/-). No es van trobar diferències significatives en les corbes de creixement o supervivència, ni en la majoria de paràmetres fisiològics o conductuals avaluats.

Quant a depressió, en l'estudi agut no es va trobar cap diferència significativa després de l'administració de fluoxetina (antidepressiu amb afinitat sigma-1) o venlafaxina (sense

afinitat sigma-1) el dia del test. Aquest era un resultat esperable, basant-nos en la literatura i resultats previs quant a la manca d'eficàcia dels antidepressius en tractament agut.

En l'estudi sub-agut la tendència a una menor immobilitat durant l'entrenament, observada en ratolí es va veure confirmada. Els dies 1 i 7 de test, ni fluvoxamina ni venlafaxina van mostrar cap eficàcia reduint el temps d'immobilitat. En canvi, a dia 14, els dos antidepressius reduïren de manera significativa la immobilitat només en les rates KO. Donat que en aquestes rates no hi ha receptor sigma-1 i que els dos antidepressius mostraren activitat, independentment de tenir o no afinitat sigma-1. tot indica que l'eficàcia podria venir per algun canvi en la seva acció sobre SERT.

Quant a l'activitat locomotora, com a mesura subrogada de potencial addictiu, el conjunt de resultats semblen indicar un major paper del receptor sigma-1 en els aixecaments, sobretot en el seu component de conducta estereotipada més que en l'activitat merament exploratòria, en tant que la diferència entre els dos genotips es dóna sobretot a les dosis altes. En l'activitat locomotora s'han trobat menys diferències i, sorprenentment, cap diferència precisament amb l'administració de cocaïna, que és la que te afinitat sigma-1. Les diferències entre mobilitat i aixecaments pot raure en les vies dopaminèrgiques implicades, meso-límbiques per la mobilitat i nigroestriatals pels estereotips.

En conclusió, en rata, la deleció del gen que codifica el receptor sigma-1 genera un fenotip viable i molt semblant a la soca WT en condicions normals. La resposta conductual en condicions d'estimulació ambiental i/o tractament farmacològic posa de manifest algunes diferències entre WT i KO, tant en ratolí com en rata. L'absència del receptor sembla comportar l'adaptació d'altres proteïnes. Els resultats donen suport a que el receptor sigma-1 podria estar implicat en el desenvolupament de la depressió i l'addicció i que el tractament amb fàrmacs que actuen sobre sigma-1 pot ésser útil en aquestes patologies.

RESUMEN

El receptor sigma-1 es una chaperona que se expresa principalmente en el retículo endoplasmático asociado a mitocondrias (MAM, del inglés Mitochondria-Associated endoplasmic reticulum Membranes). Fue clonado ya hace años a partir de diferentes tejidos de diversas especies y recientemente se ha elucidado su estructura.

En 2003 nuestro laboratorio generó el primer ratón knock-out (KO) por el receptor sigma-1. Estos ratones sirvieron para demostrar la implicación del receptor sigma-1 tanto en el dolor agudo como crónico. Aparte del dolor, el receptor sigma-1 se ha implicado en otros procesos fisiológicos y condiciones patológicas incluyendo depresión y adicción. Asimismo, se ha demostrado que el receptor sigma-1 está asociado y participa en la regulación de otras proteínas, entre ellas el transportador de dopamina (DAT).

En este trabajo, reportamos la generación de modelo de rata KO por el receptor sigma-1. Se ha caracterizado la respuesta conductual de los ratones KO sigma-1 en dos modelos de depresión y se ha procedido a hacer el fenotipado fisiológico y conductual de las ratas KO sigma-1, con especial atención a los posibles efectos de la ausencia del receptor en modelos de depresión y adicción.

Mediante la tecnología de CRISPR/Cas9 se obtuvieron dos cepas KO por el receptor sigma-1 con deleciones de 218bp y 7bp. Ninguna de las dos cepas mostró diferencias destacables en el test de Irwin, actividad locomotora espontánea, exploración en campo abierto, *startle response* o *pre-pulse inhibition* en comparación con la cepa *wild-type* (WT). En cambio, los resultados obtenidos en respuesta a la estimulación mecánica o térmica nos llevaron a seleccionar la cepa con la deleción de 218bp.

Con esta cepa se procedió al fenotipado de ratas WT (+/+), heterocigotas KO (+/-) y homocigotas KO (-/-). No se hallaron diferencias significativas en las curvas de crecimiento o supervivencia, ni en la mayoría de los parámetros fisiológicos o conductuales evaluados.

En cuanto a la depresión, en el estudio agudo no se halló ninguna diferencia significativa después de la administración de fluoxetina (antidepresivo con afinidad sigma-1) o

venlafaxina (sin afinidad sigma-1) el día de la prueba. Éste era un resultado esperable, basándonos en la literatura y resultados previos en cuanto a la falta de eficacia de los antidepresivos en tratamiento agudo.

En el estudio subagudo la tendencia a una menor inmovilidad durante el entrenamiento, observada en ratón se vio confirmada. Los días 1 y 7 de la prueba, ni fluvoxamina ni venlafaxina mostraron eficacia alguna reduciendo el tiempo de inmovilidad. En cambio, a día 14, los dos antidepresivos redujeron significativamente la inmovilidad sólo en las ratas KO. Dado que en estas ratas no existe receptor sigma-1 y que los dos antidepresivos mostraron actividad, independientemente de tener o no afinidad sigma-1. todo indica que la eficacia podría venir por algún cambio en su acción sobre SERT.

En cuanto a la actividad locomotora, como medida subrogada de potencial adictivo, el conjunto de resultados parece indicar un mayor papel del receptor sigma-1 en los levantamientos, sobre todo en su componente de conducta estereotipada más que en la actividad meramente exploratoria, en tanto que la diferencia entre los dos genotipos se da sobre todo a las dosis altas. En la actividad locomotora se han encontrado menos diferencias y, sorprendentemente, ninguna diferencia precisamente con la administración de cocaína, que es la que tiene afinidad sigma-1. Las diferencias entre movilidad y levantamientos pueden deberse a las vías dopaminérgicas implicadas, meso-límbicas por la movilidad y nigro-estriatales por los estereotipos.

En conclusión, en rata, la deleción del gen que codifica el receptor sigma-1 genera un fenotipo viable y muy similar a la cepa WT en condiciones normales. La respuesta conductual en condiciones de estimulación ambiental y/o tratamiento farmacológico pone de manifiesto algunas diferencias entre WT y KO, tanto en ratón como en rata La ausencia del receptor parece comportar la adaptación de otras proteínas. Los resultados dan soporte a que el receptor sigma-1 podría estar implicado en el desarrollo de la depresión y la adicción y que los fármacos que actúan sobre sigma-1 pueden ser útiles en el tratamiento de estas patologías.

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INTRODUCTION

The sigma receptors

The understanding of sigma receptors has been a challenging endeavour, and the story is still far from complete. Haertzen (1970) described the psychomimetic effects of the benzomorphan cyclazocine in humans. Some years later, Martin *et al.*, (1976) reported the psychomimetic effects of *N*-allyl-normetazocine (SKF 10.047), proposing there were stereochemically closely related receptors designated as μ, κ, and σ, being the prototypical ligands morphine, ketocyclazocine, and SKF-10047, respectively. However, some years later it was demonstrated that the effects of sigma-1 agonist were not blocked by classical opioid antagonists (Iwamoto, 1981; Vaupel, 1983). Moreover, sigma-1 receptors were enantio-selective for the (+)-isoforms of the benzomorphans (Brady 1982). The sigma receptor was also identified as the binding site for phencyclidine (PCP) (Mendelsohn, 1985; Zukin *et al.*, 1984). Therefore, the nature of the sigma-1 receptor was quite confusing for many years. In 1992, Quirion and colleagues conclusively identified the sigma receptor nature, classifying it as a non-opioid, non-phencyclidine unique site. The efforts in sigma research have been done in several fields, from molecular biology to medicinal chemistry, drug discovery, and clinical development. The physiological functions have been extensively studied and the potential clinical applications of sigma receptors and their ligands have been reviewed [for example, see Maurice and Su, 2009; Kim and Pasternak, 2017; Vavers *et al.*, 2019]. The number of patents has grown, led by three pharmaceutical companies, Anavex Life Sciences Corp., M's Science Corp., and Esteve Pharmaceuticals (Collina *et al.*, 2013).

Binding studies were extensively used for the characterization of the sigma receptors. As mentioned before, sigma receptors are non-opioid receptors, and different from other receptors and ion channels. In 2013 it was included in the International Union of Basic

and Clinical Pharmacology list as a ligand-regulated non-opioid intracellular receptor (Alexander *et al.*, 2013). There are two receptor subtypes, sigma-1 and sigma-2 (Hellewell and Bowen, 1990; Quirion et al, 1992; Hellewell *et al.*, 1994). Sigma-2 is also known as TMEM97 (endoplasmic reticulum-resident transmembrane protein 97; Alon *et al.*, 2017). The two receptor subtypes sigma-1 and sigma-2 show stereoselectivity for (+)-isomers and (-)-isomers, respectively. They also have differential pattern tissue distribution and subcellular localization.

Sigma-1 receptor

The sigma-1 receptor was cloned from different tissues including guinea pig liver (Hanner *et al.*, 1996), human placental choriocarcinoma cells (Kekuda *et al.*, 1996), human brain (Prasad *et al.*, 1998), rat brain (Seth *et al.*, 1998; Mei and Pasternak, 2001), and mouse brain (Pan *et al.*, 1998). The sigma-1 receptor is a 25-kDa molecular mass protein of 223 aminoacids. Its sequence has no known homology with other mammalian proteins. It is structurally different from G-protein coupled receptors and ion channels. It shares 30% identity with a yeast gene that encodes a sterol C8–C7 isomerase which is necessary for cholesterol synthesis (Moebius *et al.*, 1997). Initially, it was suggested to have a single transmembrane domain (Mei and Pasternak, 2001). Other studies suggested sigma-1 has two transmembrane domains with intracellular C- and N-terminus (Aydar *et al.*, 2002). Recently, the architecture of sigma-1 receptor was published (Schmidt *et al.*, 2016) as having 3 protomers, each one with 1 transmembrane domain (Figure 1).

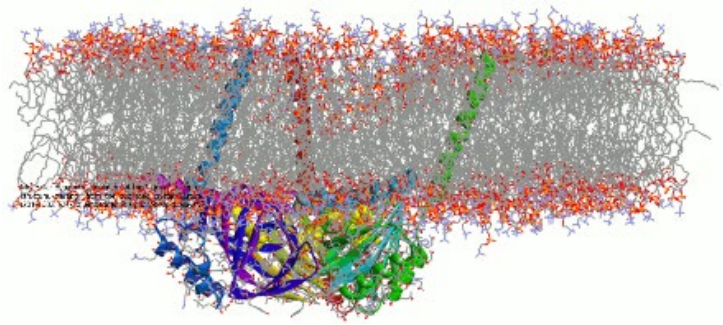


Figure 1. Molecular representation of the Sigma-1 receptor structure, starting from the published crystal (Schmidt et al., 2016) embedded in a phospholipid bilayer.

Sigma-1 receptor at cellular level

According to NCBI (National Center for Biological Information), sigma-1 receptor is a chaperone protein widely expressed encoded by the SIGMAR1 gene. This gene is in chromosome 9 with the location 9p13.3, and genomic coordinates of 9:34,634,721–34,637,825. As a chaperone, it can modulate many other proteins (Figure 2), amplifying or reducing the activity of target proteins (Hayashi T, 2007; Rodríguez-Muñoz M, 2015). Sigma-1 receptor is found in nuclear membrane, and in MAM (Mitochondria-Associated endoplasmic reticulum Membranes) (Hayashi *et al.*, 2007; Mori *et al.*, 2013; Mavlyutov *et al.*, 2015; Su *et al.*, 2016). It is involved in the conformation of IP_3 (Inositol triphosphate receptor type 3) and the Ca^{2+} signalling (Hayashi *et al.*, 2007; Boehning *et al.*, 2003; Tagashira *et al.*, 2014), and in the stress transmission and reactive oxygen species formation (Mori *et al.*, 2013; Wang *et al.*, 2015).

These activities point to a characteristic response of sigma-1 receptor to agonist or stress activation, while it has poor activity under resting conditions. Thus, upon stimulation, the receptor translocates to the plasma membrane where it interacts with other proteins, including ion channels, receptors, and kinases. (for a review, see Su *et al.*, 2016). It can also translocate to the nuclear membrane, interacting with other proteins for gene transcription regulation (Tsai *et al.*, 2015).

Of especial interest for the objectives of this thesis, the sigma-1 receptor has been linked to the serotonin transporter (Asano *et al.*, 2019), the dopamine transporter (Hong *et al.*, 2017), or some dopamine receptors (Navarro *et al.*, 2013; Aguinaga *et al.*, 2018).

Currently, the most accepted hypothesis of sigma-1 activity points to a resting state of sigma-1 receptor, forming a complex with GRP78/BiP protein in the endoplasmic reticulum (ER). In a situation of cellular stress or through agonist activation, sigma-1 receptor dissociates from BiP allowing to multiple protein interactions (Hayashi and Su, 2007).

Sigma-1 receptors exist as monomers, dimers, or even higher oligomers (Mishra *et al.*, 2015), and drug binding depends on this oligomeric state (Gromek *et al.*, 2014). Agonists and antagonist of the receptor interacts preferentially with monomeric and dimeric forms, or higher order oligomers, respectively (Mishra *et al.*, 2015; Yano *et al.*, 2019). It is an exciting feature of the sigma-1 receptor, considering its possible role in receptor functions.

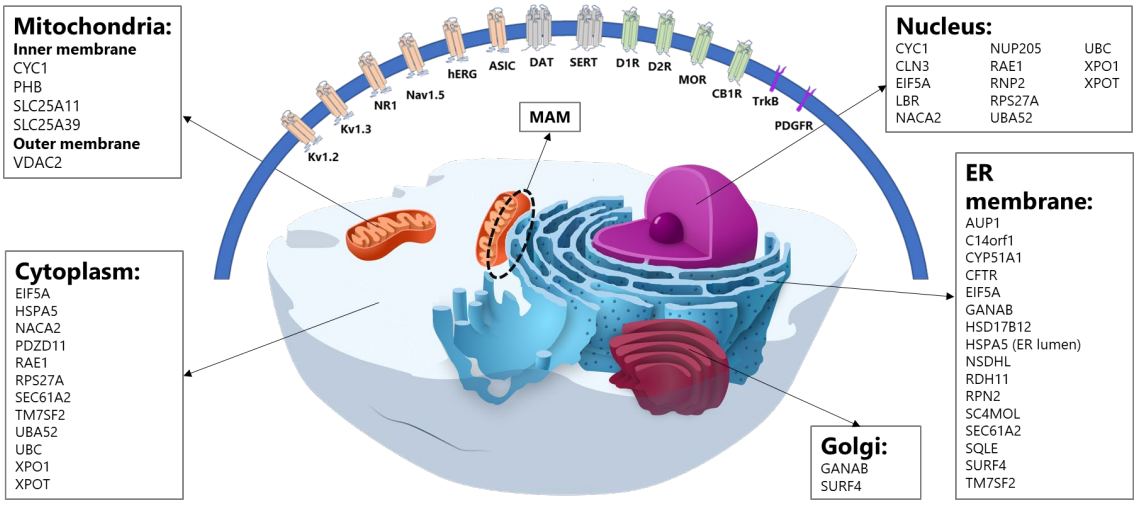


Figure 2. List of proteins interacting with sigma-1 receptor (not exhaustive; adapted from Su et al., 2016)

AUP1 (ancient ubiquitous protein 1), ASIC, acid-sensing ion channel, C14orf1 (chromosome 14 open reading frame 1), CB1R, cannabinoid receptor 1, CYC1 (cytochrome c-1), CYP51A1 (cytochrome P450, family 51, subfamily A, polypeptide 1), D1R, dopamine receptor 1, D2R, dopamine receptor 2, EIF5A (eukaryotic translation initiation factor 5A), GANAB (glucosidase, alpha; neutral AB), hERG, voltage-gated potassium channel hERG (human ether-à-go-go related gene), HSD17B12 (hydroxysteroid (17-beta) dehydrogenase 12), HSPA5 (heat shock 70kDa protein 5; glucose-regulated protein, 78kDa; BIP), Kv1.2, Kv1.3, and Kv2.1, voltage-gated potassium channel, LBR (lamin B receptor), MOR, mu opioid receptor, NACA2 (nascent polypeptide-associated complex alpha subunit 2), Nav1.5, voltage-gated sodium channel, NR1, NMDA receptor subunit 1, NSDHL (NAD(P) dependent steroid dehydrogenase-like), NUP205 (nucleoporin 205kDa), PHB (prohibitin), PDGFR, platelet-derived growth factor receptor, PDZD11 (PDZ domain containing 11), RAE1 (RAE1 RNA export 1 homolog), RDH11 (retinol dehydrogenase 11 (all-trans/9-cis/11-cis)), RPS27A (ribosomal protein S27a), RPN2 (ribophorin II), SC4MOL (sterol-C4-methyl oxidase-like), SEC61A2 (Sec61 alpha 2 subunit (S. cerevisiae)), SLC25A11 (solute carrier family 25 (mitochondrial carrier; oxoglutarate carrier), member 11), SLC25A39 (solute carrier family 25, member 39), SQLE (squalene epoxidase), SURF4 (surfeit 4), TM7SF2 (transmembrane 7 superfamily member 2), TrkB, Tropomyosin receptor kinase B for brain-derived neurotrophic factor (B), UBA52 (ubiquitin A-52 residue ribosomal protein fusion product 1), UBC (ubiquitin C), VDAC2 (voltage-dependent anion channel, 2), XPO1 (exportin 1 (CRM1 homolog, yeast)), XPOT (exportin, tRNA (nuclear export receptor for tRNAs)), Rac1, Ras-related C3 botulinum toxin substrate (Rac)-GTPase. At **MAM**: GRP78/BiP/HSPA5, glucose response protein/immunoprotein-binding protein/heat shock protein A5, IRE1, inositol-requiring enzyme 1, IP3R, inositol trisphosphate receptor, VDAC, voltage-dependent anion channel 2.

Sigma-1 receptor distribution

In early experiments, sigma-1 receptor was found to be highly expressed in several brain regions, but it is also present in other organs at high density (Tam, 1983; Largent *et al.*, 1986; Gundlach *et al.*, 1986). The use of nucleotide probes or antibodies, after the cloning of the receptor, demonstrated the expression of sigma-1 receptor mRNA in several organs, including brain, stomach, liver, adrenal gland, or testis. Surprisingly, direct measurement of protein levels showed a quite different distribution, with high levels in CNS and PNS, but lower in other organs. In the brain, a differential distribution was shown with highest levels in frontal cortex, hippocampus, and striatum (Hayashi and Su, 2002; Phan *et al.*, 2002), although in the Human Protein Atlas database, the sigma-1 receptor RNA transcripts levels are quite similar in the reported areas (Figure 3).

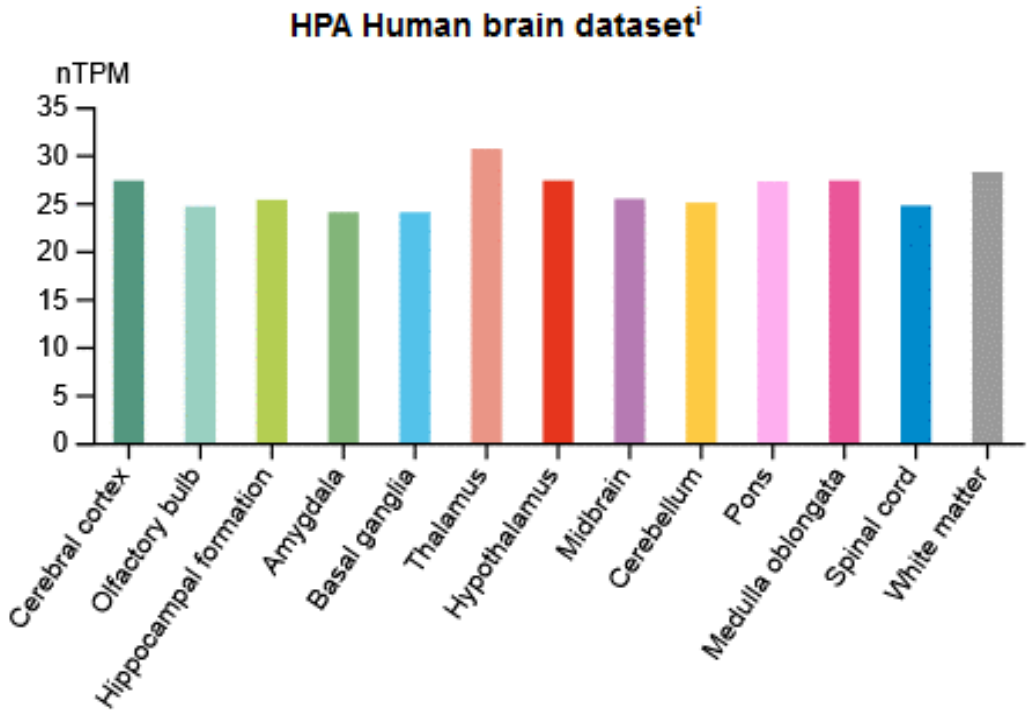


Figure 3. Brain RNA expression summary shows the consensus data based on normalized Transcripts Per Kilobase Million (nTPM) values from two different sources: internally generated Human Protein Atlas (**https://www.proteinatlas.org/about/assays+annotation - rna**) RNA-seq data and RNA-seq data from the Genotype-Tissue Expression (http://www.gtexportal.org/) project.
HPA = Human Protein Atlas.

The distribution is also accompanied by diversified effects on the proteins under sigma-1 modulation. Several lines of research demonstrated that the sigma-1 receptor differentially modulates neuronal firing and neurotransmitter release. Serotonergic neurons in the dorsal raphe increased their firing after sigma-1 receptor agonist treatment (Bermack and Debonnel, 2001), and dopamine activity is also increased in the spontaneously active dopamine neurons in the Ventral Tegmental Area (VTA) while suppressed in the Substantia Nigra pars Compacta (SNC) neurons (Minabe *et al.*, 1999).

Sigma-1 receptors distribution in the CNS was initially studied using the first available radioligands, either agonists like [^{3}H](+)-3-PPP or [^{3}H](+)-SKF 10.047, or antagonist like [^{3}H] NE-100 (Samovilova *et al.*,1988; Largent *et al.* 1986; Okuyama *et al.*, 1995). Results showed binding on several parts of the forebrain, like limbic structures such as the hippocampus or gyrus dentatus, but also the thalamus and hypothalamus, and some areas of the midbrain, like the dorsal raphe, the substantia nigra, the locus coeruleus, and in the cerebellum (hindbrain). The use of more advance techniques such us in situ hybridization and immunohistochemistry (Alonso *et al.*, 2000; McCann *et al.*, 1994; McLean and Weber, 1988) allowed a better localization of the receptor. As in binding studies, sigma-1 receptor was found to be widely distributed in the brain, with high concentration in areas involved in motor control or in limbic areas, which are particularly relevant in psychosis, drug abuse or depression.

Sigma-1 receptor has been found in several peripheral organs. Using the techniques mentioned above, the sigma-1 receptor has been found to be in the heart, kidney, liver, spleen, the digestive tract, or sexual organs (Hellewell *et al.*, 1994; Maurice *et al.*, 1996; Wolfe, 1997; Jansen *et al.*, 1992; Samovilova and Vinogradov, 1992; Hjørnevik *et al.*, 2017). In Figure 4, human body sigma-1 receptor RNA and protein expression are summarised.

Figure 4. RNA expression summary shows the consensus data based on normalized Transcripts Per Kilobase Million (nTPM) values from two different sources: internally generated Human Protein Atlas (https://www.proteinatlas.org/assays+annotation) RNA-seq data and RNA-seq data from the Genotype-Tissue Expression (http://www.gtexportal.org/) project.
Each bar represents the highest expression score found in a particular group of tissues.

Protein expression scores are based on a best estimate of the "true" protein expression from a knowledge-based annotation, described more in detail under https://www.proteinatlas.org/ assays+annotation. For genes where more than one antibody has been used, a collective score is set displaying the estimated true protein expression.
Modified from https://www.proteinatlas.org/ENSG00000147955-SIGMAR1/tissue

Ligands of the sigma receptors

Despite the increasing knowledge on the sigma-1 receptor biology the endogenous ligand has not yet been elucidated. A long list of neurotransmitters are ineffective displacing selective sigma ligands from the receptor. Serotonin, dopamine, noradrenaline, or histamine, as well as the amino acids glutamate, aspartate, cysteine, or glycine do not have binding affinity for the sigma-1 receptor. (Weber *et al.*, 1986; DeHaven-Hudkins et al, 1992; Craviso and Musacchio, 1983; Klein and Mussachio, 1989). Several peptides have been also studied with similar non-binding results (Samovilova *et al.*, 1988; (DeHaven-Hudkins and Fleissner, 1992). Among the neuropeptides, NPY deserved some interest because one study showed high affinity for sigma-1 receptor, but this was later not confirmed by another study (Tam and Mitchell, 1991). In addition, [^{35}S]GTPγS binding induced by NPY was not displaced by either agonist or antagonist of sigma-1 ligands.

Another group of endogenous ligands that show moderate affinity for sigma-1 receptor were the steroids. Among them, the most potent is progesterone with a Ki of 300nM. Testosterone, pregnenolone sulphate or deoxycorticosterone also have affinity, although in the micromolar range (McCann and Su, 1991; Maurice *et al.*, 1996). Moreover, contrary to NPY, *in vivo* binding of [^{3}H](+)-SKF 10.047 was displaced by systemic administration of steroids (Weiland, 1992).

While the endogenous ligands for sigma-1 receptor are elusive, the affinity for this receptor has been demonstrated in several drugs in diverse therapeutics fields, including antipsychotics, antidepressants, cognition enhancers or antitussives. Some drugs of abuse, like cocaine, have also shown sigma-1 receptor affinity.

Table 1. Summary of drugs with sigma-1 receptor affinity

Drugs	Affinity Ki (nM)	Function	Other activities	References
(+)-Pentazocine	16.7	Agonist		Vilner and Bowen, 2000
(–)-Pentazocine	807	Agonist	κ1 agonist, μ1. μ2, ligand, low affinity δ and κ3 opioid ligand	Vilner and Bowen, 2000 Chien and Pasternak, 1995
(+)-SKF-10.047	597	Agonist	Agonist NMDA receptor	Vilner and Bowen, 2000
Antipsychotics				
Haloperidol	6.44	Antagonist	Dopamine D2 and D3 antagonist; σ2 agonist	Vilner and Bowen, 2000 Entrena *et al.*, 2009
Chlorpromazine	453	Antagonist	Dopamine D2 antagonist	Matsumoto and Pouw, 2000 Hayashi and Su, 2004
Antidepressants				
Fluoxetine	240	Agonist	SERT	Hayashi and Su, 2008 Narita *et al.*, 1996
Fluvoxamine	36	Agonist	SERT	Hayashi and Su, 2008 Narita *et al.*, 1996
Imipramine	343	Agonist	Monoamine reuptake inhibitor	Hayashi and Su, 2008 Narita *et al.*, 1996
Sertraline	57	Agonist	SERT	Hayashi and Su, 2008 Bermack and Debonnel, 2001 Narita *et al.*, 1996
Antitussives				
Dextromethorphan	205	Agonist	NMDA receptor allosteric antagonist	LePage *et al.*, 2005 Shin *et al.*, 2007
Alzheimer's disease				
Donepezil	14.6	Agonist	Cholinesterase inhibitor	Kato *et al.*, 1999 Maurice *et al.*, 2006
Drugs of abuse				
Cocaine	2000	Agonist	Monoamine transporters inhibitor	Sharkey *et al.*, 1988 Matsumoto *et al.*, 2001 Navarro *et al.*, 2013
Metamphetamine	2160		Preferential DAT inhibitor	Nguyen *et al.*, 2005 Sambo *et al.*, 2018
MDMA	3057		Preferential SERT inhibitor	Brammer *et al.*, 2006

Worth to mention that in each therapeutic category there are representatives without sigma-1 affinity. Particularly relevant for the objectives of this thesis, venlafaxine, a dual SERT/NET inhibitor, with Ki values of 82 nM and 2480 nM, respectively, does not possess sigma-1 affinity. Similarly, the stimulant d-amphetamine, an inhibitor of the monoamine transporters and inductor of monoamine release, and apomorphine, a dopamine D2 and D1 receptor agonist, do not have sigma-1 receptor affinity.

Potential therapeutic applications of sigma receptor ligands

Since the discovery of the sigma receptor, potential therapeutic applications have been proposed (Su and Junien, 1994). Therapeutic opportunities have increasingly been described mainly for, but not limited to, nervous system disorders. Based on the peripheral distribution of the receptor, it has also been implicated in gastrointestinal disorders, vasculature, cardiac cell contractility, or regulation of hormone secretion.

However, CNS disorders have been, by far, the disorders sigma-1 receptor has been related to the most. In a recent search for sigma-1 receptor, performed in a drug discovery intelligence database (Clarivate™), the results showed up 967 molecules claimed for the treatment of several disorders, being the most relevant pain and neurological disorders (Figure 5).

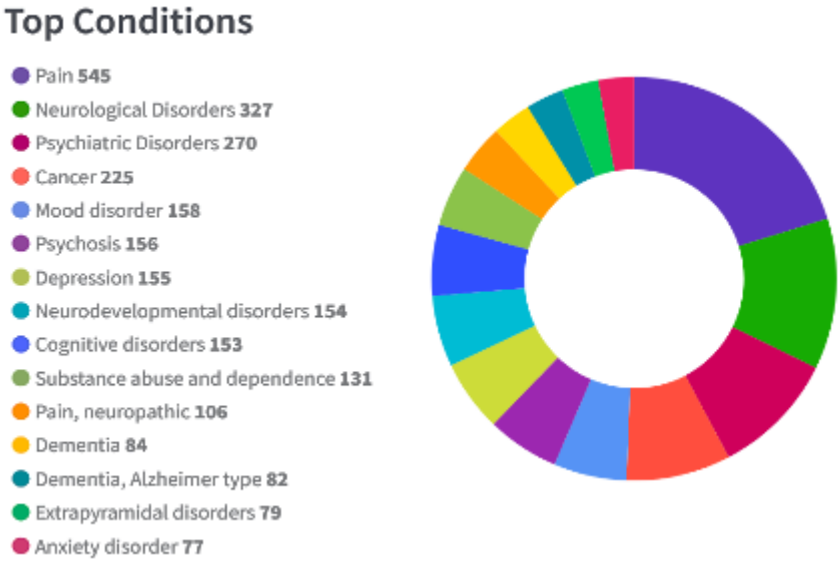


Figure 5. Therapeutic conditions claimed by sigma-1 compounds under research and development (Clarivate™ search performed on March 3rd, 2022)

As mentioned before, the discovery of the sigma receptor was around opioid research, and the discovery of sigma affinity for some psychostimulant drugs. Therefore, the potential role in drug abuse and psychosis/schizophrenia was quite evident from the beginning and it has been constantly reported since then (Borison *et al.*, 1991; Gewirtz *et al.*, 1994; Romieu *et al.*, 2002; Albayrak and Hashimoto, 2012; Hiranita *et al.*, 2013; Skuza, 2013). The potential role of sigma-1 ligands in the modulation of dopaminergic function, as key neurochemical pathway in drug rewarding properties and in positive symptoms of schizophrenia, led to the study of sigma-1 involvement in movement disorders, in particular Parkinson's disease (Francardo *et al.*, 2014; Yang et al, 2019). Alzheimer's disease (Maurice *et al.*, 1998; Jin *et al.*, 2015; Maurice and Goguadze, 2017), anxiety and depression (Albayrak and Hashimoto, 2017; Mandelli *et al.*, 2017; Yang *et al.*, 2019), pain (Vela *et al.*, 2015; Merlos *et al.*, 2017; Bruna and Velsco, 2018; Shin S*et al.*, 2022) or amyotrophic lateral sclerosis (Guzmán-Lenis *et al.*, 2009; Mancuso *et al.*, 2012; Mavlyutov *et al.*, 2015; Couly *et al.*, 2020; Herrando-Grabulosa *et al.*, 2021) have been some of the other CNS disorders for which sigma-1 receptor has been proposed to play a role.

Sigma-1 receptors and depression

Current treatments for depression and depressive-like states belong to several pharmacological classes. The efficacy of such treatments is variable, with high level of non-responders and a long-lasting delay for reaching a significant improvement. The most widely use therapeutic class of drugs are the non-selective tricyclic antidepressants (TCA), like imipramine, desipramine, and amitriptyline, the selective serotonin-reuptake inhibitors (SSRI), fluoxetine, fluvoxamine, or sertraline, and dual noradrenaline-serotonin reuptake inhibitors (SNRI), like duloxetine or venlafaxine.

The development of new drugs with different mechanisms of action that improve the efficacy, the onset of the action, and with a better side-effects profile is a clear unmet need.

Sigma-1 receptor has been involved in the antidepressant effects of several types of drugs. Sigma-1 agonists like (+)-pentazocine or (+)-SKF-10.047 were active decreasing the immobility time in widely used models of depression in rodents, like the forced swimming test and in the tail suspension test (Matsuno *et al.*, 1996; (Ukai *et al.*, 1998; Urani *et al.*, 2001). Other compounds developed as sigma-1 agonist showed antidepressant-like activity in pre-clinical tests, such as OPC-14523 (Oshiro *et al.*, 2000; Tottori *et al.*, 2001) or igmesine (JO 1784) (Matsuno *et al.*, 1996), which decreased the immobility time in the forced swimming test in rats and mice. It has been shown that the antidepressant-like activity of igmesine, a sigma-1 receptor agonist, requires modulation of intracellular calcium mobilization (Urani *et al.*, 2002). Neurosteroids were suggested to play a role on depressive states through the sigma-1 receptor (van Broekhoven and Verkes, 2003; Maurice *et al.*, 2006; Guo *et al.*, 2020; Sałaciak and Pitka, 2021). Interestingly, several antidepressants have affinity for sigma-1 receptor (Table. 2) (Narita *et al.*, 1996; (Ishima *et al.*, 2014)) and it has been proposed to play a role in their antidepressant activity (Takebayashi et al., 2004; Villard *et al.*, 2011; Sugimoto *et al.*, 2012).

Table 2. Sigma-1 affinities and functionality of several antidepressants.

Antidepressant	**Ki (nM)**	
	Sigma-1	**Functionality**
Selective Serotonin Reuptake Inhibitors		
Fluvoxamine	36[1] - 17[2]	Agonist
Sertraline	57[1] - 31.6[2]	Putative antagonist or inverse agonist
Fluoxetine	240[1] - 191.2[2]	Putative agonist
Citalopram	292[1] - 403.8[2]	Agonist
Paroxetine	1893[1]	Antagonist?[3]
Tricyclic antidepressants		
Imipramine	343[1] - 332.1[2]	Agonist
Desipramine	1987[1]	Antagonist?[4]

[1] Narita *et al.*, 1996; [2] Ishima *et al.*, 2014. [3]Nishimura et al., 2008; [4]Dhir and Kulkarni, 2008.

The monoamine hypothesis of depression, based on the efficacy of antidepressant drugs increasing the serotonin levels in the synapsis, has some limitations, and several other mechanisms of action have been described to play a role. Neurotrophic factors like BDNF or NGF (Duman *et al.*, 2019; Jaggar *et al.*, 2019), lipid peroxidation processes (Sowa-Kucma *et al.*, 2018; Zhang *et al.*, 2018), or the involvement of the glutamatergic system (Sanacora *et al.*, 2012), are some examples of mechanisms acting through pathways other than the serotonergic or the noradrenergic ones. Sigma-1 receptor has been described to interact with a long list of proteins (see above), including the pathways involved in the new hypothesis of depression.

Moreover, some clinical studies on sigma-1 receptor gene have revealed the association of some polymorphisms of the gene with major or bipolar depressive disorders in some population (Kishi *et al.*, 2010; Ikeda *et al.*, 2018).

Sigma-1 receptors and addiction

Early studies on sigma-1 receptor already suggested a dopaminergic involvement in sigma-1 compound effects (Martin *et al.*, 1976). Later, the affinity of cocaine for sigma-1 and its involvement in the actions of cocaine was described (Sharkey *et al.*, 1988; Ritz and George, 1993; Matsumoto *et al.*, 2001; Maurice *et al.* 2002). Moreover, the locomotor activity effects of cocaine were antagonised with sigma-1 antagonists like BMY-14802 or BD-1063 (Menkel *et al.*, 1991; McCraken *et al.*, 1999; Matsumoto *et al.*, 2001). Other effects, like behavioural sensitization induced by cocaine (Ujike *et al.*, 1996)) or the conditioned place preference were modified by sigma-1 antagonists (Romieu *et al.*, 2000). The use of sigma-1 antisense oligodeoxynucleotides further supports the involvement of sigma-1 receptors in cocaine effects (Matsumoto *et al.*, 2001; Matsumoto *et al.*, 2002). Taken together, there is a huge amount of data associating sigma-1 receptor with short- and long-term effects of cocaine. In addition, due to its relationship with the dopaminergic system, a role in the effects of other psychostimulant drugs has been suggested. Changes in sigma-1 binding sites after methamphetamine treatment (Itzhak,

1993), blockade of methamphetamine-induced sensitization (Ujike *et al.*, 1992) or antagonism of d-amphetamine-induced hyperactivity (Clissold *et al.*, 1993) have been reported.

Sigma-1 receptor knock-out rodents

Sigma-1 receptor KO mice are available through EMMA repository (https://www.infrafrontier.eu/search?keyword=sigma&category=strains) A high number of studies have been performed with those mice in a long list of different therapeutic fields, and using pharmacological, behavioural, physiological, electrophysiological, and other technical approaches (Nieto *et al.*, 2012; Valenza *et al.*, 2016; Snyder *et al.*, 2016; Castany *et al.*, 2018; Liu *et al.*, 2018; Bravo-Caparrós *et al.*, 2019; Crouzier *et al.*, 2020; López-Estévez *et al.*, 2021; Wang *et al.*, 2021). The studies on KO mice have been highly useful for profiling the sigma-1 receptor mediated effects of drugs, or the role of sigma-1 receptor in several diseases and pathological processes. However, mouse is just an animal model, and as such, it has the limitations when translating the observations to a human situation.

Differences in receptor distribution, physiology or pharmacology of mouse, rat, and human have been reported (Cunningham, 2002; Hirst *et al.*, 2003; Hok *et al.*, 2016; Netser *et al.*, 2020). For this reason, pharmaceutical research requires to assure the observations in more than one species. In fact, for regulatory purposes, studies should be done in rodent and non-rodent species. In early drug discovery, non-rodent species are not an alternative. Therefore, for lead finding and profiling, the preferred option for confirming results obtained in mice is the rat. No sigma-1 KO rats are available to our knowledge. Thus, we have had access to a unique tool to further investigate the role of sigma-1 receptor in several physiological and pathological processes, including depression and addiction.

OBJECTIVES

The overall objective of this PhD thesis was to **characterise the physiological and behavioural phenotype of sigma-1 KO rodents**, with a specific interest on the involvement of this receptor in the development and potential treatment of depression and addiction. The specific objectives to meet this overall objective were:

1. Behavioural phenotyping of sigma-1 KO mice in the tail suspension test and the forced swimming test, as measures of the potential involvement of the sigma-1 receptor in the development of depression.

2. Use of antidepressants for assessing the involvement of sigma-1 receptor on the pharmacological treatment of depression in mice.

3. Phenotypic profiling of sigma-1 KO rats, at physiological level. Use standard test for the assessment of the motor and sensory function at behavioural level.

4. Behavioural phenotyping of sigma-1 KO rats in the forced swimming test, as measure of the potential involvement of the sigma-1 receptor in the development of depression.

5. Use of antidepressants for assessing the involvement of sigma-1 receptor on the pharmacological treatment of depression in rats.

6. Behavioural phenotyping of sigma-1 KO rats in a test of locomotor activity, after pharmacological treatment with drugs active on the dopaminergic system, as a measure of the potential role in addiction.

7. Study of the mRNA expression levels of the dopamine, serotonin, and noradrenaline transporters in the brain, as relevant proteins involved in depression and addiction.

METHODS

Ethics

All experimental procedures and animal husbandry were conducted according to ethical principles for the evaluation of pain in conscious animals (Zimmermann 1983), and they were approved by the Committee on Animal Research Ethics of Parc Científic of Barcelona. Moreover, it was authorised by the Animal Research Commission in agreement with the established in law 5/1995, 21st June, developed by Decree 214/1997, 30th July (Generalitat de Catalunya, Spain), the European Communities Council Directive, 22nd September 2010, and Royal Decree 53/2013, 1st February (Spain). At the end of the experiments, rats were euthanized by exposure to a CO2 saturated environment. All effort was taken to minimize the number of animals used and their suffering.

Animals

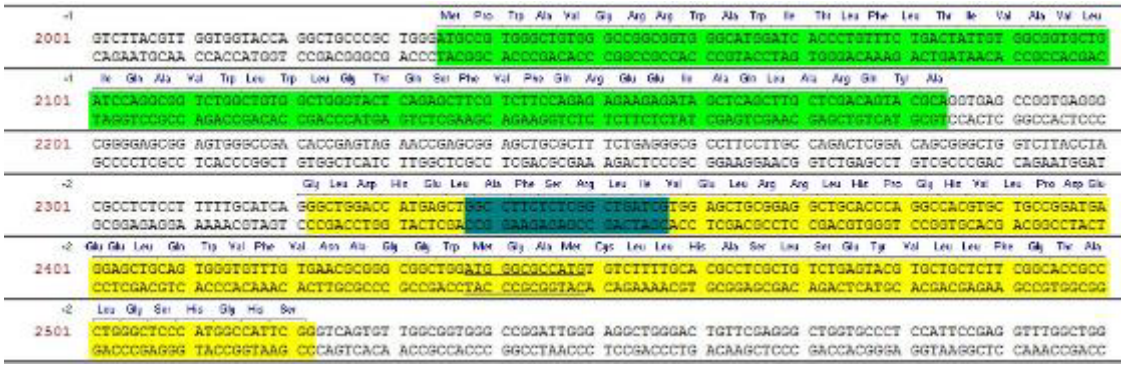
Male C57BL/6J WT mice and male C57BL/6J/129Sv KO mice 10-14 weeks old from Envigo were used for the Tail Suspension Test (TST), and the Forced Swimming Test (FST). C57BL/6J/129Sv mice were established by mating germ-line transmitting chimeras ([129Svx1/SvJ x 129S1/Sv]F1) with C57BL/6J females, resulting in mixed 129SvxC57BL/6J genetic background mice (Langa *et al.*, 2003).

Using CRISPR/Cas9 technology (Doudna and Charpentier, 2014) the company HORIZON created a Sigma-1 receptor knockout rat in the Wistar background. Subsequent colony generation and breeding was performed at Envigo. CRISPR/Cas9 technology enable

targeted mutagenesis in single cell embryos directly, completely bypassing the culture and manipulation of embryonic stem (ES) cells. Briefly, CRISPR/Cas9 technology is adapted from the pathogen, *Streptococcus pyogenes*, and belongs to the CRISPR/Cas9 system that provide immunity to bacteria and archaea against invading phage or plasmid DNA. An active CRISPR/Cas9 complex contains two components: a single guide RNA (sgRNA) designed to be partially complementary to one strand of a DNA target, and Cas9 protein providing nuclease activity. The sgRNA/CRISPR/Cas9 complex binds and cleaves specific DNA sequences in a given genome, generating double strand breaks. A cell then repairs these double strand breaks, generating a fully operative DNA with a deletion on the chose gene.

The WT DNA sequence of the sigma-1 receptor is shown in Figure 6, with the two exon sequences and the site for the sgRNA.

Figure 6. Sigma-1 **wild-type** genomic sequence. Exon1 (green), and Exon2 (yelow) which includes the sgRNA target site (teal)



The selected sigma-1 receptor deletion sequences of 218bp and 7bp obtained are shown in Figure 7 and 8. The sgRNA target site in the exon-2 produced a 218bp deletion involving 46bp of exon-1 and 34 bp of exon-2, in addition to 137bp of an intron. The 7bp deletion only involved exon-2.

Figure7. 218bp deletion (blue) Sigma-1 knock-out genomic sequence. Exon1 (green), and Exon2 (yelow), and the rest of the sgRNA target site (teal).

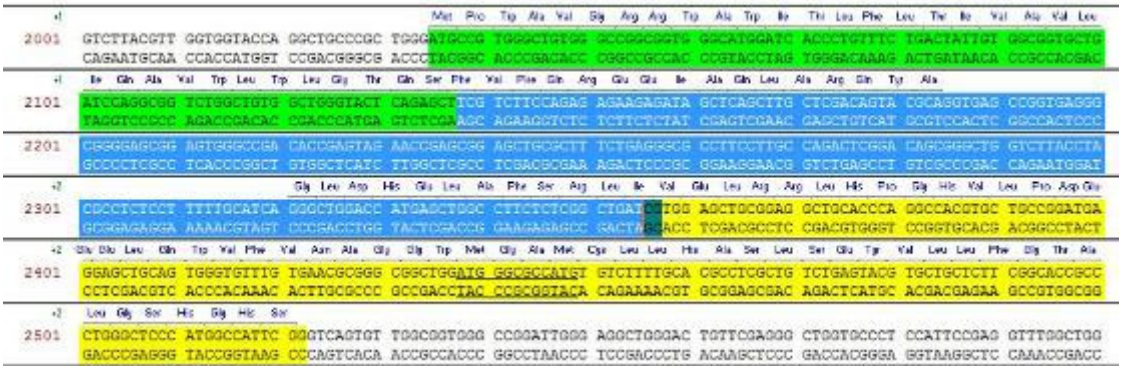
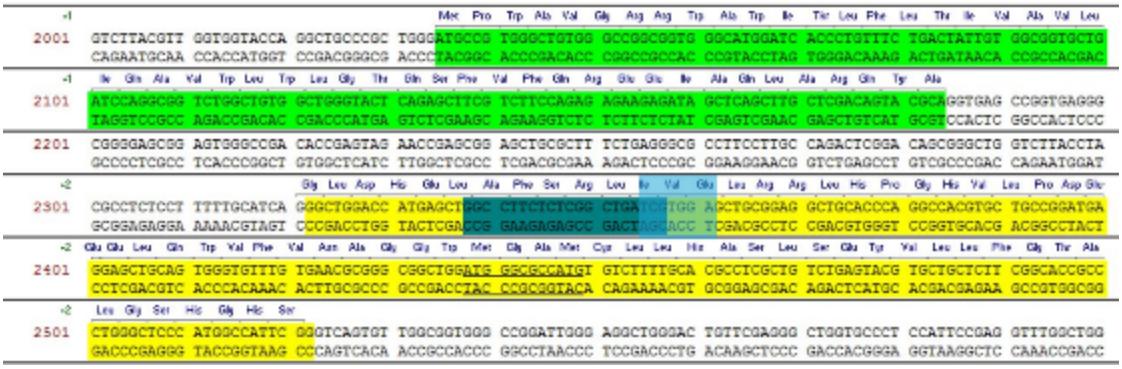


Figure8. 7bp deletion (blue box) Sigma-1 knock-out genomic sequence. Exon1 (green), and Exon2 (yelow), and the rest of the sgRNA target site (teal).



Male WT Wistar rats (Charles-River) and male KO rats (Envigo) were used for strain comparison experiments, and for FST and Locomotor activity (in this later experiments, female rats were also used). In the heterozygous line phenotyping WT and KO (heterozygous and homozygous) rats were obtained from Envigo. All the WT rats were matched in age with their heterozygous or homozygous counterparts.

Drugs

- Apomorphine hydrochloride (CAS Number: 41372-20-7) from Esteve Química
- BD-1063 dihydrochloride (CAS Number: 206996-13-6) from TOCRIS
- Cocaine hydrochloride (CAS Number: 53-21-4) from Johnson Matthey
- d-Amphetamine sulphate (CAS 51-63-8) from SIGMA-ALDRICH
- Sertraline hydrochloride (CAS Number: 79559-97-0) from CHEMOS
- Fluoxetine hydrochloride (CAS Number: 56296-78-7) from TOCRIS
- Fluvoxamine maleate (CAS Number: 61718-82-9) from TOCRIS
- Venlafaxine hydrochloride (CAS Number: 99300-78-4) from Esteve Química

Doses of all drugs are expressed as the corresponding salt, except for FST test in rats where doses of antidepressant drugs are expressed as free base. Correction factors were 1.1., 1.13, and 1.36 for fluoxetine, venlafaxine, and fluvoxamine, respectively.

Reagents

- (Hydroxypropyl)methyl cellulose (HPMC) CAS Number: 9004-65-3 (Sigma-Aldrich Ref.: H8384).
- Anti-Sigma Receptor Antibody (B-5) mouse monoclonal IgG1 κ (Santa Cruz Biotechnology, Inc. Ref.: SC-137075).
- Anti-Sigma Receptor Antibody (F-5) mouse monoclonal IgG2b κ (Santa Cruz Biotechnology, Inc. Ref.: SC-166392)
- Formalin (Formaldehyde solution 37%; formaldehyde CAS Number: 50-00-0 (Sigma Aldrich, Ref.: HT501128)
- RIPA Buffer (Sigma Aldrich Ref.: R0278)
- Protease inhibitor (Sigma Aldrich Ref.: P8340)

- Phosphatase inhibitor (Sigma Aldrich Ref.: P5726)
- Protein Standard Assay (BioRad)
- Reagent A (Biorad Ref: 500-0113)
- Reagent B (Biorad Ref: 500-0114)
- Laemmli Sample Buffer 2x (Biorad, Ref: 161-0737)
- 2-Mercaptoethanol (Sigma Aldrich, Ref.: L6250)
- Sodium dodecyl sulfate (Sigma Aldrich, Ref.: L4509)
- Acrylamide/Bis-acrylamide, 30% solution (Sigma Aldrich, Ref.: A3574)
- 10xTGS (10x Tris/Glycine/SDS, Biorad, Ref.: 161-0772).
- Precision Plus Protein™ Dual Color Standards, Biorad Ref.: 161-0374)
- TTBS (Tween-Tris-buffered saline) (Sigma Aldrich Ref.: 91414).
- Immun-Blot® PVDF Membrane (Biorad Ref.: 162-0177).
- 10x Tris/Glycine Buffer (Biorad Ref.: 162-0771)
- Non-fat milk (Biorad Ref.: 1706404).
- MultiScreen filter 96-well plate GFC (Millipore, Ref.: MSFCN6B50)
- Haloperidol (Sigma-Aldrich, Ref.: H-1512)
- [^{3}H](+)-pentazocine (NEN, Ref.: NET-1056)
- Polyethylenimine (Sigma-Aldrich, Ref.: P.3143)
- Dimethyl sulfoxide (DMSO) (Carlo Erba, Ref.: 444926)
- Liquid scintillation cocktail Ecoscint H (National Diagnostics, Ref.: LS-275)
- Liquid scintillation cocktail Ultima Gold (PerkinElmer, Ref.: 6013329)
- Tris(hydroxymethyl)methylamine (Merck, Ref.: 8382)
- Sucrose (Sigma-Aldrich, Ref.: S9378)
- Kit for the determination of total protein (Sigma-Aldrich, Ref.: P-5656)

IRWIN

The used methodology was a modification of the protocol described by Samuel Irwin (Irwin, 1962; Mathiasen and Moser, 2018; Redfern *et al.*, 2019), including a systematic and quantitative methodology for measuring the behavioural changes and/or the physiological modifications produced by chemical entities in rodents.

Before starting the experiments, all the animals were observed and checked for their healthy status, The animals were introduced in makrolon cages (approx. 17x17x17 cm) with wood shavings on the floor, and remained there for at least 1 hour, before the experiment started.

The standard procedure consists in administering the animals with vehicle or the substance to be evaluated, and then proceed with the scoring or measurement of the different parameters at several time points. In this study, we only performed the assessment of the different parameters at time 0, after the acclimatization period, without any administration.

The evaluated parameters were:

CNS Depression: passivity, postration and sedation; ataxia; palpebral ptosis; reflexes reduction; corneal, pupilar, auricular (ear) and righting reflex; muscular tone reduction; hypnosis; hypothermia; catalepsy.

CNS Stimulation: excitation; stereotypy; exophtalmia; tremors; muscular tone increase; convulsions (clonic or tonic); hyperthermia; straub tail.

PNS: lacrimation and salivation; diarrhoea or urination; myosis or mydriasis; piloerection.

Toxicity: lethality

Any other parameters not described above: e.g. coloured urine, dyspnea, corneal opacity, priapism, hyperemia, ischaemia, etc… were also annotated.

The score for each parameter, except for body temperature and pupil diameter, was semi-quantitative and the values were:

value	Effect detected
0	Absent
1	Light or doubtful
2	Clear
3	Intense

Rectal temperature was measured with a electronic thermometer (Cibertec, Mod. CITER) attached to a rectal probe.

Mydriasis or myosis, was assessed measuring the pupil diameter using USB Digital Microscope with LED dimming control, connected to a computer. In the screen of the computer a transparency with a scale in tenths of a millimeter was attached.

The mean of each score obtained for each parameter and animal evaluated was obtained, and then also the mean and the % of activity for each treatment group and for each parameter.

Rotarod

The rotarod test (Dunham and Miya, 1957; Curzon *et al.*, 2009) is a standard procedure for measuring coordination. A standard rotarod apparatus for rats (Rota-Rod / RS LE8500, Panlab S.L.U.) was used.

On Phase 1. rats were trained to stay in the rod for 240 sec. At a constant r.p.m. of 10. Number of falls were recorded. Any animal that did not learn to stay moving on the rod for 240 s was discarded for the study. On phase

II, just after the last animal in a group of 4 finalise phase I, rats were placed again on the rod for recording the time on equilibrium on an acceleration speed procedure. The rod constantly increased the r.p.m. from 0 up to 40r.p.m., that is, 1r.p.m every 1.5 seconds. The latency to fall was recorded.

Open field

We used a modified version of the already described procedure (Ramos *et al.*, 1997). The open field arena (70x70x30 cm) was made on white plexiglass material. The activity of the rats was measure by means of a video tracking system (SMART) that allowed to virtually divide the open field in several areas (centre, wide-centre, side-walls and corners for a refined analysis of the animal trajectory. This trajectory was captured by a digital camera and analysed by the SMART software (Panlab, Barcelona), allowing to obtain several parameters (permanence time, speed, transitions, in a global manner or for each area, and as absolute values or percentage. The exploration time was 10 min, and the studies were performed under attenuated light conditions (<200 lux).

Startle response and Pre-pulse inhibition

Four SR-LAB startle boxes (San Diego Instruments) were used. SR-LAB boxes are sound attenuated and provided with a ventilation fan, light, and viewing lenses. A complete sound system generates white noise and software-controlled sound stimuli for measuring startle response and pre-pulse

inhibition. Animal adjustable enclosures allows free movements, which are monitored with a closely coupled accelerometer sensor underneath.

We used a standard procedure (Swerdlow *et al.*, 1992). Briefly, after 5 min acclimation period in the startle chamber (background noise of 65 dB), rats were exposed to a sequence of combined stimuli: Pulse alone (120dB), no-stimulus (only background), and 3 pre-pulse intensities of 3, 6 and 12dB followed, after 100ms, by the 120dB pulse. In a session, each 5 types of stimuli were presented 10 times in a pseudorandom order. The time between the different stimuli was also variable, 15 s in average. The SR-LAB system is associated to a PC with a software that registers the intensity of the startle response under each stimulus condition.

Locomotor activity

Eight Linton AM548 Standard (Dual Layer) X, Y, Z IR Activity Monitor (Linton Instrumentation), provided with infrared beams, together with transparent polycarbonate Tecniplast rat cages, model 2154F, measuring 482 x 267 x 210mm were used. The top metal grid was not used, to allow rats to perform rearing in full extension. Each system was provided with two level of beams that allows to measure forward locomotion (bottom level) and rearing activity (upper level). These two parameters were recorded as mobile time, and rearing time, both in seconds.

In the studies comparing 218bp and 7bp deletions, and the drug-induced hyperactivity, locomotor activity (mobile time) and rearing (rearing time) were recorded for one hour at 5min intervals. Drugs were administered immediately before locomotion recording.

In the heterozygous line phenotyping, locomotor activity was measured for 24 hours at 30min intervals. These experiments were performed adding a running wheel on each cage, making not possible to measure rearing activity and blocking the measurement of locomotor activity in central area of the cage, due to the interference of the lower part of the wheel.

Running wheels

Eight Makrolon® cages provided with stainless steel wheels were used (Ugo Basile). Data is collected by connecting the wheels to a PC, through a Multifunction Interface. ANYmaze software (Ugo Basile) collected rotations every 30min for a total 24h period.

The cages were placed in the Activity Monitors for measuring running wheel and locomotion at the same time, with the limitations described above. In this test we only used females because males were too big. Experiments were performed from 9:00p.m. to 9:00a.m.

Pain sensitivity

The four pain models used were spaced one week, and testing order was decided from the minor stressing one (von Frey filaments) up to the one administering chemicals into the rat (formalin test).

MECHANICAL SENSITIVITY – VON FREY FILAMENTS

Mechanical sensitivity was quantified as previously described (Chaplan *et al.,* 1994). To assess pain sensitivity, rats were placed on a metal grid in a transparent methacrylate cylinder (200mm diameter, 300mm high, 3mm thick) then allowed to acclimate to their new environment, at least 15min before testing. Tactile sensitivity was assessed by determination of the paw withdrawal threshold (PWT) to von Frey filaments stimulation, starting 1 to 26 grams, on the plantar surface of one hind paw (Touch-Test von Frey monofilaments, North Coast Medical, Inc., Morgan Hill, CA, USA). Each filament was applied 3s until a withdrawal response occurred. A single response indicated a positive response.

MECHANICAL SENSITIVITY – PAW PRESSURE TEST

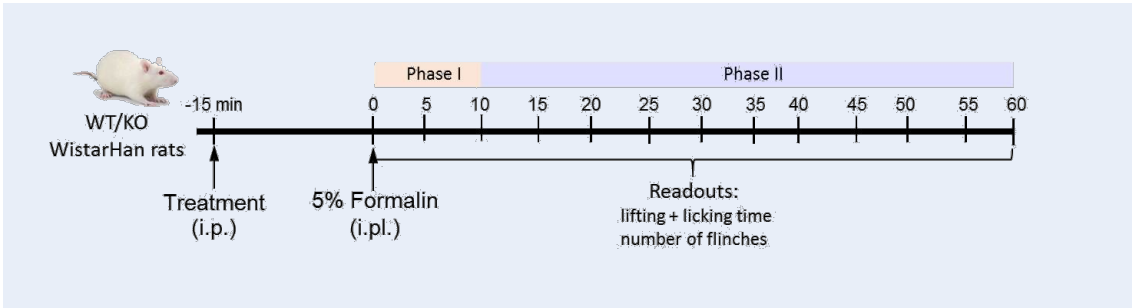
The test was performed as previously described by Randall and Selitto (1957). Briefly, the animals were gently restrained with a cloth and an increasing mechanical nociceptive stimulus using a cone-shaped paw-presser with a rounded tip (Analgesy-meter, Ugo-Basile) was applied to the dorsal surface of the right hind paw. The paw pressure was defined as the pressure at which the rat voluntarily withdrew its hindpaw. The test was done twice at an interval of 1 min between each stimulation with a 1000 g cut-off to avoid tissue injury.

THERMAL SENSITIVITY - TAIL FLICK TEST

The test was performed as previously described by D'Amour and Smith (1941). Briefly, the animals were gently restrained with a cloth to orient their tails toward the source of heat of the tail-flick apparatus (Panlab, LE 7106, Spain). A noxious beam of light was focussed on the tail about 5 cm from the tip, and the tail-flick latency (TFL, latency to remove the tail as of the onset of the radiant heat stimulus) was recorded automatically to the nearest 0.1 s. The intensity of the radiant heat source was adjusted to yield baseline latencies between 2 and 5 s and a cut-off time was set at 10 s to avoid heat-related damage.

CHEMICAL SENSITIVITY - FORMALIN TEST

Following the method described by Dubuisson and Dennis (1977) , diluted formalin was prepared from a stock solution of 37% formaldehyde in water to obtain 5% formalin. 50 µl of 5% formalin were injected into the mid-plantar surface of the right hind paw of the rats. The intraplantar injection was made with a 0.3 ml U-100 Insulin microsyringe (30 G needle). Formalin-induced pain was quantified evaluating the number of flinches and the total lifting+licking time along 1h post-formalin administration. Formalin response follows two phases (phase I and phase II) described in the scheme below:



Tail suspension test

The test was performed as previously described (Steru *et al.*, 1985). Two racks of MED-Associates Tail suspension device were used. Mice were suspended above the ground by their tails using a piece of tape. The system registers, through the sensor attached to the tape, the quantity of movements a mouse does trying to escape during the 6 min session. The software allows to measure the activity (moving/immobility), energy and power of the movements, but for the purposes of these experiments only immobility time was used.

Forced Swimming Test

MICE

The procedure was based on the test previously described (Porsolt *et al.*, 1977). In the fluoxetine experiments, mice were individually forced to swim for 6 min in an open cylindrical container (diameter 15 cm, height 25 cm) containing 20 cm of water, to prevent an animal from touching the bottom of the container with their tail. Water temperature was set at 28±1°C. Once the pre-test session had finished, mice were removed and dried with a towel. Twenty-four hours later, they were placed again in the container for 6 min (test session), and the total duration of immobility was recorded. Resting was set for speed values ≤ 2,5 cm/s, a value that was established during the setting up of the procedure, in comparison with the immobility time measured by an

experimented observer using standard criteria of considering immobility as the absence of any movement beyond those necessary to stay floating.

In the fluvoxamine experiment, training and test time were 1 latency time + 5 min reading, container was 24 cm diameter and 35 depth, with 20 cm water depth. Water temperature was maintained between 23-25 Cº. Moreover, this experiment was previous to scheduled pharmacological antagonism experiments and, therefore, mice received 05% HPMC and 05% HPMC or fluvoxamine 15 min later. The test started 30 min after the second administration.

As we had the 6 min recording split in 1-minute timelapses, for the analysis of the fluoxetine experiments we only use the immobility time from minute 1 to 5, for a better comparison to the fluvoxamine experiment.

RATS

The test was performed as previously described (Porsolt *et al.,* 1978). During the training session rats were individually forced to swim for 15 min in a glass beaker (diameter 22 cm, height 36 cm) containing water at 24±1°C and 22 cm depth. Once the pre-test session had finished, rats were removed and dried with a towel. Twenty-four hours later, they were placed again in the beaker for 5 min (test session), and the total duration of immobility was recorded. In the acute study, in both the training session and the test session immobility and struggling was recorded by an observer using two stopwatches. A rat was judged to be immobile when it ceased struggling and remained floating, making only the necessary movements to keep its head above the water. In the sub-acute experiments, training session (day 1) was recorded with a digital video camera and then analysed with Smart software using the same criteria used in mice. The whole 15 min period was considered. Drugs were administered i.p. every day in the morning, and 30 mins before the test session on days 2, 7, and 14, following the scheme below:

Day 1	Day 2	3	4	5	6	day 7	8	9	10	11	12	13	day 14	day 15
	treatment													
Training	TEST					TEST							TEST	sampling

Growth

For registering the growth of young animals (2-4 months old) rats were weighted once a week, for adult rats (1-1.5 years old) were weighted once a month, and for old rats (2 years old) twice a month, using a Mettler-Toledo precision balance.

Food consumption

In young and old animals, one week for each group was selected for weighting food every day, from Monday to Friday. The difference in the amount of food from one day to the next, was annotated and divided by the number of animals on each cage, for obtaining 24h food consumption per rat.

Polymerase chain reaction (PCR) genotyping

To ensure the proper selection of the genetic background (WT or sigma-1 receptor KO), genotype was periodically studied at the end of the behavioural testing. Genomic deoxyribonucleic acid (DNA) was obtained from tail tips using the DNeasy Blood & Tissue kit (QIAGEN, cat number: 203643) according to the manufacturer's instructions. Amplifications for PCR were performed with HotStarTaq Plus Master Mix Kit (QIAGEN) and with 0.5 µM of each primer (Invitrogen Ltd, Paisley, UK). The PCR was done with a thermal controller using an initial template denaturation at 94ºC, followed by 35 cycles: 30 s at 94 ºC, 20 s at 57 ºC and 30 s at 70 ºC; and, as a final extension step, 10 min at 72 ºC. Two DNA amplifications (set of 3 primers, one repeated) for each sample were done

to ensure genetic background. The oligonucleotide primer (5′– 3′) sequences specific for the genes examined were as follows: 5′-ACG TTG GTG GTA CCA GGC TGC-3′ and 5′-GCA CGT ACT CAG ACA GCG AGG-3′ to detect a DNA fragment of 476bp, 258bp and 469 bp for wild type, 218bp line and 7bp line, respectively. Another pair of primers were used as follows: 5′- ACG TTG GTG GTA CCA GGC TGC -3′ and 5′- AGC CTC CGC AGC TCC ACG-3′ to detect a DNA fragment of 368bp, 150bp for wild type and 218bp line, respectively and not detectable for the 7bp line. This last pair of primers were also used for genotyping the heterozygous line, which was from the 218bp deletion line. Amplified products were analysed by electrophoresis on 4% agarose gel containing ethidium bromide. The gels were then photographed with a UV transilluminator to visualize the ethidium bromide-stained bands.

Protein expression using Western blot

Dissected spinal cord, dorsal root ganglia (DRG) and liver samples from WT, 218bp, and 7bp rats (n=9-10 per group; 4 months old) were homogenized by mechanical disruption in radioimmunoprecipitation assay (RIPA) buffer and the supernatant was obtained.

Equal amounts of protein (50, 20, and 30µg for spinal cord, DRG and liver samples, respectively) were fractionated by 12% (w/v) SDS–PAGE and transferred onto a polyvinylidene difluoride membrane, blocked with 5% non-fat dry milk in Tris–Tween 20-buffered Saline (T–TBS) for 1 h. Membranes were then incubated overnight at 4ºC in 1% non-fat dry milk in T–TBS with the mouse sigma-1 monoclonal antibody (IgG1 κ; SC-137075), and the mouse sigma-1 monoclonal (IgG2b κ, SC-166392) from Santa Cruz Biotechnology, Inc.

The immunoreactive bands were detected by a peroxidase reaction using an enhanced chemiluminescence method (Clarity Western ECL Substrate, Ref.: 170-5061) and ChemiDoc Imaging System (Bio-Rad). Quantification was realized with Image Lab Software (Bio-Rad).

Binding experiments

Membrane preparation

Male from WT, KO 218bp deletion and KO 7bp deletion Wistar rat genotypes were used in all the experiments. Rats were killed by decapitation and brains (minus cerebellum) were rapidly removed and frozen at -80ºC, until use. The frozen rat brains where thawed and then homogenized with 10 strokes, 900 r.p.m. in Potter in about 10 volumes of ice-cold Tris-HCl buffer (10 mM, sucrose 0.32 M, pH 7.4). The homogenate was then centrifuged at 5000 g for 10 min at 4ºC. The obtained supernatant was then centrifuged at 60000 g for 20 min at 4ºC. Finally, the obtained membrane pellet was collected and frozen at -80ºC until use.

Protein concentrations were determined by using the Bradford protein assay (Bradford, 1976) (Kit for the determination of total protein Sigma, Ref.: P-5656).

Binding assay

We used the method described by Cagnotto and colleagues (1994) with minor modifications. The incubation was performed in 96-well flat bottom plates in Tris-HCl 50 mM, pH 8.0 as binding buffer. Each well contained 25 µL of [3H](+)-pentazocine (spec. act. 26.9 Ci/mmol, NEN) at a final concentration of 5 nM, 25 µL of the membrane suspension to a final assay volume of 250 µL (final tissue concentration of approximately 160 µg protein/well). Non-specific binding was defined by addition of a final concentration of 10 µM haloperidol. Plates were incubated at 37ºC for 120 min. 200 µl of the reaction mixture was transferred to a pre-treated 0.5% PEI, Millipore filter 96-well plate (GFC) (Millipore, Ref.: MSFCN6B50), filtered and washed 3-times with buffer solution Tris-HCl 10 mM, pH 7.4. Filters were allowed to dry at 60ºC during 2h and 30 µl of scintillation liquid was dispensed into the wells. Following addition of scintillation cocktail, the samples were allowed to equilibrate overnight. The amount of bound radioactivity was determined by liquid scintillation spectrometry using a Microbeta (PerkinElmer) liquid scintillation counter.

Haematology and Biochemistry

Whole blood and plasma of the 3 different genotypes were obtained from young (2-4 months old) and old rats (around 2 years old). A total of 8 young males and 8 young females of each genotype, and 5 WT, 8 heterozygous, 7 homozygous old female rats were used. Old male rat samples were also obtained but their analysis is pending.

Samples were obtained through the jugular vein in young animals, and by cardiac puncture extraction under isoflurane anaesthesia in old animals. Whole blood was collected in K2-EDTA. For plasma, blood was collected in lithium-heparin tubes and then centrifugated at 2060g, at 4ºC for 10 min. Samples were sent to PCB Animal Facility laboratories for the analysis. For whole blood analysis, Spincell5 (Spinreact™) equipment, a 5-part differential haematology instrument based on the principle of flow cytometry to differentiate white blood cells (WBC) into their five major sub-populations—neutrophils, lymphocytes, monocytes, eosinophils, and basophils—based on cell size and complexity (granularity), was used.

Measurement of cholesterol and triglycerides in plasma was done with the auto-analyser Spinlab100 (Spinreact™).

Plasma biochemistry measurements were performed using a Vetscan V2 analyser (Abaxis).

Data processing and statistical analysis

Data were represented as mean ± S.E.M. The differences between the different experimental groups were analysed using Student's t-test, One-way ANOVA, Two-Way ANOVA, or Two-Way Repeated measures, depending on the test and parameter analysed, and followed by the appropriate comparison test. Data statistical analysis and graphs were done using GraphPad Prism software (v9.0; GraphPad Software, Inc., San Diego, CA, USA). The criterion for statistical significance was set at a p value of less than 0.05.

RESULTS

Mouse

TAIL SUSPENSION TEST

Initially, we performed two experiments without any treatment, for assessing basal WT and KO mice behaviour in this test. In Figure 9 (experiment 1) and Figure 10 (experiment 2), no differences between WT and KO mice during the first or second day were found, although a tendency to a lower immobility for KO mice in the second day was observed. Immobility time during the second day was highly increased in both genotypes, from 107.8±10.6s (exp.1) or 97.8±15.5s (exp.2) up to 178.8±11.2s or 191.7±14.4s, respectively, in WT mice, while for KO mice the increase was from 101.2±10.7s or 112.9±13.7s up to 158.6±14.6s or 167.8±21.0s, respectively.

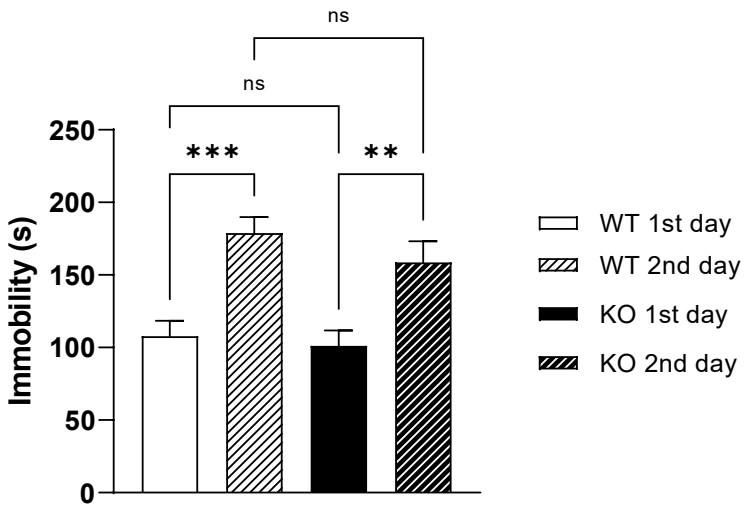


Figure 9. First experiment without any treatment. Immobility time for WT and KO mice during the 1st day (training) and 2nd day (test) session. One-way ANOVA followed by Tukey's Multiple comparisons, ** p<0.01; *** p<0.001

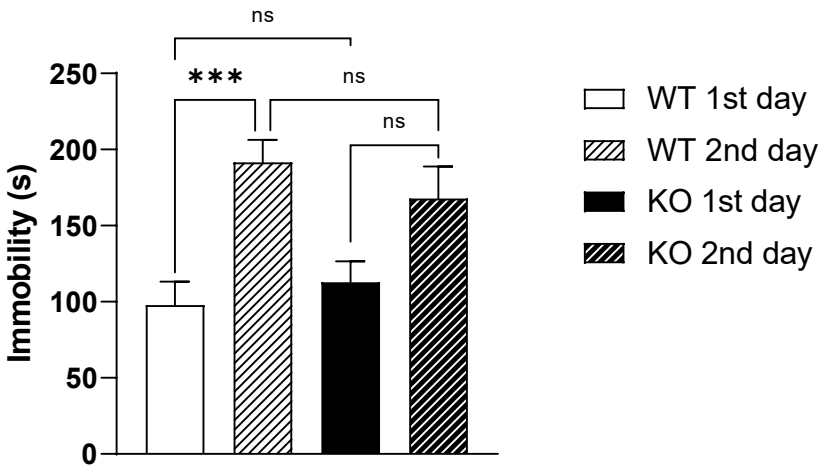


Figure 10. Second experiment without any treatment. Immobility time for WT and KO mice during the 1st day (training) and 2nd day (test) session. One-way ANOVA followed by Tukey's Multiple comparisons, *** p<0.001

In the cumulated data (Figure 11) the increase in immobility time was from 104.5±8.7s to 183.1±8.9s in WT mice (75% increase), and from 105.1±8.4s to 161.7±11.8s in KO animals (53.8% increase). However, despite this lower increase, no differences were found between WT and KO mice in the second day

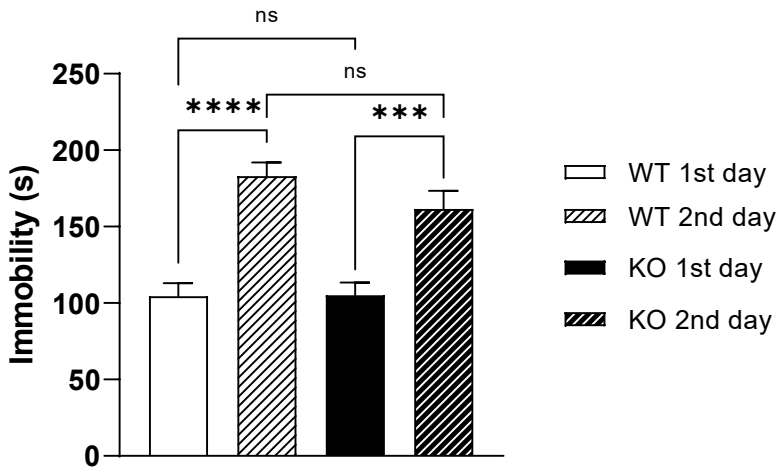


Figure 11. Experiments 1 and 2 without any treatment. Cumulated immobility time for WT and KO mice during the 1st day (training) and 2nd day (test). One-way ANOVA followed by Tukey's Multiple comparisons, ***p<0.001; ****p<0.0001

In the experiments with pharmacological treatment, during the first day of the test (training session), KO mice of the groups treated in the second day with fluoxetine (Figure 12), sertraline (Figure 13) or fluvoxamine (Figure 14), spent less time in immobility than WT mice, although not always it reached statistically significance. Overall (Figure 15), WT mice were immobile for 162.6±6.6s and KO mice 135.6±7.6s ($p<0.0082$).

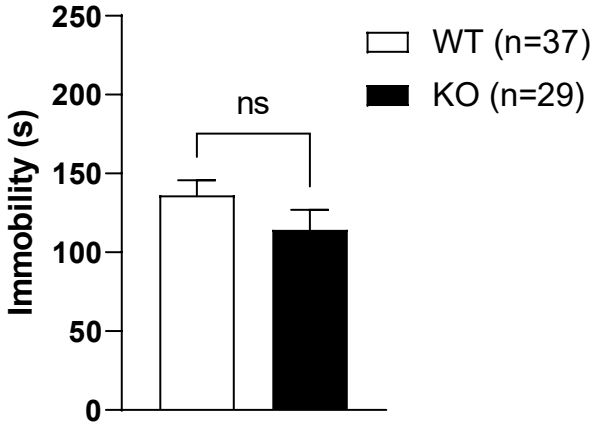


Figure 12. Immobility time during the training session (first day, no treatment) in the group that were later treated with fluoxetine 30 mg/kg, i.p. in the second day (test day). Unpaired t-test.

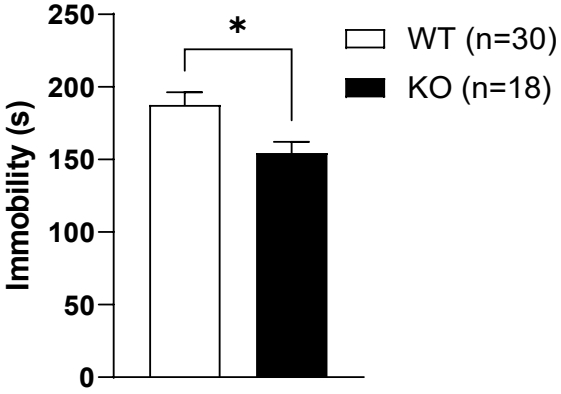


Figure 13. Immobility time during the training session (first day, no treatment) in the groups that were later treated with sertraline 30 and 10 mg/kg, i.p. in the second day (test day). Unpaired t-test * $p<0.05$.

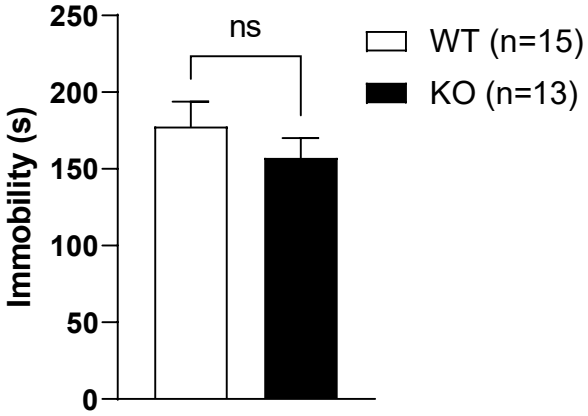


Figure 14. Immobility time during the training session (first day, no treatment) in the group that were later treated with fluvoxamine 10 mg/kg, i.p. Unpaired t-test.

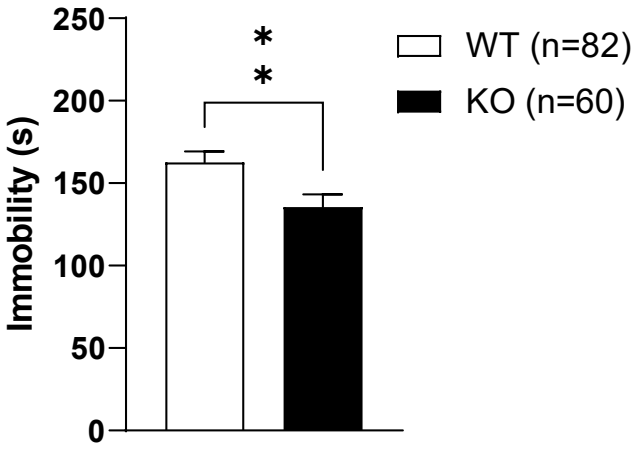


Figure 15. Immobility time during the training session (first day, no treatment) for all the animals used in TST experiments with pharmacological treatment. Unpaired t-test. **p<0.01.

On the test day, although on each experimental group the differences between WT and KO mice treated with saline did not reach statistically significance (p= 0.2671 (Figure 16); p= 0.3161 (Figure 17); p=0.1489 (Figure 18)), the tendency of vehicle-treated KO mice to have higher immobility times was repeatedly shown. In fact, the direct comparison between the WT and KO saline-treated groups using an unpair t-test reveal statistically significant differences in all of them (t-test p= 0.0438 (Figure 16); p= 0.0244 (Figure 17); p=0.0414 (Figure 18)).

The administration of the antidepressant drugs did not induce a significant reduction in the immobility time of WT mice, although a tendency to reduce immobility was observed. Sertraline 10mg/kg (Figure 17) did not induce any effect either in WT or in KO animals. Interestingly, in KO mice all the antidepressants, fluoxetine 30 mg/kg (Figure 16), sertraline 30 mg/kg (Figure 17), and fluvoxamine 10 mg/kg (Figure 18) induced a statistically significant reduction in the immobility time in comparison with saline-treated mice.

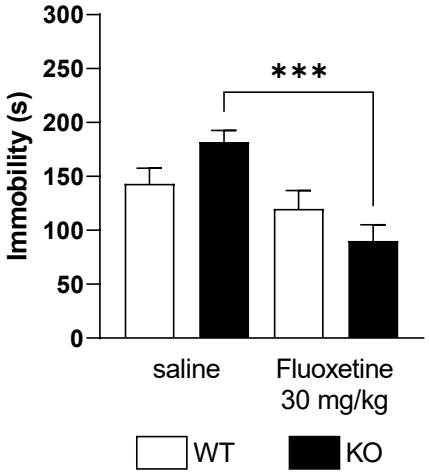


Figure 16. Immobility time during the test session (second day) after treatment with saline or fluoxetine 30 mg/kg, i.p. One-way ANOVA followed by Tukey's Multiple comparisons, *** $p<0.001$

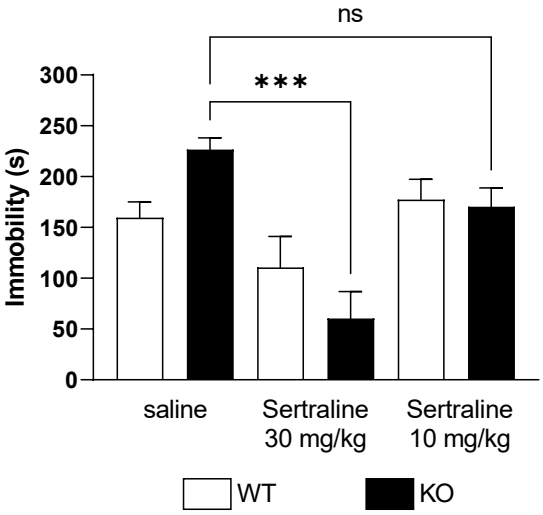


Figure 17. Immobility time during the test session (second day) after treatment with saline or sertraline 10 and 30 mg/kg, i.p. One-way ANOVA followed by Tukey's Multiple comparisons, *** $p<0.001$

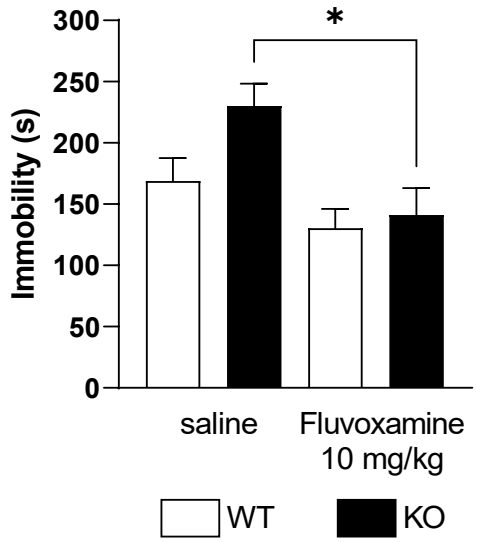


Figure 18. Immobility time during the test session (second day) after treatment with saline or fluvoxamine 10 mg/kg, i.p. One-way ANOVA followed by Tukey's Multiple comparisons, * $p<0.05$

In this widely used model of depression, during the training day (no treatment) KO mice showed a lower immobility time in every single experiment, although statistical significance was hardly obtained due to the test variability.

In four independent fluoxetine experiments (Figures 19, 20, 21, and 22) WT mice showed an immobility time of 230.3±14.1. 214±5.9, 256.1±9.2 and 263.6±11.4 respectively, while KO mice showed 217.9±10.9, 187.1±13.9, 176.2±10.1. and 221.3±16.9, which means a reduction of 5.3%, 12.6%, 31.2%, and 16% versus the corresponding WT controls. Only in Exp. 3 a statistically significant lower immobility time was reached. When grouped (Figure 23), a lower immobility time was shown by KO mice during training day (237.7±5.3 for WT vs 205.4±7.1in KO mice, p=0.0212). Similar profile was shown in the fluvoxamine experiment (Figure 24), despite different experimental conditions were used (24±1ºC vs 28±1ºC used in the fluoxetine experiments).

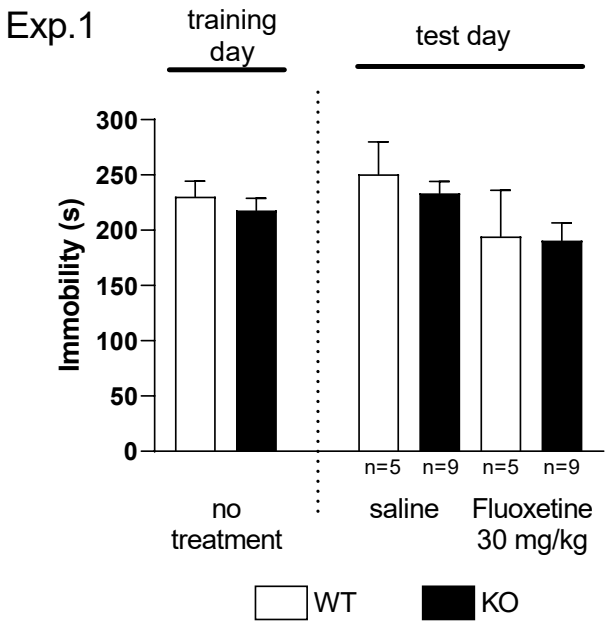


Figure 19. Immobility time during the 5 min training and test sessions for Fluoxetine experiments. Exp.1. ANOVA Multiple comparisons.

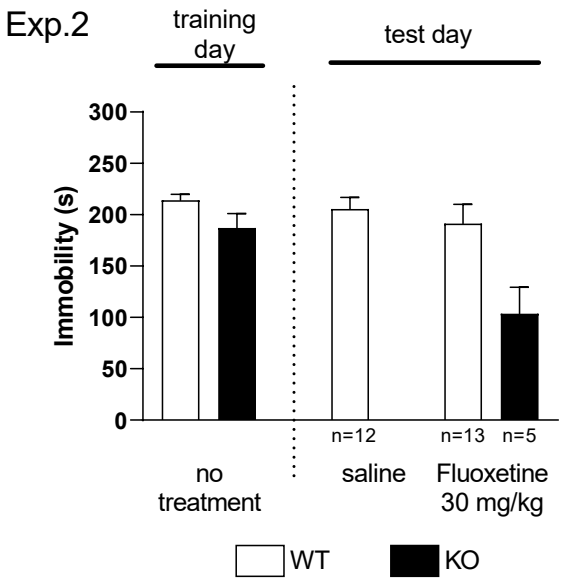


Figure 20. Immobility time during the 5 min training and test sessions for Fluoxetine experiments. Exp.2.
One-way ANOVA Multiple comparisons.

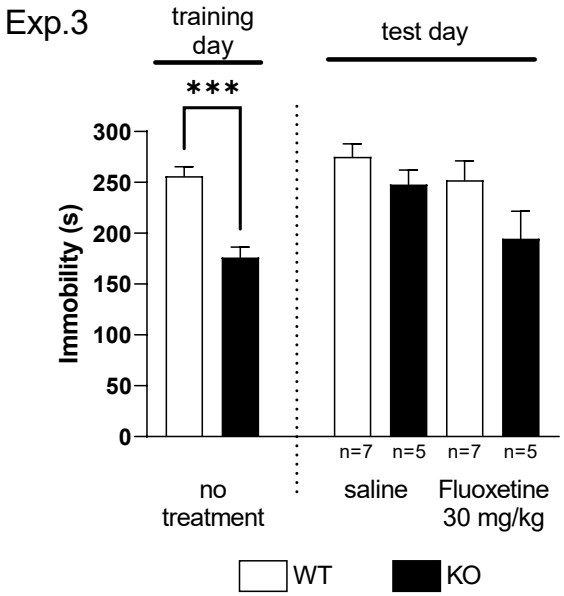


Figure 21. Immobility time during the 5 min training and test sessions for Fluoxetine experiments. Exp.3.
One-way ANOVA Multiple comparisons.
*** $p<0.001$

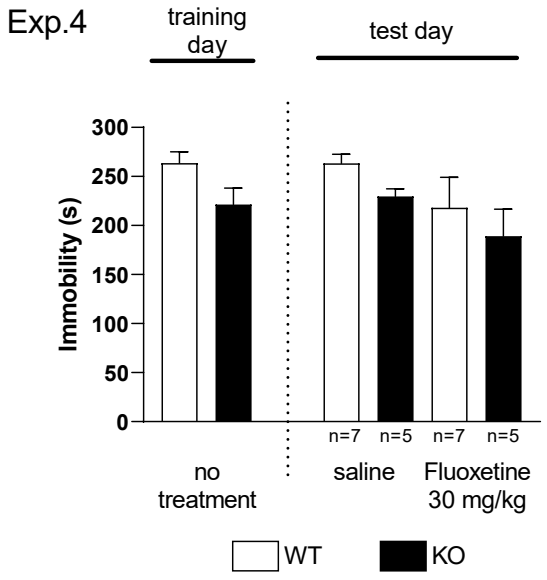


Figure 22. Immobility time during the 5 min training and test sessions for Fluoxetine experiments. Exp.4.
One-way ANOVA Multiple comparisons.

On test day, we did not observe an increase in the immobility of KO animals in comparison to WT after saline treatment, as we did in the TST. Interestingly, there was no statistically significant effect of fluoxetine or fluvoxamine in WT-treated mice but a reduction in the immobility time in KO mice, that was highly significant (P=0.0006) for fluoxetine 30 mg/kg (Figure 23), with KO mice showing an immobility time of 179.3±12.7s vs 241.5±6.3s in WT mice. The effect of fluvoxamine at 10 mg/kg did not reach a statistically significant effect (P=0.2958), although the reduction in the immobility time was from 161.5±26.8s in saline-treated KO mice to 113.5±15.4s in fluvoxamine-treated KO animals (Figure 24).

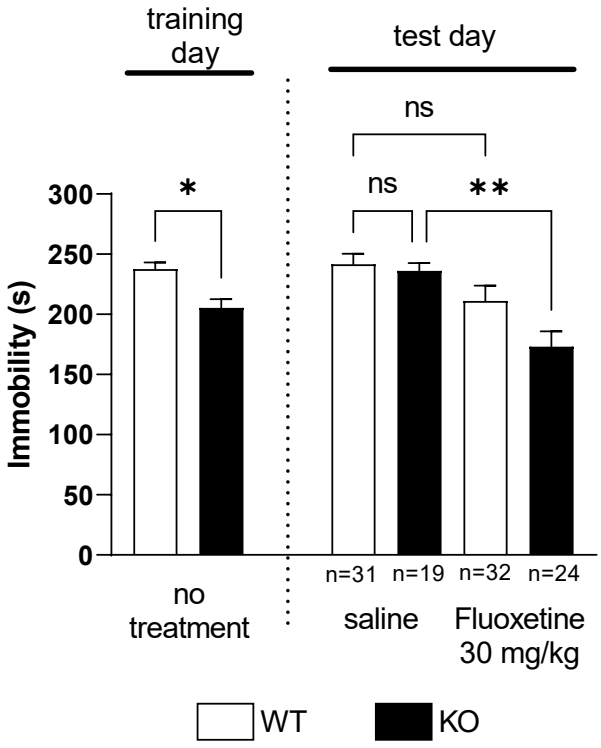


Figure 23. Immobility time during the 5 min training and test sessions for cumulated fluoxetine experiments. A lower immobility time was shown by KO mice during training day, and the reduction induced by fluoxetine was also significant in KO mice on the test day. ANOVA Multiple comparisons, followed by Tukey's test. * p<0.05; ** p<0.01.

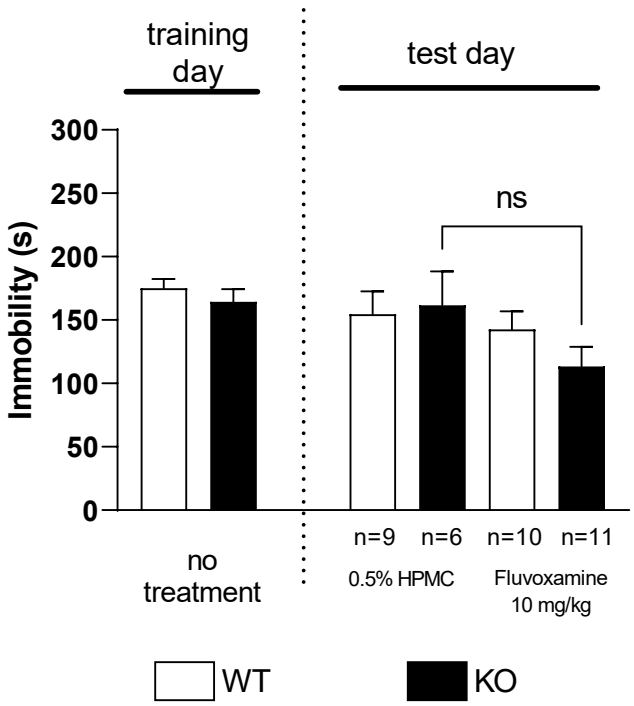


Figure 24. Immobility time during the 5 min training and test sessions for fluvoxamine experiment. A lower immobility time was shown by KO mice during the test session, but no statistical significance was reached. ANOVA Multiple comparisons.

Rat

SIGMA-1 RECEPTOR KO RAT GENERATION

Using CRISPR/Cas9 technology the company HORIZON created a sigma-1 receptor knockout rat in the Wistar background. During the process of knocking-out the sigma-1 receptor gene Horizon obtained two different deletions of the sigma-1 receptor gene, one with a deletion of 218bp and another one of 7bp.

In both strains the absence of the sigma-1 protein was confirmed by PCR using tail samples from females and males used in the strain selection studies. The bands for WT, 218bp line and 7bp line, either in males or females, were clearly detected (Figure 25).

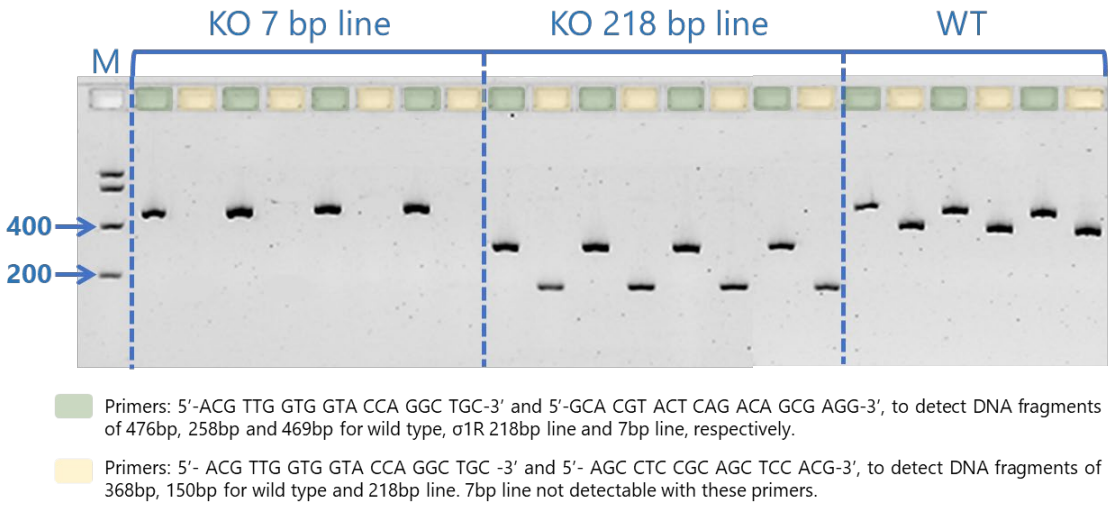


Figure 25. Representative PCR gels obtained for the genotyping of WT and KO rats with 218bp or 7bp deletions.
M= molecular-weight size marker

Genotype confirmation was also performed routinely when using the heterozygous line (Figure 26).

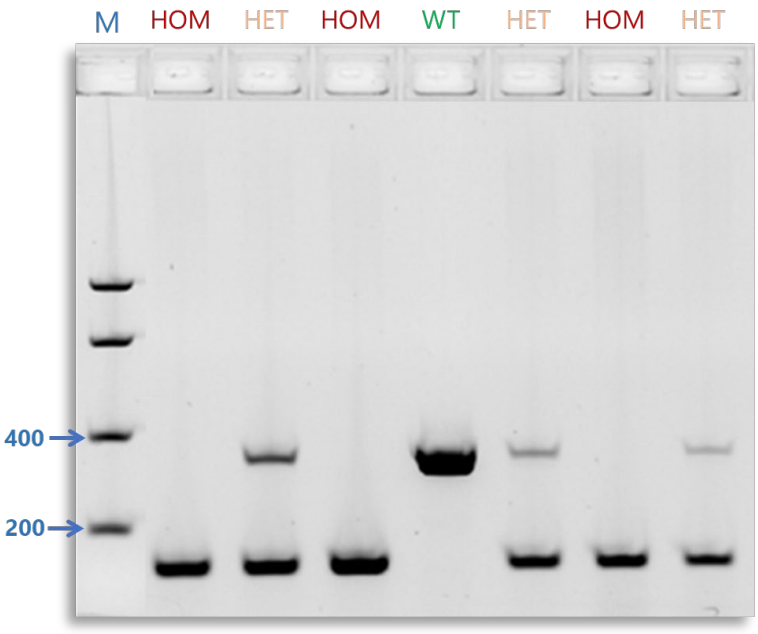


Figure 26. Representative PCR gels obtained for the genotyping of WT, heterozygous (HET), and homozygous (HOM) 218bp KO rats.
M= molecular-weight size marker

We also verified the presence or the absence of the sigma-1 protein by western blot using spinal cord, DRG, and liver samples using monoclonal antibodies raised against sigma-1 receptor (Figure 27).

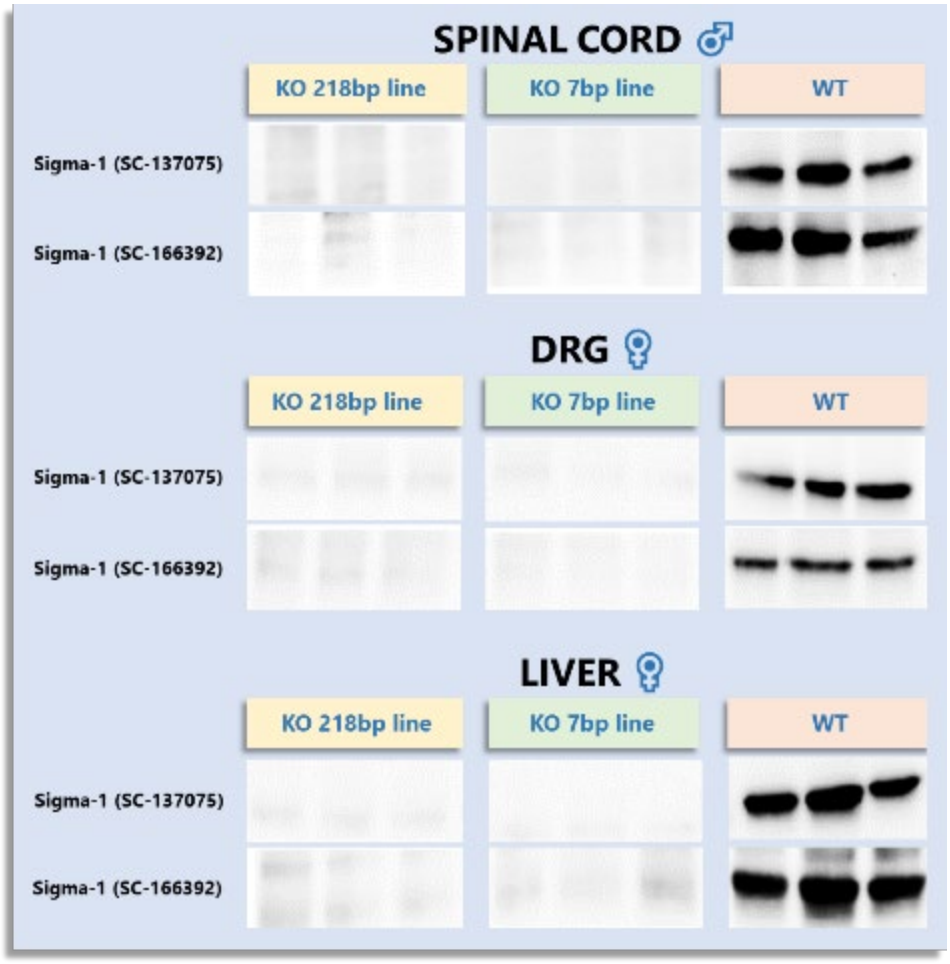


Figure 27. Representative western blots obtained for WT, 218bp, and 7bp rat identification.

Moreover, binding experiments using [^{3}H]-(+)-pentazocine as radioligand showed no specific binding activity in brain membranes in either the 218bp or the 7bp mutants, while in WT rats, [^{3}H]-(+)-pentazocine showed a saturation curve with a calculated Kd of 4nM for [^{3}H]-(+)-pentazocine (Figure 28).

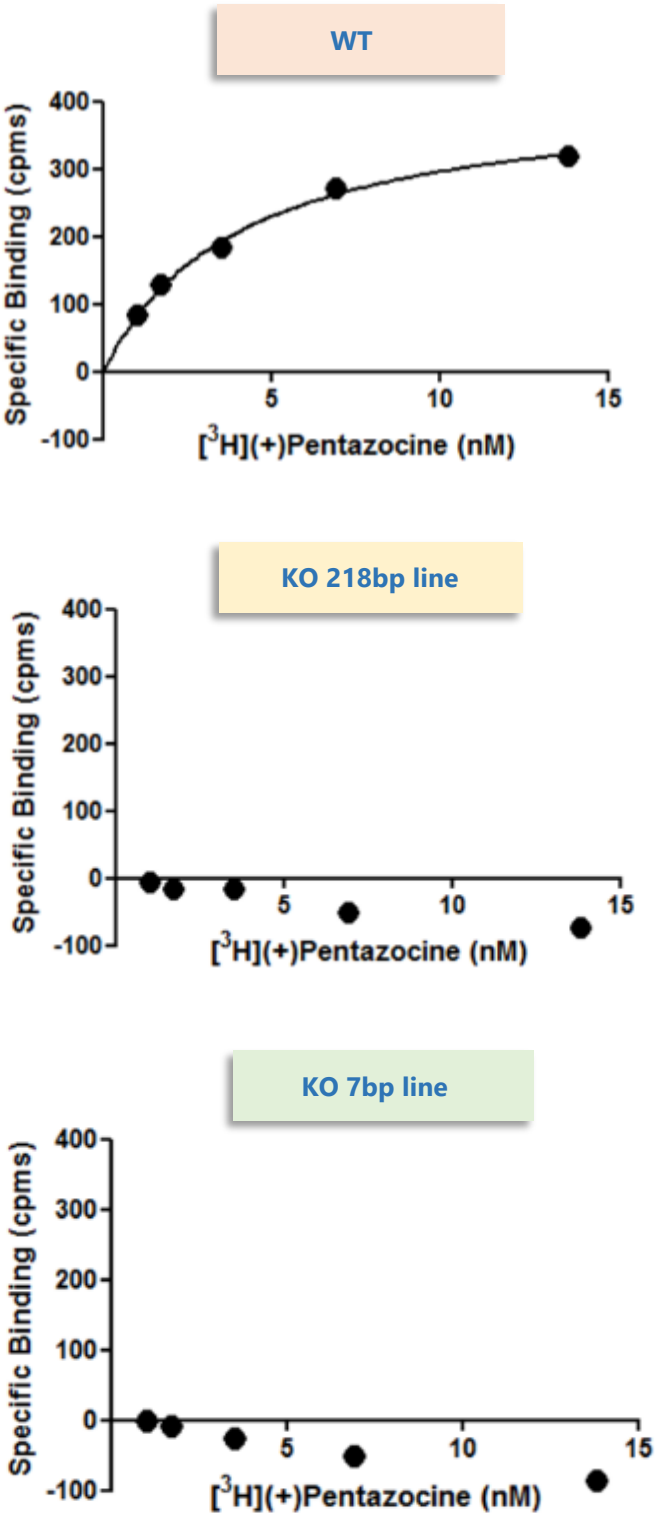


Figure 28. Representative binding curves of radiolabelled sigma-1 ligand [3H]-(+)-Pentazocine in rat brain. Specific Binding = (Total Binding – Total Binding without membrane) – (Non-Specific Binding – Non-Specific Binding without membrane).

STRAIN SELECTION

CRISPR/Cas9 system is widely used technology because is cheap and highly specific, but mutations other than expected can be generated. These off-target effects are random and can influence other genes or regions of the genome. Moreover, the breaks generated by Cas9 cannot be anticipated. Therefore, it was mandatory that the first work was to evaluate the absence of overt differences in comparison to WT genotype that may suggest deletions other than the sigma-1 receptor gene, and to select the best strain for performing the phenotyping.

WT rats (n =10 males and n=10 females), 218bp deletion KO rats (10 males and 9 females) and 7bp deletion KO rats (9 males and 9 females) were used for these experiments in a sequential way. At the beginning of the experiments all the rats were ~4 months old. The criteria for strain selection included:

- Behaviour in the Irwin test and in the open field
- Behaviour in the startle response and pre-pulse inhibition
- Changes in locomotor activity
- Changes in pain sensitivity
- Gender differences

IRWIN

In the functional observation test battery (Irwin's test) no differences in the scored parameters were found, either between genotypes, or between gender within each genotype, except when comparing excitation or vocalisations. In these two parameters, females showed higher values with a score of 2 (clear) in all of them, irrespective of the genotype. In the quantified parameters, a slightly higher rectal temperature was found

in females (Figure 29), irrespective of the genotype. No differences were found in pupil diameter (Figure 30).

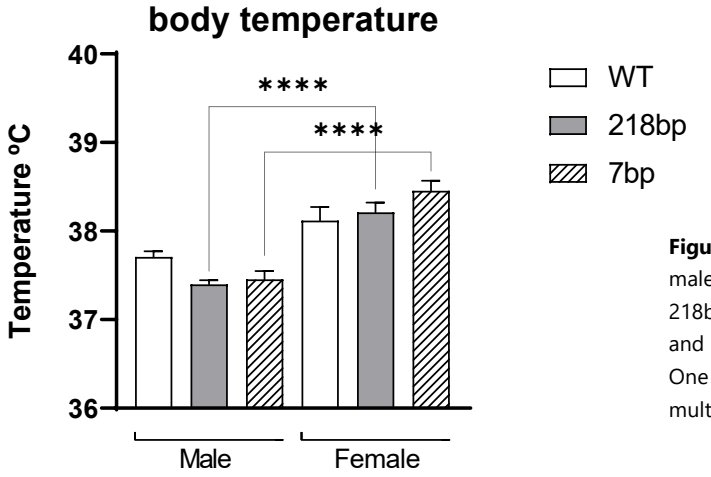


Figure 29. Body temperature of WT (n=10 males and 10 females) and KO rats of the 218bp deletion (n=10 males and 9 females) and 7bp deletion (n=9 males and 9 females). One Way ANOVA. followed by Tukey's multiple comparisons test. ****p<0.0001.

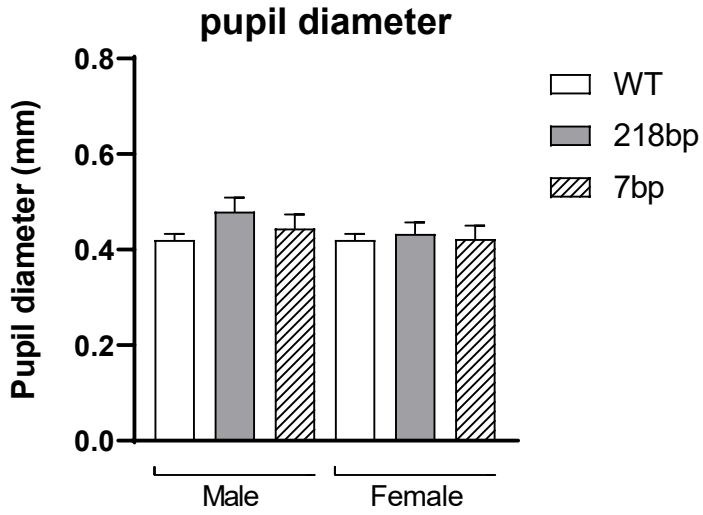


Figure 30. Pupil diameter of WT (n=10 males and 10 females) and KO rats of the 218bp deletion (n=10 males and 9 females) and 7bp deletion (n=9 males and 9 females). One Way ANOVA.

LOCOMOTOR ACTIVITY

The analysis of the time course results in the locomotor activity assessment showed slightly lower mobile times for the 218bp genotype either in males (Figure 31) or females (Figure 32), at the last reading points. Similar results were obtained for the rearing activity (Figures 33 and 34, for males and females, respectively), although for this parameter the

unusual high values of WT males in the last reading points were the main responsible for the statistical significance.

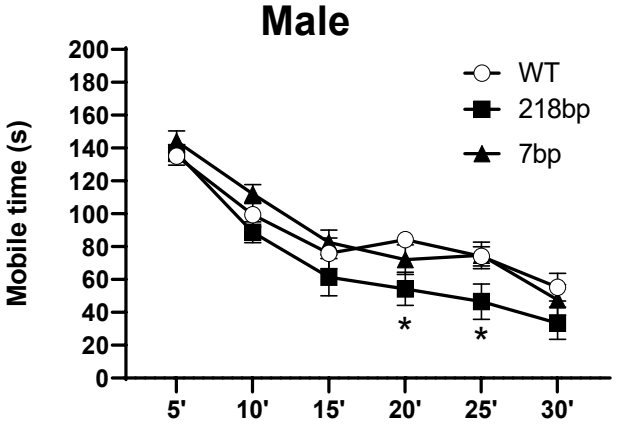


Figure 31. Locomotor activity in male rats. No significant differences were found between genotypes. WT (n=10) and KO rats of the 218bp deletion (n=10) and 7bp deletion (n=9). Two-way ANOVA followed by Tukey's multiple comparisons test. *p<0.05.

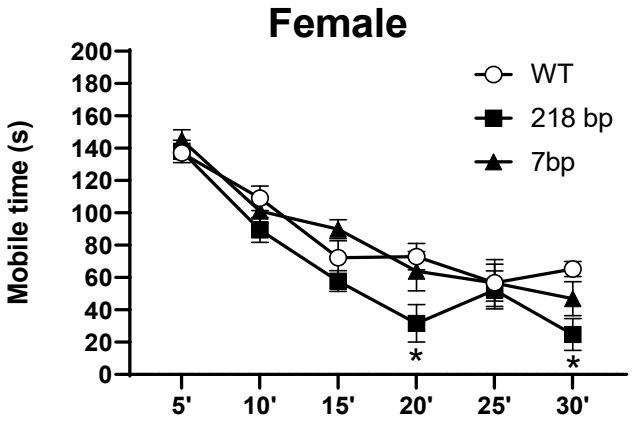


Figure 32. Locomotor activity in female rats. WT (n=10) and KO rats of the 218bp deletion (n=9) and 7bp deletion (n=9). Two-way ANOVA followed by Tukey's multiple comparisons test. *p<0.05.

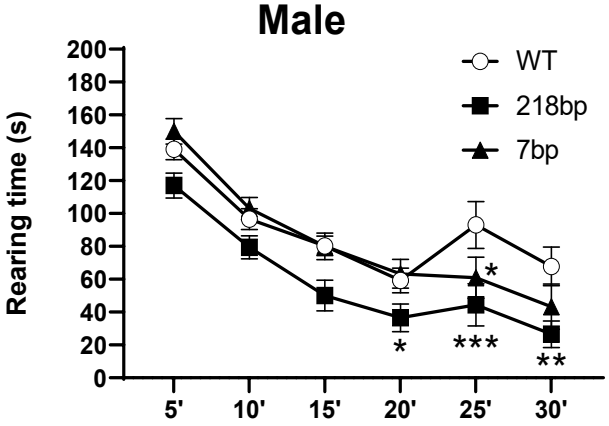


Figure 33. Rearing activity in male rats. WT (n=10) and KO rats of the 218bp deletion (n=10) and 7bp deletion (n=9). Two-way ANOVA followed by Tukey's multiple comparisons test. *p<0.05; **p<0.01; ***p<0.001.

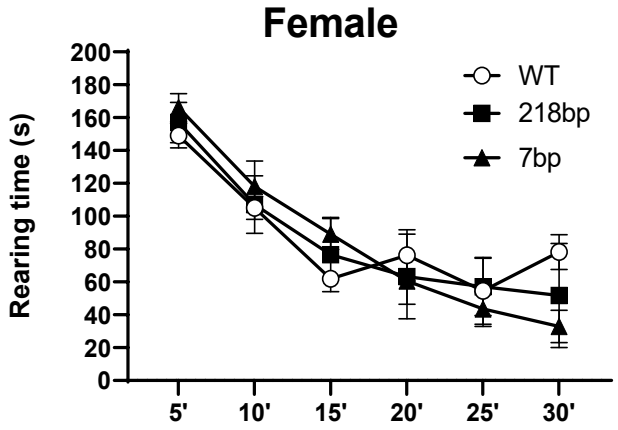


Figure 34. Rearing activity in female rats. No significant differences were found between genotypes. WT (n=10) and KO rats of the 218bp deletion (n=9) and 7bp deletion (n=9). Two-way ANOVA followed by Tukey's multiple comparisons test.

The analysis of the area under curve for the whole reading period showed a tendency to lower values for the 218bp genotype for both mobile (Figure 35) and rearing time (Figure 36) in either males or females, although statistical significance was only reached for males in the rearing activity.

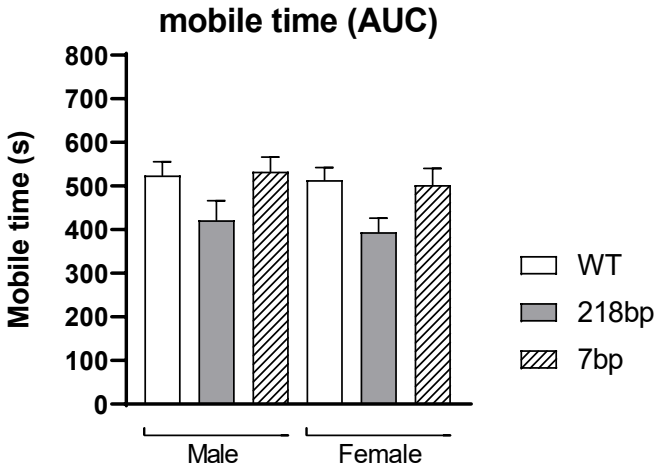


Figure 35. Locomotor for the total 60' reading period, in male and female rats. No significant differences were found between genotypes. WT (10 males and 10 females) and KO rats of the 218bp deletion (10 males and 9 females) and 7bp deletion (n=9 males and 9 females). One way ANOVA.

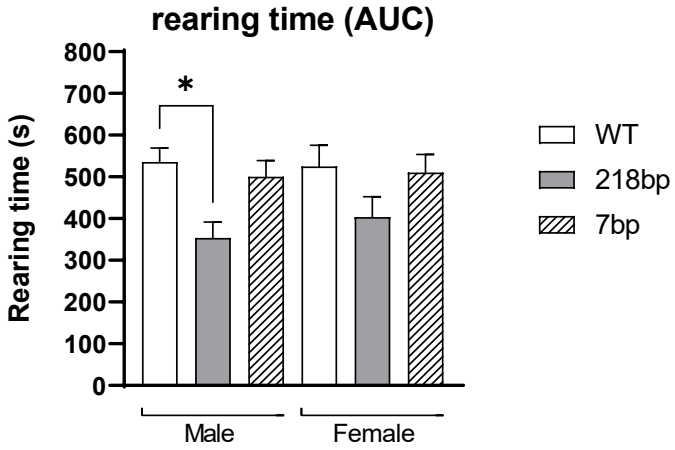


Figure 36. Rearing time for the total 60' reading period, in male and female rats. No significant differences were found between genotypes. WT (10 males and 10 females) and KO rats of the 218bp deletion (10 males and 9 females) and 7bp deletion (n=9 males and 9 females). One way ANOVA followed by Tukey's multiple comparison test. *<0.05.

OPEN FIELD

The open field test allows to assess the level of anxiety of the rodents, along with locomotor activity parameters. Anxiety can be assessed by the permanence time in the centre, the more anxiogenic area, or the thigmotaxis, as the time in the side-walls and the corners, the safer zones. In males, no differences were found for the travelled distance (Figure 37) and the number of entries (Figure 38) in any area analysed. In females, the travelled distance, and the number of entries in the corners and the centre were the same for all the genotypes, but in the side-walls area a longer distance and higher number of entries were recorded for 7bp genotype (Figure 39 and Figure 40).

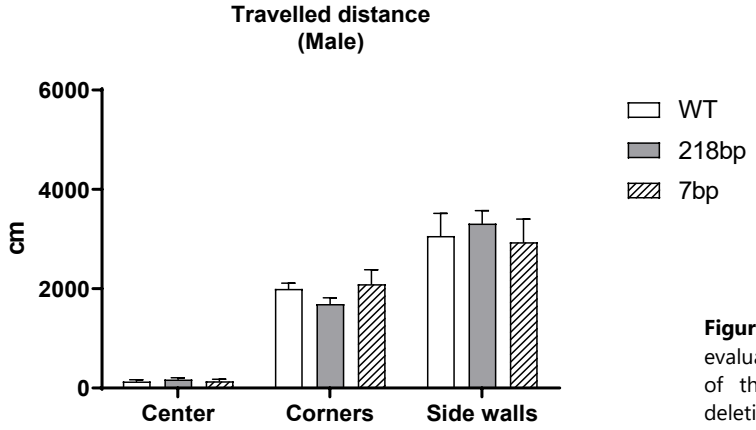


Figure 37. Open field distance travelled evaluated in 3 different areas in male rats of the WT, 218bp deletion, and 7bp deletion genotypes. One-way ANOVA.

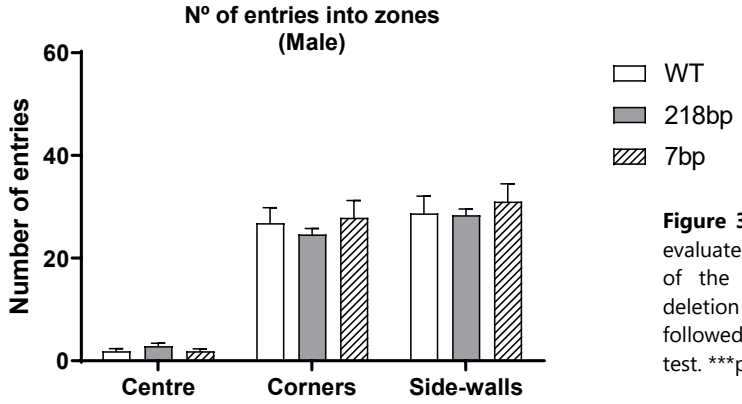


Figure 38. Open field number of entries evaluated in 3 different areas, in male rats of the WT, 218bp deletion, and 7bp deletion genotypes. One-way ANOVA followed by Dunnett's multiple comparison test. ***p<0.001

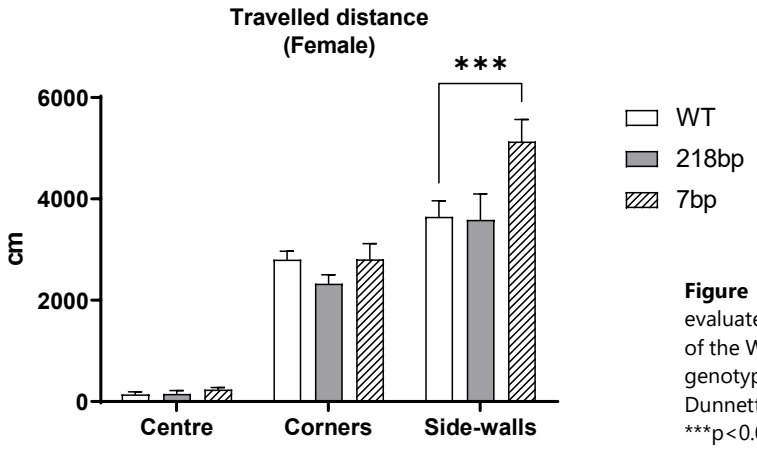


Figure 39. Open field distance travelled evaluated in 3 different areas in female rats of the WT, 218bp deletion, and 7bp deletion genotypes. One-way ANOVA followed by Dunnett's multiple comparison test. ***p<0.001

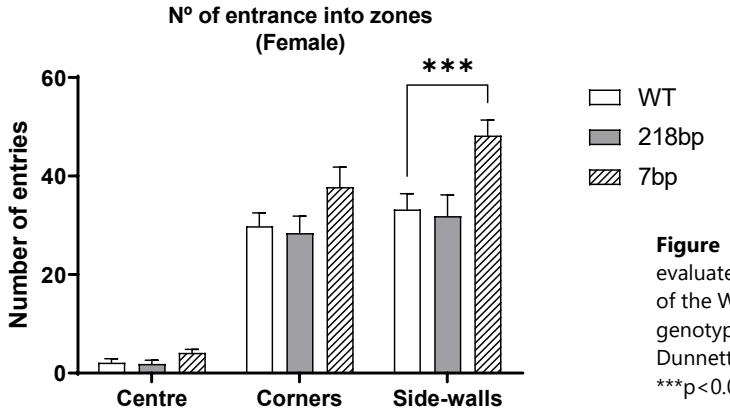


Figure 40. Open field number of entries evaluated in 3 different areas in female rats of the WT, 218bp deletion, and 7bp deletion genotypes. One-way ANOVA followed by Dunnett's multiple comparison test. ***p<0.001

STARTLE RESPONSE AND PRE-PULSE INHIBITION

Startle response measures the force of the reaction of the animal to a sudden noise. For these experiments the force sensor under the restrainer was not adjusted to each gender, that is, to different weights. Therefore, considering the differences in the weight between males and females it was not surprising that the force of the startle response was higher for males than females. However, within each gender there were no differences between genotypes (Figure 41).

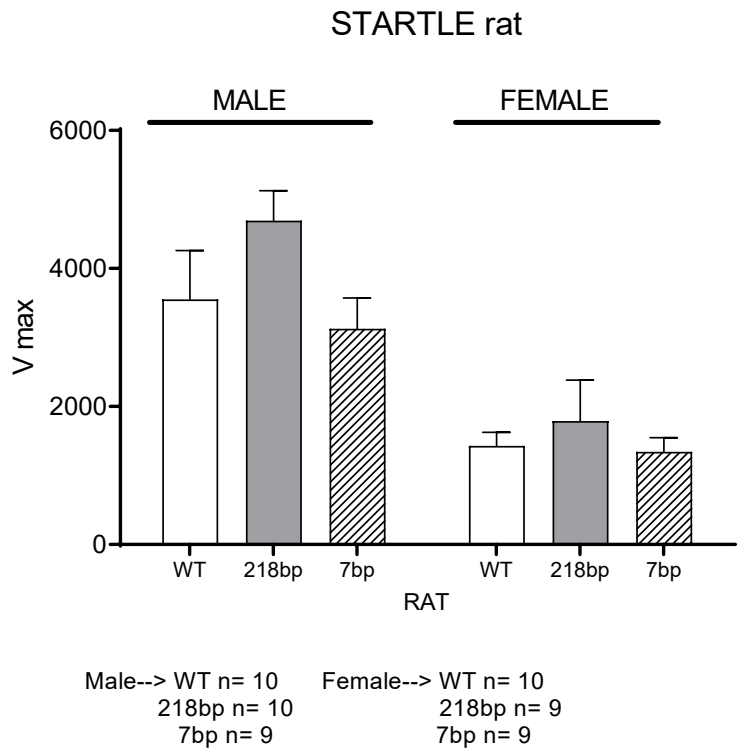


Figure 41. Startle response to a high intensity pulse (120db) in male and female rats.

In the pre-pulse inhibition trials, 3 different pulses of low intensity were applied before the pulse of high intensity used for measuring the startle response. These pre-pulses have the capacity to reduce or to inhibit the force of the startle response in a pre-pulse intensity-dependent manner.

In WT animals, 3, 6, and 12dB pre-pulses were able to inhibit the startle response, with similar percentages for the different genotypes but higher values for males, accordingly with the startle values. Males showed an inhibition of 46.2%, 56.5%, and 68.2% for 3, 6, and 12db pre-pulse, respectively (Figure 42). For the females, the values of inhibition for 3, 6, and 12db pre-pulse intensities were 29.5%, 40.1%. and 53.6%, respectively (Figure 43).

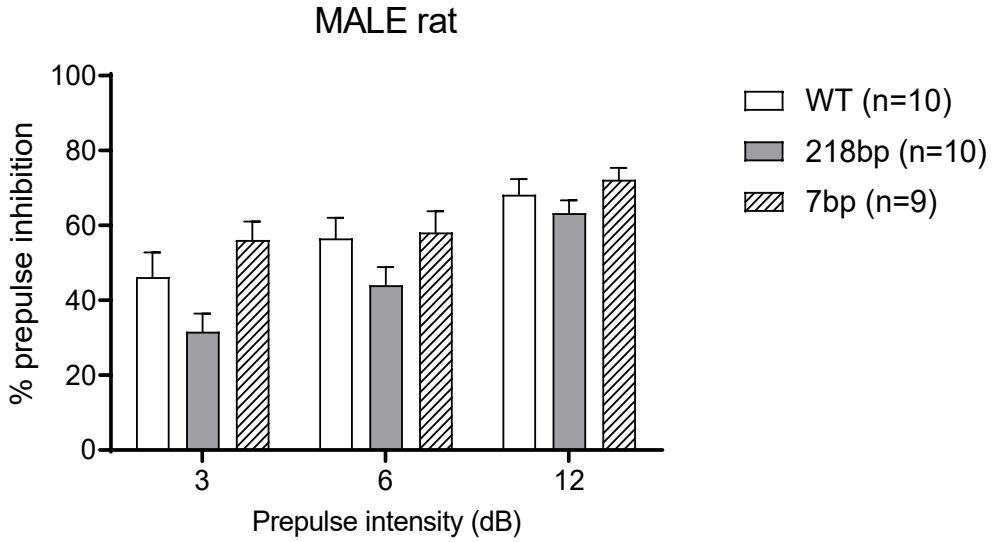


Fig. 42. Pre-pulse inhibition in male rats. Two-way ANOVA multiple comparisons.

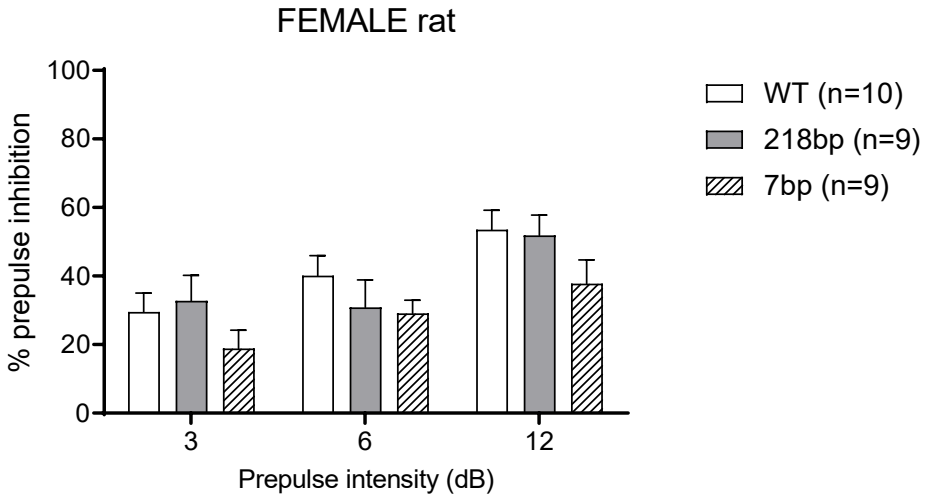


Fig. 43. Pre-pulse inhibition in female rats. Two-way ANOVA multiple comparisons.

The weight of the animals was the same for each genotype within each gender, with the only exception of females of the 7bp deletion that had slight lower weight the first two weeks (Figure 44). No differences were found in male rats (Figure 45).

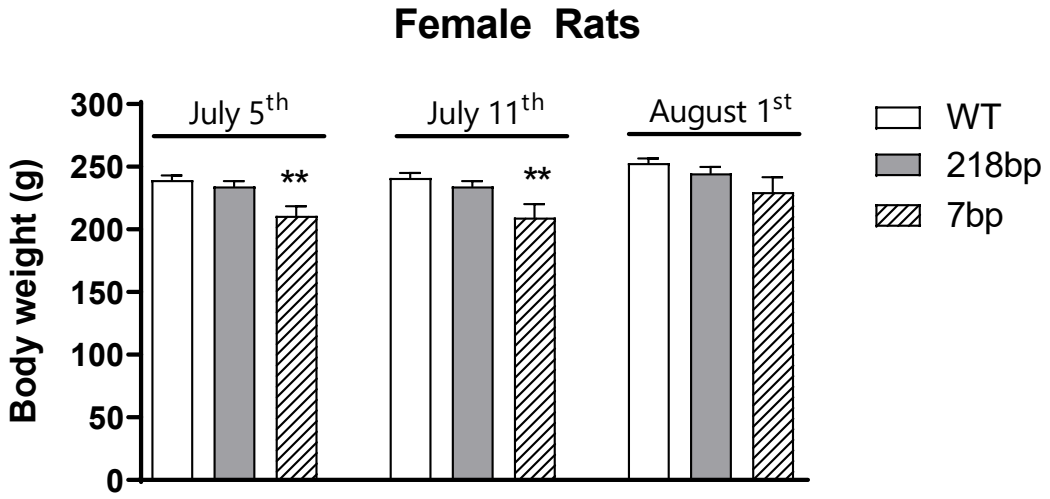


Figure 44. Body weight of female rats of the different genotypes during the sensitivity/pain assessment. For each week, one-way ANOVA followed by Dunnett's multiple comparison vs WT group. **p<0.01

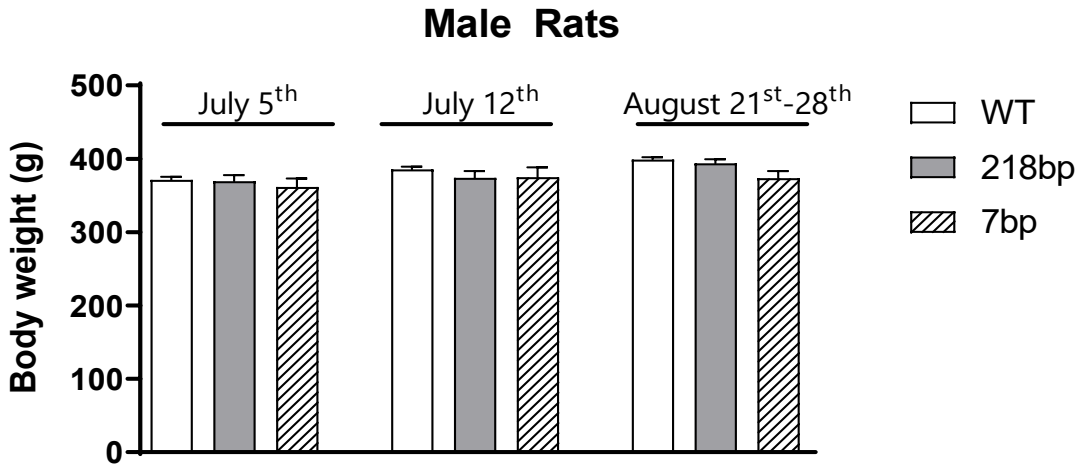
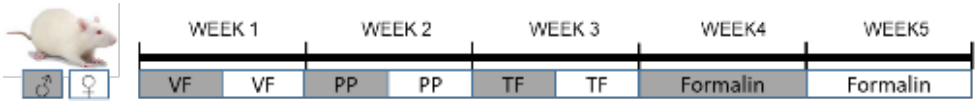


Figure 45. Body weight of male rats of the different genotypes during the sensitivity/pain assessment. For each week, one-way ANOVA.

The evaluation of pain sensitivity was performed in a sequential manner, following the scheme:



We started with the von Frey filaments that, without sensitization, measures touch sensitivity. One week later we did the paw pressure test that measures the pain response to an increasing pressure on the hind paw. The week after the tail flick was performed, by applying a high-intensity beam of light to the tail, which produces a painful heat sensation, and measuring the latency to withdraw the tail. Finally, the last week we did the formalin test. In this test the injection of formalin into the hind paw induces several pain responses in the rat, lifting, licking and flinches.

MECHANICAL SENSITIVITY – VON FREY

Male and female rats showed a different level of mechanical sensitivity to von Frey filaments. Male rats of WT and 218bp deletion genotypes did not withdraw the paw until von Frey filament of 19.2±1.6 and 18.9±2 g was applied, respectively. Surprisingly, males from the 7bp deletion genotype withdrew the paw when 8.8±0.6 g filament was applied, which was highly significant vs WT rats ($p=0.0002$) (Figure 46).

WT female rats behaved quite similar, with no differences between WT and 218bp deletion groups (10.7±0.7 g and 9±0.2 g, respectively) but much lower paw withdrawal threshold in the 7bp deletion females (5.2±0.4 g) $p<0.0001$ vs WT group) (Figure 47).

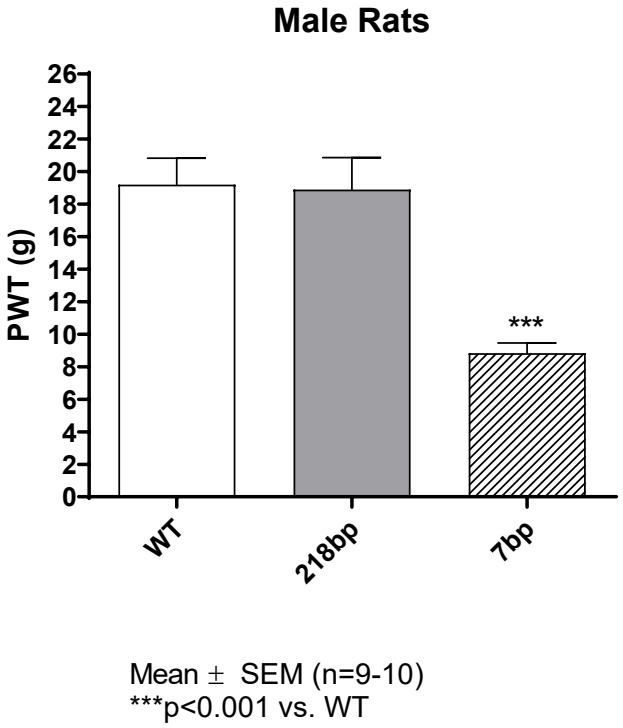


Figure 46. Mechanical sensitivity measured by von Frey filaments in male rats. One way ANOVA followed by Dunnett's multiple comparisons test. *** p <0.001.

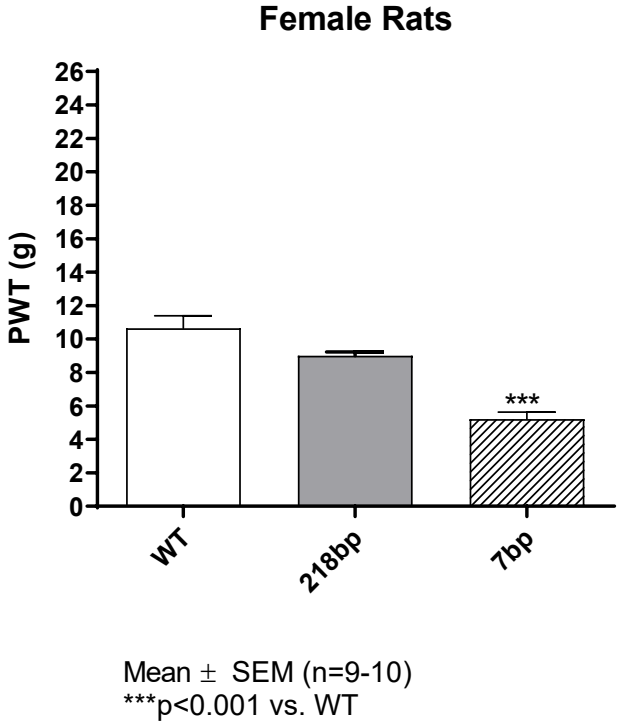


Figure 47. Mechanical sensitivity measured by von Frey filaments in female rats. One way ANOVA followed by Dunnett's multiple comparisons test. *** p <0.001.

Contrary to von Frey filaments test, which was originally setup for measuring allodynia, that is, for measuring the response to a non-painful stimulus unless a sensitisation process has been established, in the paw pressure test the stimulus reached a painful level. Under these circumstances, neither male (Figure 48) nor female KO rats (Figure 49) of any genotype showed a significant difference versus WT rats.

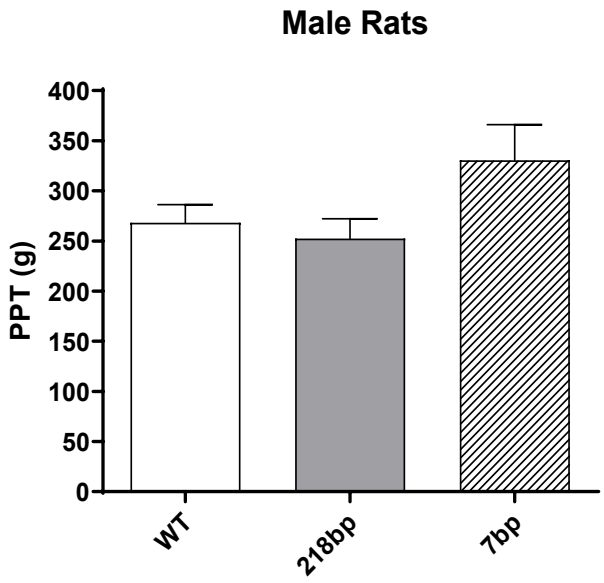


Figure 48. Pain threshold measured by mechanical sensitivity in the paw pressure test in male rats. One way (n=9-10)

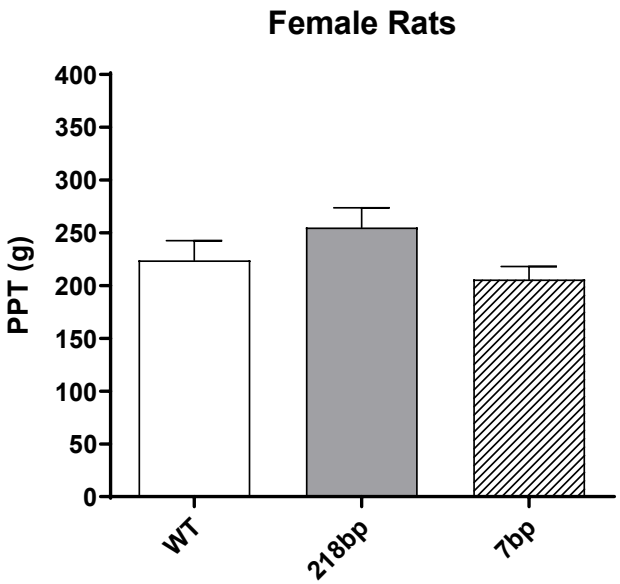


Figure 49. Pain threshold measured by mechanical sensitivity in the paw pressure test in female rats. One way ANOVA. (n=9-10)

No tail withdrawal differences were found in thermal sensitivity to a noxious stimulus in male rats (Figure 50). In males, the three genotypes withdrew at almost the same latency time (WT = 2.8±0.1s; 218bp = 2.9±0.1s; 7bp = 2.4±0.1s). However, in females (Figure 51) both KO genotypes showed lower withdrawal latencies (2.8±0.2s and 2.3±0.2s, for 218bp and 7bp genotypes, respectively) in comparison to WT rats (3.4±0.1s). These differences were statistically significant.

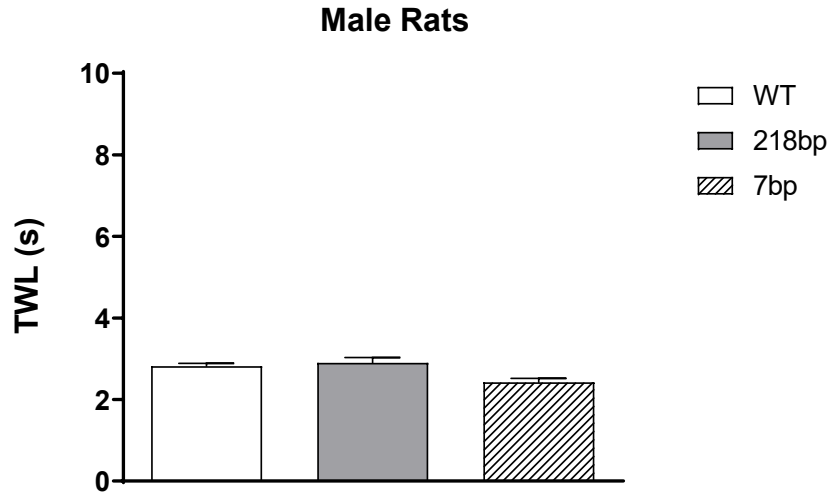


Figure 50. Pain threshold measured by thermal sensitivity in the tail flick test in male rats. One way ANOVA.

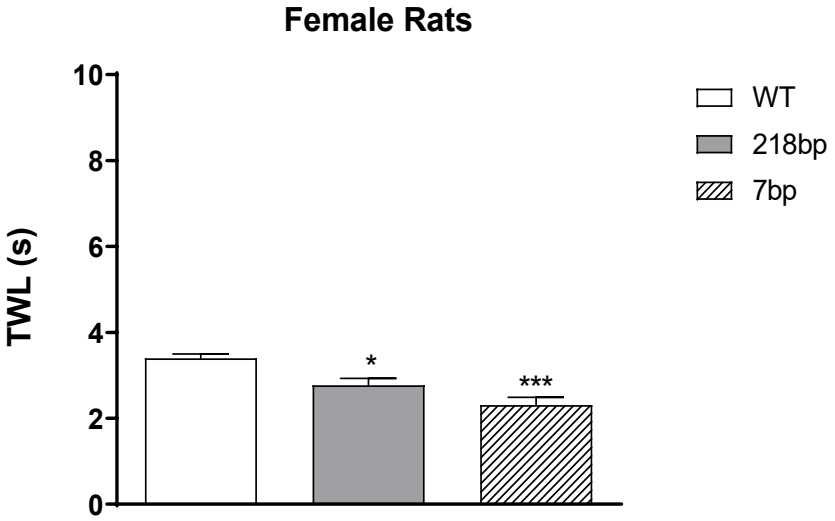


Figure 51. Pain threshold measured by thermal sensitivity in the tail flick test in female rats. One way ANOVA followed by Dunnett's multiple comparison test. *p<0.05; ***p<0.001

Formalin-induced lifting and licking clearly showed the two-phase pattern of response, in both males (Figure 52) and females (Figure 53), with no differences between genotypes. The two phases were much clear in males than in females, and particularly more diffuse in KO females of both deletions.

Lifting + Licking time

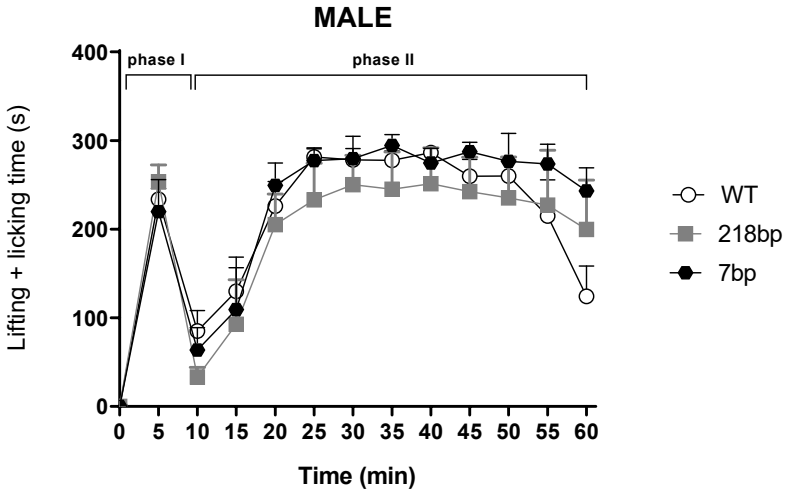


Figure 52. Pain threshold measured by chemical sensitivity to formalin in male rats. Lifting + Licking time in male rats of the WT, 218bp, and 7bp genotypes. A main effect of time ($p<0.0001$) was found, but no genotype differences, either for the whole curve or for any single time point. Two-way ANOVA repeated measures followed by Dunnett's multiple comparison test.

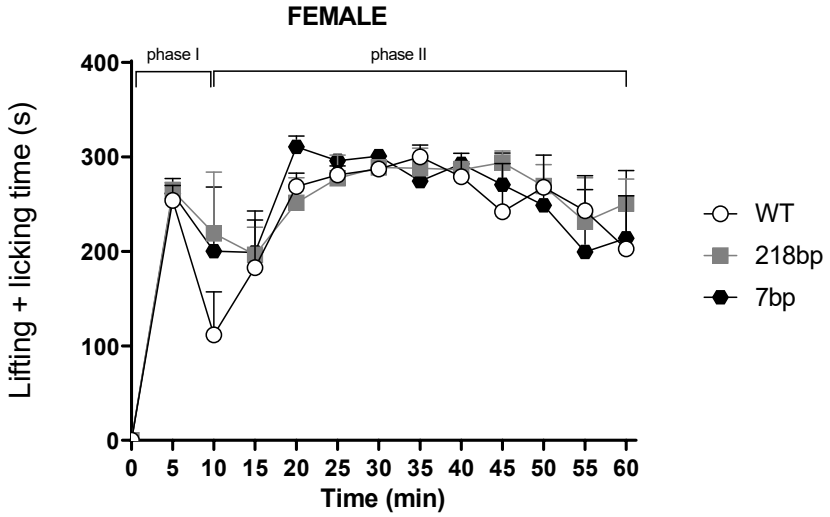


Figure 53. Pain threshold measured by chemical sensitivity to formalin in female rats. Lifting + Licking time in female rats of the WT, 218bp, and 7bp genotypes. A main effect of time ($p<0.0001$) was found, but no genotype differences, neither for the whole curve nor for any single time point. Two-way ANOVA repeated measures followed by Dunnett's multiple comparison test.

Number of flinches

In males (Figure 54), a main effect of time ($p<0.0001$) and genotype ($p< 0.0159$) was found for the number of flinches, although the analysis of each time points did not reveal any significant effect. In females (Figure 55), a main effect was found on time ($p<0.0001$) and genotype ($p<0.0001$), but the analysis of each time points only revealed a significant effect at 40' for both deletions and at 55' for 7bp deletion.

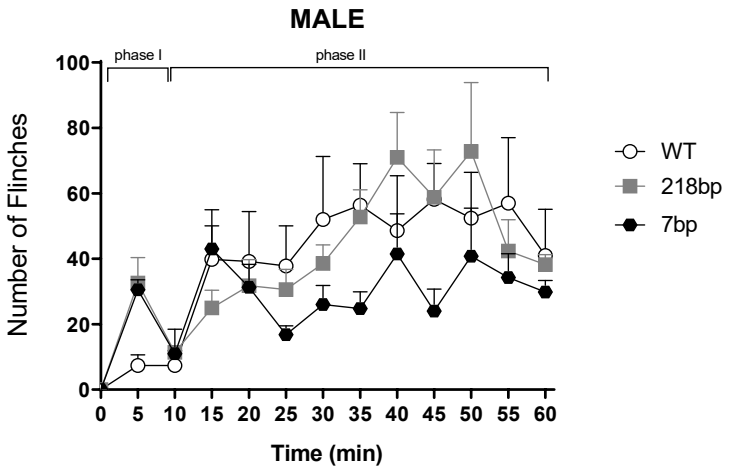


Figure 54. Chemical sensitivity measured in the formalin test in male rats. Number of flinches in male rats of the WT, 218bp, and 7bp genotypes. In males, a main effect of time ($p<0.0001$) and genotype ($p< 0.0159$) was found, although the analysis of each time points did not reveal any significant effect. Two-way ANOVA repeated measures.

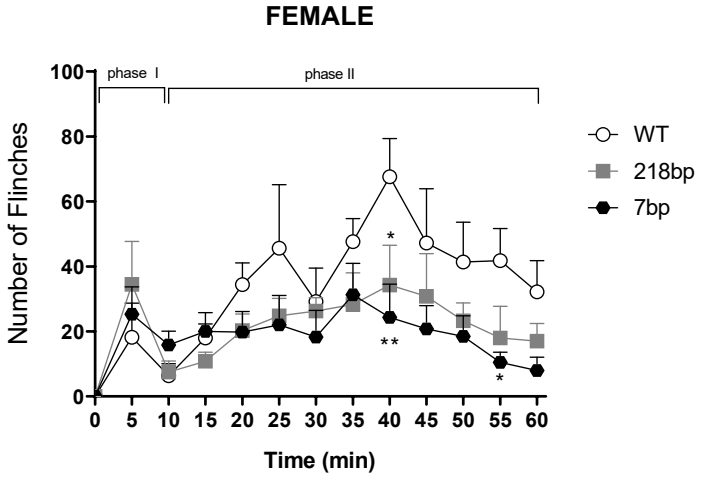


Figure 55. Chemical sensitivity measured in the formalin test in female rats. Number of flinches in female rats of the WT, 218bp, and 7bp genotypes. A main effect of time ($p<0.0001$) and genotype ($p<0.0001$) was found, and the analysis of each time points only revealed a significant effect at minutes 40 for both deletions, and at minute 55 for the 7bp deletion. Two-way ANOVA followed by Dunnett's multiple comparison test (* $p<0.05$; ** $p<0.01$).

In summary, the results obtained in the comparison between the 218bp and 7bp strains (Table. 3) made us to select the 218bp strain for the phenotyping of the heterozygous line. A main reason for this decision was the reduction in pain sensitivity found in the 7bp strain, mainly in the von Frey test under non-sensitizing conditions, which was clearly unexpected, based on all the literature available on sigma-1 KO mice and sigma-1 pharmacological antagonism.

Table 3. Summary of the results of initial phenotyping of the two sigma-1 KO deletion lines.

	7bp deletion	**218bp deletion**
Body weight	Reduced BW increase ($p<0.01$ vs WT, Female)	n.s.*
Locomotor activity	n.s.	No relevant slight reduction
Open field	n.s.	n.s.
Prepulse inhibition	n.s.	n.s.
Mechanical sensitivity (VF)	PWT reduction ($p<0.001$ vs WT, Male & Female)	n.s.
Mechanical sensitivity (PP)	n.s.	n.s.
Thermal sensitivity (TF)	TWL reduction ($p<0.001$ vs WT, Female)	n.s.
Formalin test	Lower number of flinches	Lower number of flinches

*n.s. = not significant

PHENOTYPIC ANALYSIS OF THE HETEROZYGOUS LINE

After the selection of the sigma-1 KO line, we performed the phenotyping of the heterozygous line, so we had WT (+/+), heterozygous (HET; +/-) and homozygous (HOM; -/-) subjects for the 218bp deletion.

Table 4. Number of animals used in the phenotyping of the heterozygous line are summarised.

Genotype	Males	Females
HOM	12 (4 euth*.)	10 (4 euth.)
HET	12 (3 euth.)	10 (4 euth.)
WT	10 (2 euth.)	10 (6 euth.)
Total (At the beginning of the experiment)	34	30
Remaining	25	16

* Euthanised for welfare reasons

PHYSIOLOGICAL PARAMETERS

GROWTH

The analysis of the growth curve of male rats revealed a main effect of time ($p<0.0001$) and genotype ($p<0.0001$). The growth of both HET and HOM male rats was slightly lower compared to WT rats, reaching statistical significance in the HET subjects from week 38 onwards, and for HOM subjects from week 71 (Figure 56).

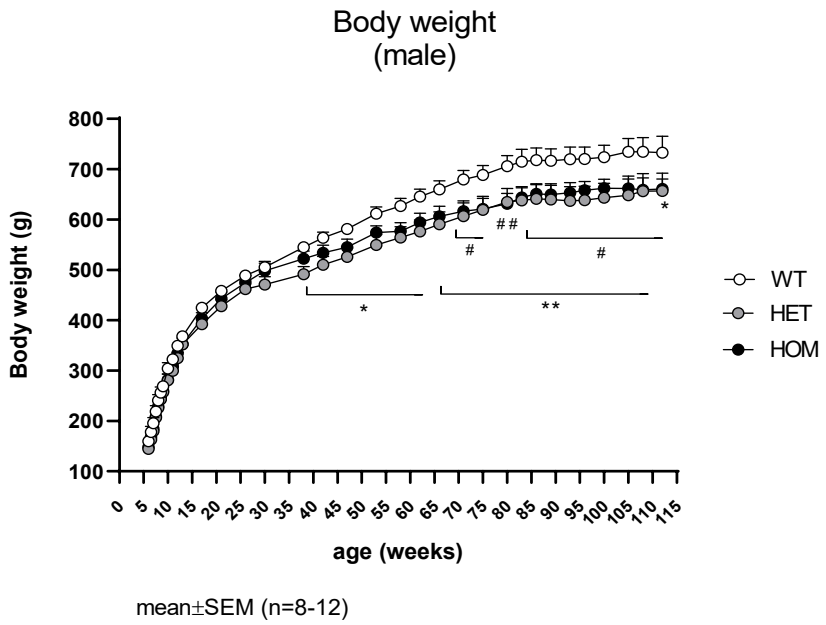


Figure 56. Growth of male WT and KO rats. Two-way ANOVA followed by Dunnett's multiple comparison test (* $p<0.05$; ** $p<0.01$ for HET line; # $p<0.05$; ## $p<0.01$ for HOM line).

Food consumption in young rats was significantly higher than in old rats, for WT ($p<0.0034$) and HET rats ($p< 0.0012$), but not significant in HOM (0.3492) (Figure 57). However, for each range of age, food consumption was not different between genotypes.

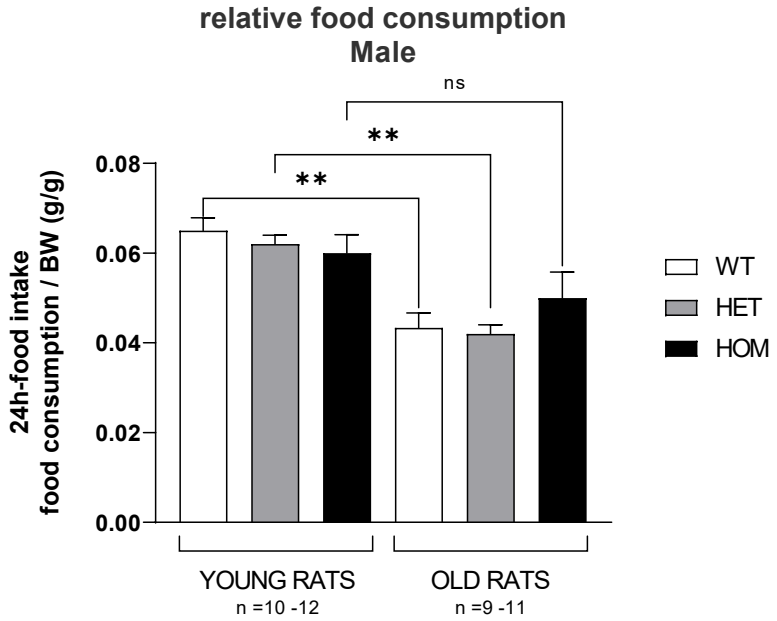


Figure 57. Food consumption of young and old male WT and KO rats. One-way ANOVA followed by Tukey's multiple comparison test. **$p<0.01$

In females no differences in the pattern of growth between genotypes was shown, with only a main effect of time ($p<0.0001$) (Figure 58). Food consumption in female young rats was significantly higher than in old rats in WT ($p< 0.0087$), HET rats ($p< 0.0253$), and HOM rats ($p< 0.0422$) (Figure 59). However, for each range of age, food consumption was not different between genotypes.

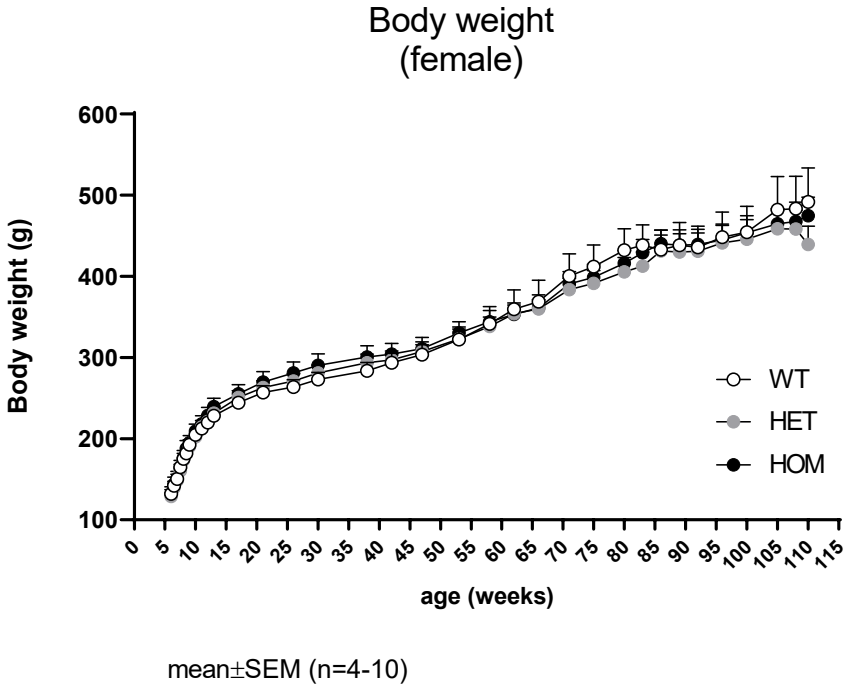


Figure 58. Growth of female WT and KO rats. Two-way ANOVA followed by Dunnett's multiple comparison test.

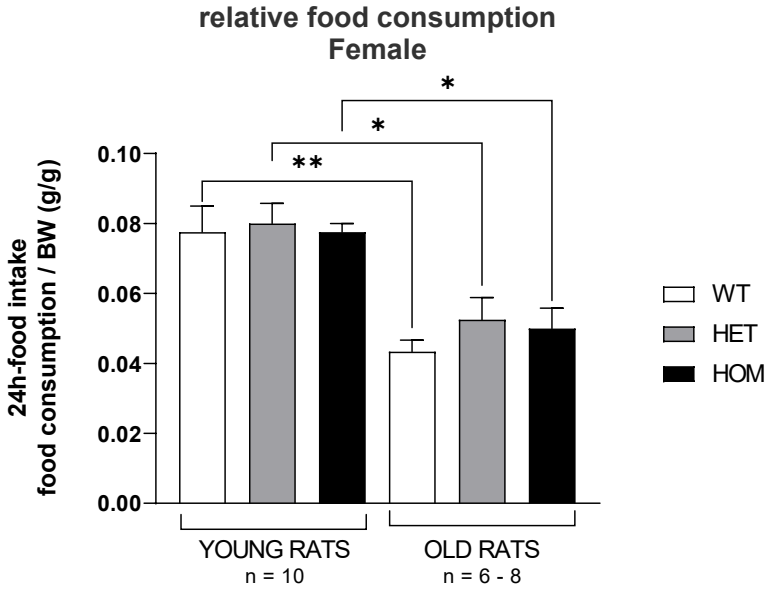


Figure 59. Food consumption of young and old female WT and KO rats. One-way ANOVA followed by Tukey's multiple comparison test. *p<0.05: **p<0.01

Survival curves at 24 months of the WT, HET, and HOM genotypes were not different, either in male (Figure 60) or in female rats (Figure 61).

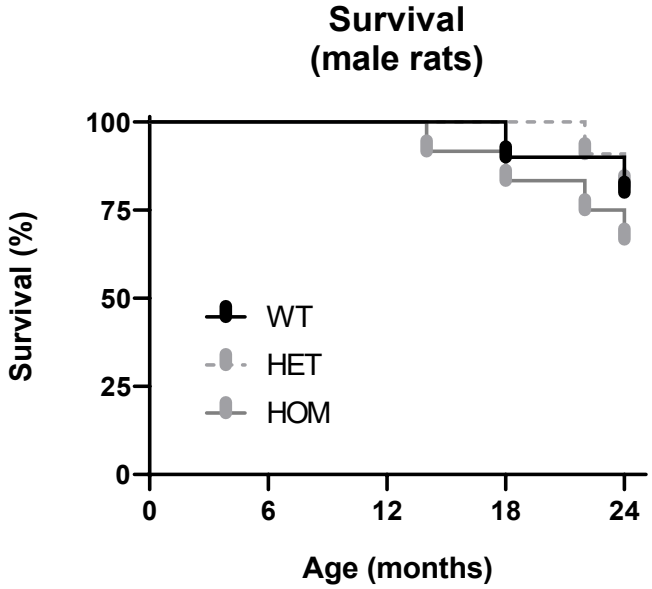


Figure 60. Survival curve for male rats of the WT, HET, and HOM genotypes (n=8-12). No differences in the survival curves were found. Log-rank (Mantel-Cox) test, p = 0.5968.

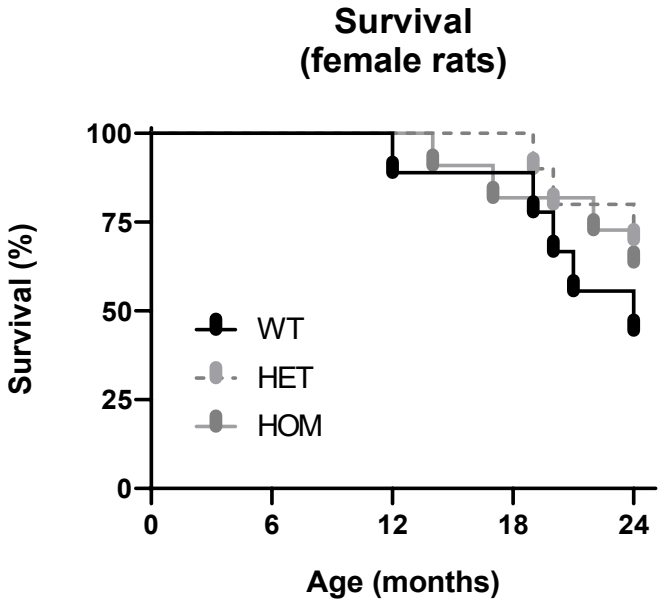


Figure 61. Survival curve for female rats of the WT, HET, and HOM genotypes (n=4-10). No differences in the survival curves were found. Log-rank (Mantel-Cox) test, p = 0.4698.

HEMATOLOGY

The parameters analysed in whole blood samples from young (Table 5) and old female rats (Table 6), and young male rats (Table 7), revealed no differences between genotypes, in any gender or age. The haematological analysis of old male rats was cancelled. Young animals were 2-4 months old. Old rats were around 2 years old.

Table 5. Haematological values for young WT, HET, and HOM female rats. N = 8/group. One-way ANOVA for each parameter, with WT as control. Parameters analysed in the Abbreviations list.

Young FEMALE		5-Part-Diff Auto Hematology Analyzer (whole blood, K2-EDTA)					
		BASO	**BASO**	**M.C.H.C**	**EOS**	**EOS**	
		10^9/L	**%**	**g/dL**	**10^12/L**	**%**	
WT	mean	0.003	0.118	34.04	0.005	0.15	
	SEM	0.001	0.045	0.15	0.002	0.053	
HET	mean	0.004	0.139	34.21	0.004	0.154	
	SEM	0.002	0.058	0.23	0.001	0.026	
HOM	mean	0.008	0.236	34.13	0.01	0.24	
	SEM	0.002	0.086	0.14	0.004	0.08	
Young FEMALE		**M.C.H.**	**R.B.C.**	**H.C.T.**	**H.G.B.**	**W.B.C.**	
		pg	**10^12/L**	**%**	**g/dL**	**10^9/L**	
WT	mean	18.65	8.10	44.35	15.11	3.13	
	SEM	0.22	0.25	1.07	0.34	0.28	
HET	mean	19.21	7,99	44.86	15.36	3.01	
	SEM	0.18	0.15	0.69	0.18	0.20	
HOM	mean	19.14	7.80	43.71	14.94	3.75	
	SEM	0.25	0.13	0.49	0.16	0.29	
Young FEMALE		**LYM**	**LYM**	**MON**	**MON**	**NEU**	**NEU**
		g/dL	**%**	**10^9/L**	**%**	**10^9/L**	**%**
WT	mean	2.25	71.03	0.43	14.09	0.44	14.61
	SEM	0.25	2.80	0.09	2.41	0.09	2.98
HET	mean	2,06	66.74	0.50	17.17	0.44	15.80
	SEM	0.22	3.16	0.08	2.81	0.09	3.66
HOM	mean	2,66	70.25	0.45	12.54	0.62	16.73
	SEM	0.26	2.01	0.07	2.22	0.09	2.38
Young FEMALE		**PLT**	**PLT**	**R.D.W.**	**M.C.V.**	**M.P.V..**	
		10^9/L	**%**	**%**	**fL**	**fL**	
WT	mean	628.50	0.37	10.86	54.89	6.01	
	SEM	118.13	0.07	0.19	0.49	0.10	
HET	mean	863.71	0.47	10.78	56,29	5.53	
	SEM	85.63	0.05	0.16	0.40	0.09	
HOM	mean	835.00	0.47	10.96	56.16	5.65	
	SEM	22.36	0.01	0.15	0.60	0.12	

Table 6. Haematological values for old WT, HET, and HOM female rats. N = 8/group. One-way ANOVA for each parameter with WT as control. Parameters analysed in the Abbreviations list.

Old FEMALE		5-Part-Diff Auto Hematology Analyzer (whole blood, K2-EDTA)					
		BASO	BASO	M.C.H.C.	EOS	EOS	
		10^9/L	%	g/dL	10^12/L	%	
WT	mean	0.0086	0.22	32.50	0.04	1.05	
	SEM	0.0030	0.08	0.12	0.01	0.34	
HET	mean	0.0098	0.26	32.85	0.03	0.97	
	SEM	0.0014	0.03	0.18	0.02	0.51	
HOM	mean	0.0054	0.18	32.60	0.01	0.52	
	SEM	0.0024	0.09	0.16	0.01	0.36	
Old FEMALE		M.C.H.	R.C.B.	H.C.T.	H.G.B.	W.B.C.	
		pg	10^12/L	%	g/dL	10^9/L	
WT	mean	18.42	8.18	46.32	15.08	3.87	
	SEM	0.26	0.17	0.44	0.16	0.50	
HET	mean	18.65	7.28	41.38	13.60	3.63	
	SEM	0.11	0.25	1.40	0.41	0.39	
HOM	mean	18.59	8.09	46.24	15.09	3.55	
	SEM	0.10	0.16	0.97	0.26	0.24	
Old FEMALE		LYM	LYM	MON	MON	NEU	NEU
		g/dL	%	10^9/L	%	10^9/L	%
WT	mean	2.24	59.05	0.84	22.04	0.75	17.64
	SEM	0.26	4.95	0.15	3.85	0.26	5.03
HET	mean	2.13	58.56	0.60	16.45	0.86	23.76
	SEM	0.28	3,47	0.08	1.43	0.11	2.29
HOM	mean	2.30	64.93	0.72	19.95	0.51	14.43
	SEM	0.19	3.79	0.10	1.83	0.08	2.16
Old FEMALE		PLT	P.C.T.	R.D.W.	M.C.V.	M.P.V.	
		10^9/L	%	%	fL	fL	
WT	mean	760.00	0.44	11.24	56,80	6,30	
	SEM	50.90	0.04	0.31	0.75	0.32	
HET	mean	849,00	0.46	11.66	56,95	5,53	
	SEM	50.74	0.03	0.19	0.19	0.12	
HOM	mean	782,43	0.46	11.20	57,21	5,94	
	SEM	48,35	0.03	0.27	0.25	0.11	

Table 7. Haematological values for young WT, HET, and HOM male rats. N = 8/group. One-way ANOVA for each parameter with WT as control. Parameters analysed in the Abbreviations list.

Young MALE		5-Part-Diff Auto Hematology Analyzer (whole blood, K2-EDTA)				
		BASO	BASO	M.C.H.C..	EOS	EOS
		10^9/L	%	g/dL	10^12/L	%
WT	mean	0.002	0.05	33,90	0.005	0.084
	SEM	0.001	0.04	0.14	0.002	0.035
HET	mean	0.004	0.06	33,40	0.005	0.084
	SEM	0.002	0.03	0.15	0.002	0.022
HOM	mean	0.005	0.09	33,60	0.003	0.059
	SEM	0.002	0.03	0.16	0.001	0.011

Table 7 (Cont.). Haematological values for young WT, HET, and HOM male rats. N = 8/group. One-way ANOVA for each parameter with WT as control. Parameters analysed in the Abbreviations list.

Young MALE		M.C.H. pg	R.B.C. 10^12/L	H.C.T. %	H.G.B. g/dL	W.B.C. 10^9/L
WT	mean	18,10	8,80	47,00	16,00	6,20
	SEM	0.17	0.14	0.62	0.18	0.60
HET	mean	18,10	9,00	48,90	16,40	5,80
	SEM	0.09	0.14	0.68	0.21	0.37
HOM	mean	17,90	9,20	48,90	16,40	6,00
	SEM	0.16	0.14	0.73	0.21	0.30

Young MALE		LYM g/dL	LYM %	MON 10^9/L	MON %	NEU 10^9/L	NEU %
WT	mean	4,30	68,90	0.90	13,80	1.00	17,20
	SEM	0.68	7,10	0.19	3,33	0.28	4,97
HET	mean	4,10	71.20	0.90	15,40	0.80	13,20
	SEM	0.37	3,91	0.19	3,79	0.18	2,67
HOM	mean	4,50	73,80	0.90	15,90	0.60	10.10
	SEM	0.34	2,48	0.12	2,18	0.09	1.81

Young MALE		PLT 10^9/L	PLT %	R.D.W. %	M.C.V. fL	M.P.V. fL
WT	mean	865,60	0.50	10.90	53,60	5,60
	SEM	72,48	0.04	0.16	0.43	0.11
HET	mean	822,40	0.50	10.90	54,40	5,60
	SEM	75,94	0.04	0.15	0.33	0.12
HOM	mean	847,90	0.50	11.00	53,30	5,60
	SEM	64,62	0.04	0.17	0.39	0.07

BIOCHEMISTRY

The analysis of the cholesterol and triglyceride levels showed no differences between any genotype or sex, either in young females (Figures 62 and 63) or in old females (Figures 64 and 65), or young males (Figure 66), with the only exception of lower levels of cholesterol in HOM young male rats ($p < 0.0109$). No differences in triglyceride levels in young males were found. For old males, the samples were collected but the analysis is pending.

The levels of the other biochemical parameters analysed were similar in the three genotypes (Tables 8, 9, and 10).

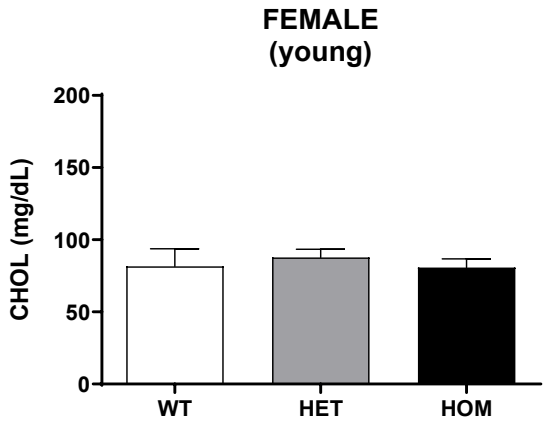


Figure 62. Cholesterol levels for young female rats.
N= 8/group. One-way ANOVA, WT as control.

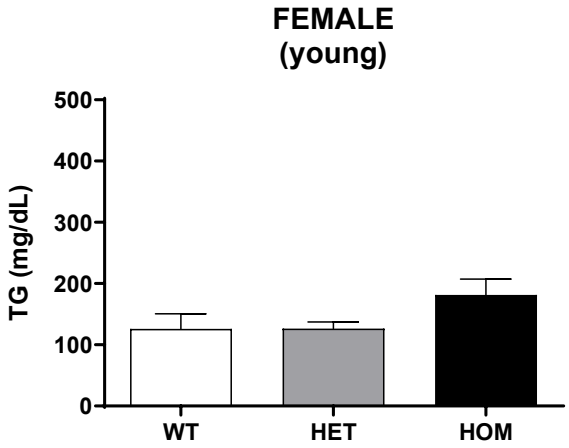


Figure 63. Triglycerides levels for young female rats. N = 8/group. One-way ANOVA. WT as control.

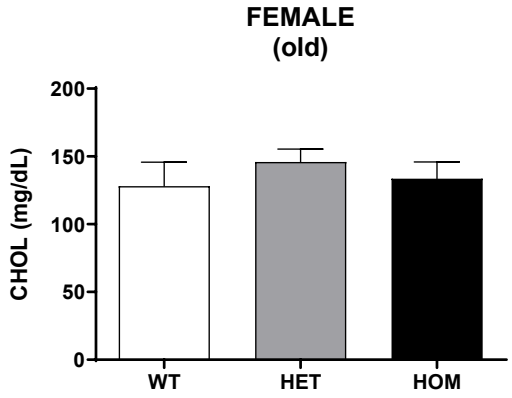


Figure 64. Cholesterol levels for old female rats. N= 8/group. One-way ANOVA. WT as control.

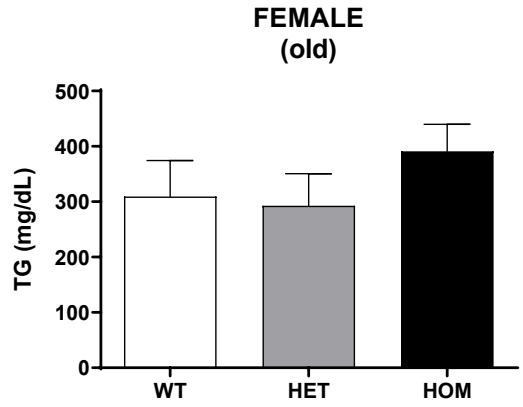


Figure 65. Triglycerides levels for old female rats. N=8/group. One-way ANOVA. WT as control.

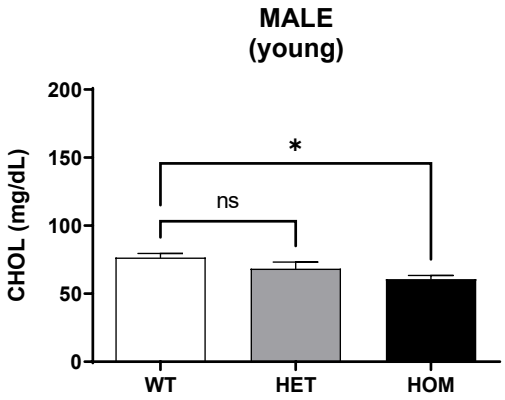


Figure 66. Cholesterol levels for young male rats. N = 8/group. One-way ANOVA followed by Dunnett's Multiple comparisons vs WT group; * $p<0.05$.

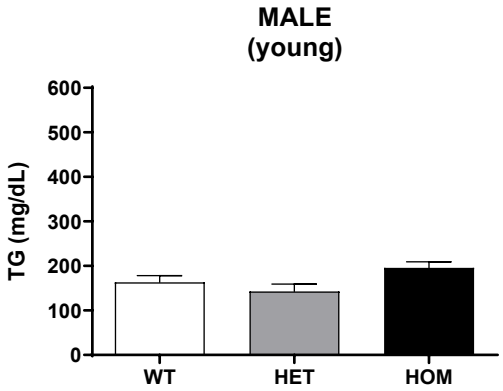


Figure 67. Triglycerides levels for young male rats. N = 8/group. One-way ANOVA. WT as control.

Table 8. Biochemistry results obtained in young WT, HET, and HOM female rats. Parameters analysed in the Abbreviations list.

		Biochemistry (Hep-Li plasma)								
Young female rats		CHOL	TG	BUN	CRE	ALT	ALP	AST	TBIL	GLU
		mg/dL	mg/dL	mg/dL	mg/dL	U/L	U/L	U/L	mg/dL	mg/dL
WT	mean	76,6	163,1	14,1	0.4	41.5	200.4	67,3	0.3	127,8
	s.e.m.	2,80	14,29	0.37	0.03	2,54	17,07	2,32	0.00	3,69
HET	mean	68,4	142,5	13,6	0.5	36,8	193,1	71.1	0.3	131.6
	s.e.m.	4,51	15,58	0.35	0.03	1.57	14,16	2,84	0.00	4,47
HOM	mean	60.8	195,9	13,6	0.4	37,4	163,4	64,6	0.3	133,8
	s.e.m.	2,50	12,31	0.43	0.03	2,02	10.60	1.84	0.01	2,21

Young female rats		Ca	TP	ALB	GLOB	Na+	K+	Cl-	tCO2
		mg/dL	g/dL	g/dL	g/dL	mmol/L	mmol/L	mmol/L	mmol/L
WT	mean	11.8	7,2	5,9	1.1	141.5	5,2	101.4	26,0
	s.e.m.	0.12	0.15	0.06	0.10	1.19	0.10	0.88	0.77
HET	mean	11.5	7,0	5,9	1.1	140.5	5,3	102,5	25,8
	s.e.m.	0.14	0.11	0.07	0.07	0.73	0.14	1.06	0.84
HOM	mean	11.6	7,1	5,9	1.2	141.1	5,4	101.4	26,6
	s.e.m.	0.18	0.13	0.06	0.09	0.65	0.15	0.81	0.68

Table 9. Biochemistry results obtained in old WT, HET, and HOM female rats.

		Biochemistry (Hep-Li plasma)								
		CHOL	TG	BUN	CRE	ALT	ALP	AST	TBIL	GLU
		mg/dL	mg/dL	mg/dL	mg/dL	U/L	U/L	U/L	mg/dL	mg/dL
WT	mean	81.5	125,9	16,4	0.5	30.5	94,0	68,1	0.3	142,5
	s.e.m.	11.39	23,17	0.86	0.03	0.94	7,68	3,22	0.03	8,68
HET	mean	87,8	126,6	16,6	0.4	30.5	121.8	61.5	0.3	135,3
	s.e.m.	5,20	9,99	0.98	0.03	1.83	17,48	2,49	0.00	3,94
HOM	mean	80.8	181.6	16,1	0.4	27,4	76,6	54,3	0.3	119,9
	s.e.m.	5,61	24,04	0.65	0.04	3,36	10.67	5,60	0.00	14,06

		Ca	TP	ALB	GLOB	Na+	K+	Cl-	tCO2
		mg/dL	g/dL	g/dL	g/dL	mmol/L	mmol/L	mmol/L	mmol/L
WT	mean	11.3	7,0	n.a.	n.a.	138,0	5,2	102,9	21.5
	s.e.m.	0.17	0.07	n.a.	n.a.	1.22	0.26	1.60	0.31
HET	mean	11.6	7,5	n.a.	n.a.	141.6	5,1	104,1	24,1
	s.e.m.	0.20	0.15	n.a.	n.a.	2,38	0.16	2,29	0.84
HOM	mean	11.7	7,5	n.a.	n.a.	142,1	4,4	101.3	25,3
	s.e.m.	0.16	0.15	n.a.	n.a.	1.50	0.59	0.72	0.26

n.a. = not available

Table 10. Biochemistry results obtained in young WT, HET, and HOM male rats.

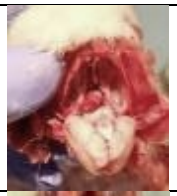
		Biochemistry (Hep-Li plasma)								
		CHOL	TG	BUN	CRE	ALT	ALP	AST	TBIL	GLU
		mg/dL	mg/dL	mg/dL	mg/dL	U/L	U/L	U/L	mg/dL	mg/dL
WT	mean	128,2	309,2	10.8	0.4	31.0	13,0	71.0	0.3	126,2
	s.e.m.	15,7	58,4	1.0	0.1	4,1	1.8	14,5	0.0	10.8
HET	mean	145,9	292,5	13,1	0.3	28,0	13,0	69,0	0.4	99,9
	s.e.m.	8,9	54,3	0.8	0.0	3,9	1.1	11.5	0.0	12,7
HOM	mean	133,6	391.1	11.0	0.3	24,7	10.4	63,0	0.4	98,6
	s.e.m.	11.33	45,20	0.81	0.03	1.45	1.09	7,13	0.03	15,68

		Ca	TP	ALB	GLOB	Na+	K+	Cl-	tCO2
		mg/dL	g/dL	g/dL	g/dL	mmol/L	mmol/L	mmol/L	mmol/L
WT	mean	n.a.	7,0	6,2	0.6	160.3	n.a.	91.6	28,2
	s.e.m.	n.a.	0.2	0.1	0.1	0.4	n.a.	1.6	2,2
HET	mean	n.a.	7,4	5,6	0.9	162,9	n.a.	88,3	23,6
	s.e.m.	n.a.	0.2	0.0	0.0	1.4	n.a.	1.7	2,9
HOM	mean	n.a.	7,3	6,2	1.0	163,7	n.a.	88,4	27,1
	s.e.m.	n.a.	0.21	0.09	0.12	1.22	n.a.	1.61	2,53

n.a. = not available

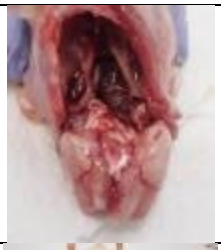
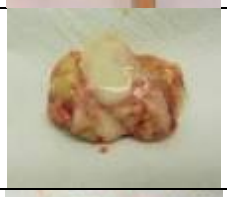
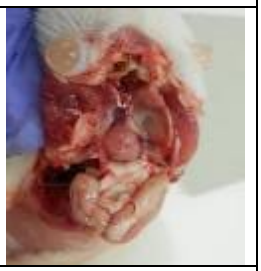
Finally, in the clinical observations and histopathological analysis performed we found several health incidences without any correlation with genotype or gender. Results are summarised in Table 11. Such observations are within those expected for old animals of the same strain and no clear correlation of findings and genotype was found.

Table 11. Summary of clinical observations during the phenotyping of WT rats.

ID	Genotype	Clinical findings	Age/Observations/Histology	
23F	WT	Ocular prolapse, keratitis	8 months. Retrobulbar mass	
2F	WT	Axilar nodule (5x5 cm)	12 months. Encapsulated mass, adhered to skin. Mammary ductal fibroadenoma (NMT)	
49M	WT	Death	18 months. Absence of clinical symptoms. No macroscopical alterations.	
4F	WT	Wellbeing affectation, head tilt	19 months. Intracranial mass, adhered to skull*	
5F	WT	Wellbeing affectation	20 months. Intracranial mass, adhered to skull *	
14F	WT	Wellbeing affectation	21 months. Intracranial mass, adhered to skull*	
10F	WT	Wellbeing affectation, head tilt	24 months. Intracranial mass, adhered to skull*	
44M	WT	Wellbeing affectation, head tilt	24 months. Intracranial mass, adhered to skull*	

* Mass with macroscopic appearance and location similar to that found in HOM animal 15F (compatible with pituitary adenoma). NMT: non-malignant tumor

Table 12. Summary of clinical observations during the phenotyping of HET rats.

ID	Genotype	Clinical findings	Age/Observations/Histology	
2M	HET	Severe dental malocclusion	9 months. Dental malformation.	
17F	HET	Wellbeing affectation, head tilt, circling	19 months Intracranial mass, adhered to skull*	
28F	HET	Abdominal nodule (4x3 cm)	20 months Abscess, adhered to abdominal wall	 
42M	HET	Subcutaneous nodule (5x5 cm)	22 months. Encapsulated mass, adhered to skin	
4M	HET	Wellbeing affectation, black stools	24 months. Generalized intestinal bleeding (melena), absence of GI masses.	
43F	HET	Wellbeing affectation	24 months. Intracranial mass, adhered to skull*	
29F	HET	Inguinal nodule (5x5 cm)	24 months.Encapsulated mass, solid	

* Mass with macroscopic appearance and location similar to that found in HOM animal 15F (compatible with pituitary adenoma).

Table 13. Summary of clinical observations during the phenotyping of HOM rats.

ID	Genotype	Clinical findings	Age/Observations/Histology	
55M	HOM	Axilar nodule (5x5 cm)	14 months. Encapsulated mass, adhered to skin Fibrosarcoma (MT)	
15F	HOM	Wellbeing affectation, head tilt	14 months. Intracranial mass adhered to skull. Choroid plexus tumor (MT)	
13F	HOM	Wellbeing affectation	17 months. Intracranial mass, adhered to skull*	
11M	HOM	Facial mass (3x3 cm) Wellbeing affectation	18 months. Abscess, adhered to facial muscles	 
21F	HOM	Wellbeing affectation, head tilt	22 months. Intracranial mass, adhered to skull*	
3M	HOM	Abdominal mass (5x5 cm)	22 months. Encapsulated fatty mass (lipoma?)	
33M	HOM	Death	23 months. Abdominal mass, hemorrhagic (gut)	
35F	HOM	Axilar nodules, ulcerated (3x3 cm)	24 months. Encapsulated mass adhered to skin. Solid contents.	

* Mass with macroscopic appearance and location similar to that found in animal 15F (compatible with pituitary adenoma). MT: malignant tumor.

MOTOR COORDINATION (ROTAROD)

The study of motor coordination was done only in females because males were too big for the apparatus. Both young and old female rats learnt the task normally, with a better performance in 1 year old rats of any genotype in the number of falls during the training session (Figure 68) but no significant differences in the latency to fall during test session, although a tendency to felt earlier in old rats (Figure 69).

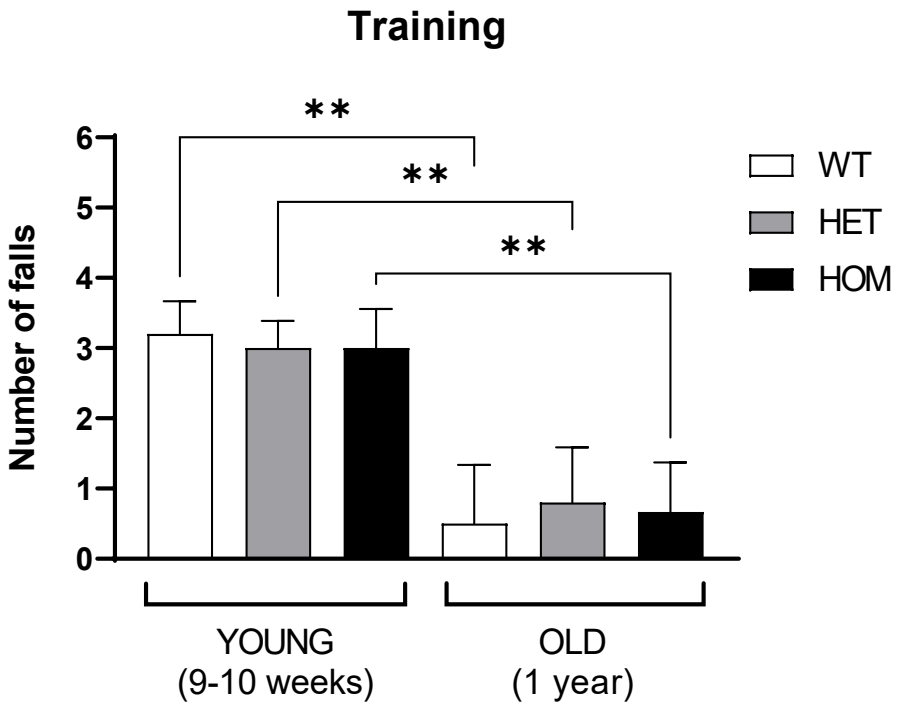


Figure 68. Motor coordination in young and 1 year old female rats measured by number of falls. One way ANOVA followed by Tukey's multiple comparison test. ** $p<0.01$.

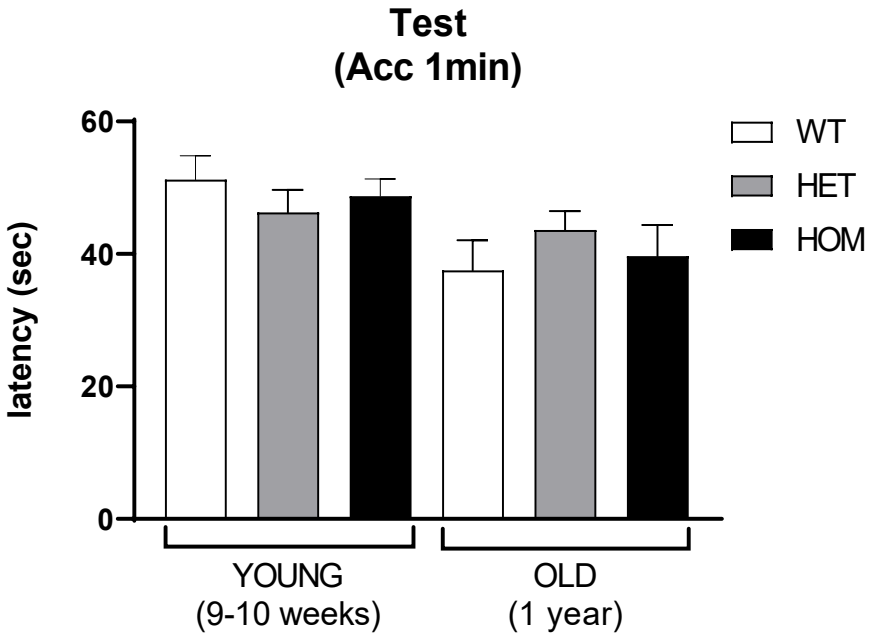


Figure 69. Motor coordination in young and 1 year old female rats measured by the latency to fall in 1-minute accelerated procedure. One way ANOVA followed by Tukey's multiple comparison test.

LOCOMOTOR ACTIVITY & RUNNING WHEELS

Twenty-four hour locomotor activity and running wheel activity were assessed in young and old female rats and in young male rats. Old male rats were discarded for this study due to their body size.

After the initial high level of mobile time due to novelty exploration activity, the 24h recording of the locomotor activity showed a circadian pattern with an increased activity during the dark phase, coming back to low levels of mobile time in the light phase. This pattern of activity was similar in WT, HET, and HOM sigma-1 KO female rats, both in young (Figures 70 and 72) and old female animals (Figures 73 and 74). No differences were found in the 24h time-course analysis, either in

the light or in the dark phase. The analysis of the AUC of the whole period did not show any difference between genotypes.

Overall profile of activity for young and old rats was similar, with old rats showing lower levels of activity, although they did not reach statistical significance for any genotype comparison.

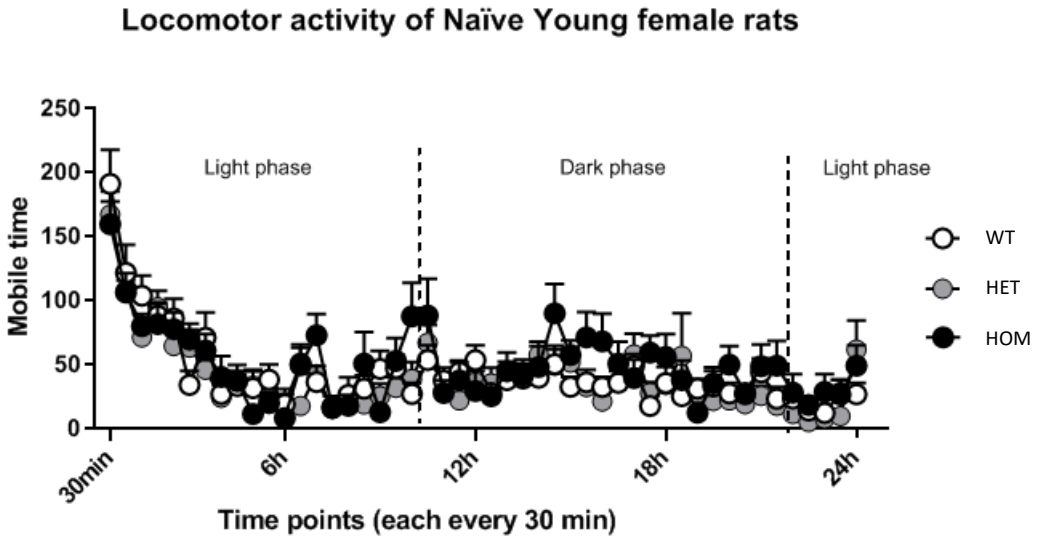


Figure 70. Time-course locomotor activity of young female rats of the 3 genotypes. Two-way repeated measures.

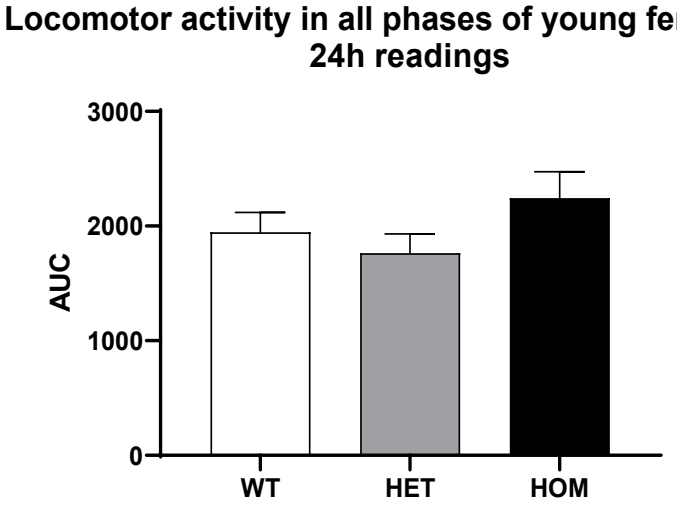


Figure 71. Overall 24 h activity of young female rats. One way ANOVA followed by Tukey's multiple comparison test.

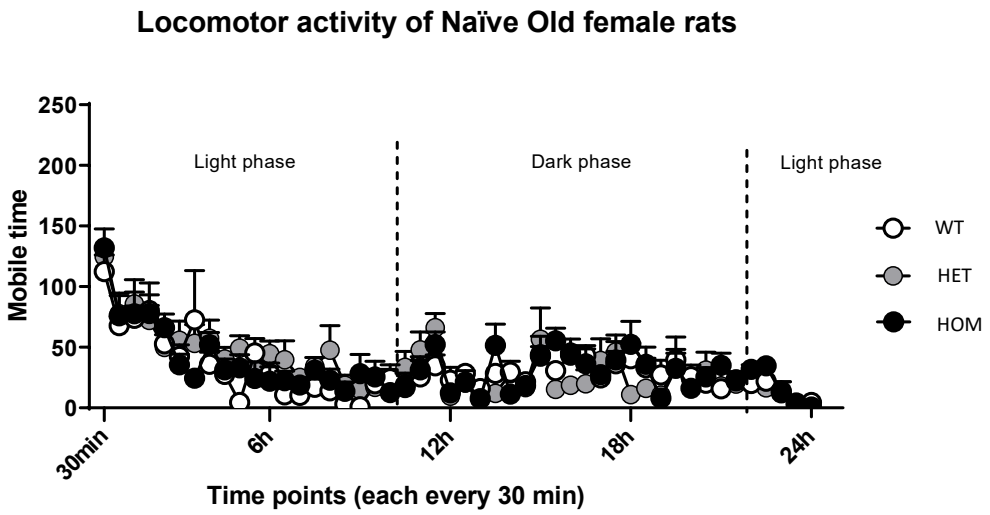


Figure 72. Time-course locomotor activity of old female rats of the 3 genotypes. Two-way repeated measures.

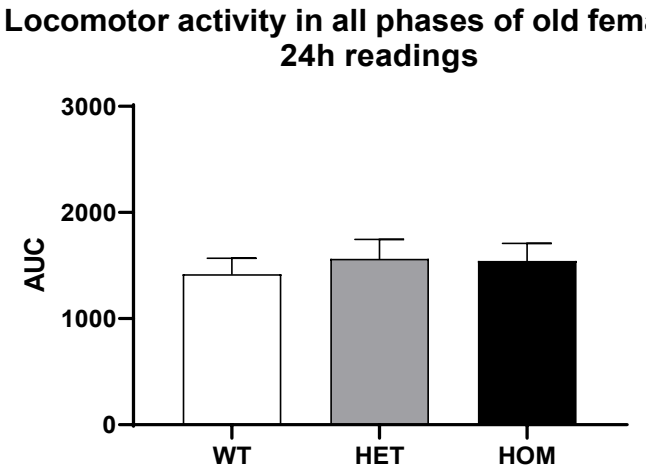


Figure 73. Overall 24 h activity of old female rats. One way ANOVA followed by Tukey's multiple comparison test.

Table 14. Comparison of the locomotor activity of young and old female rats the whole 24h period. Student's t-Test.

Genotype	Statistic	Mobile time (24h-AUC) (Young female rats)	Mobile time (24h-AUC) (Old female rats)	Significance
WT	Mean±s.e.m.	1945 ± 173.5	1418±150.1	p=0.0404 (*)
HET	Mean±s.e.m.	1763±168.1	1564±181.3	p=0.4314
HOM	Mean±s.e.m.	2244±229.3	1544±165	p=0.0234 (*)

In young male rats, the locomotor activity of WT subjects, and the HET, and HOM sigma-1 KO subjects was also similar, without any differences in the 24h time-course analysis or in the overall 24h analysis (Figures 74 and 75).

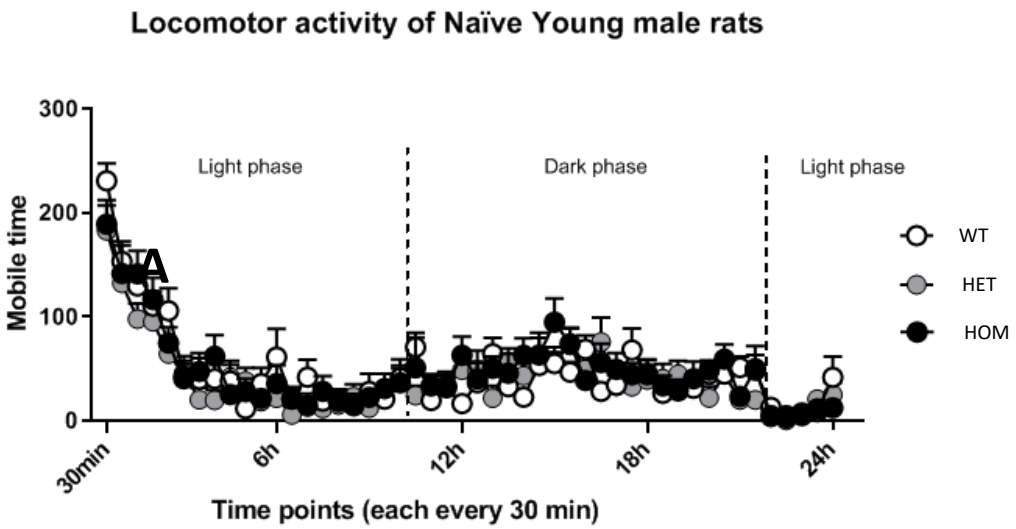


Figure 74. Time-course locomotor activity of young male rats of the 3 genotypes. Two-way repeated measures.

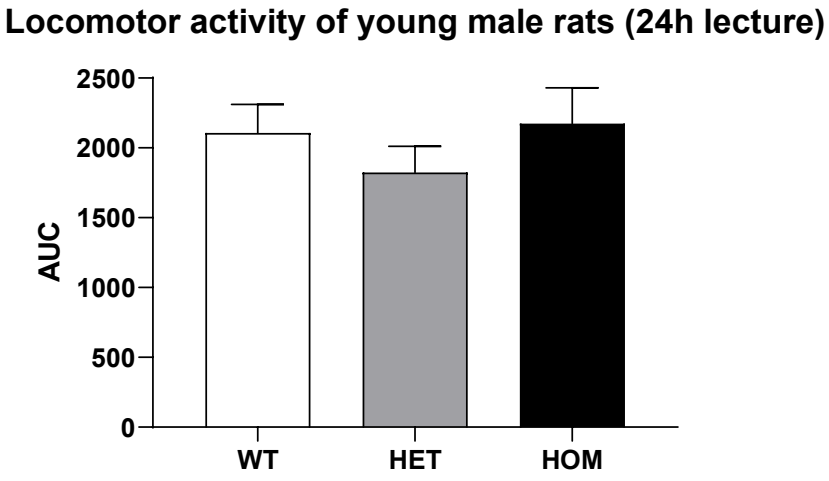


Figure 75. Overall 24 h activity of young male rats. One way ANOVA followed by Tukey's multiple

In relation to running wheel activity, the results were similar to those observed in locomotor activity. For both young (Figures 76 and 77) and old female (Figures 78 and 79) rats, no differences were found between WT subjects, and the HET, and HOM sigma-1 KO subjects, either in the 24h time-course analysis or in the overall 24h analysis. The level of activity for young male rats was similar to young female rats, with no significant difference between any of the genotypes.

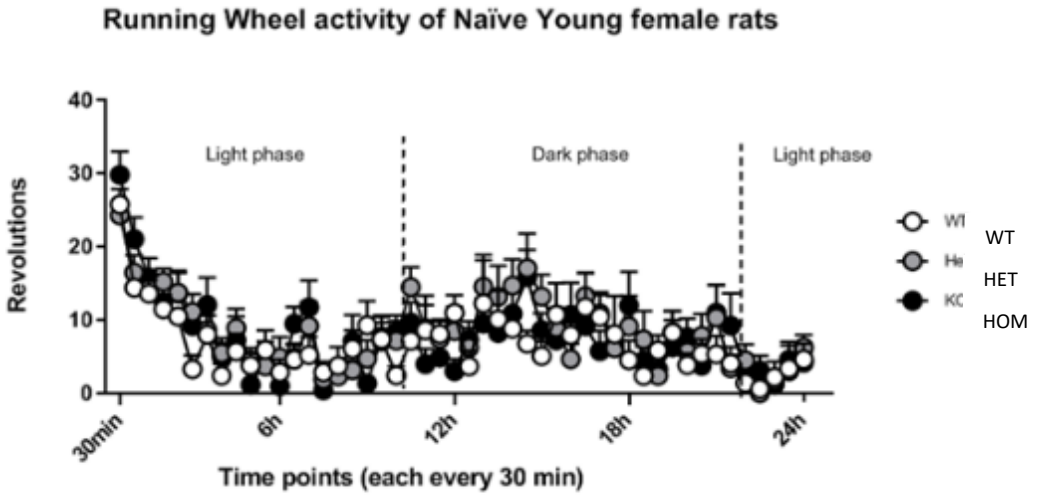


Figure 76. Time-course of running wheel activity of young female rats of the 3 genotypes. Two-way repeated measures.

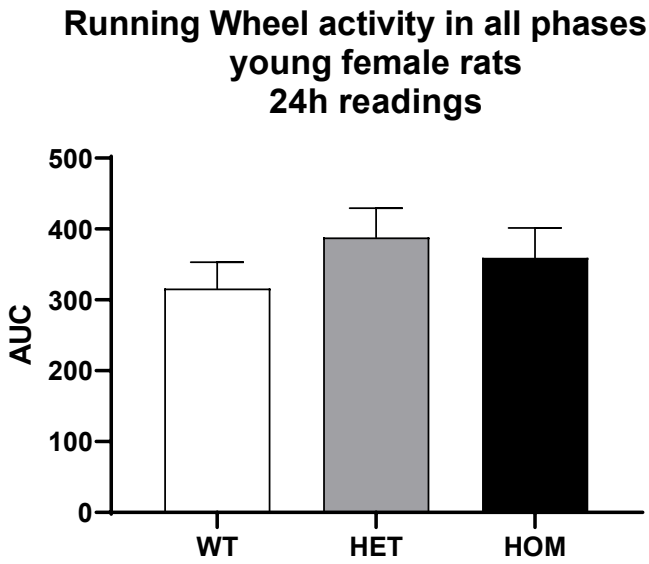


Figure 77. Overall 24 h running wheel activity of young female rats. One way ANOVA followed

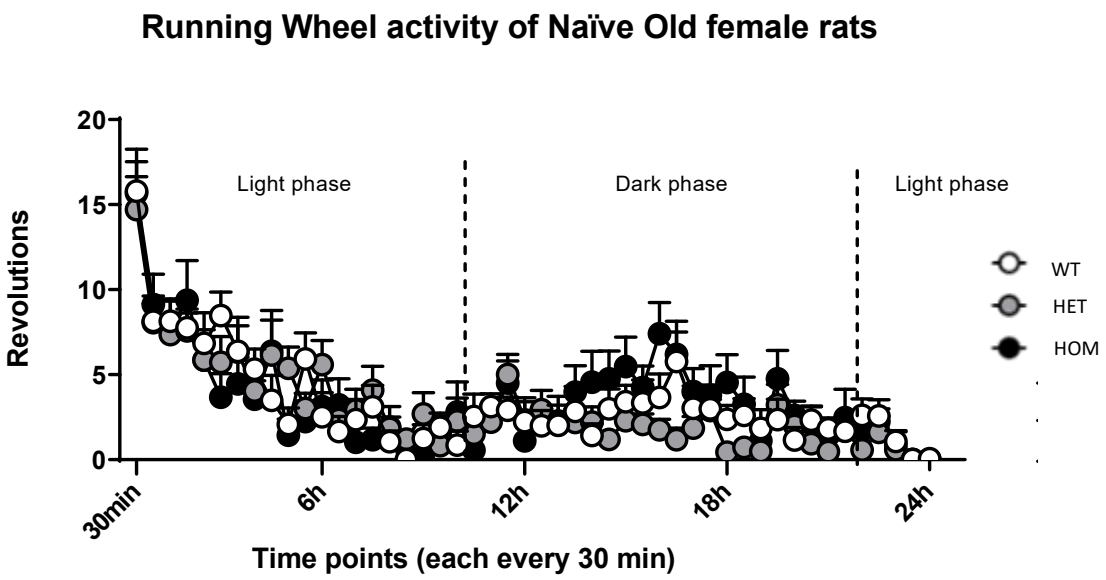


Figure 78. Time-course of running wheel activity of old female rats of the 3 genotypes. Two-way repeated measures.

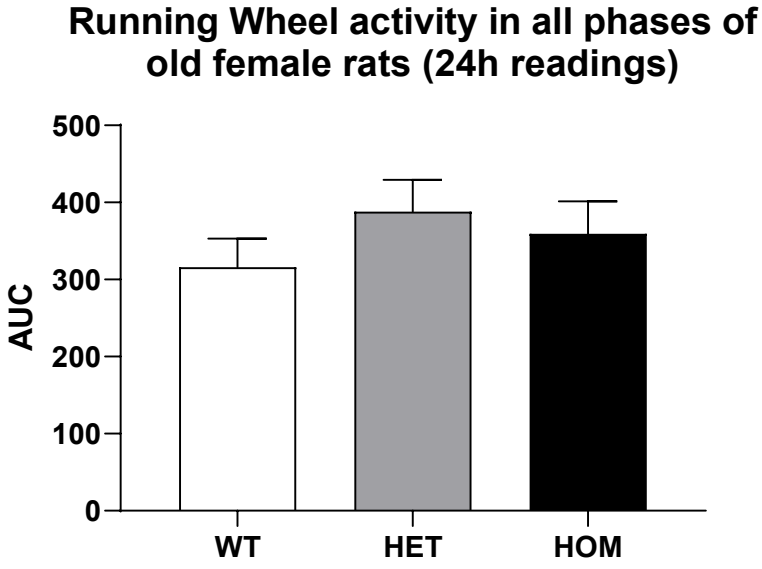


Figure 79. Overall 24 h running wheel activity of old female rats. One way ANOVA followed by

Table 15. Comparison of the running wheel activity of young and old female rats the whole 24h period. Student's t-Test.

Genotype		Statistic	Revolutions (24h-AUC) (Young female rats)	Revolutions (24h-AUC) (Old female rats)	Significance
WT		Mean±s.e.m.	316.1±36.94	151.9±15.44	p=0.0017 (**)
HET		Mean±s.e.m.	388.1±41.21	137.1±15.03	p<0.001 (***)
HOM		Mean±s.e.m.	359.2±42.42	161±21	P<0.001 (***)

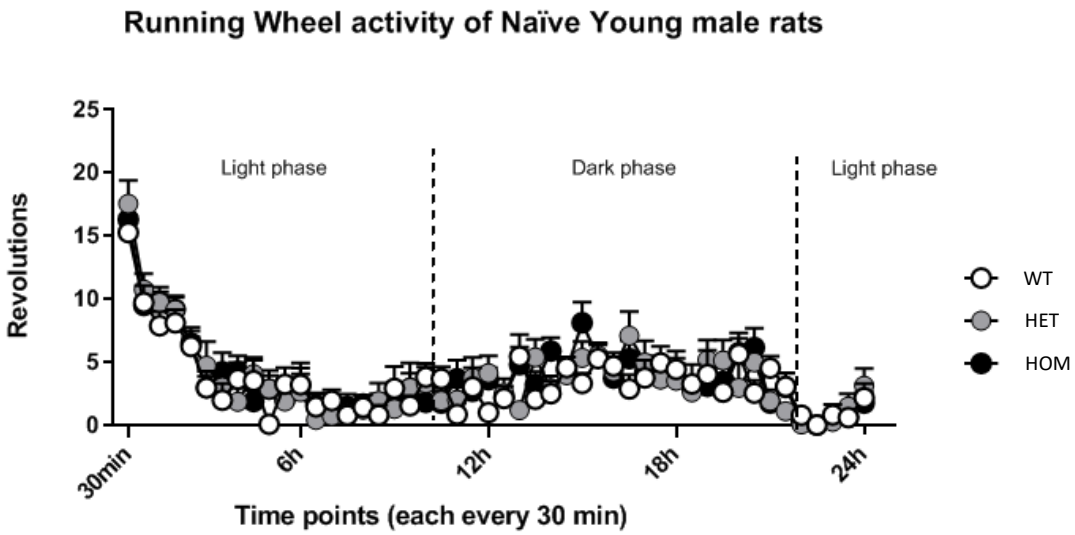


Figure 80. Time-course of running wheel activity of young male rats of the 3 genotypes. Two-way repeated measures.

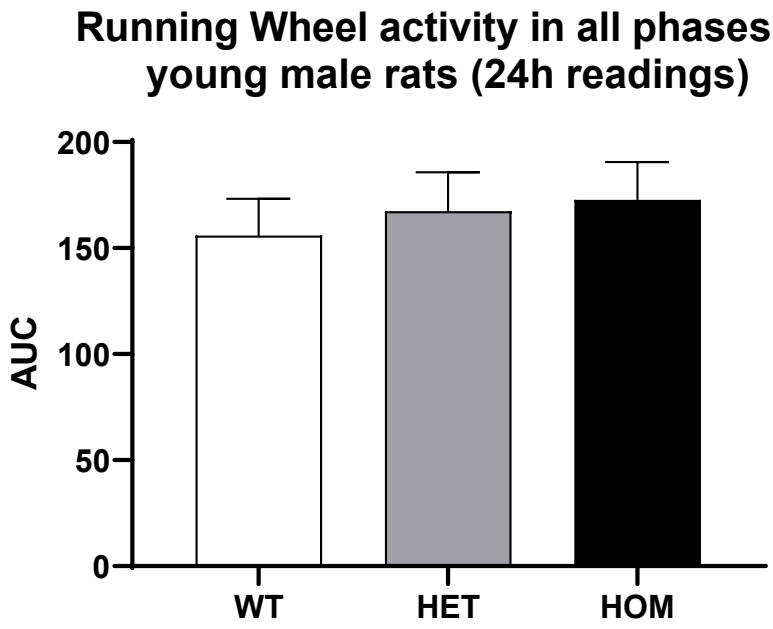
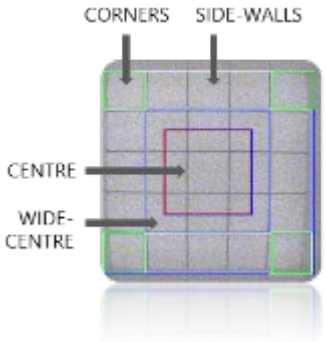


Figure 81. Overall 24 h running wheel activity of young male rats. One way ANOVA followed by Tukey's multiple comparison test.



Open field test results comparing the three genotypes are shown in Figures 82 to 93. For all the parameters, the statistical analysis results are presented in Table 14. The comparison between young and old animals is shown in Figures 94 to 97, including the statistical analysis results.

The behaviour of WT young male rats in the open field showed the normal rodent pattern with a clear preference for safer zones, that is, the side-walls and corners. This behaviour is called thigmotaxis, and it is defined as the natural tendency of rats to stay near the perimeters of a novel environment.

In young male rats, thigmotaxis was mainly observed in the permanence time parameter (Figure 82), with a statistically significant difference between side-walls or corner values vs centre or wide-centre values (Table 12), independently of the genotype. The thigmotaxis was less evident in the travelled distance (Figure 83) or the number of entries (Figure 84).

Young male rats (9-10 weeks old)

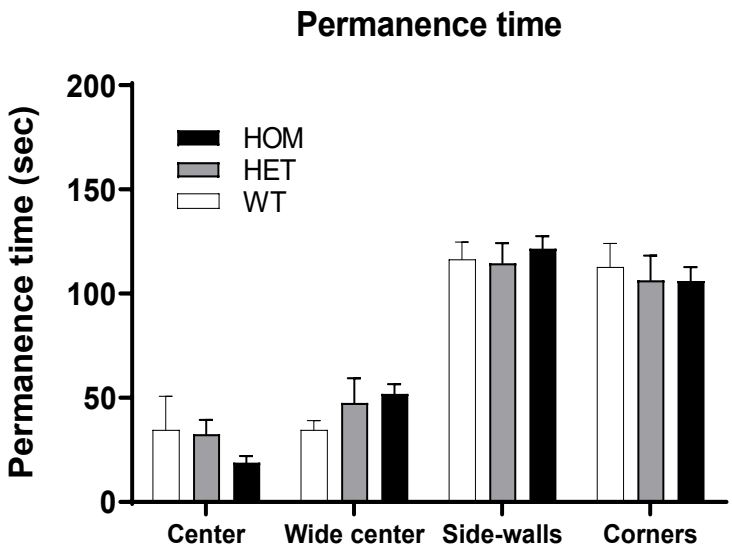


Figure 82. Permanence time in the different zones by young male rats in the open field test. Two-way ANOVA followed by Tukey's multiple comparisons test. For clarity reasons, significances are described in Table 12.

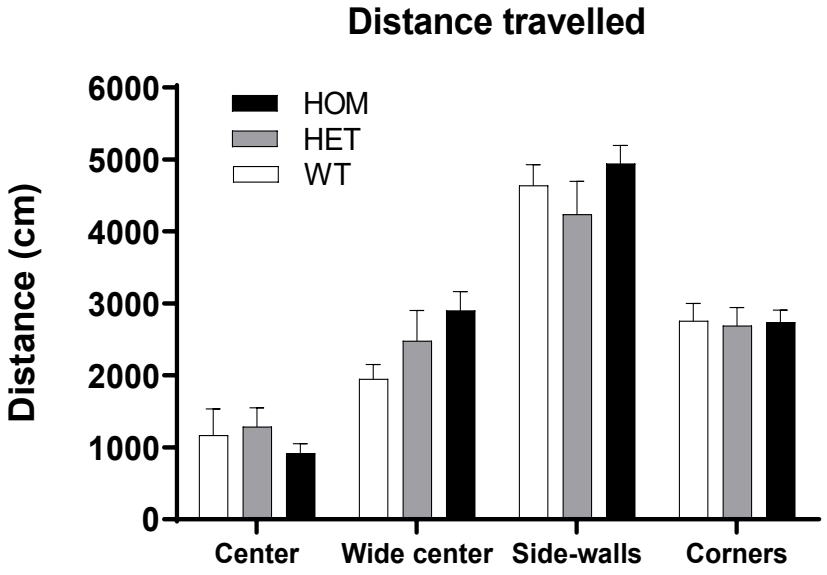


Figure 83. Distance travelled by young male rats in the open field test. Two-way ANOVA followed by Tukey's multiple comparisons test. Statistics are described in Table 12.

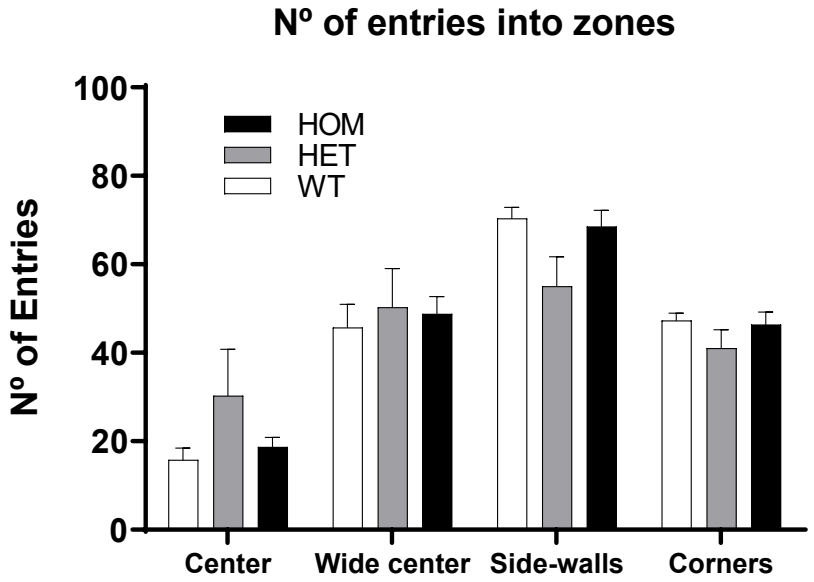


Figure 84. Number of entries in the different zones by young male rats in the open field test. Two-way ANOVA followed by Tukey's multiple comparisons test. Statistics are described in Table 12.

Old male rats (Figure 85) moved much less in the open field, with very low travelled distances (Figure 86) or the number of entries in all the zones, spending most of the time in the corners (Figure 87).

Old male rats (20 months old)

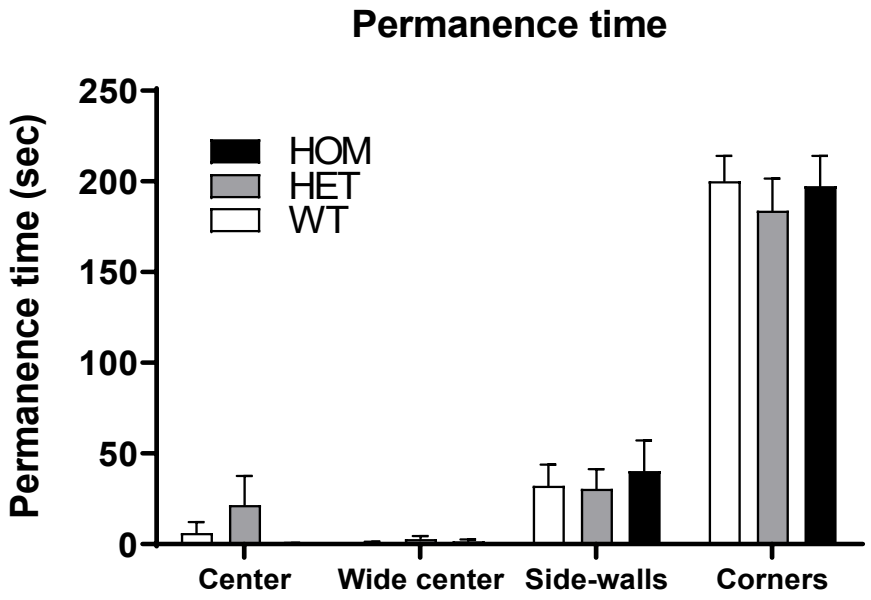


Figure 85. Permanence time in the different zones by old male rats in the open field test. Two-way ANOVA followed by Tukey's multiple comparisons test. Statistics are described in Table 12.

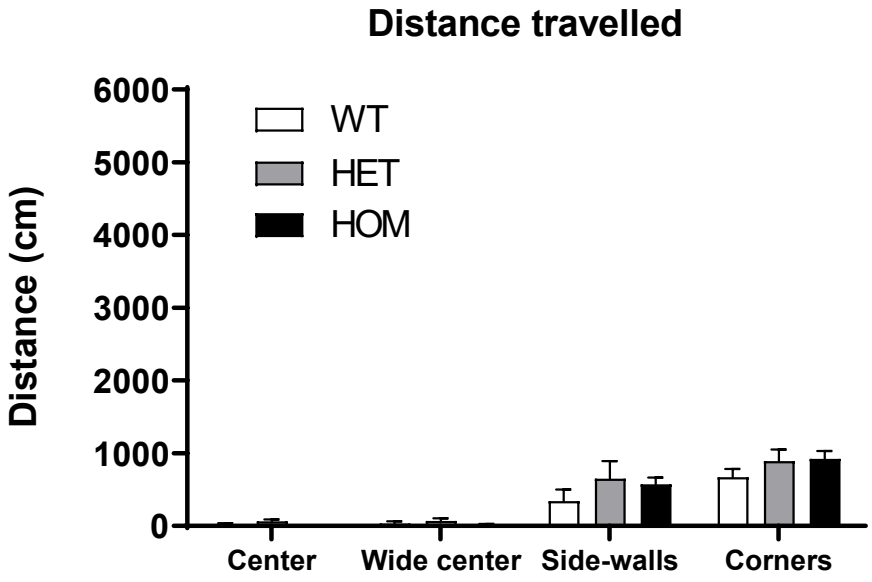


Figure 86. Distance travelled in the different zones by old male rats in the open field test. Two-way ANOVA followed by Tukey's multiple comparisons test. Statistics are described in Table 12.

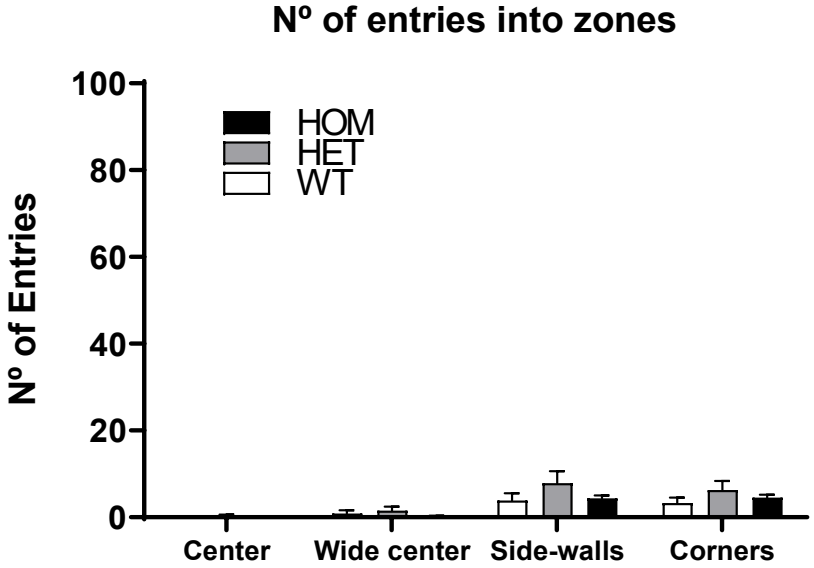


Figure 87. Number of entries in the different zones by old male rats in the open field test. Two-way ANOVA followed by Tukey's multiple comparisons test. Statistics are described in Table 12.

The same pattern was found for young (Figs. 88, 89, and 90) and old (Figs. 91, 92, and 93) female rats. At both ages, rats spent most of the time in the safe zones and travelling more in these areas, as shown by the distance travelled on each area. Old rats moved much less in the open field, with very low travelled distances or number of entries irrespective of the zone and spending most of the time in the corners.

The level of thigmotaxis was higher in old than in young rats, as shown in the permanence time in the side-walls area (Figure 94) and in the corners (Figure 95), although no differences were observed between the three genotypes.

Young female rats (9-10 weeks old)

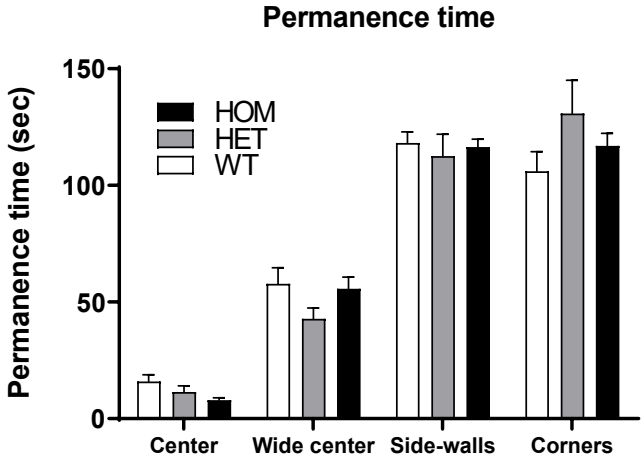


Figure 88. Permanence time in the different zones by young female rats in the open field test. Two-way ANOVA followed by Tukey's multiple comparisons test. Statistics are described in Table 12.

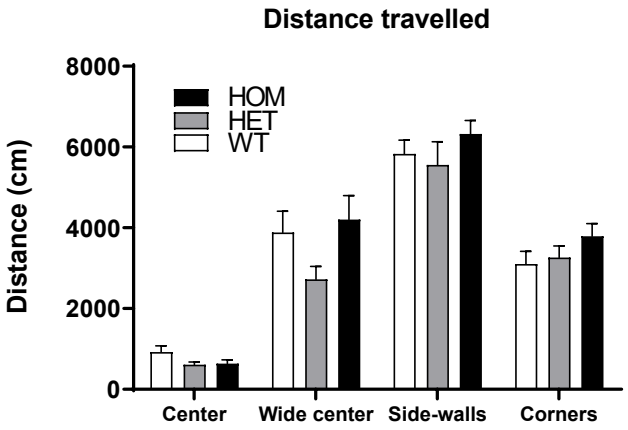


Figure 89. Distance travelled in the different zones by young female rats in the open field test. Two-way ANOVA followed by Tukey's multiple comparisons test. Statistics are described in Table 12.

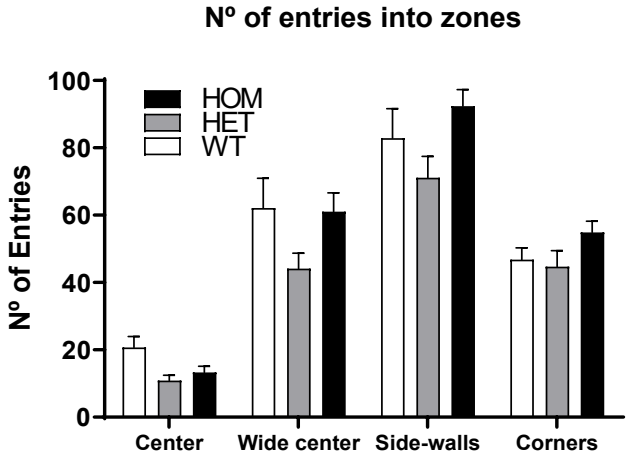


Figure 90. Number of entries in the different zones by young female rats in the open field test. Two-way ANOVA followed by Tukey's multiple comparisons test. Statistics are described in Table 12.

Old female rats (20 months old)

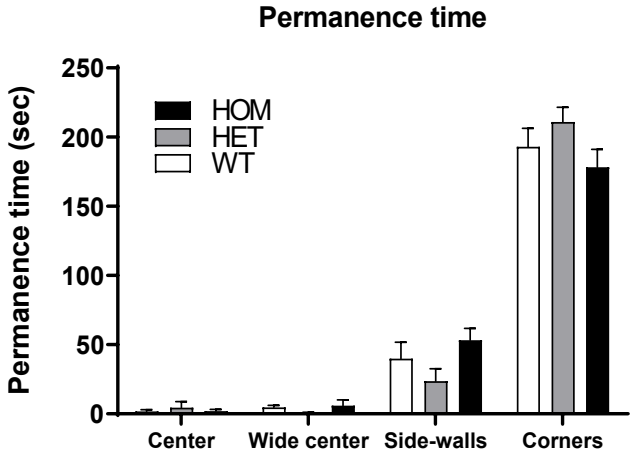


Figure 91. Activity profile of old female rats in the open field test, measured by permanence time in the different zones of the field. Two-way ANOVA followed by Tukey's multiple comparisons test. Statistics are described in Table 12.

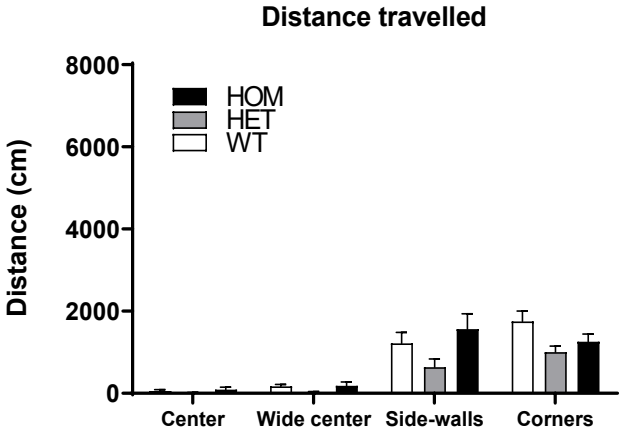


Figure 92. Activity profile of old female rats in the open field test, measured travelled distance in the different zones of the field. Two-way ANOVA followed by Tukey's multiple comparisons test. For clarity reasons, significances are described in Table 12.

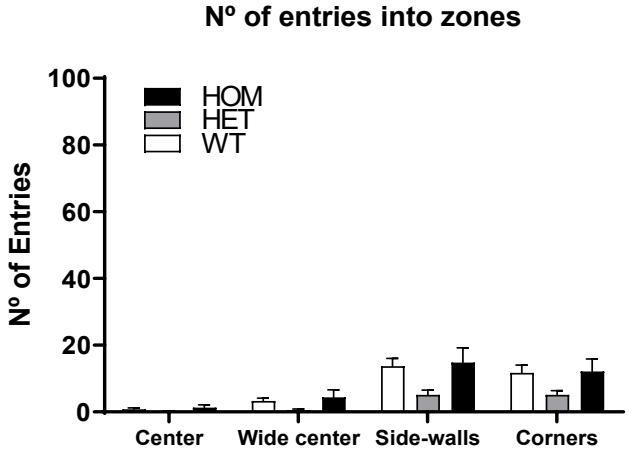


Figure 93. Activity profile of old female rats in the open field test, measured by number of entries in the different zones of the field. Two-way ANOVA followed by Tukey's multiple comparisons test. For clarity reasons, significances are described in Table 12.

Further analysis of the thigmotaxis behaviour, a clear different between young and old rats was shown when comparing the permanence time or the distance travelled in the side-walls or the corners, either in females or males. Old animals stayed more time in the corners (Figure 95) than in the side-walls area (Figure 94), just the contrary than young rats did. This behaviour was also reflected as distance travelled on each area, with old rats moving much less as shown by lower distances travelled in the side-walls (Figure 95) and the corners (Figure 96). This pattern was similar in females and males, and no differences between genotypes were observed.

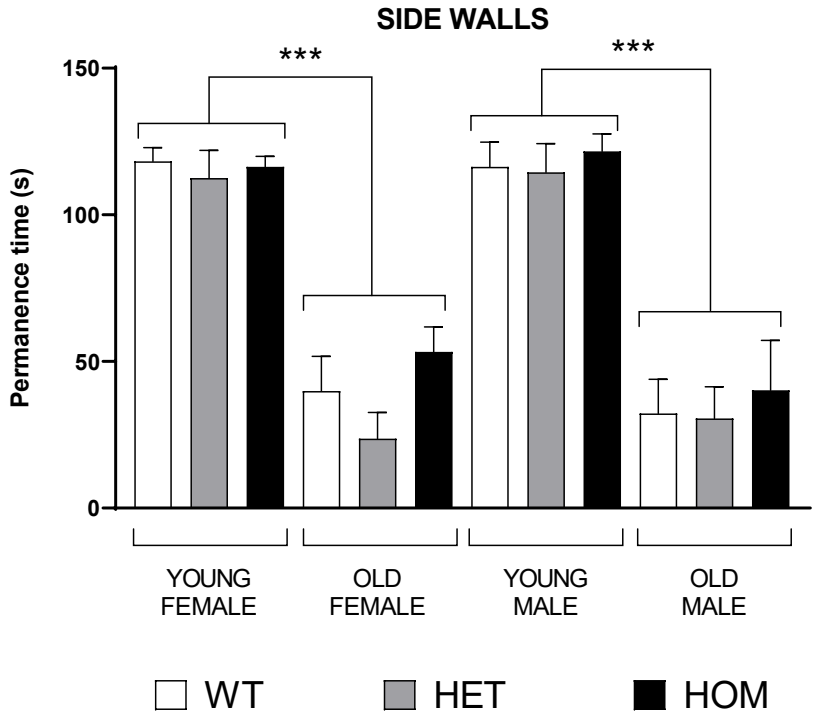


Figure 94. Thigmotaxis of young and old female and male rats in the open field test, measured by permanence time in the side-walls area. Two-way ANOVA followed by Tukey's multiple comparisons test. ***P<0.001

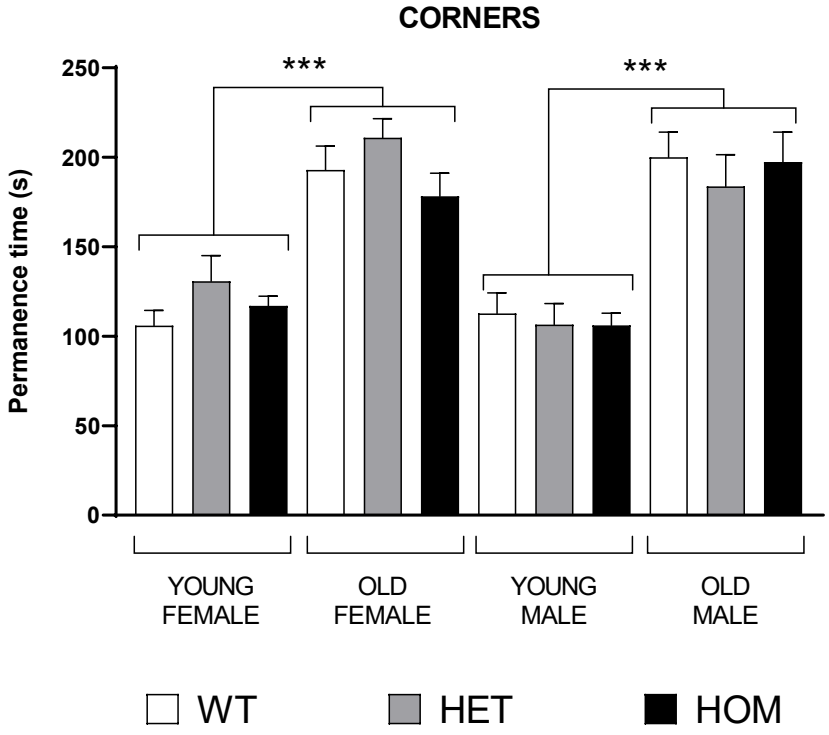


Figure 95. Thigmotaxis of young and old female and male rats in the open field test, measured by permanence time in the corners area. Two-way ANOVA followed by Tukey's multiple comparisons test.

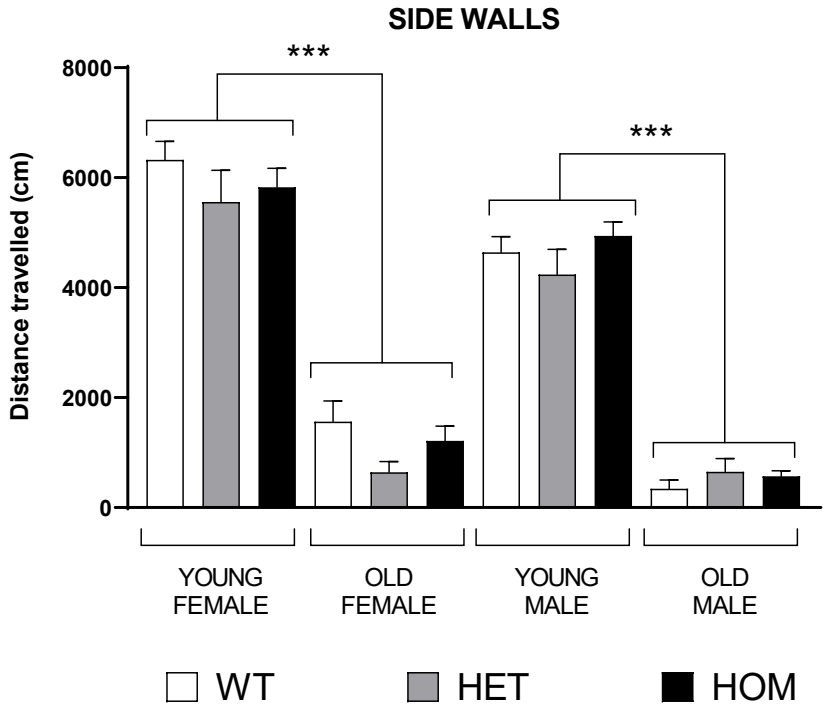


Figure 96. Thigmotaxis of young and old female rats in the open field test, measured by distance travelled in the side-walls area. Two-way ANOVA followed by Tukey's multiple comparisons test. ***P<0.001

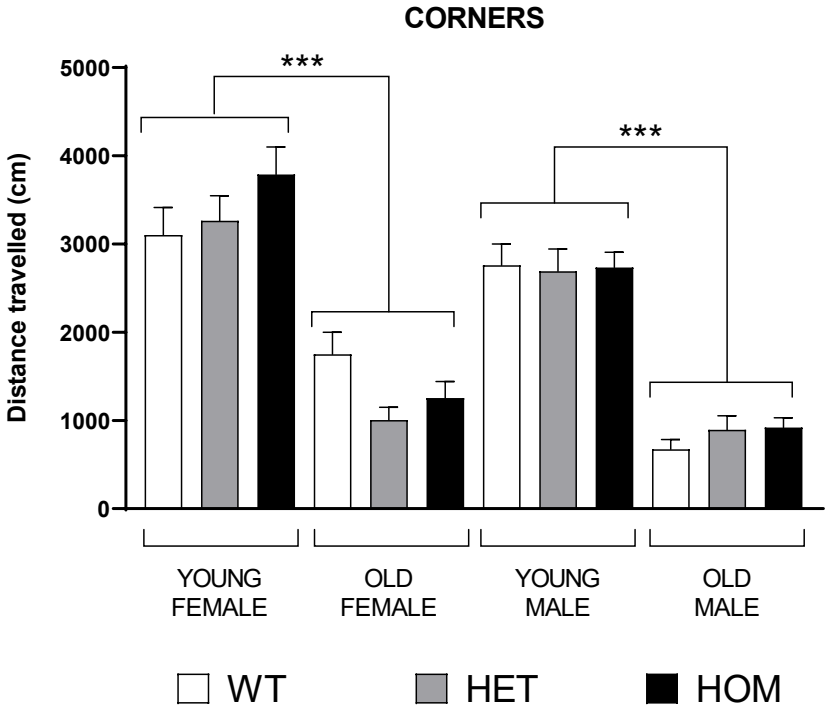


Figure 97. Thigmotaxis of young and old female rats in the open field test, measured by distance travelled in the corners area. Two-way ANOVA followed by Tukey's multiple comparisons test. ***P<0.001

Table 16. Statistical analysis of open field results. Two-way ANOVA followed by Tukey's multiple comparison test. (n.s. = not significant). Marked in blue values different from WT results for each age range.

	Young male rats			Old male rats			Young female rats			Old female rats		
	Permanence Time	Distance travelled	Number of entries	Permanence Time	Distance travelled	Number of entries	Permanence Time	Distance travelled	Number of entries	Permanence Time	Distance travelled	Number of entries
	Adjusted P Value											
WT												
Center vs. Wide center	n.s.	n.s.	<0.0001	n.s.	n.s.	n.s.	0.0001	<0.0001	<0.0001	n.s.	n.s.	n.s.
Center vs. Side-walls	<0.0001	<0.0001	<0.0001	n.s.	n.s.	n.s.	<0.0001	<0.0001	<0.0001	0.0169	0.0006	0.0026
Center vs. Corners	<0.0001	0.0007	<0.0001	<0.0001	0.0013	n.s.	<0.0001	0.0003	0.0038	<0.0001	<0.0001	0.0146
Wide center vs. Side-walls	<0.0001	<0.0001	0.0018	n.s.	n.s.	n.s.	<0.0001	0.0016	0.0316	0.0317	0.0023	0.0217
Wide center vs. Corners	<0.0001	n.s.	n.s.	<0.0001	0.0018	n.s.	<0.0001	n.s.	n.s.	<0.0001	<0.0001	n.s.
Side-walls vs. Corners	n.s.	<0.0001	0.0041	<0.0001	n.s.	n.s.	n.s.	<0.0001	<0.0001	<0.0001	n.s.	n.s.
HET												
Center vs. Wide center	n.s.	0.042	0.0411	n.s.	n.s.	n.s.	0.0065	0.0005	0.0001	n.s.	n.s.	n.s.
Center vs. Side-walls	<0.0001	<0.0001	0.0065	n.s.	0.0014	0.0002	<0.0001	<0.0001	<0.0001	n.s.	n.s.	n.s.
Center vs. Corners	<0.0001	0.011	n.s.	<0.0001	<0.0001	0.0059	<0.0001	<0.0001	<0.0001	<0.0001	0.0006	n.s.
Wide center vs. Side-walls	<0.0001	0.0007	n.s.	n.s.	0.0016	0.0026	<0.0001	<0.0001	0.0025	n.s.	n.s.	n.s.
Wide center vs. Corners	0.0002	n.s.	n.s.	<0.0001	<0.0001	0.0415	<0.0001	n.s.	n.s.	<0.0001	0.0008	n.s.
Side-walls vs. Corners	n.s.	0.0039	n.s.	<0.0001	n.s.	n.s.	n.s.	0.0001	0.0033	<0.0001	n.s.	n.s.
HOM												
Center vs. Wide center	0.007	<0.0001	<0.0001	n.s.	n.s.	n.s.	<0.0001	<0.0001	<0.0001	n.s.	n.s.	n.s.
Center vs. Side-walls	<0.0001	<0.0001	<0.0001	n.s.	0.0039	n.s.	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0002
Center vs. Corners	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	n.s.	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0033
Wide center vs. Side-walls	<0.0001	<0.0001	0.0024	n.s.	0.005	n.s.	<0.0001	0.0005	0.0003	0.0003	<0.0001	0.0056
Wide center vs. Corners	<0.0001	n.s.	n.s.	<0.0001	<0.0001	n.s.	<0.0001	n.s.	n.s.	<0.0001	0.0002	n.s.
Side-walls vs. Corners	n.s.	<0.0001	0.0005	<0.0001	n.s.	n.s.	n.s.	<0.0001	<0.0001	<0.0001	n.s.	n.s.

Overall, the sigma-1 KO rats did not show any difference in the anxiety behaviour in comparison to WT rats, either in males or in females, and the behaviour was also similar between genotypes when comparing young and old animals. In addition, the differences due to the age were the same in all the genotypes.

DEPRESSION

The depression studies were performed before heterozygous line creation, so they were done with WT Wistar rats and sigma-1 homozygous KO rats.

ACUTE STUDY

First, we performed an acute administration study for assessing the behaviour of KO rats in this environmental condition during the training session, and to assess whether the efficacy of antidepressant treatments was different in KO subjects, in comparison to the described poor efficacy of antidepressants in WT rats after acute treatment.

The size of the rats was comparable between WT and KO males (Figure 98). In females a statistically significant difference was obtained for KO females, which were slightly bigger than WT females (Figure 99). Nevertheless, none of the rats were able to touch the bottom of the water container with the hindlimbs, which would be an important bias that could interfere with the measurement of the immobility.

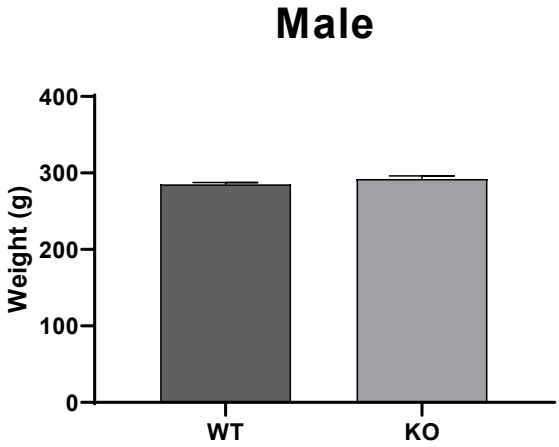


Figure 98. Body weight of male rats of WT and sigma-1 KO genotypes. Student-t test.

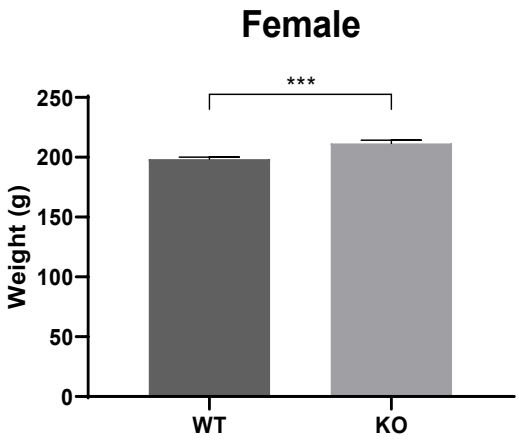


Figure 99. Body weight of female rats of WT and sigma-1 KO genotypes. Student-t test ***p<0.001

No differences were observed in the immobility time of WT and sigma-1 KO rats, either in males (Figure 100) or in females (Figure 101), during the last 5 min of the 15 min training session.

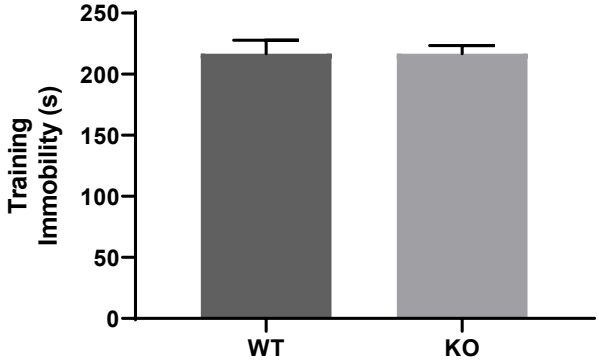


Figure 100. Immobility time of male rats of WT and sigma-1 KO genotypes. Student-t test.

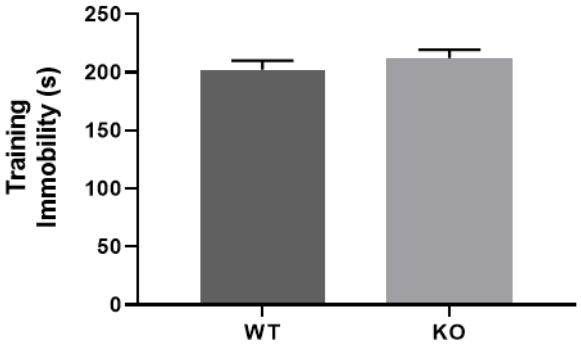


Figure 101. Immobility time of female rats of WT and sigma-1 KO genotypes. Student-t test.

It has been reported that, after acute administration, WT rats respond poorly to antidepressant treatment, or high doses are needed to obtain a significant efficacy. Moreover, previous studies in this work showed some better activity of antidepressant drugs in KO mice. Therefore, we decide to use fluoxetine, with sigma-1 receptor affinity, and venlafaxine, without sigma-1 receptor affinity, for assessing their efficacy in WT and KO rats after acute treatment.

The administration of 0.5% HPMC did the same effect in WT and KO rats. The administration of fluoxetine (30 mg/kg, i.p.) or venlafaxine (30 mg/kg, i.p.) did not produce any significant reduction in the immobility time during test session, 24h after training, either in WT males (Figure 102), KO males (Figure 103), WT females (Figure 104) and KO females (Figure 105).

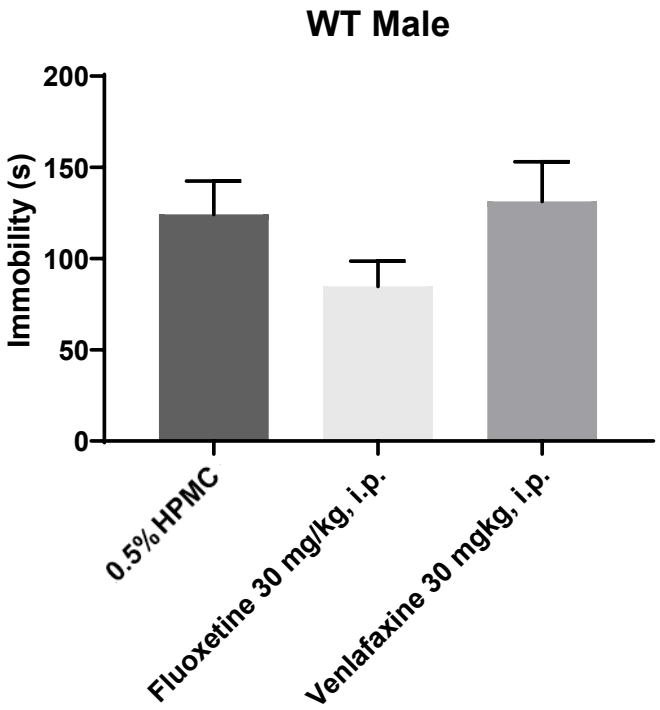


Figure 102. Immobility time of WT male rats after 0.5% HPMC, fluoxetine 30mg/kg, or venlafaxine 30mg/kg administration.

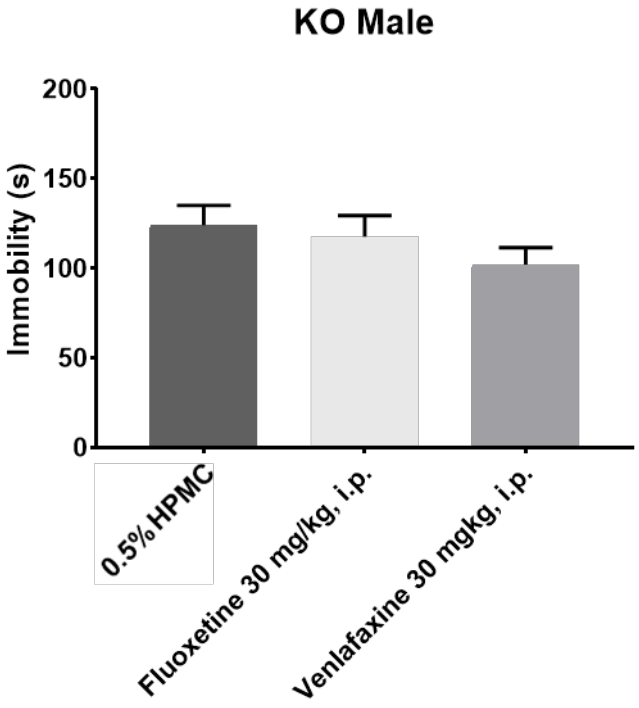


Figure 103. Immobility time of KO male rats after 0.5% HPMC, fluoxetine 30mg/kg, or venlafaxine 30mg/kg administration.

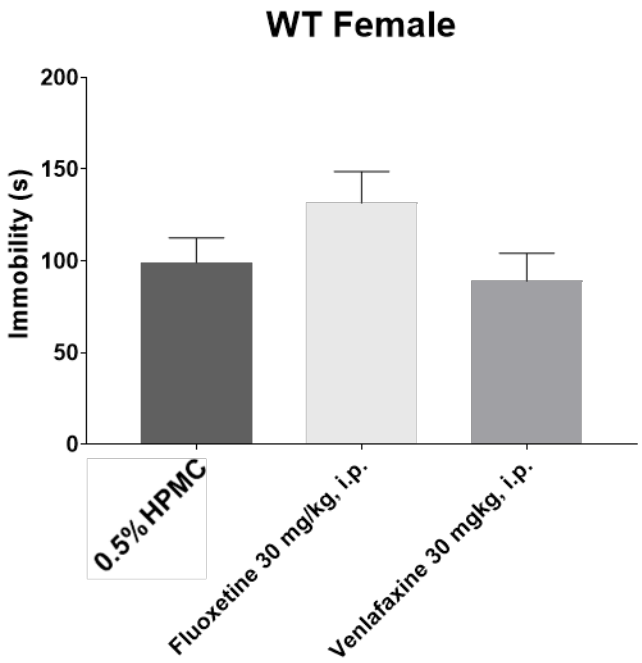


Figure 104. Immobility time of WT female rats after 0.5% HPMC, fluoxetine 30mg/kg, or venlafaxine 30mg/kg administration.

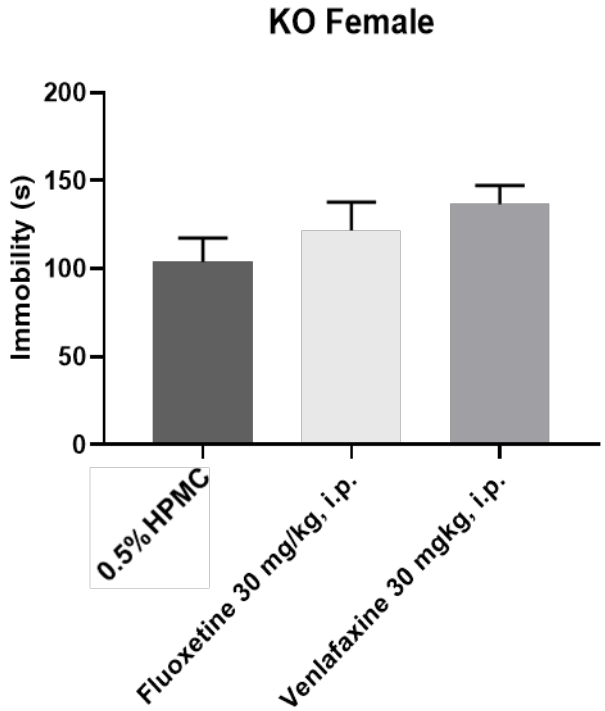


Figure 105. Immobility time of KO female rats after 0.5% HPMC, fluoxetine 30mg/kg, or venlafaxine 30mg/kg administration.

The FST acute treatment study do not revealed any difference between WT and KO rats, either in the spontaneous behaviour during the training session or in the antidepressant effects during the test session.

After the acute treatment study, we scheduled a sub-acute study with the antidepressant with the highest sigma-1 receptor affinity, fluvoxamine, and with an antidepressant without sigma-1 receptor affinity, venlafaxine. In the acute study, no differences in the training session or in response to antidepressant treatment were observed between male and female rats. Therefore, sub-acute experiments were done only with male rats. The change from fluoxetine to fluvoxamine was because the highest affinity for the sigma-1 receptor of fluvoxamine.

The availability of sigma-1 KO rats conditioned the range of ages and weights used in these experiments, making difficult to match with WT animals from another provider. This explains the slight difference in the weight of the different groups we worked with. However, a correlation analysis performed on the results obtained during the training session showed that immobility time was not dependent on the weight of the animals, either in the WT (Figure 106) or in the KO (Figure 107) rats. The analysis of all the data available during training session did not show any correlation either (Figure 108).

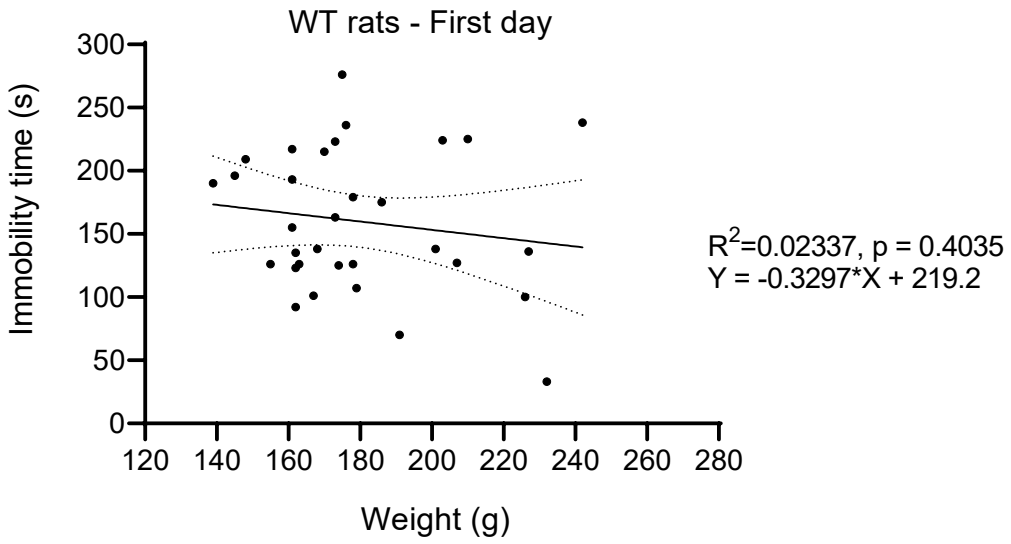


Figure 106. Simple linear regression analysis between weight and immobility time of WT rats.

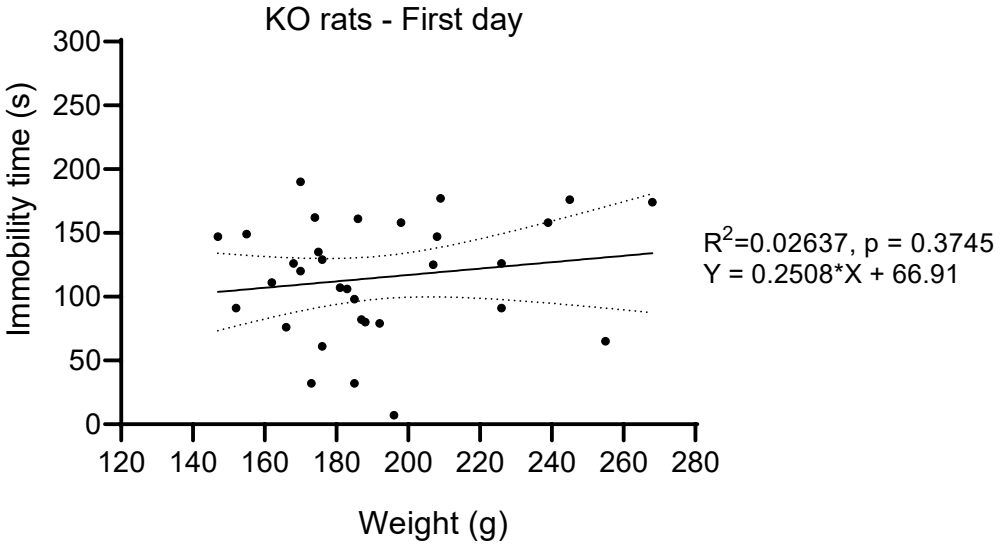


Figure 107. Simple linear regression analysis between weight and immobility time of KO rats.

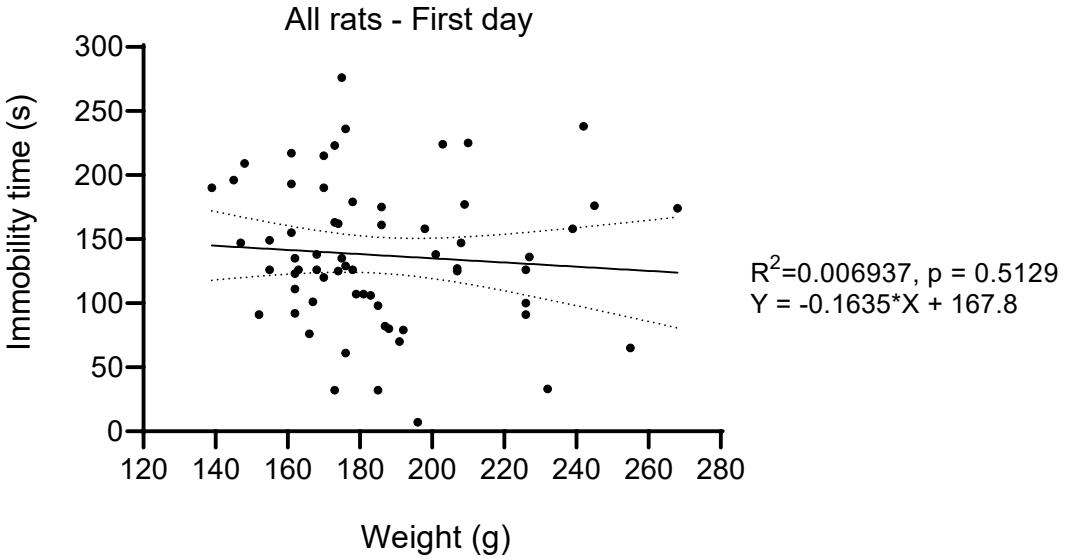


Figure 108. Simple linear regression analysis between weight and immobility time during the training session of all used rats.

The use of the digital image analysis system SMART allowed us to further evaluate the pattern of immobility time throughout the session, and additional parameters like speed,

slow movements, or fast movements. The immobility during the last 5′ of the session was not different for WT and KO rats, in agreement with what we found in the acute study. However, sigma-1 KO rats' immobility was significantly lower during the 5′ and 10′ readings. The reduction in the resting time (immobility; Figure 109) was partially due to an increase in slow movements (Figure 110), but mainly to the increase in fast movements (Figure 111), that could be correlated with struggling to escape behaviour. Consequently, average speed was found to be higher in KO rats than in WT rats (Fig 112). All the differences found disappeared in the last 5′ of the session. The differences in the swimming behaviour, as already mentioned, cannot be assigned to a difference in the weight of the animals (Figure 113).

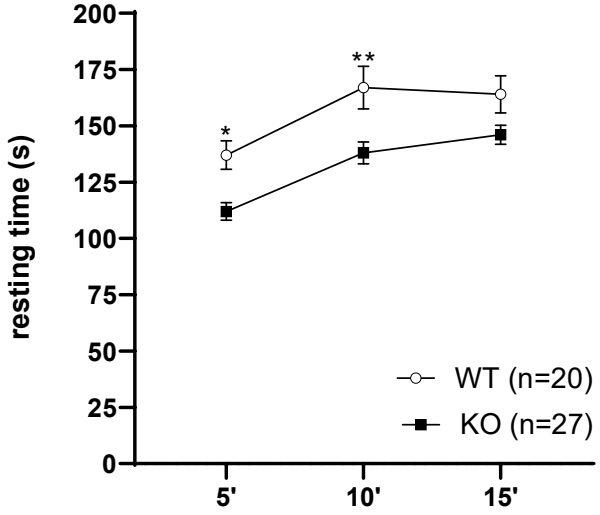


Figure 109. Forced swimming test. Resting time during the 15 min training session, split in 5 min reading points. Two-way ANOVA followed by Šídák's multiple comparisons test. *p<0.05; **p<0.01.

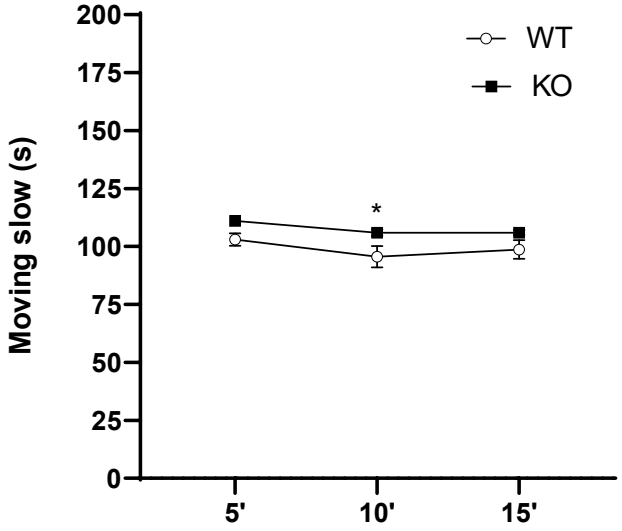


Figure 110. Forced swimming test. Moving slow time during the 15 min training session, split in 5 min reading points. Two-way ANOVA followed by Šídák's multiple comparisons test. *p<0.05.

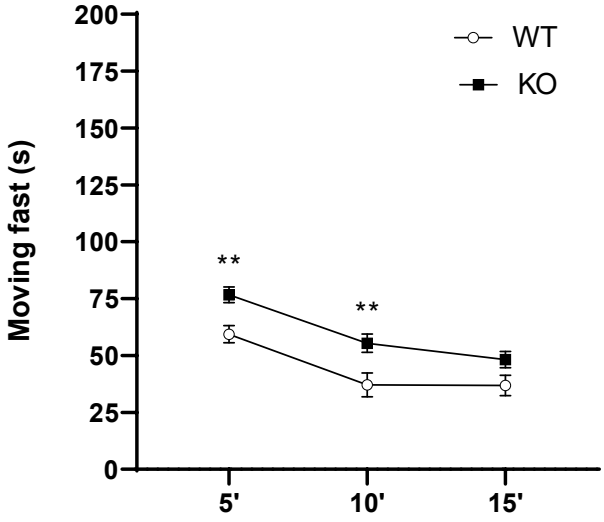


Figure 111 Forced swimming test. Moving fast time during the 15 min training session, split in 5 min reading points. Two-way ANOVA followed by Šídák's multiple comparisons test. **p<0.01.

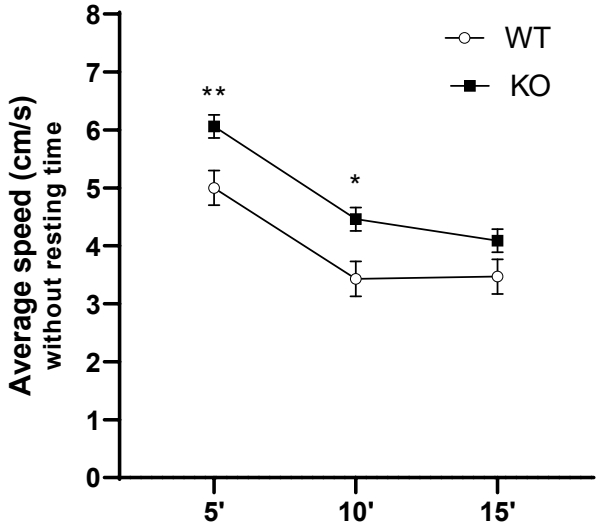


Figure 112. Forced swimming test. Average speed during the 15 min training session, split in 5 min reading points. Two-way ANOVA followed by Šídák's multiple comparisons test. *p<0.05; **p<0.01.

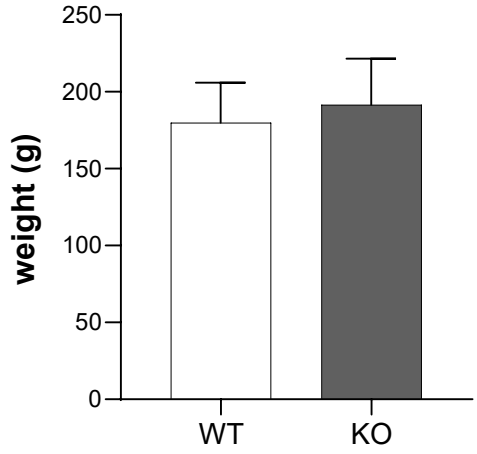


Figure 113. Forced swimming test. Weight of rats at training session. t-Student's test.

Twenty-four hours after the training session (day 1) we did the first administration and 30 min later the first test session (day 2). 0.5% HPMC, venlafaxine (10 mg/kg, i.p.) or fluvoxamine (10 mg/kg, i.p.) were administered daily, in the morning, and additional test sessions were performed on day 7 and day 14. Vehicle-treated rats showed an increasing immobility time throughout test sessions (Figure 114) in both, WT and KO subjects. Venlafaxine and fluvoxamine did not modify WT rats' behaviour showing a similar profile in the three test sessions (Figure 115; Table 17).

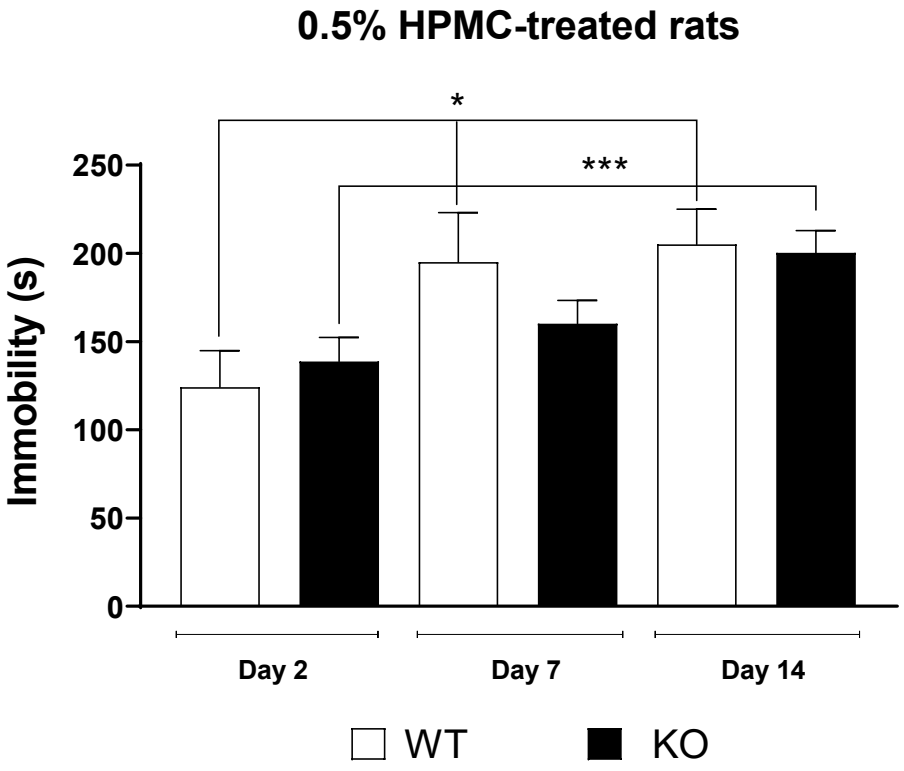


Figure 114. Immobility time of WT and KO rats in the FST, after sub-acute i.p. administration of 0.5% HPMC. One-way ANOVA followed by Dunnett's multiple comparison test vs respective day 2. *p<0.05; ***p<0.001. Unpaired t-test was applied for WT and KO groups on each day.

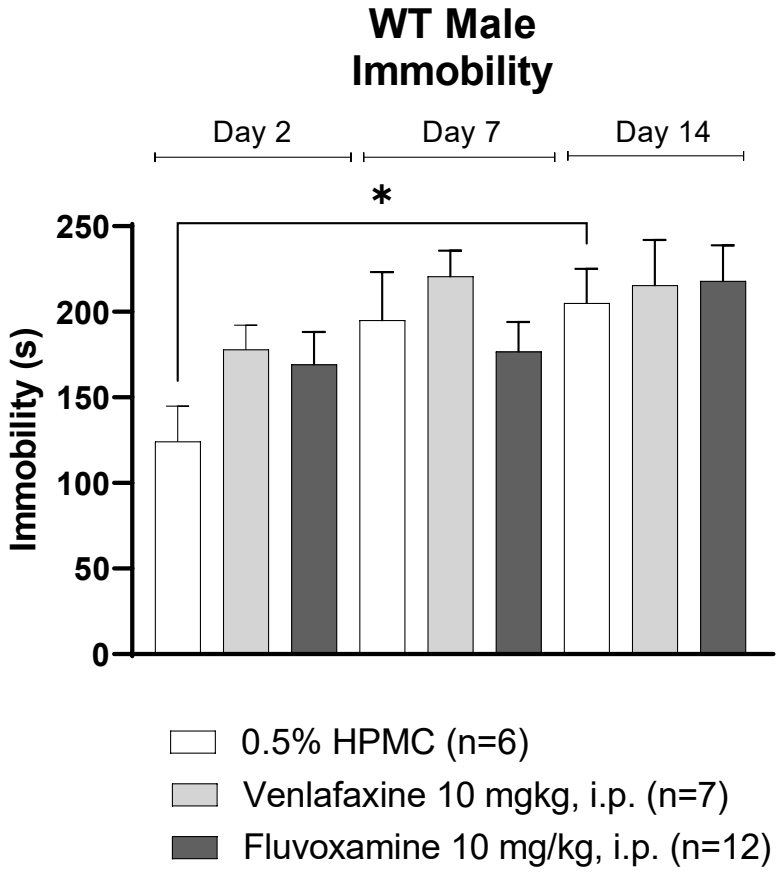


Figure 115. Immobility time of WT rats in the FST, after sub-acute administration of vehicle or the antidepressants venlafaxine and fluvoxamine. Two-way repeated measures ANOVA followed by Tukey's multiple comparison test.

Table. 17. Immobility time of WT rats in the FST, after sub-acute administration of vehicle or the antidepressants venlafaxine and fluvoxamine.

	1st test session	**2nd test session**	**3rd test session**
0.5% HPMC	124±20.7	195±28.1	205.2±20
Venlafaxine 10 mg/kg, i.p.	178±14.2	220.7±15	215.4±26.6
Fluvoxamine 10 mg/kg, i.p.	169.3±18.9	176.8±17.2	218.1±20.7

In sigma-1 KO rats, the administration of 0.5% HPMC did not change the pattern of resting time observed in WT animals, with increasing immobility over time (Figure 116). In the first test session, the administration of the antidepressants venlafaxine or fluvoxamine, both at 10 mg/kg, did not induce any statistically significant effect on the immobility of KO rats, although a slight reduction was observed. The same was shown during the second test session on day 7, with non-significant slight immobility time reduction. However, during the 3rd test session on day 14, both antidepressants induced a significant reduction of immobility in comparison to vehicle-treated rats (Figure 116, Table 18).

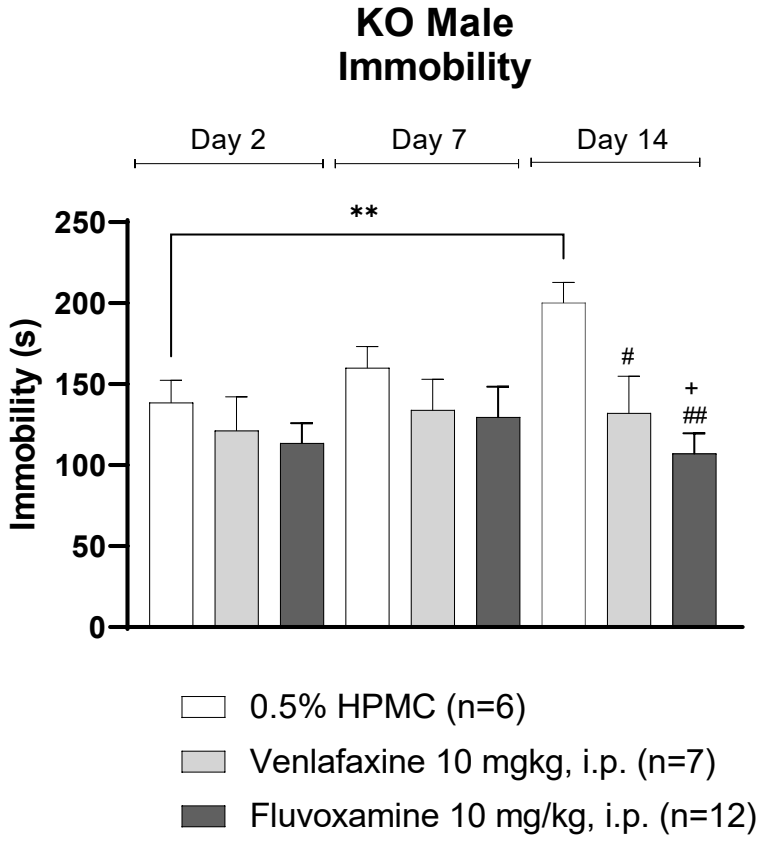


Figure 116. Immobility time of sigma.1 KO rats in the FST, after sub-acute administration of vehicle or the antidepressants venlafaxine and fluvoxamine. Two-way repeated measures ANOVA followed by Tukey's multiple comparison test $^{+}p<0.05$ vs 0.5% HPMC on day 14; One-way ANOVA followed by Dunnett's multiple comparisons $^{**}p<0.01$ between days for each treatment; $^{\#}p<0.05$; $^{\#\#}p<0.01$ vs 0.5% HPMC on day 14.

Table. 18. Immobility time of sigma-1 KO rats in the FST, after sub-acute administration of vehicle or the antidepressants venlafaxine and fluvoxamine. One-way ANOVA followed by Dunnett's multiple comparisons *p<0.05; **p<0.01 vs 0.5% HPMC on each test session.

	1st test session	2nd test session	3rd test session
0.5% HPMC	138.7±13.7	160.2±13.2	200.3±12.6
Venlafaxine 10 mg/kg, i.p.	121.3±21	134.0±19	132.3±22.6 *
Fluvoxamine 10 mg/kg, i.p.	113.6±12.3	129.7±18.8	107.2±12.6 **

In addition to the immobility time, we measured the struggling behaviour, quick movements of the forelimbs surpassing the surface of the water trying to escape. We observed a decrease in this behaviour within the session and between sessions, both in WT (Figure 117) and KO (Figure 118) rats. However, we found a high variability for this read-out and no differences were found for any treatment or genotype.

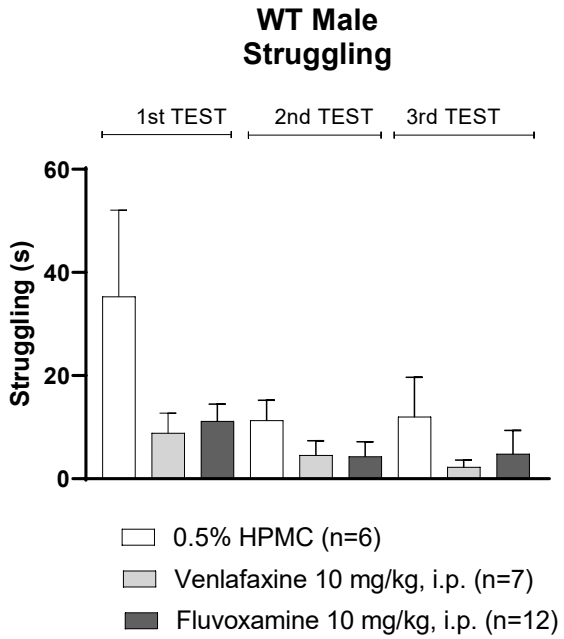


Figure 117. Struggling time of WT rats in the FST, after sub-acute administration of vehicle or the antidepressants venlafaxine and fluvoxamine. Two-way repeated measures ANOVA followed by Tukey's multiple comparisons test.

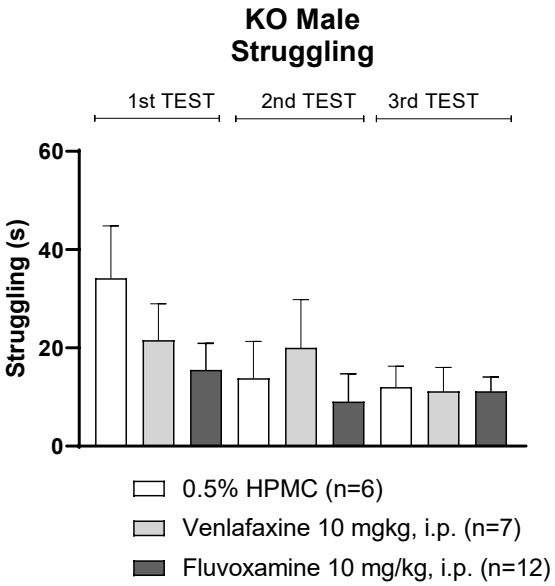


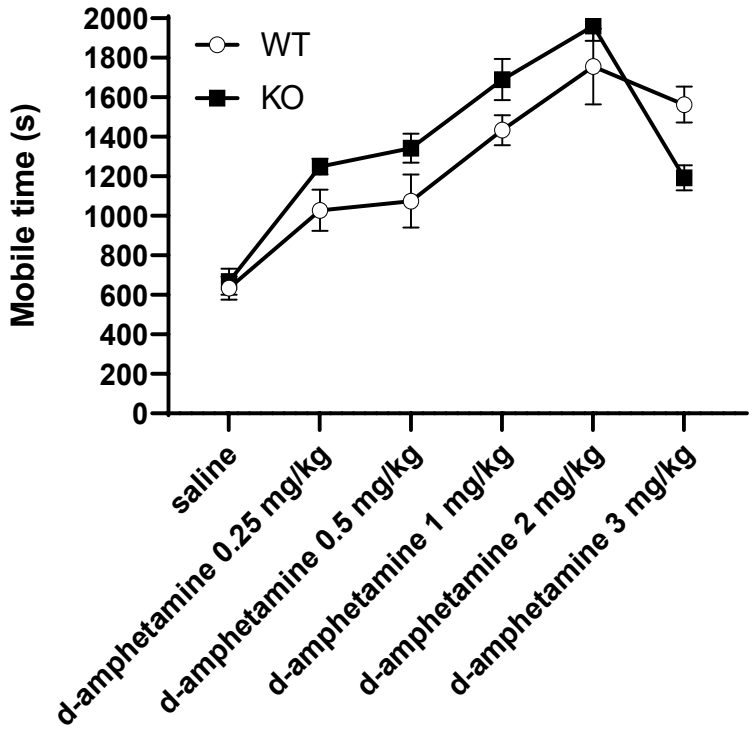
Figure 118. Struggling time of sigma-1 KO rats in the FST, after sub-acute administration of vehicle or the antidepressants venlafaxine and fluvoxamine. Two-way repeated measures ANOVA followed by Tukey's multiple comparisons test.

DRUG-INDUCED HYPERACTIVITY

D-AMPHETAMINE

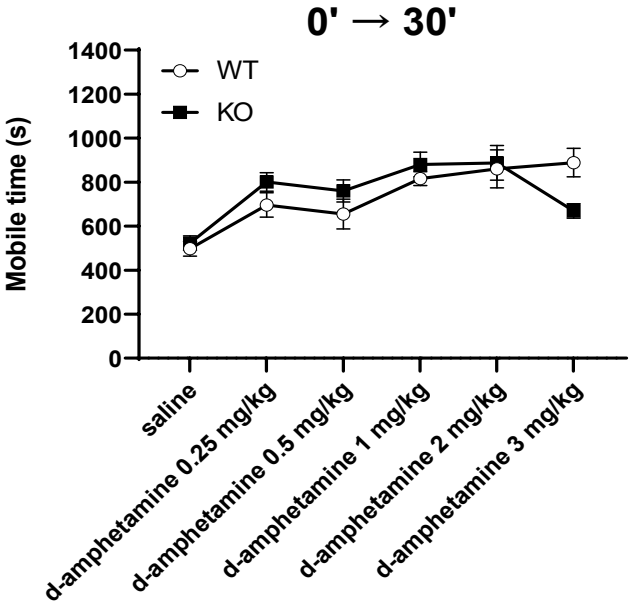
The administration of d-amphetamine (0.25-3 mg/kg, s.c.) induced hyperactivity in a dose-dependent manner (Figure 119) from 0.25 mg/kg up to 2 mg/kg. At 3 mg/kg locomotion decreased due to an increase in the stereotyped behaviours (sniffing, licking, head weaving, gnawing...) that blocked the expression of forward locomotion. At all doses tested, the effect induced by d-amphetamine was lower in WT rats than in KO counterparts, but no statistical significance was reached in any of them. The analysis of the time-course of d-amphetamine-induced hyperactivity at different doses for WT (Figure 122) and KO rats (Figure 123) revealed a significant effect of time ($p<0.0001$) and treatment ($p<0.0001$), with a significant interaction between the variables, so the time effect depended on the treatment given ($p<0.0001$).

Splitting the analysis of the dose-dependent effect in the first (Figure 120) and second half (Figure 121) of the session revealed that most of the effect was obtained during the second half. Although the pattern of response was the same, with higher response in KO subjects at all d-amphetamine doses, in the second half a statistically significant difference was reached for the genotype (p=0.012).



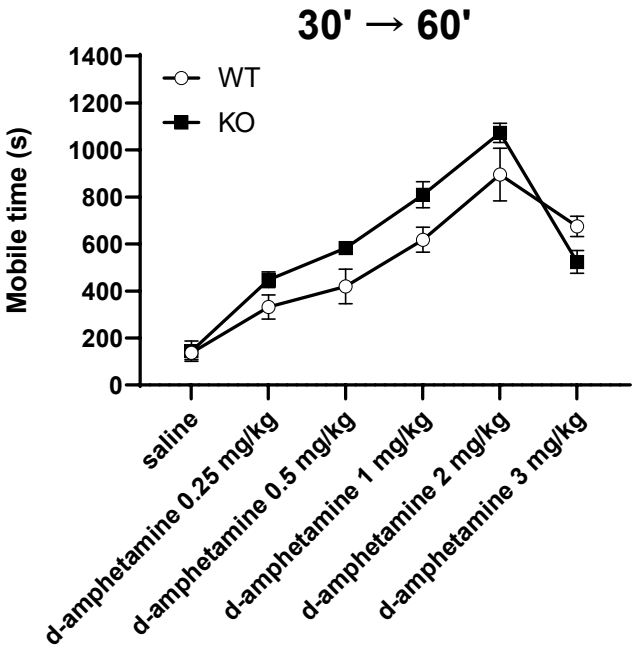
Source of Variation	% of total variation	P value	P value summary
Interaction	4.782	0.0173	*
Treatment	75.36	<0.0001	****
Genotype	1.079	0.0701	ns

Figure 119. d-amphetamine-induced dose-response effects on forward locomotion (mobile time) in WT and KO rats. Two-way ANOVA followed by Tukey's multiple comparison test.



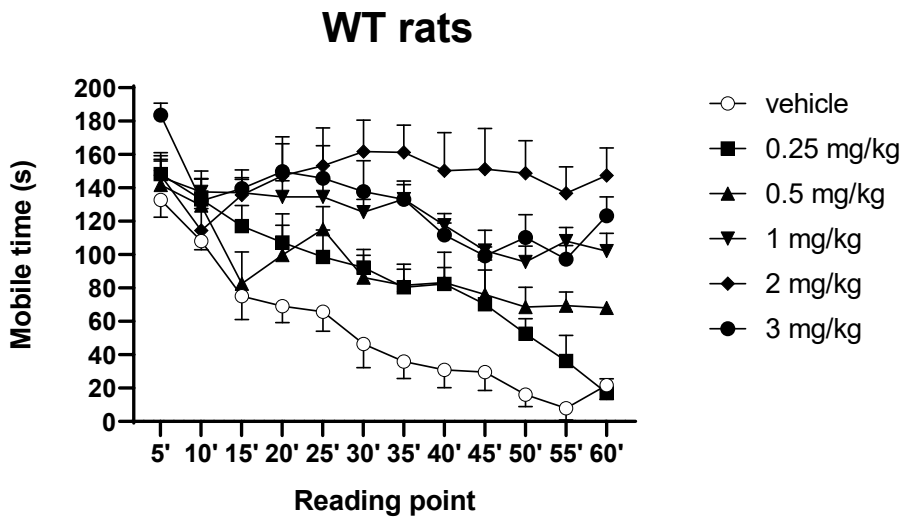
Source of Variation	% of total variation	P value	P value summary
Interaction	8.044	0.0482	*
Treatment	55.15	<0.0001	****
Genotype	0.2396	0.5521	ns

Figure 120. d-amphetamine-induced dose-response effects on forward locomotion (mobile time) during the first half of the session. Two-way ANOVA followed by Tukey's multiple comparison test.



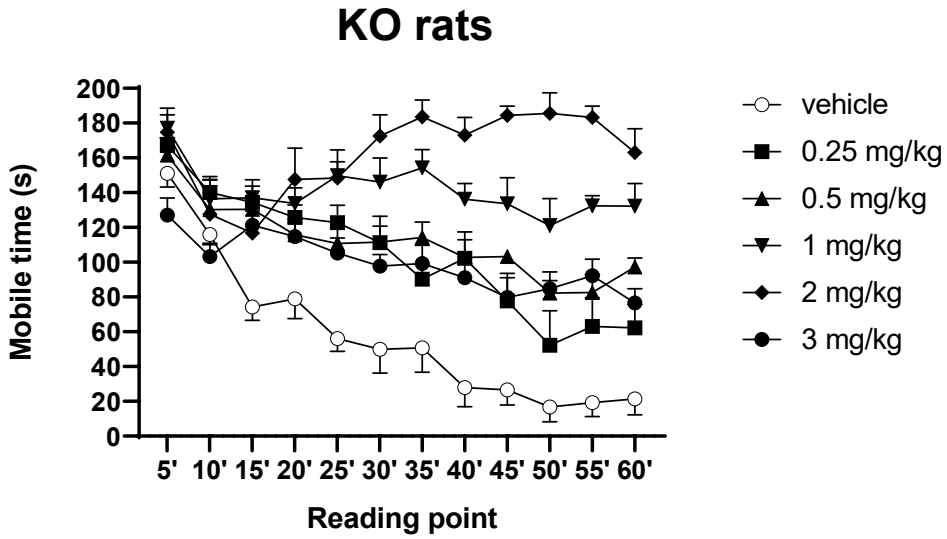
Source of Variation	% of total variation	P value	P value summary
Interaction	3.319	0.0292	*
Treatment	80.04	<0.0001	****
Genotype	1.656	0.0120	*

Figure 121. d-amphetamine-induced dose-response effects on forward locomotion (mobile time) during the first half of the session. Two-way ANOVA followed by Tukey's multiple comparison test.



Source of Variation	% of total variation	P value	P value summary
Interaction	9.935	0.0005	***
Reading point	16.02	<0.0001	****
Treatment	42.66	<0.0001	****

Figure 122. d-amphetamine-induced time-course effects on forward locomotion (mobile time) in WT rats. Two-way ANOVA followed by Dunnett's multiple comparison test.

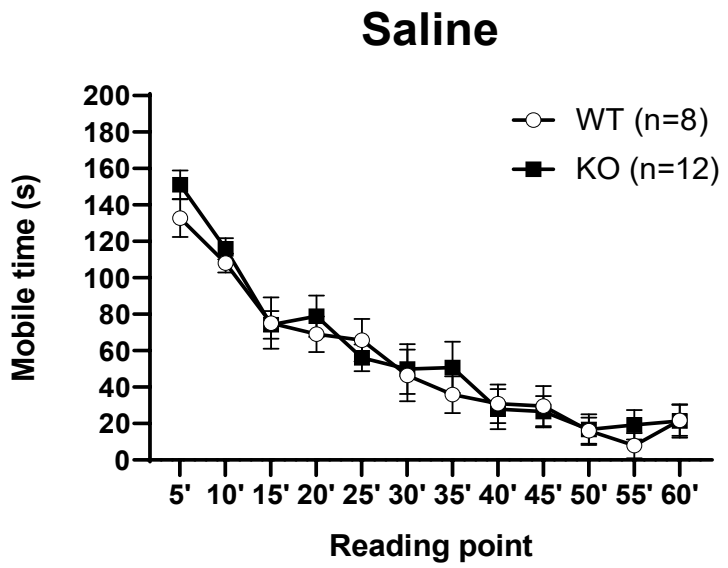


Source of Variation	% of total variation	P value	P value summary
Interaction	13.1	<0.0001	****
Reading point	10.2	<0.0001	****
Treatment	46.6	<0.0001	****

Figure 123. d-amphetamine-induced time-course effects on forward locomotion (mobile time) of KO rats. Two-way ANOVA followed by Dunnett's multiple comparison test.

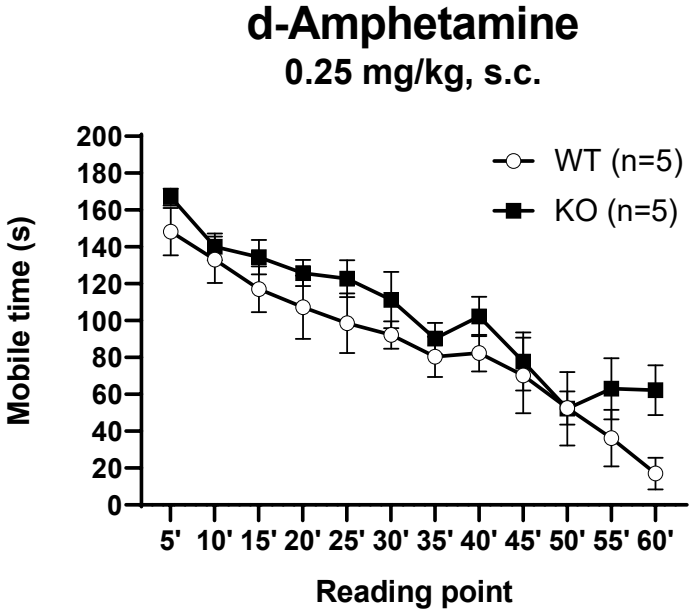
The pattern of locomotor activity after saline administration (Figure 124) was the same in WT and KO rats, with a higher values of forward locomotion during the first half of the session, a clear sign of novelty exploration, and a progressive reduction of the activity until an almost complete resting behaviour in the second half part of the session. A main effect of time was reported after two-way repeated measures ANOVA ($p<0.0001$) and no effect of genotype ($p=0.333$).

As mentioned above, d-amphetamine induced a dose-dependent increase in locomotor activity that was higher in KO rats, and more evident in the second half of the session. This can be observed already at the lowest dose used of 0.25 mg/kg (Figure 125), and for 0.5 mg/kg (Figure 126), 1 mg/kg (Figure 127), and 2 mg/kg (Figure 128). Conversely, at dose of 3 mg/kg, WT rats showed higher hyperactivity (Figure 129).



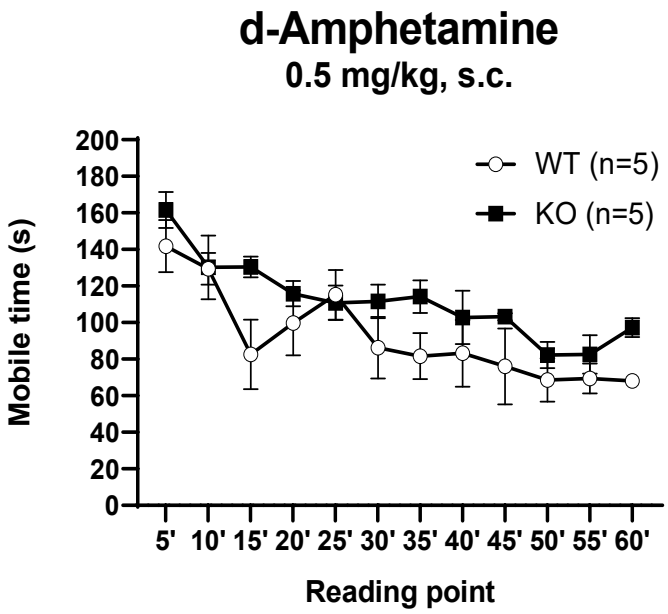
Source of Variation	% of total variation	P value	P value summary
Interaction	0.6385	0.9791	ns
Reading point	57.47	<0.0001	****
Genotype	0.1661	0.3339	ns

Figure 124. Time-course of forward locomotion (mobile time) in saline-treated rats. Two-way ANOVA followed by Tukey's multiple comparison test.



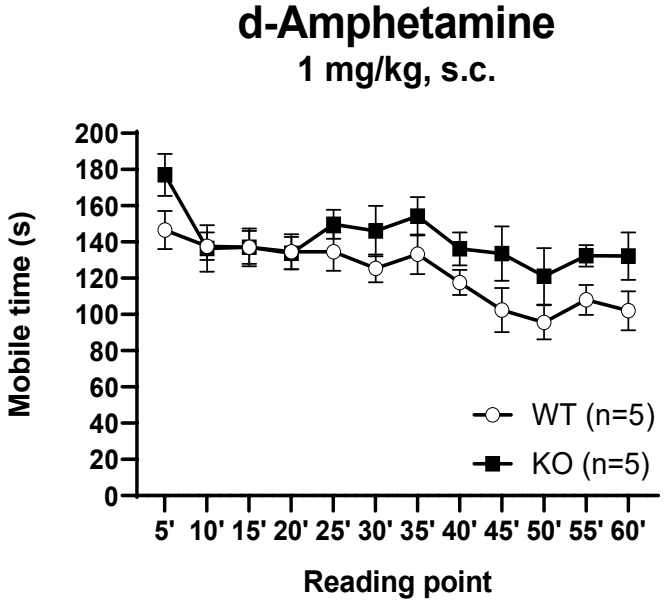
Source of Variation	% of total variation	P value	P value summary
Interaction	1.438	0.9685	ns
Reading point	61.90	<0.0001	****
Genotype	4.086	0.0012	**

Figure 125. Time-course of d-amphetamine (0.25mg/kg) effects on forward locomotion (mobile time). Two-way ANOVA followed by Tukey's multiple comparison test.



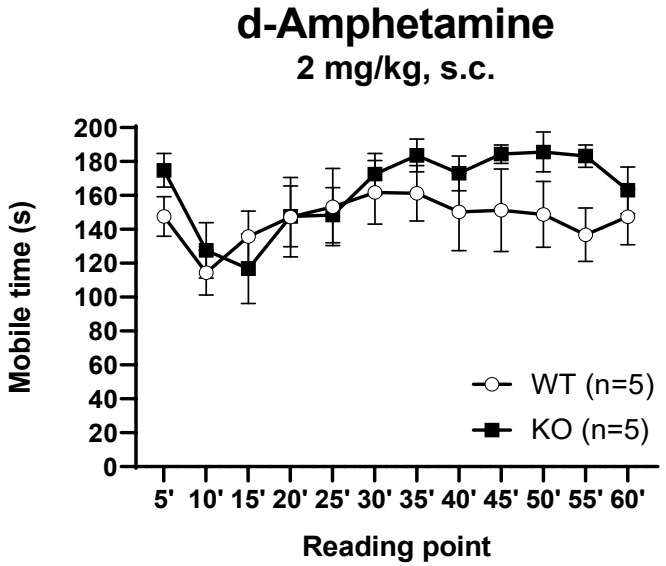
Source of Variation	% of total variation	P value	P value summary
Interaction	3.714	0.7905	ns
Reading point	37.33	<0.0001	****
Genotype	8.292	0.0001	***

Figure 126. Time-course of d-amphetamine (0.5mg/kg) effects on forward locomotion (mobile time). Two-way ANOVA followed by Tukey's multiple comparison test.



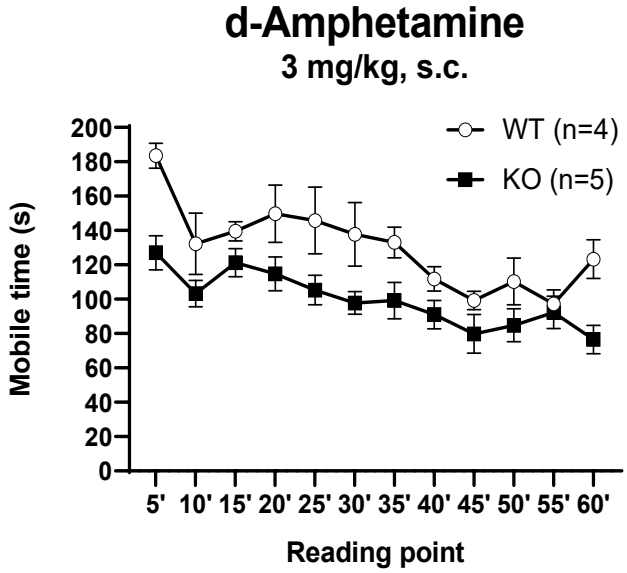
Source of Variation	% of total variation	P value	P value summary
Interaction	4.558	0.7804	ns
Reading point	25.99	0.0002	***
Genotype	10.65	<0.0001	****

Figure 127. Time-course of d-amphetamine (1mg/kg) effects on forward locomotion (mobile time).. Two-way ANOVA followed by Tukey's multiple comparison test.



Source of Variation	% of total variation	P value	P value summary
Interaction	5.427	0.7809	ns
Reading point	16.77	0.0354	*
Genotype	5.018	0.0116	*

Figure 128. Time-course of d-amphetamine (2mg/kg) effects on forward locomotion (mobile time).. Two-way ANOVA followed by Tukey's multiple comparison test.



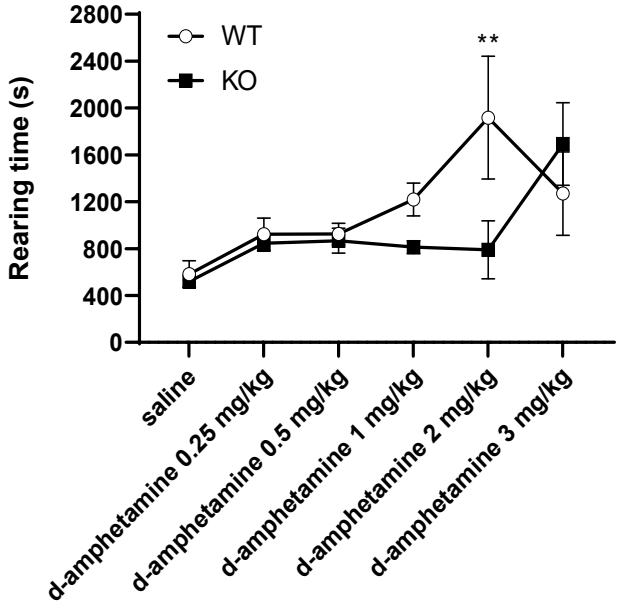
Source of Variation	% of total variation	P value	P value summary
Interaction	4.313	0.5703	ns
Reading point	31.66	<0.0001	****
Genotype	22.34	<0.0001	****

Figure 129. Time-course of d-amphetamine (3mg/kg) effects on forward locomotion (mobile time). Two-way ANOVA followed by Tukey's multiple comparison test.

The rearing time (Figure 130) was higher in WT than in KO rats. This was the contrary at 3 mg/kg, which agrees with the results observed in Mobile time, and with a higher efficacy of d-amphetamine in KO rats. Dose-response curves for rearing time from 0 min to 30 min (Figure 131) or from 3 min to 60 min (Figure 132) showed that there is a significant effect of genotype only in the second half of the reading period.

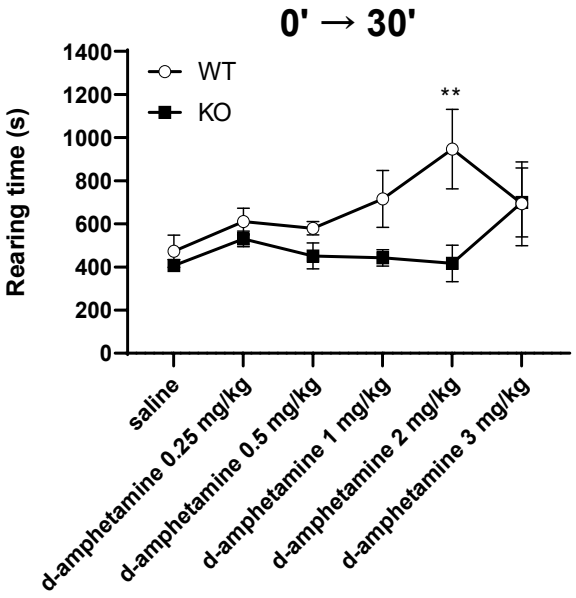
Overall, d-amphetamine was more active in KO rats than in WT counterparts.

The pattern of rearing activity after saline administration (Figure 133) was also similar for WT and KO rats, with a higher rearing values in the first half of the session, and a progressive reduction of the activity in the second half part of the session. A main effect of time was reported after two-way repeated measures ANOVA ($p<0.0001$) and no effect of genotype ($p=0.1626$).



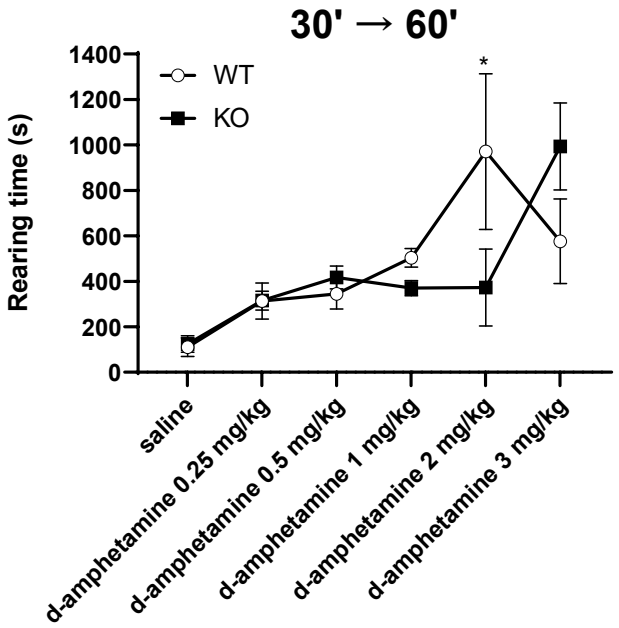
Source of Variation	% of total variation	P value	P value summary
Interaction	12.86	0.0317	*
Treatment	27.77	0.0002	***
Genotype	2.936	0.0871	ns

Figure 130. d-amphetamine-induced dose-response effects on rearing time in WT and KO rats. Two-way ANOVA followed by Tukey's multiple comparison test.



Source of Variation	% of total variation	P value	P value summary
Interaction	10.84	0.1152	ns
Treatment	13.22	0.0595	ns
Genotype	11.18	0.0030	**

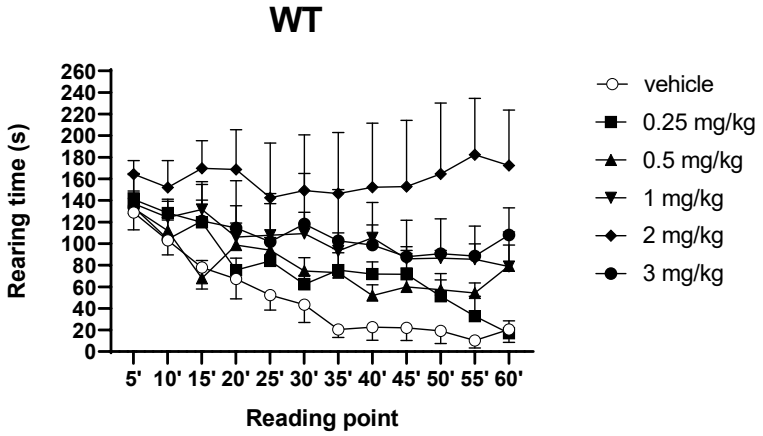
Figure 131. d-amphetamine-induced dose-response effects on rearing time from 0 to 30 min. Two-way ANOVA followed by Šídák's multiple comparisons test.



Source of Variation	% of total variation	P value	P value summary
Interaction	12.94	0.0175	*
Treatment	35.15	<0.0001	****
Genotype	0.2187	0.6153	ns

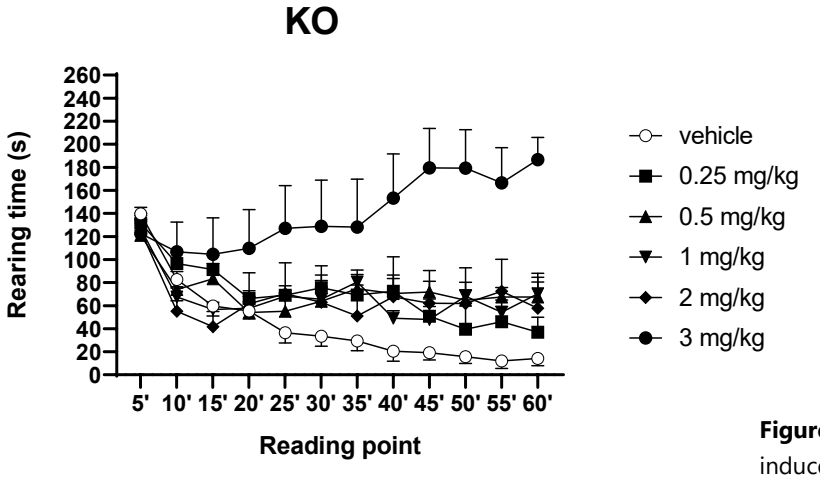
Figure 132. d-amphetamine-induced dose-response effects on rearing time from 30 to 60 min. Two-way ANOVA followed by Šídák's multiple comparisons test.

Time-course analysis showed the dose-response effect in both, the WT and KO rats (Figures 133 and 134, respectively), although the highest effect in WT was observed at 2 mg/kg, and in KO rats at 3 mg/kg.



Source of Variation	% of total variation	P value	P value summary
Interaction	5.36	0.9987	ns
Reading point	7.40	0.0001	***
Treatment	26.6	<0.0001	****

Figure 133. d-amphetamine-induced dose-response effects on rearing time in WT rats. Two-way ANOVA followed by Dunnett's multiple comparison test.



Source of Variation	% of total variation	P value	P value summary
Interaction	12.9	0.0003	***
Reading point	6.51	<0.0001	****
Treatment	30.2	<0.0001	****

Figure 134. d-amphetamine-induced dose-response effects on rearing time in KO rats. Two-way ANOVA followed by Dunnett's multiple comparison test.

No differences in the rearing time were observed between WT and KO rats for vehicle-treated rats, or cocaine at 0.25 and 0,5 mg/kg doses (Figures 135, 136 and 137). However, the administration of d-amphetamine induced an increase in the rearing activity that was

genotype-dependent for the doses of 1, 2 and 3 mg/kg (Figures. 138, 139, and 140, respectively).

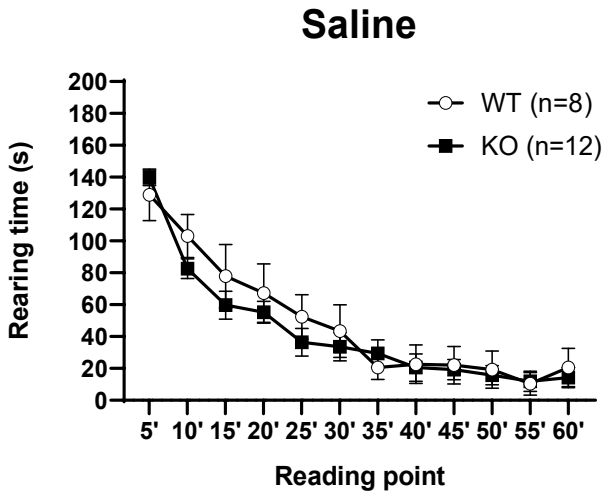
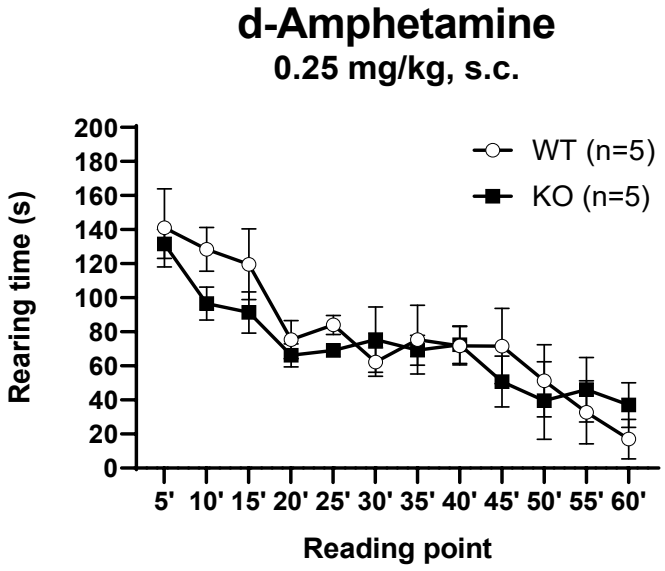


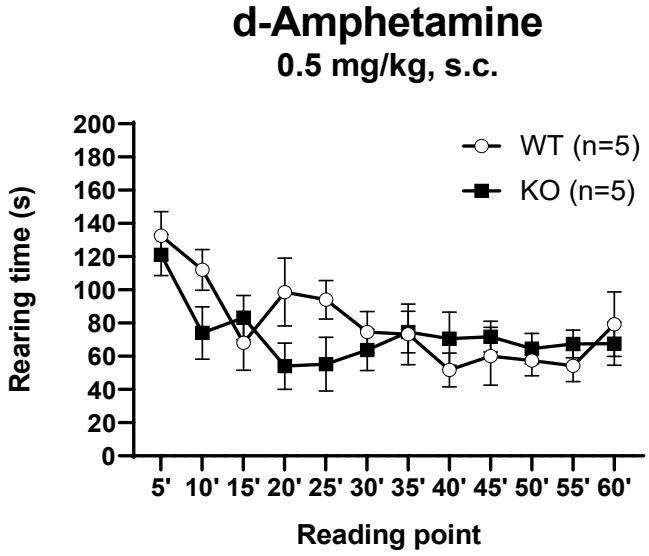
Figure 135. d-amphetamine-induced dose-response effects on rearing time. Two-way ANOVA followed by Tukey's multiple comparison test.

Source of Variation	% of total variation	P value	P value summary
Interaction	1.014	0.9084	ns
Reading point	55.94	<0.0001	****
Genotype	0.3694	0.1626	ns



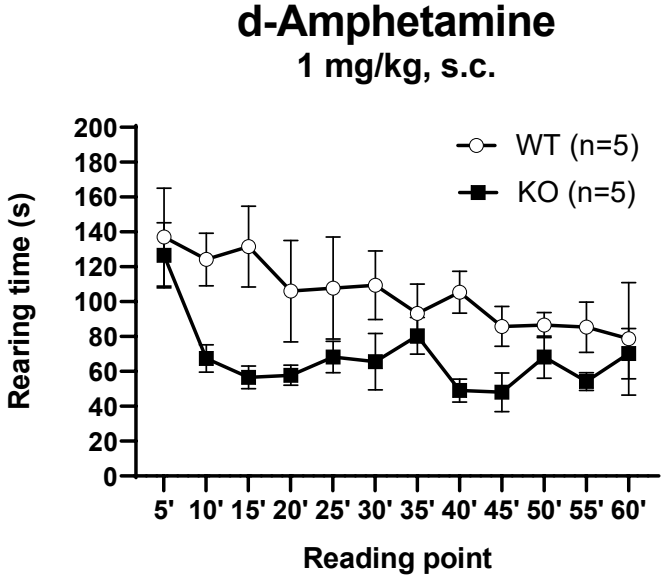
Source of Variation	% of total variation	P value	P value summary
Interaction	3.174	0.8647	ns
Reading point	46.95	<0.0001	****
Genotype	0.6753	0.2599	ns

Figure 136. d-amphetamine-induced dose-response effects on rearing time. Two-way ANOVA followed by Tukey's multiple comparison test.



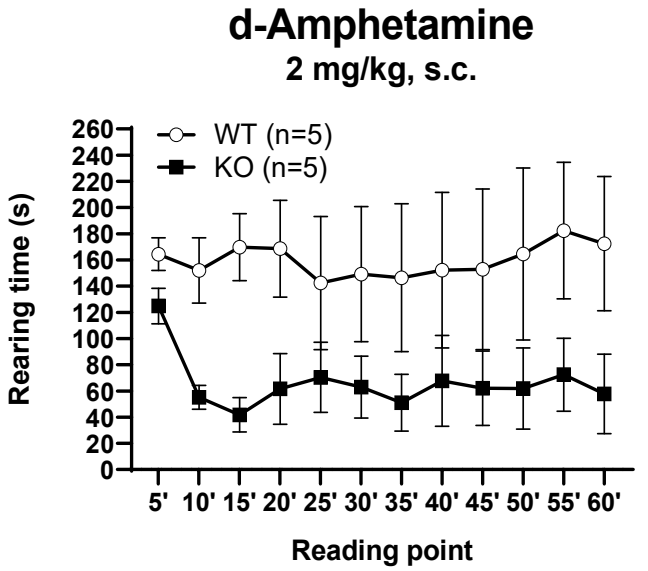
Source of Variation	% of total variation	P value	P value summary
Interaction	9.694	0.2208	ns
Reading point	25.50	0.0004	***
Genotype	1.109	0.1992	ns

Figure 137. d-amphetamine-induced dose-response effects on rearing time. Two-way ANOVA followed by Tukey's multiple comparison test.



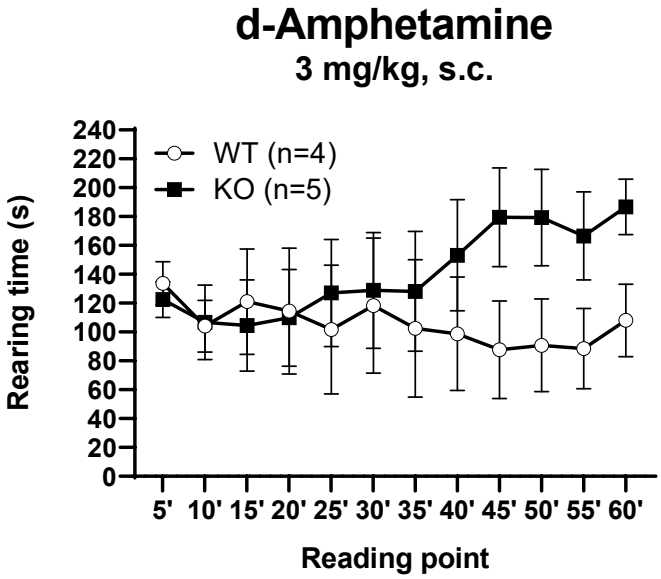
Source of Variation	% of total variation	P value	P value summary
Interaction	5.586	0.6409	ns
Reading point	15.11	0.0231	*
Genotype	18.66	<0.0001	****

Figure 138. d-amphetamine-induced dose-response effects on rearing time. Two-way ANOVA followed by Tukey's multiple comparison test.



Source of Variation	% of total variation	P value	P value summary
Interaction	1.410	0.9986	ns
Reading point	1.673	0.9968	ns
Genotype	26.21	<0.0001	****

Figure 139. d-amphetamine-induced dose-response effects on rearing time. Two-way ANOVA followed by Tukey's multiple comparison test.



Source of Variation	% of total variation	P value	P value summary
Interaction	6.589	0.7520	ns
Reading point	2.249	0.9946	ns
Genotype	5.341	0.0154	*

Figure 140. d-amphetamine-induced effects on time-course rearing time. Two-way ANOVA followed by Tukey's multiple comparison test.

Table 19. Adjusted p values for the mobile and rearing time comparison between vehicle- and d-amphetamine-treated WT and KO rats. Two-way ANOVA followed by Dunnett's multiple comparison test.

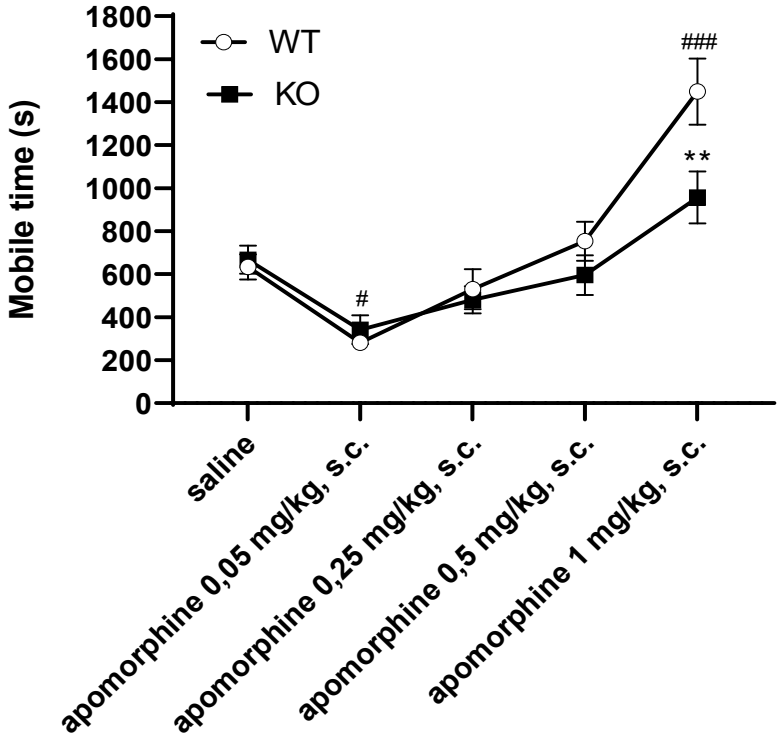
		Adjusted P Values			
		Mobile time		Rearing time	
Reading point	Comparison	WT	KO	WT	KO
5'	vehicle vs. 0.25 mg/kg	0.8757	0.7962	0.997	0.9981
10'		0.5159	0.4182	0.9333	0.9749
15'		0.077	0.0004	0.6535	0.572
20'		0.1268	0.0106	0.9997	0.9918
25'		0.2418	<0.0001	0.8488	0.5454
30'		0.0428	0.0003	0.9802	0.2876
35'		0.0522	0.0461	0.3819	0.3374
40'		0.0168	<0.0001	0.4948	0.1145
45'		0.0924	0.004	0.486	0.5779
50'		0.158	0.0899	0.8436	0.8076
55'		0.3839	0.0204	0.9586	0.499
60'		0.9997	0.0356	0.9999	0.8354
5'	vehicle vs. 0.5 mg/kg	0.9857	0.9558	0.9999	0.9204
10'		0.6645	0.8569	0.9996	0.997
15'		0.9941	0.0012	0.9986	0.8159
20'		0.3011	0.0713	0.8533	>0.999
25'		0.0238	0.0018	0.6556	0.9174
30'		0.1023	0.0003	0.8553	0.6196
35'		0.0434	0.0002	0.4238	0.2169
40'		0.0146	<0.0001	0.8858	0.137
45'		0.0392	<0.0001	0.7313	0.107
50'		0.0142	0.0001	0.7282	0.1547
55'		0.0026	0.0002	0.6084	0.0785
60'		0.0604	<0.0001	0.3682	0.0985
5'	vehicle vs. 1 mg/kg	0.9165	0.3433	0.9997	0.9819
10'		0.343	0.5854	0.968	0.9648
15'		0.0024	0.0002	0.4041	0.9999
20'		0.0011	0.0017	0.716	0.9999
25'		0.0005	<0.0001	0.3723	0.5705
30'		<0.0001	<0.0001	0.209	0.563
35'		<0.0001	<0.0001	0.1373	0.1256
40'		<0.0001	<0.0001	0.0674	0.6733
45'		0.0002	<0.0001	0.2382	0.6656
50'		<0.0001	<0.0001	0.1929	0.1081
55'		<0.0001	<0.0001	0.1175	0.2771
60'		0.0013	<0.0001	0.5366	0.0744

Table 19 (Cont.). Adjusted p values for the mobile and rearing time comparison between vehicle- and d-amphetamine-treated WT and KO rats. Two-way ANOVA followed by Dunnett's multiple comparison test.

		Adjusted P Values			
		Mobile time		Rearing time	
Reading point	Comparison	WT	KO	WT	KO
5'	vehicle vs. 2 mg/kg	0.892	0.4358	0.78	0.9684
10'		0.9969	0.9319	0.501	0.7076
15'		0.003	0.0252	0.0326	0.9318
20'		<0.0001	<0.0001	0.0144	0.9996
25'		<0.0001	<0.0001	0.0382	0.502
30'		<0.0001	<0.0001	0.0095	0.6447
35'		<0.0001	<0.0001	0.0012	0.8627
40'		<0.0001	<0.0001	0.0008	0.179
45'		<0.0001	<0.0001	0.0007	0.2666
50'		<0.0001	<0.0001	0.0001	0.2006
55'		<0.0001	<0.0001	<0.0001	0.0443
60'		<0.0001	<0.0001	0.0002	0.2496
5'	vehicle vs. 3 mg/kg	0.0342	0.3531	0.9998	0.9268
10'		0.6137	0.8859	>0.999	0.7576
15'		0.0035	0.0053	0.687	0.1721
20'		0.0001	0.0578	0.605	0.0587
25'		0.0001	0.0031	0.5625	0.0002
30'		<0.0001	0.0043	0.1667	<0.0001
35'		<0.0001	0.0038	0.1074	<0.0001
40'		0.0001	<0.0001	0.1547	<0.0001
45'		0.0013	0.0011	0.2756	<0.0001
50'		<0.0001	<0.0001	0.2025	<0.0001
55'		<0.0001	<0.0001	0.137	<0.0001
60'		<0.0001	0.0007	0.0993	<0.0001

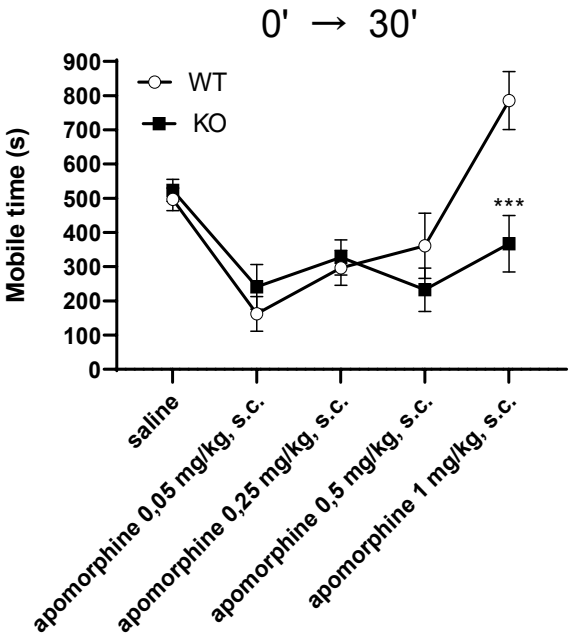
APOMORPHINE

Apomorphine induced an increase n mobile time only at 1 mg/kg (Figure 141). Apomorphine produced high level of stereotyped behaviour, mainly gnawing and sniffing, during the first half of the experiment. After 30 min rats started to do some grooming behaviour and rearings (observer annotations). Consequently, mobile time was low during the first half of the experiment, at all doses tested (0.05-1 mg/kg, s.c.) in both WT and KO rats, except for 1 mg/kg in WT subjects, that showed a pronounced hyperactivity (Figure 142).



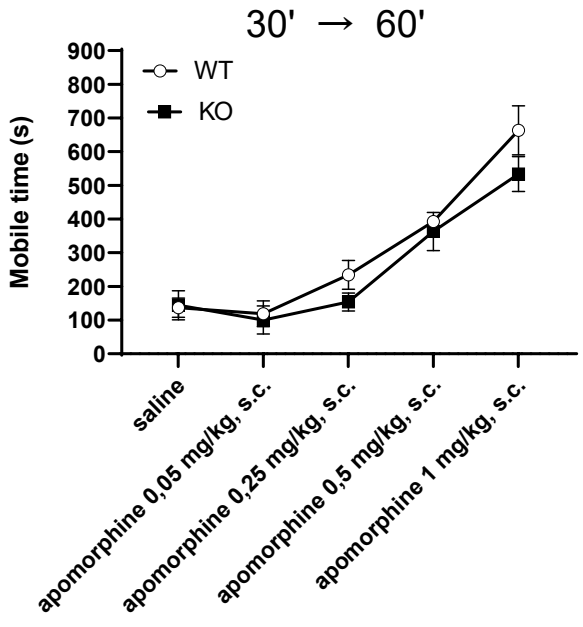
Source of Variation	% of total variation	P value	P value summary
Interaction	6.702	0.0303	*
Treatment	52.79	<0.0001	****
Genotype	2.061	0.0649	ns

Figure 141. Apomorphine-induced dose-response effects on forward locomotion (mobile time). Two-way ANOVA followed by Šídák's multiple comparisons test. ** $p<0.01$ vs WT; $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ vs respective saline group.



Source of Variation	% of total variation	P value	P value summary
Interaction	6.702	0.0303	*
Treatment	52.79	<0.0001	****
Genotype	2.061	0.0649	ns

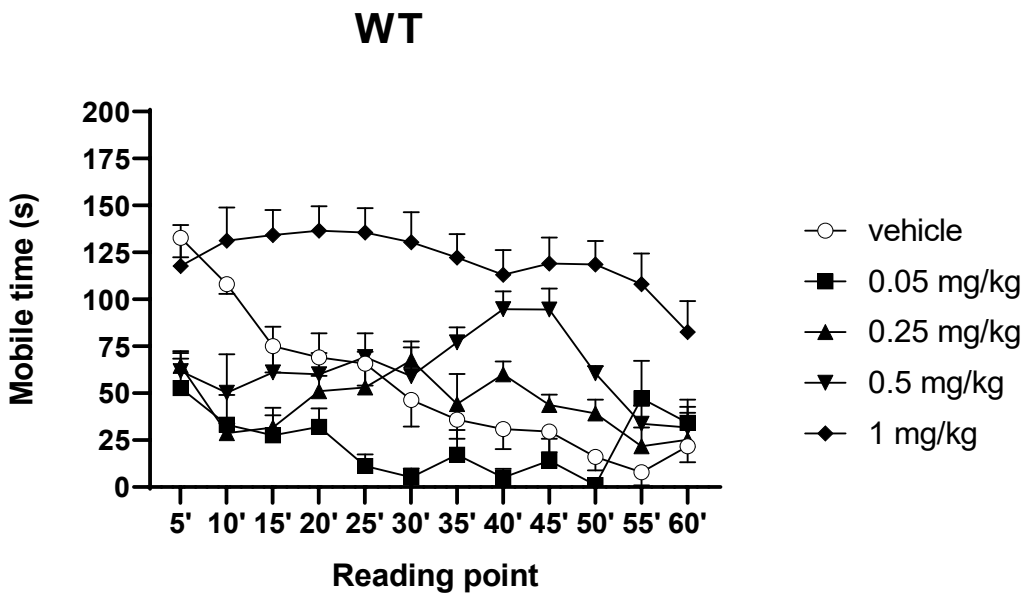
Figure 142. Apomorphine-induced dose-response effects on forward locomotion (mobile time). Two-way ANOVA followed by Šídák's multiple comparisons test.



Source of Variation	% of total variation	P value	P value summary
Interaction	1.269	0.6250	ns
Treatment	65.64	<0.0001	****
Genotype	1.068	0.1427	ns

Figure 143. Apomorphine-induced dose-response effects on forward locomotion (mobile time). Two-way ANOVA followed by Šídák's multiple comparisons test.

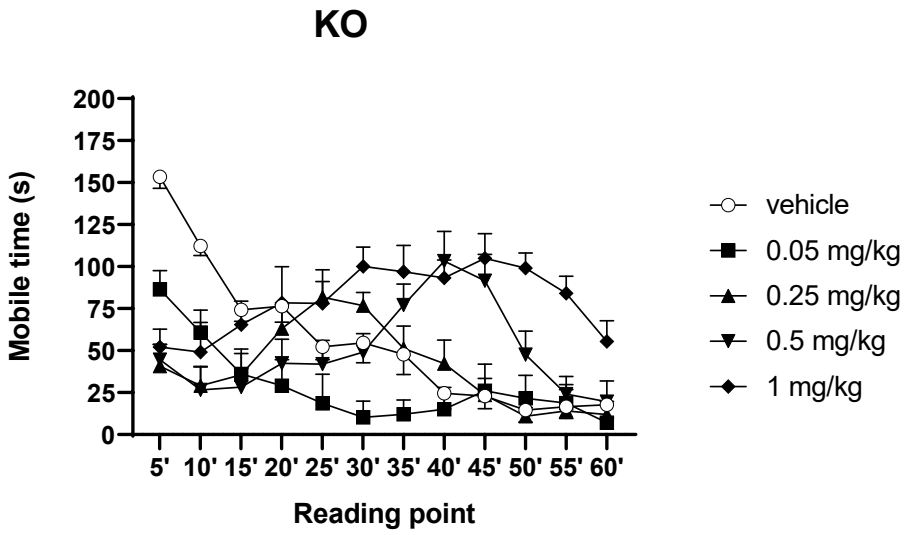
In WT rats, mobile time was initially inhibited at all apomorphine doses except 1mg/kg, and 30' after the administration hyperactivity was observed for 0.5 and 1mg/kg. Dose of 0.25mg/kg only induced a slight increase in the mobile time in the second half of the session.



Source of Variation	% of total variation	P value	P value summary
Interaction	12.97	<0.0001	****
Reading point	26.37	<0.0001	****
Genotype	10.93	<0.0001	****

Figure 144. Apomorphine-induced dose-response effects on mobile time in WT rats. Two-way ANOVA followed by Dunnett's multiple comparison test.

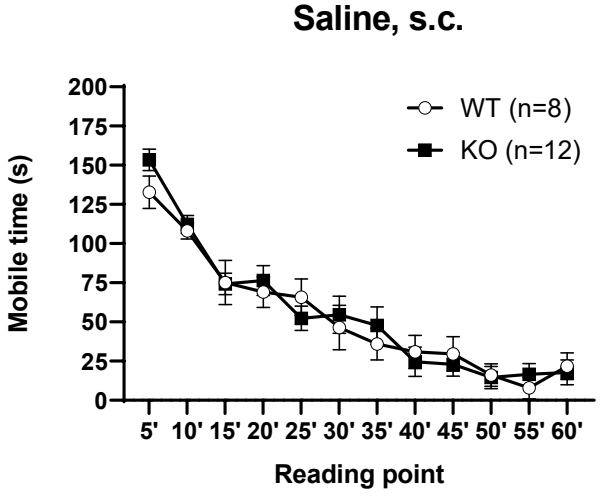
In KO rats, the effects induced by apomorphine were like those observed in WT animals, but apomorphine 1mg/kg was not able to induce hyperactivity in the first half of the session, contrary to what we observed in WT animals. In both genotypes, the lowest dose used, 0.05mg/kg induced the higher level of hypoactivity, reaching statistically significant effect in KO rats (Figure 141).



Source of Variation	% of total variation	P value	P value summary
Interaction	5.646	0.0022	**
Reading point	38.91	<0.0001	****
Genotype	7.504	<0.0001	****

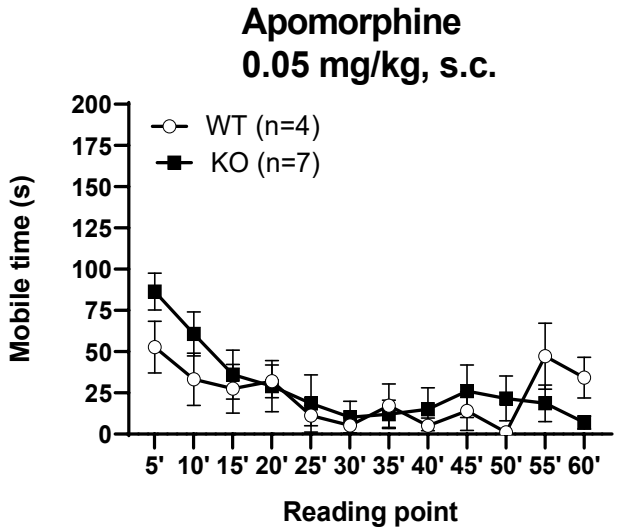
Figure 145. Apomorphine-induced dose-response effects on mobile time in WT rats. Two-way ANOVA followed by Dunnett's multiple comparison test.

The pattern of locomotor activity after saline or apomorphine administration (Figure 146, 147, 148, and 149) was the same in WT and KO rats, except for apomorphine 1mg/kg, with lower values of forward locomotion in KO rats during the first half of the session.



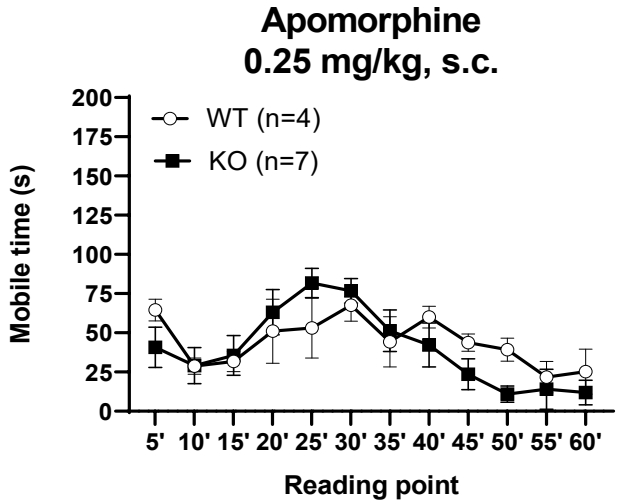
Source of Variation	% of total variation	P value	P value summary
Interaction	0.8611	0.8980	ns
Reading point	61.50	<0.0001	****
Genotype	0.05521	0.5508	ns

Figure 146. vehicle effects on forward locomotion (mobile time). Two-way ANOVA followed by Tukey's multiple comparison test.



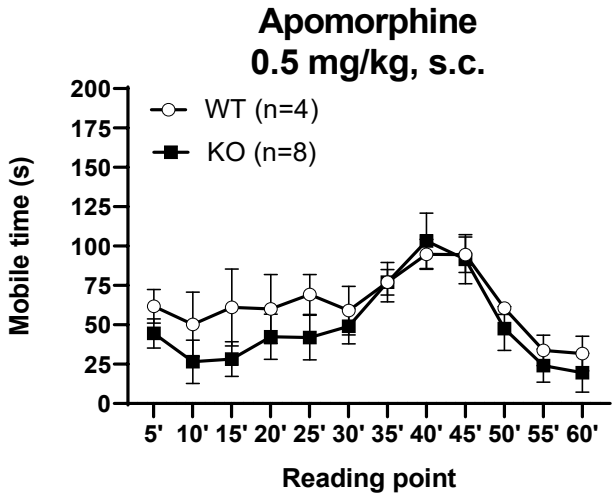
Source of Variation	% of total variation	P value	P value summary
Interaction	6.402	0.4853	ns
Reading point	22.82	0.0004	***
Genotype	0.4926	0.3689	ns

Figure 147. Apomorphine-induced dose-response effects on forward locomotion (mobile time). Two-way ANOVA followed by Tukey's multiple comparison test.



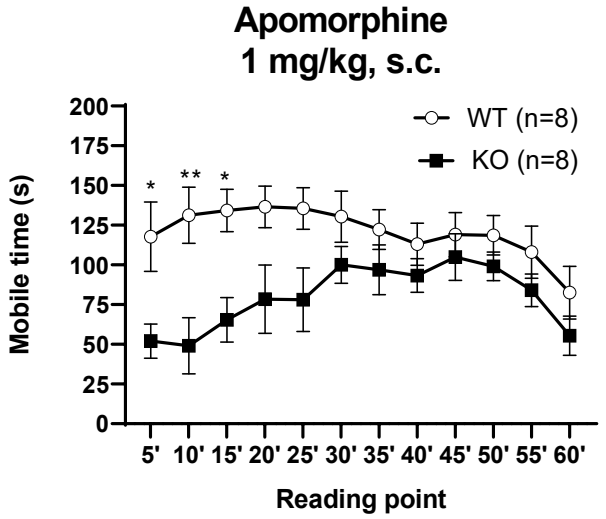
Source of Variation	% of total variation	P value	P value summary
Interaction	5.853	0.4902	ns
Reading point	26.96	<0.0001	****
Genotype	0.3741	0.4140	ns

Figure 148. Apomorphine-induced dose-response effects on forward locomotion (mobile time). Two-way ANOVA followed by Tukey's multiple comparison test.



Source of Variation	% of total variation	P value	P value summary
Interaction	1.711	0.9853	ns
Reading point	26.38	<0.0001	****
Genotype	2.360	0.0357	*

Figure 149. Apomorphine-induced dose-response effects on forward locomotion (mobile time). Two-way ANOVA followed by Tukey's multiple comparison test.



Source of Variation	% of total variation	P value	P value summary
Interaction	5.626	0.2562	ns
Reading point	7.203	0.1015	ns
Genotype	18.59	<0.0001	****

Figure 150. Apomorphine-induced dose-response effects on forward locomotion (mobile time). Two-way ANOVA followed by Tukey's multiple comparison test.

Rearing activity after apomorphine administration was different in WT and KO rats (Figure 151), with an effect of genotype ($p<0.0003$), but no dose-dependency. Within

each genotype, no differences vs saline-treated group were found.

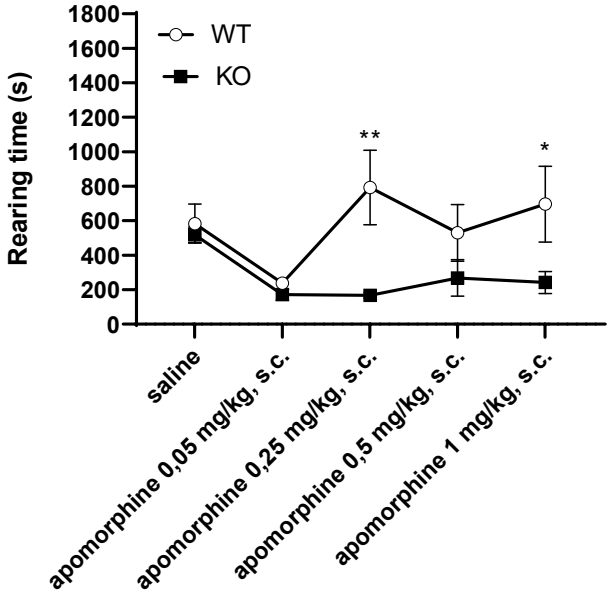


Figure 151. Apomorphine-induced dose-response effects on rearing time. Two-way ANOVA followed by Šídák's multiple comparisons test.

Source of Variation	% of total variation	P value	P value summary
Interaction	9.663	0.0877	ns
Treatment	10.85	0.0604	ns
Genotype	16.97	0.0003	***

Despite the huge variability found, WT rats showed a higher rearing time in both half-parts of the session in comparison to KO rats (Figures 152 and 153), but a main effect was observed during the first half.

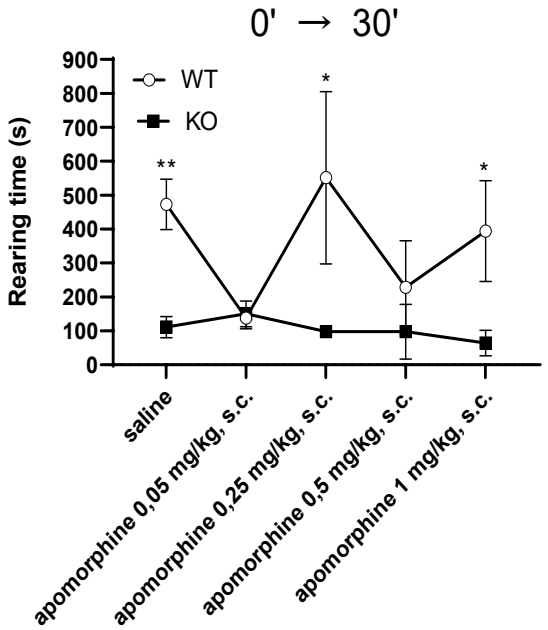
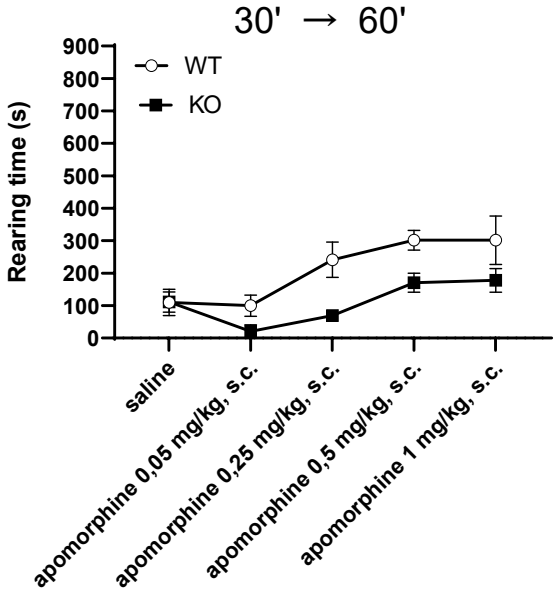


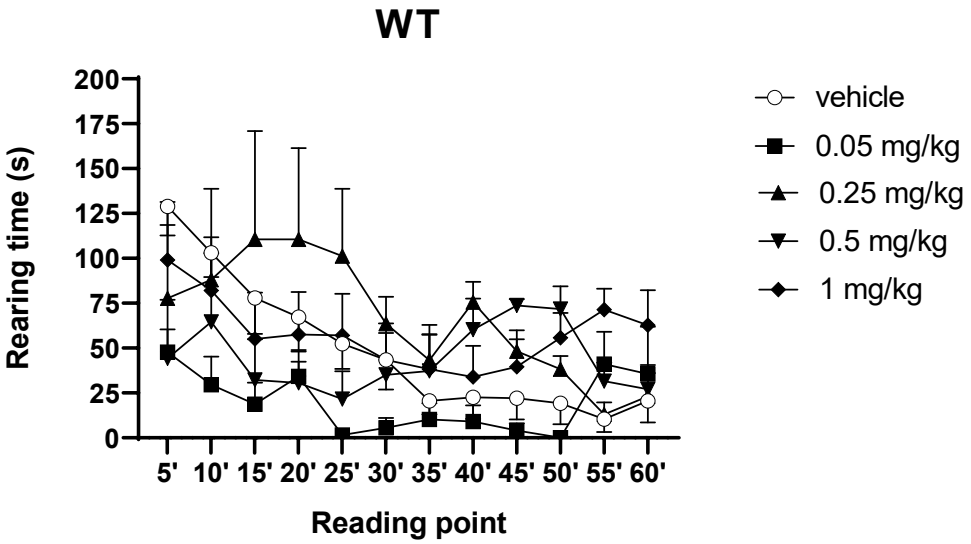
Figure 152. Apomorphine-induced dose-response effects on rearing time. Two-way ANOVA followed by Šídák's multiple comparisons test.

Source of Variation	% of total variation	P value	P value summary
Interaction	8.240	0.1305	ns
Treatment	5.987	0.2628	ns
Genotype	20.41	<0.0001	****



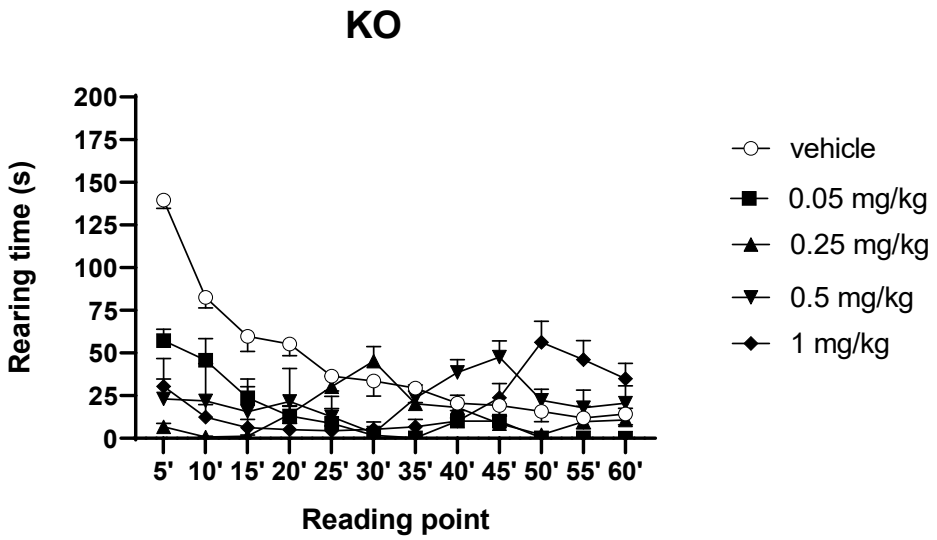
Source of Variation	% of total variation	P value	P value summary
Interaction	5.757	0.2191	ns
Treatment	26.22	0.0002	***
Genotype	13.51	0.0004	***

Figure 153. Apomorphine-induced dose-response effects on rearing time. Two-way ANOVA followed by Šídák's multiple comparisons test.



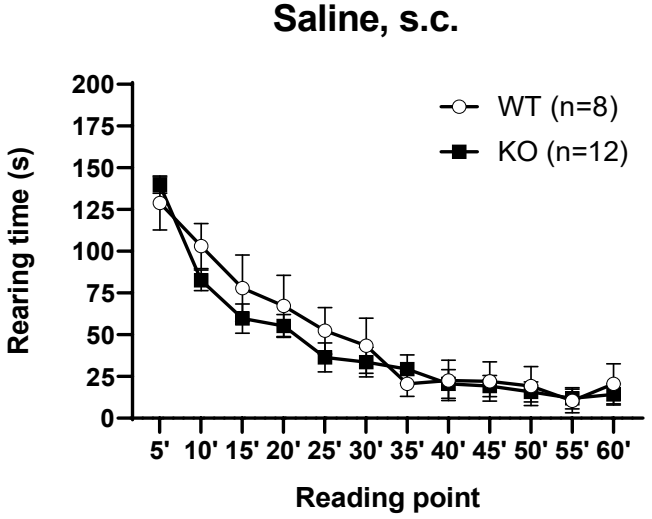
Source of Variation	% of total variation	P value	P value summary
Interaction	10.41	0.0108	*
Reading point	21.80	<0.0001	****
Genotype	8.625	<0.0001	****

Figure 154. Apomorphine-induced dose-response effects on mobile time in WT rats. Two-way ANOVA followed by Dunnett's multiple comparison test.



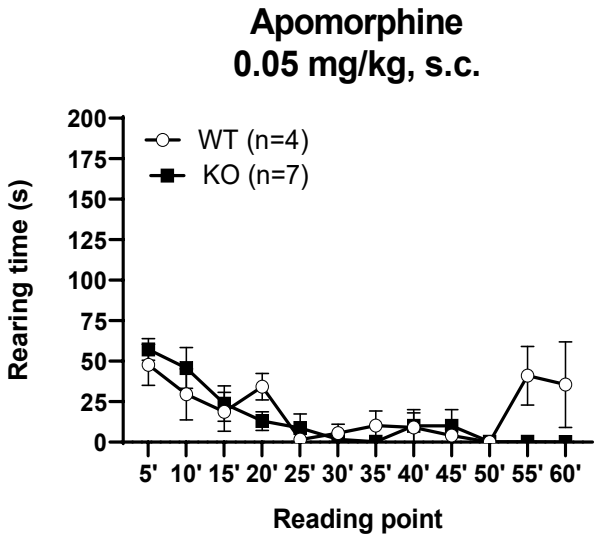
Source of Variation	% of total variation	P value	P value summary
Interaction	5.231	0.0007	***
Reading point	38.63	<0.0001	****
Genotype	11.47	<0.0001	****

Figure 155. Apomorphine-induced dose-response effects on mobile time in KO rats. Two-way ANOVA followed by Dunnett's multiple comparison test.



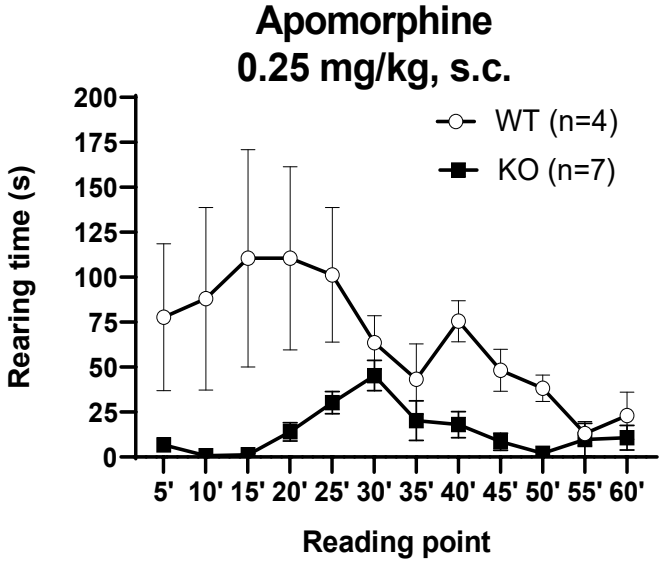
Source of Variation	% of total variation	P value	P value summary
Interaction	1.013	0.9088	ns
Reading point	55.94	<0.0001	****
Genotype	0.3696	0.1625	ns

Figure 156. Apomorphine-induced dose-response effects on rearing time. Two-way ANOVA followed by Tukey's multiple comparison test.



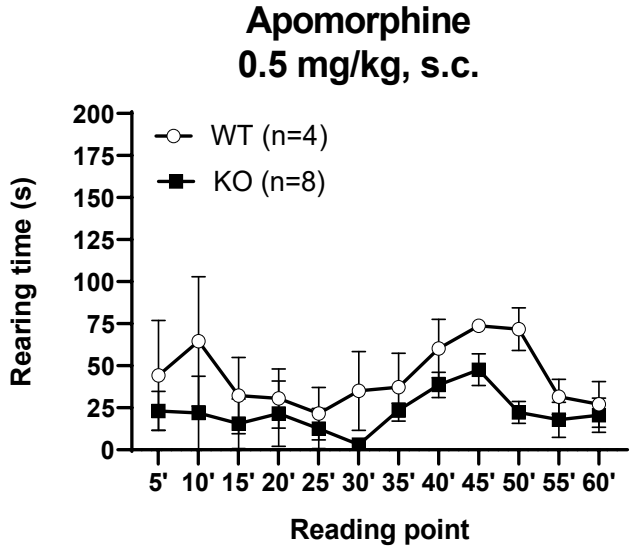
Source of Variation	% of total variation	P value	P value summary
Interaction	10.98	0.0603	ns
Reading point	32.55	<0.0001	****
Genotype	1.107	0.1582	ns

Figure 157. Apomorphine-induced dose-response effects on rearing time. Two-way ANOVA followed by Tukey's multiple comparison test.



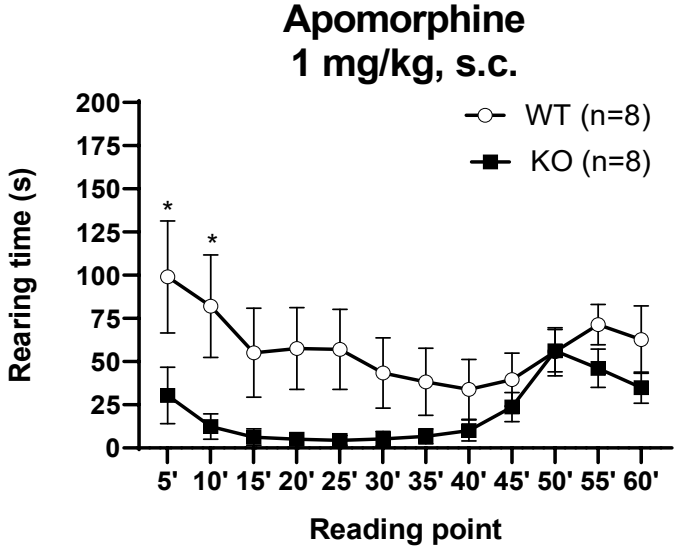
Source of Variation	% of total variation	P value	P value summary
Interaction	10.56	0.0920	ns
Reading point	11.32	0.0662	ns
Genotype	25.34	<0.0001	****

Figure 158. Apomorphine-induced dose-response effects on rearing time. Two-way ANOVA followed by Tukey's multiple comparison test.



Source of Variation	% of total variation	P value	P value summary
Interaction	2.660	0.9673	ns
Reading point	10.81	0.1478	ns
Genotype	7.395	0.0011	**

Figure 159. Apomorphine-induced dose-response effects on rearing time. Two-way ANOVA followed by Tukey's multiple comparison test.



Source of Variation	% of total variation	P value	P value summary
Interaction	4.463	0.6175	ns
Reading point	9.083	0.0848	ns
Genotype	15.44	<0.0001	****

Figure 160. Apomorphine-induced dose-response effects on rearing time. Two-way ANOVA followed by Tukey's multiple comparison test.

Table 20. Adjusted p values for the mobile and rearing time comparison between vehicle- and apomorphine-treated WT and KO rats. Two-way ANOVA followed by Dunnett's multiple comparison test.

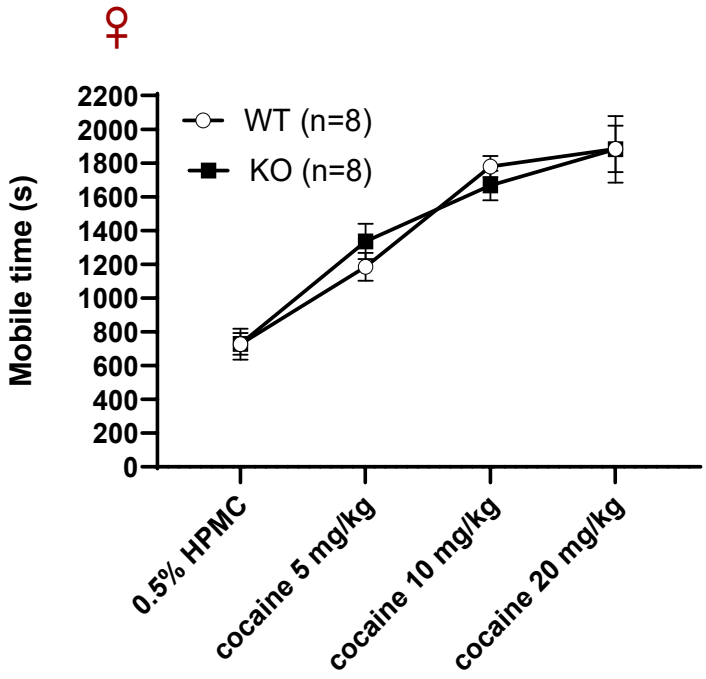
		Adjusted P Values			
		Mobile time		Rearing time	
Reading point	**Comparison**	**WT**	**KO**	**WT**	**KO**
5'	vehicle vs. 0.05 mg/kg	0.0004	0.0002	0.024	<0,0001
10'		0.001	0.0064	0.2686	<0,0001
15'		0.0681	0.0695	0.0169	<0,0001
20'		0.2209	0.0142	0.6067	<0,0001
25'		0.0274	0.1322	0.0486	0,0127
30'		0.1446	0.0252	0.9694	<0,0001
35'		0.7929	0.1005	0.529	<0,0001
40'		0.545	0.9506	0.8421	<0,0001
45'		0.8761	0.9991	0.1567	0,0159
50'		0.888	0.9824	0.6731	<0,0001
55'		0.1741	0.9998	0.3719	0,0005
60'		0.9458	0.9226	0.8006	<0,0001
5'	vehicle vs. 0.25 mg/kg	0.0032	<0.0001	0.664	0,003
10'		0.0004	<0.0001	0.4224	0,0039
15'		0.1127	0.0643	0.5735	0,0121
20'		0.8119	0.8519	0.9891	0,0001
25'		0.9357	0.2309	0.2732	0,0941
30'		0.712	0.488	0.3079	0,9722
35'		0.9856	0.9986	0.7141	0,1273
40'		0.4352	0.6798	0.9991	0,0268
45'		0.91	>0.9999	0.5464	0,0409
50'		0.6425	0.9987	0.9181	0,7844
55'		0.915	0.9997	0.9966	0,0285
60'		0.9997	0.991	>0.9999	0,0631
5'	vehicle vs. 0.5 mg/kg	0.002	<0.0001	0.9927	0,0714
10'		0.0169	<0.0001	0.8791	0,8941
15'		0.9098	0.013	0.9559	0,9697
20'		0.9811	0.1038	0.9051	0,1866
25'		0.9993	0.9217	0.9788	0,8425
30'		0.9379	0.9916	0.2408	0,999
35'		0.1448	0.1998	0.5525	0,3445
40'		0.0067	<0.0001	0.9805	0,8227
45'		0.0057	<0.0001	0.9436	0,893
50'		0.1007	0.119	0.8149	0,8311
55'		0.5452	0.975	0.2587	0,0478
60'		0.9752	0.9999	0.9085	0,9902

Table 20 (Cont.). Adjusted p values for the mobile and rearing time comparison between vehicle- and apomorphine-treated WT and KO rats. Two-way ANOVA followed by Dunnett's multiple comparison test.

5'	vehicle vs. 1 mg/kg	0.8008	<0.0001	0.9292	0,5718
10'		0.4605	0.0003	0.9323	0,6749
15'		0.0016	0.9551	0.2466	0,954
20'		0.0002	0.9998	0.4251	0,0027
25'		0.0001	0.311	0.72	0,7895
30'		<0.0001	0.0144	0.9999	0,9993
35'		<0.0001	0.0067	0.9043	0,9667
40'		<0.0001	<0.0001	0.055	0,0156
45'		<0.0001	<0.0001	0.9725	0,6466
50'		<0.0001	<0.0001	0.9999	0,9966
55'		<0.0001	<0.0001	0.9988	0,9587
60'		0.0029	0.0595	0.3422	0,2632

COCAINE

Due to a reduced number of male KO rats available, we performed cocaine experiments using female rats only. Considering that the comparison we would like to perform was between genotypes, we considered it was justified doing the cocaine experiments in females.



Source of Variation	% of total variation	P value	P value summary
Interaction	0.7529	0.7036	ns
Treatment	69.41	<0.0001	****
Genotype	0.007485	0.9061	ns

Figure 161. Cocaine-induced dose-response effects on forward locomotion (mobile time). Two-way ANOVA followed by Šídák's multiple comparisons test.

Cocaine induced hyperactivity in both genotypes in a dose-dependent manner, and with similar efficacy (Figure 161). No differences were found when analysing the activity in the first and second half of the session (Figures 162 and 163).

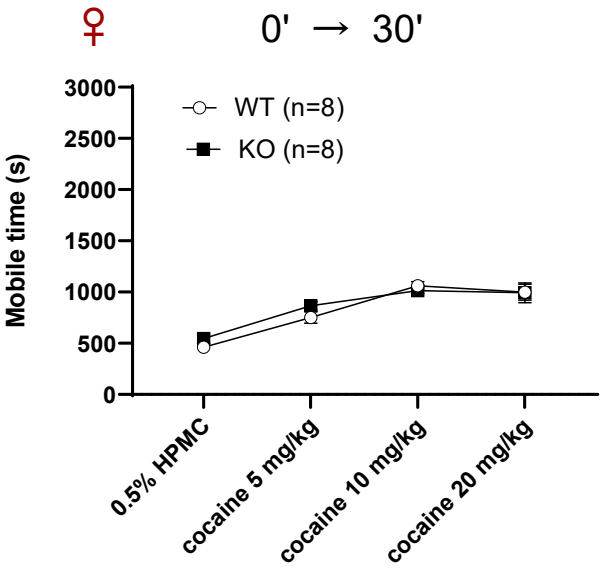


Figure 162. Cocaine-induced dose-response effects on forward locomotion (mobile time) during the first half of the session. Two-way ANOVA followed by Šídák's multiple comparisons test.

Source of Variation	% of total variation	P value	P value summary
Interaction	1.56	0.4684	ns
Treatment	64.1	<0.0001	****
Genotype	0.478	0.3777	ns

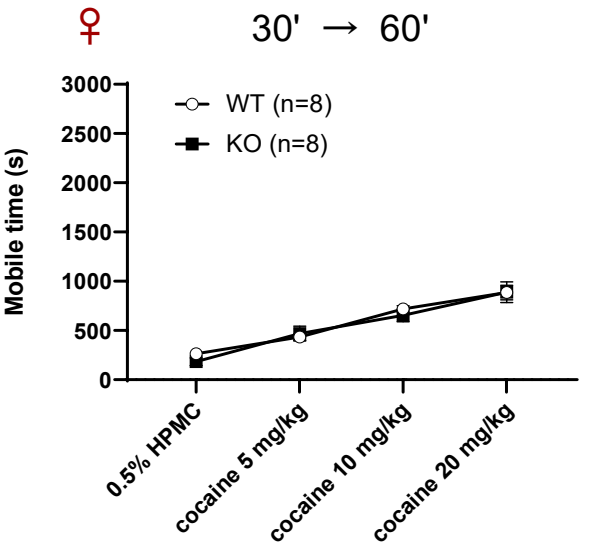
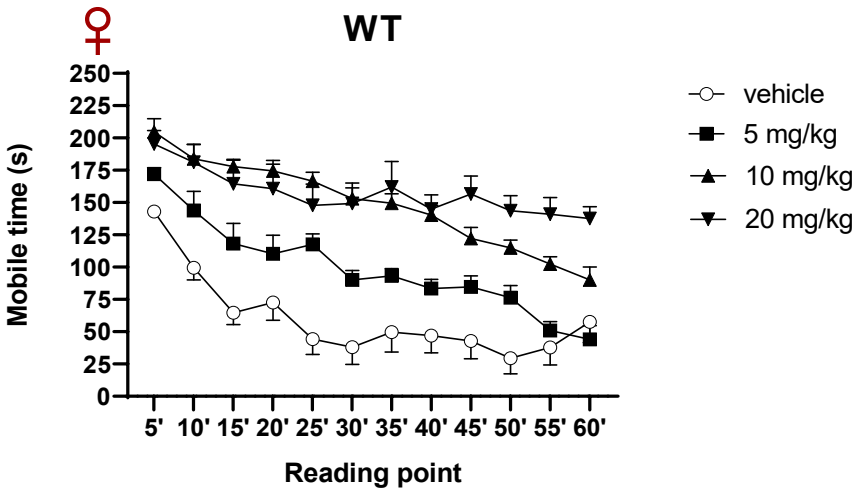


Figure 163. Cocaine-induced dose-response effects on forward locomotion (mobile time) during the second half of the session. Two-way ANOVA followed by Šídák's multiple comparisons test.

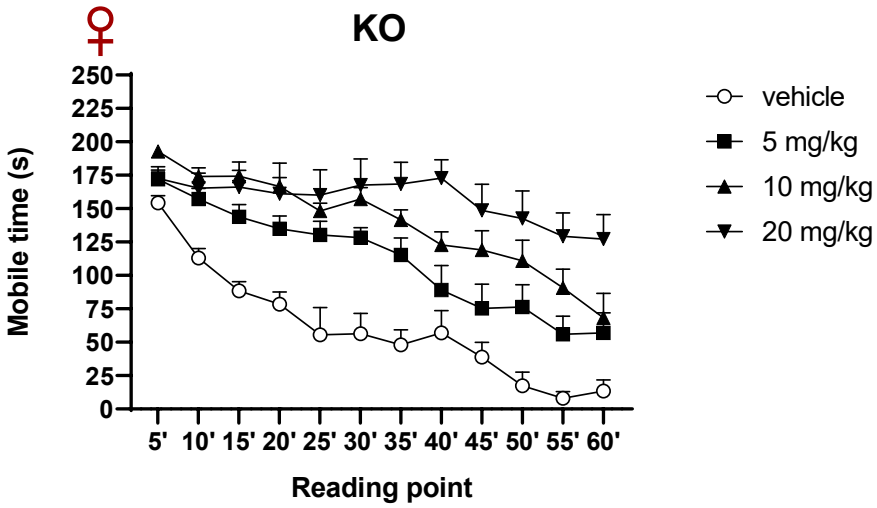
Source of Variation	% of total variation	P value	P value summary
Interaction	0.643	0.7647	ns
Treatment	67.9	<0.0001	****
Genotype	0.198	0.5534	ns

The profile of the time-course of vehicle-treated or cocaine-treated animals, in WT and KO rats is similar (Figures 164 and 165).



Source of Variation	% of total variation	P value	P value summary
Interaction	4.43	0.0224	*
Reading point	22.1	<0.0001	****
Treatment	45.3	<0.0001	****

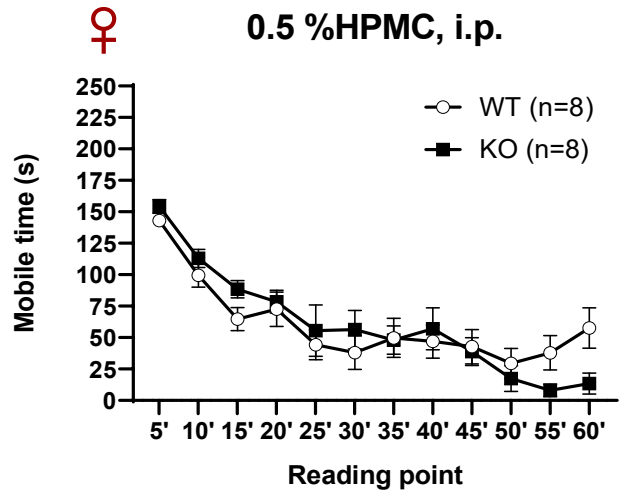
Figure 164. Cocaine-induced dose-response effects on forward locomotion (mobile time) in WT rats. Two-way ANOVA followed by Tukey's multiple comparison test.



Source of Variation	% of total variation	P value	P value summary
Interaction	4.97	0.0426	*
Reading point	26.0	<0.0001	****
Treatment	35.3	<0.0001	****

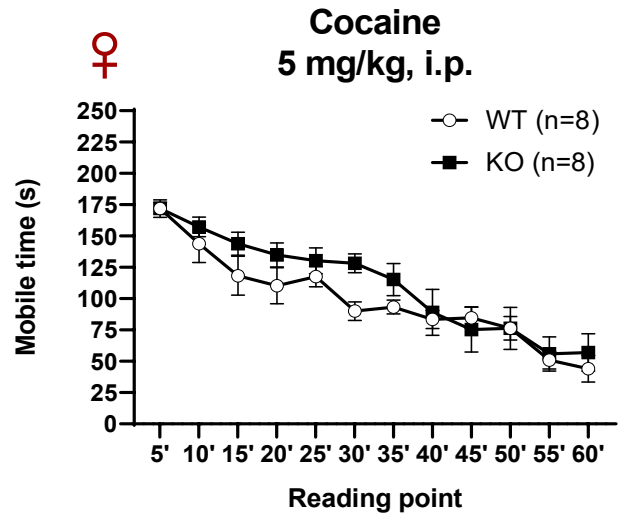
Figure 165. Cocaine-induced dose-response effects on forward locomotion (mobile time) in KO rats. Two-way ANOVA followed by Tukey's multiple comparison test.

Mobile time was increased dose-dependently (5-20 mg/kg i.p.) by cocaine administration in WT and KO rats (Figures 167, 168, and 169).



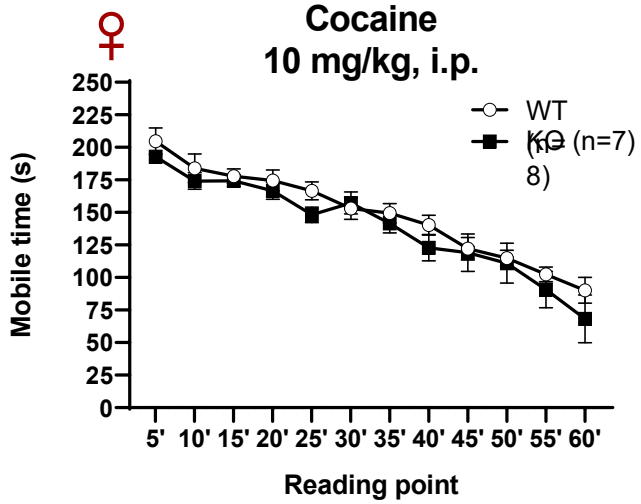
Source of Variation	% of total variation	P value	P value summary
Interaction	4.006	0.1742	ns
Reading point	52.47	<0.0001	****
Genotype	0.0004666	0.9662	ns

Figure 166. Effects on forward locomotion (mobile time) after cocaine 5mg/kg administration. Two-way ANOVA followed by Tukey's multiple comparison test.



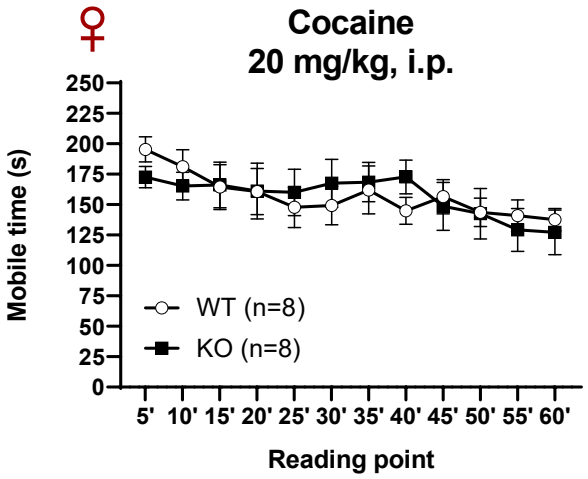
Source of Variation	% of total variation	P value	P value summary
Interaction	1.796	0.7587	ns
Reading point	55.98	<0.0001	****
Genotype	1.724	0.0082	**

Figure 167. Effects on forward locomotion (mobile time) after cocaine 5mg/kg administration. Two-way ANOVA followed by Tukey's multiple comparison test.



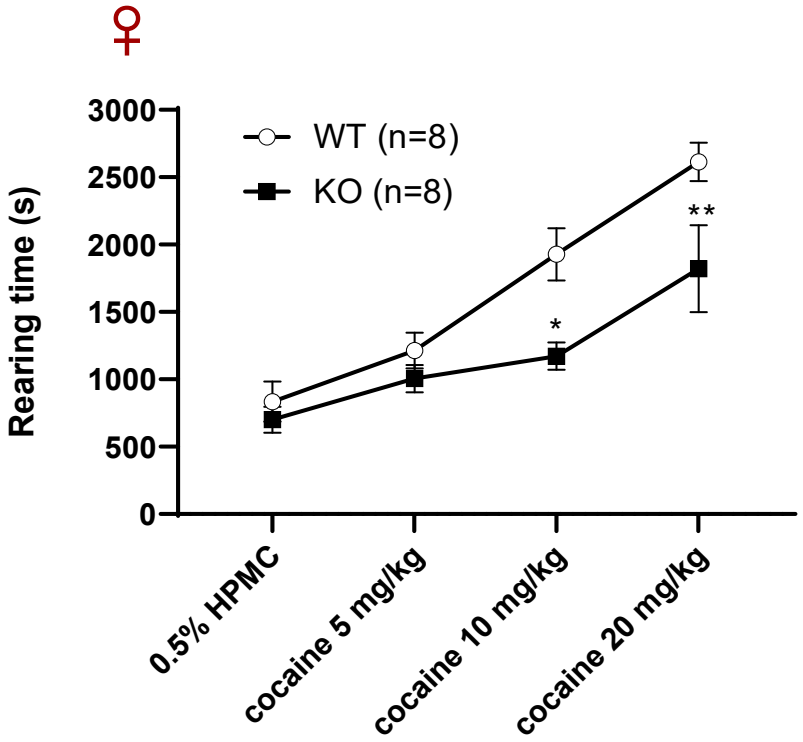
Source of Variation	% of total variation	P value	P value summary
Interaction	0.6891	0.9822	ns
Reading point	64.58	<0.0001	****
Genotype	1.196	0.0154	*

Figure 168. Effects on forward locomotion (mobile time) after cocaine 10mg/kg administration. Two-way ANOVA followed by Tukey's multiple comparison test.



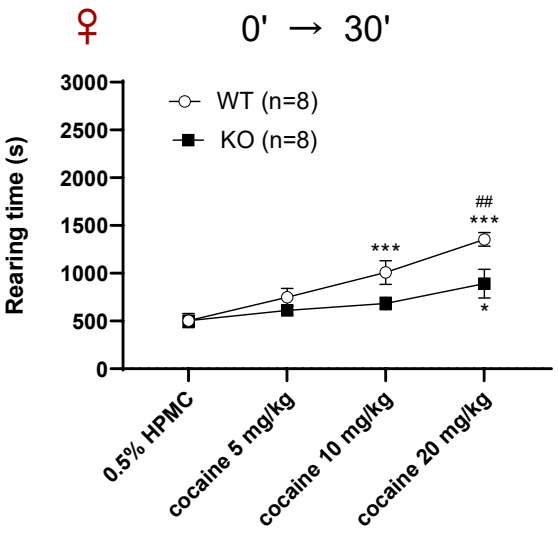
Source of Variation	% of total variation	P value	P value summary
Interaction	2.351	0.9511	ns
Reading point	9.678	0.0816	ns
Genotype	0.0006238	0.9725	ns

Figure 169. Effects on forward locomotion (mobile time) after cocaine 20mg/kg administration. Two-way ANOVA followed by Tukey's multiple comparison test.



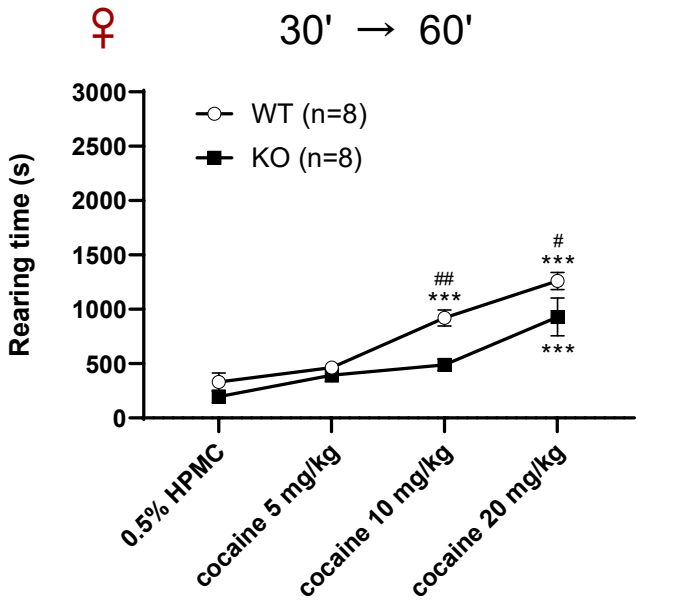
Source of Variation	% of total variation	P value	P value summary
Interaction	4.003	0.1071	ns
Treatment	51.13	<0.0001	****
Genotype	9.725	0.0002	***

Figure 170. Effects of the administration of vehicle or cocaine at 5, 10, and 20mg/kg on rearing time. Two-way ANOVA followed by Tukey's multiple comparison test.



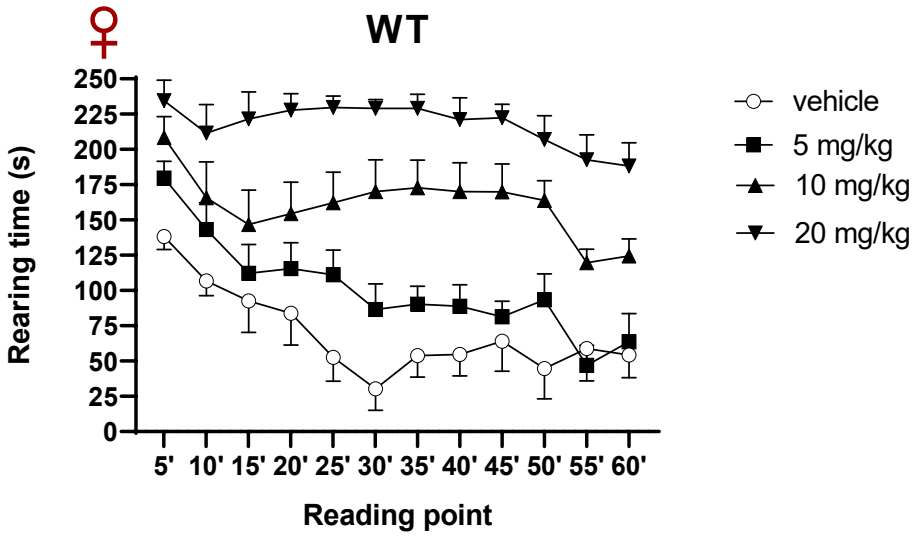
Source of Variation	% of total variation	P value	P value summary
Interaction	5.95	0.0675	ns
Treatment	39.7	<0.0001	****
Genotype	10.2	0.0007	***

Figure 171. Effects of the administration of vehicle or cocaine at 5, 10, and 20mg/kg on rearing time during the first half of the session. Two-way ANOVA followed by Tukey's multiple comparison test.



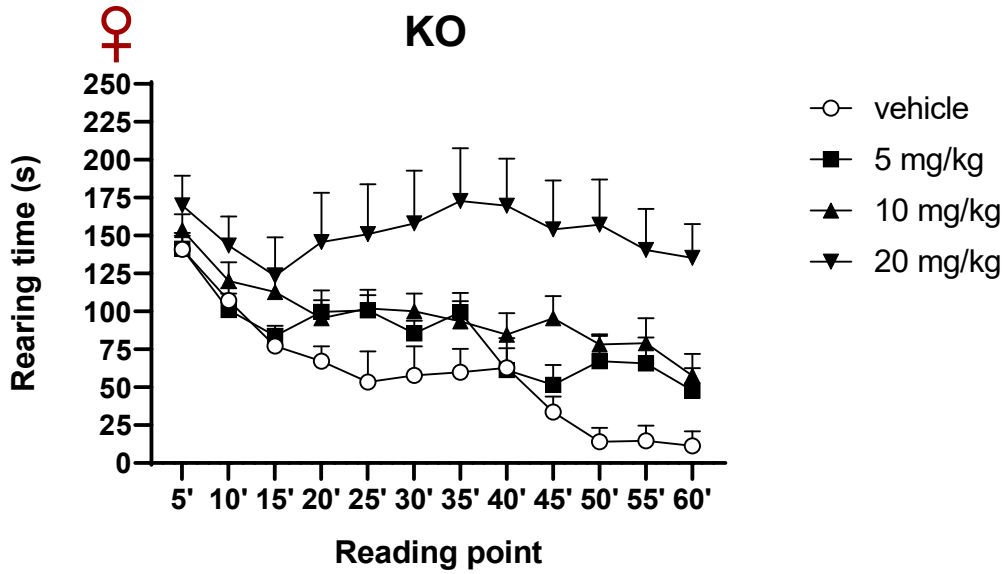
Source of Variation	% of total variation	P value	P value summary
Interaction	3.06	0.1518	ns
Treatment	57.3	<0.0001	****
Genotype	8.47	0.0003	***

Figure 172. Effects of the administration of vehicle or cocaine at 5, 10, and 20mg/kg on rearing time during the second half of the session. Two-way ANOVA followed by Tukey's multiple comparison test.



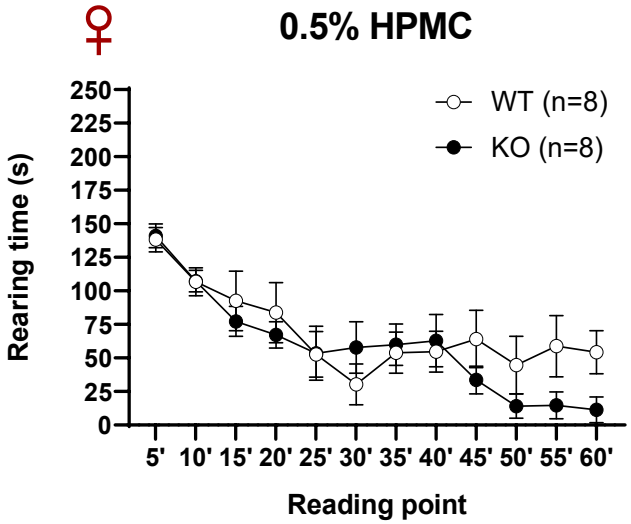
Source of Variation	% of total variation	P value	P value summary
Interaction	3.65	0.3710	ns
Row Factor	7.55	<0.0001	****
Column Factor	54.0	<0.0001	****

Figure 173. Time-course of vehicle or cocaine-induced effects on rearing time in WT female rats. Two-way ANOVA followed by Tukey's multiple comparison test.



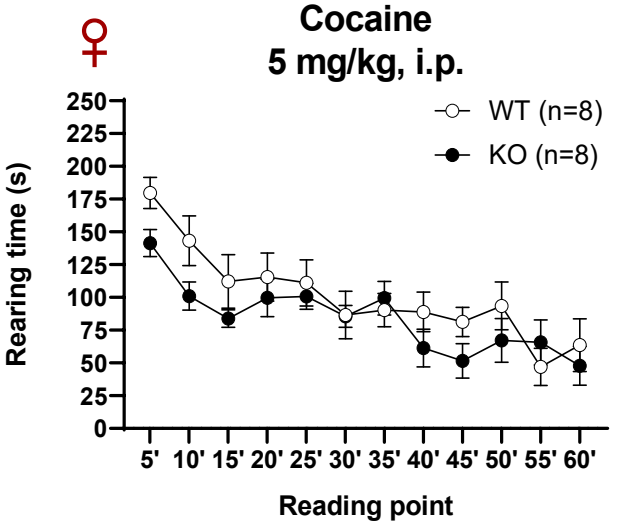
Source of Variation	% of total variation	P value	P value summary
Interaction	5.11	0.5861	ns
Row Factor	11.2	<0.0001	****
Column Factor	27.6	<0.0001	****

Figure 174. Cocaine-induced dose-response effects on rearing time. Two-way ANOVA followed by Tukey's multiple comparison test.



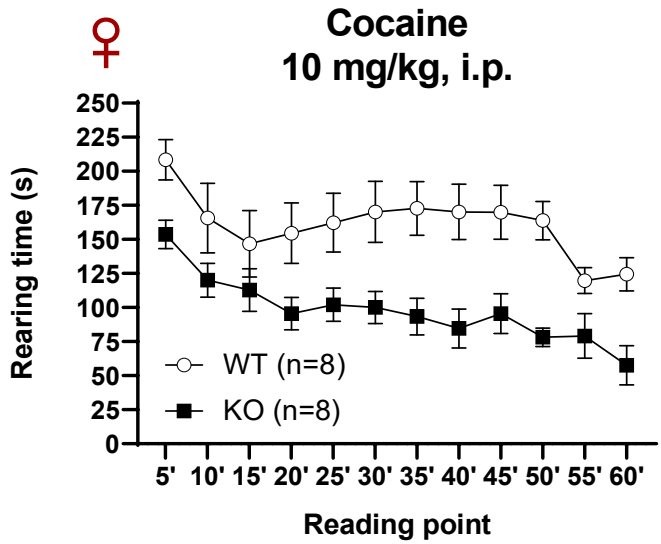
Source of Variation	% of total variation	P value	P value summary
Interaction	3.993	0.4440	ns
Reading point	34.26	<0.0001	****
Genotype	1.078	0.0858	ns

Figure 175. Effects on rearing time after 0.5% HPMC administration. Two-way ANOVA followed by Tukey's multiple



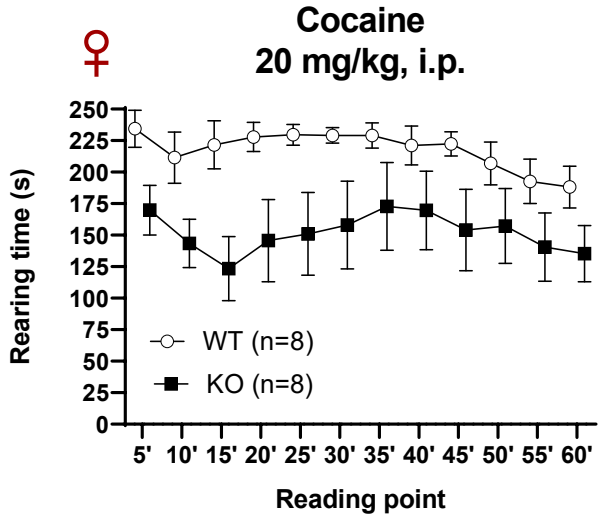
Source of Variation	% of total variation	P value	P value summary
Interaction	3.164	0.6537	ns
Reading point	32.38	<0.0001	****
Genotype	2.978	0.0049	**

Figure 176. Effects on rearing time after cocaine 5mg/kg administration. Two-way ANOVA followed by Tukey's multiple comparison test.



Source of Variation	% of total variation	P value	P value summary
Interaction	1.952	0.8797	ns
Reading point	13.07	0.0002	***
Genotype	28.94	<0.0001	****

Figure 177. Effects on rearing time after cocaine 10mg/kg administration. Two-way ANOVA followed by Tukey's multiple comparison test.



Source of Variation	% of total variation	P value	P value summary
Interaction	1.019	0.9968	ns
Reading point	3.127	0.7870	ns
Genotype	21.95	<0.0001	****

Figure 178. Effects on rearing time after cocaine 20mg/kg administration. Two-way ANOVA followed by Tukey's multiple comparison test.

Table 21. Adjusted p values for the mobile and rearing time comparison between vehicle- and cocaine-treated WT and KO rats. Two-way ANOVA followed by Dunnett's multiple comparison test.

		Adjusted P Values			
		Mobile time		Rearing time	
Reading point	**Comparison**	**WT**	**KO**	**WT**	**KO**
5'	vehicle vs. 5 mg/kg	0.1912	0.6745	0.2123	>0.9999
10'		0.0208	0.0535	0.3094	0.9904
15'		0.0037	0.0105	0.7592	0.9887
20'		0.0581	0.0089	0.4212	0.4544
25'		<0.0001	0.0003	0.0453	0.1712
30'		0.0049	0.0005	0.0572	0.5813
35'		0.0237	0.0013	0.312	0.2979
40'		0.0712	0.2175	0.3598	0.9999
45'		0.0304	0.1372	0.8237	0.8296
50'		0.0128	0.0059	0.1157	0.1074
55'		0.7694	0.0326	0.932	0.127
60'		0.7444	0.059	0.9634	0.3631
5'	vehicle vs. 10 mg/kg	0.0006	0.1092	0.0117	0.9306
10'		<0.0001	0.004	0.0435	0.9288
15'		<0.0001	<0.0001	0.0698	0.3827
20'		<0.0001	<0.0001	0.0111	0.5613
25'		<0.0001	<0.0001	<0.0001	0.1553
30'		<0.0001	<0.0001	<0.0001	0.2494
35'		<0.0001	<0.0001	<0.0001	0.4297
40'		<0.0001	0.0017	<0.0001	0.7384
45'		<0.0001	<0.0001	<0.0001	0.0476
50'		<0.0001	<0.0001	<0.0001	0.0386
55'		0.0003	<0.0001	0.0347	0.0367
60'		0.1236	0.0116	0.012	0.1849
5'	vehicle vs.20 mg/kg	0.0046	0.6468	0.0003	0.5477
10'		<0.0001	0.0171	<0.0001	0.3686
15'		<0.0001	0.0002	<0.0001	0.1849
20'		<0.0001	<0.0001	<0.0001	0.0077
25'		<0.0001	<0.0001	<0.0001	0.0006
30'		<0.0001	<0.0001	<0.0001	0.0004
35'		<0.0001	<0.0001	<0.0001	<0.0001
40'		<0.0001	<0.0001	<0.0001	0.0002
45'		<0.0001	<0.0001	<0.0001	<0.0001
50'		<0.0001	<0.0001	<0.0001	<0.0001
55'		<0.0001	<0.0001	<0.0001	<0.0001
60'		<0.0001	<0.0001	<0.0001	<0.0001

DISCUSSION

Sigma-1 receptor is a chaperone that modulates many other proteins, amplifying or reducing the activity of these proteins (Hayashi and Su, 2007; Zamanillo *et al.*, 2013; Rodríguez-Muñoz *et al.*, 2015). The activity of the receptor is mainly revealed after agonist or stress activation, while it has poor activity under resting conditions. Upon stimulation, the receptor translocates to plasma membrane where it interacts with other proteins, including ion channels, receptors, and kinases (Su *et al.*, 2016). In particular, the interaction of sigma-1 receptor with DAT or DA receptors has been reported (Navarro *et al.*, 2013; Hong *et al.*, 2017; Aguinaga *et al.*, 2018 Asano *et al.*, 2019). Due to its broad interaction with several neurochemical pathways, a role for sigma-1 receptor in several CNS disorders has been described (for a review Merlos *et al.*, 2017), including drug abuse and psychosis/schizophrenia (Borison *et al.*, 1991; Gewirtz *et al.*, 1994; Romieu *et al.*, 2002; Hiranita *et al.*, 2013; Skuza, 2013), Parkinson's disease (Francardo *et al.*, 2014; Yang *et al.*, 2019). Alzheimer's disease (Maurice et al, 1998; Jin *et al.*, 2015; Ruscher and Wieloch, 2015; Jia *et al.*, 2019), anxiety and depression (Albayrak and Hashimoto, 2012; Mandelli *et al.*, 2017; Wang *et al.*, 2019), pain (Bruna *et al.*, 2018; Shin *et al.*, 2020) or amyotrophic lateral sclerosis (Mancuso *et al.*, 2012; Ono *et al.*, 2014).

Sigma-1 receptor KO mice have been widely characterised in a variety of physiological processes under normal and pathological conditions (Nieto *et al.*, 2012; Valenza *et al.*, 2016; Snyder *et al.*, 2016; Castany *et al.*, 2018; Liu *et al.*, 2018; Bravo-Caparrós *et al.*, 2019; Crouzier *et al.*, 2020; López-Estévez *et al.*, 2021; Wang *et al.*, 2021). The studies performed so far have been highly useful for the discovery of sigma-1 receptor role in several diseases, and for the identification of the mechanism of action of a long list of compounds already used in the clinical practice or under development.

However, the mouse has some limitations as animal model of human disorders or illnesses. Pharmaceutical research tries to reduce the risk of misleading results adding more than one species in the drug discovery process. For lead finding and profiling, the preferred alternative to mouse is the rat. No sigma-1 KO rats were available to our knowledge. Thus, the access to a sigma-1 receptor KO rat model was a unique tool to further investigate the role of sigma-1 receptor in several physiological and pathological processes, including depression and addiction.

Despite the broad potential involvement of sigma-1 receptor in the neurochemical pathways behind the described CNS disorders, in our study no overt behavioural changes have been observed in sigma-1 KO rats, which is in line with what has been described in sigma-1 receptor KO mice (Table 22).

In the functional observation test battery (Irwin's test) no differences in the scored parameters were found in comparison to WT animals. Females of both genotypes showed an increase in excitation and vocalisations. The increase in these parameters could be explained by a higher level of anxiety in female rats, although controversial results can be found when comparing anxiety-related behaviours in male and female rats using tests of anxiety, like the open field or the elevated plus maze (Johnston and File, 1991; Peña *et al.*, 2006; Mazor *et al.*, 2009). An increase in body temperature was also found. Differences between male and female rats on the stress induced by the experimental manipulation, necessary for the assessment of the different parameters of the Irwin's test, could account for this increase in female body temperature. In any case, the differences we found were gender-related and not between genotypes, pointing to a minor role of sigma-1 receptor in this anxiety-related responses.

In relation to the motor function, sigma-1 KO rats behaved equally to WT rats in the locomotor activity assessment, running wheels, rotarod or open field test. We only found a slight lower mobile times for the males and females of the 218bp genotype when analysing the time-course, but no differences for the overall locomotor activity in the 218bp and 7bp strain comparison studies, or in the analysis of the heterozygous line, were obtained. Similar results were observed for the rearing activity. Therefore, we consider that the differences between genotypes in locomotor activity were not relevant.

Next, we performed an experiment for assessing the reactivity of rats. The startle response measures the force of the reaction of the animal to sudden noise (Geyer *et al.*, 1990). For these exploratory experiments the force sensor was not adjusted to different weights. Therefore, considering the differences in the weight between males and females it was not surprising that the force of the startle response was higher for males than females. However, within each gender there were no differences between genotypes.

The main differences between the 218bp and the 7bp strains appeared when analysing pain sensitivity. The role of sigma-1 receptor in pain has been widely demonstrated at pharmacological level with selective agonists and antagonists, both in mice and rats, and using sigma-1 receptor KO mice (for a review Merlos *et al.*, 2017). Therefore, based on the literature we do not expect to have differences in pain sensitivity. For females of the 218bp genotype we found a slight but significant difference in the Tail Withdrawal Latency (TWL), but no other differences were found either in males or in females in the other tests. However, in the 7bp genotype lower paw withdrawal threshold was found in mechanical allodynia, both in males and females, and a highly significant reduction of TWL in females in the tail flick test. Overall, the 218bp genotype behaved more consistently through the different tests, maintaining pain sensitivity, and therefore it was the selected genotype for further profiling.

Next phenotyping step was done in (+/+), (+/-), and (-/-) rats for the sigma-1 receptor, in rats generated by heterozygous mating. We used male and female rats in almost every study, except when this was not possible due to the size of the male rats. In this heterozygous line, we did not observed differences in the growth curve, feeding or survival percentage. The haematological and biochemical parameters evaluated were in the normal range with no differences versus WT animals, either for the heterozygous or the homozygous genotype. Finally, health incidences were present with similar ratio, independently of the genotype or gender.

Overall, the results obtained with the 218bp sigma-1 receptor KO rats, either in the strain selection studies or in the heterozygous phenotyping studies, for the selected 218bp line, did not show any differences between WT and sigma-1 KO rats in motor function, anxiety or auditive sensory processing, pain processing, haematology, biochemistry, or health

incidences. These results agree with the results reported for the sigma-1 KO mice, with no grossly behavioural changes in comparison to WT animals (Table 22), and with the demonstrated poor activity of sigma-1 receptor under resting conditions.

These results suggest that no interference of unspecific effects on the behavioural outputs could be expected in the depression or drug-induced hyperactivity experiments.

Depression

Sigma-1 receptor has been widely reported to be involved in depression (Guo *et al.*, 2021; Salaciak *et al.*, 2021). Antidepressant activity has been described for reference sigma-1 agonists, compounds under development, neurosteroids, or antidepressants with sigma-1 affinity. Some of the reported activities have been antagonised with BD-1047 or NE-100, two reference sigma-1 antagonists (Matsuno *et al.*, 1996; Ukai *et al.* 1998; Urani *et al.* 2001; Oshiro *et al.* 2000; Tottori *et al.* 2001). Therefore, the investigation on how the absence of sigma-1 receptor in mice or rats has an influence on the development of a depressive-like state or whether it modifies the efficacy of standard anti-depressant treatments was considered of great interest.

Sigma-1 KO mouse model Oprs1$^{Gt(IRESBetageo)33Lex}$/Oprs1 $^{Gt(IRESBetageo)33Lex}$ (Langa *et al.*, 2003) has been widely used for studying the involvement of the receptor in pain modulation and pain sensitization (Nieto *et al.*, 2012; Castany *et al.*, 2018; Entrena *et al.*, 2009; Tejada *et al.*, 2014; Lopez-Estevez *et al.*, 2021) but nothing has been published in relation to depression, despite the evidences found in the literature on the potential role of sigma-1 receptor in this neuropsychiatric disease.

Sigma-1 receptor KO mice developed so far display, under normal conditions, a phenotype that is very similar to that of the WT animals (Table 22) (Langa *et al.*, 2003, Sabino *et al.*, 2009; Hong *et al.*, 2015). However, under stimulation conditions, like the stress induced in the Morris water maze (MWM) or the forced swimming test (FST) or the anxiety in the Elevated Plus Maze (EPM), KO mice behave different than the WT counterparts, showing a higher level of anxiety (EPM and MWM) or depression (FST)

(Chevallier *et al.*, 2011; Sabino *et al.*, 2009; Di *et al.*, 2017). Our results in sigma-1 receptor KO mice also showed a different behaviour in comparison to WT counterparts in two tests of depression, the Tail suspension test (TST; Steru *et al.*, 1985) and FST (Porsolt *et al.*, 1977; Petit *et al.*, 2005). However, in our studies, during training session, the first exposure to a new stressing environmental condition, KO mice showed less immobility time in both tests, contrary to what was previously described (Chevallier *et al.*, 2011; Sabino *et al.*, 2009; Di *et al.*, 2017) using the Oprs1$^{Gt(IRESBetageo)33Lex}$/Oprs1 $^{Gt(IRESBetageo)33Lex}$ KO mouse model. Using these mice, during the first exposure to the situation, a higher immobility time for KO mice was reported. In our hands, this increase in immobility time only was observed during test session after vehicle administration. Besides the different KO mouse model, the experimental conditions used could account for the differences observed. In Sabino et al (2009) the age of the mice was 6-8 months old and reversed light cycle stablished. Moreover, the FST was done after two anxiety test, the elevated plus maze test and the light/dark box text, were performed in the same subjects. In fact, in these anxiety models no differences between WT and KO mice were reported, but an effect on the depressive state of these mice, observed in the FST, cannot be discarded. Similarly, in Di et al. (2017), the animals were evaluated in an open field test before the FST. Interestingly, in the TST mice were hanged at 60 cm above the floor, which is quite unusual. In our experimental setting were hanged at less than 5 cm. To which extend this difference influence the mouse response requires further analysis. Moreover, it should be mentioned that in Chevalier et al. (2011) the increased immobility time in the FST was only observed in male mice, not in females, and the difference versus WT male mice was mainly due to a very low immobility time of WT subjects during the training session.

It should be considered that in tests of depression a false-positive result can be obtained if a compound stimulates locomotion (Bourin *et al.*, 2001). Similarly, a false-positive effect could be obtained if sigma-1 KO mice were spontaneously more active than WT animals. However, the spontaneous locomotor activity in the KO mice strain we have used has been reported to be lower than the spontaneous locomotor activity of WT mice (Langa *et al.*, 2003) which would produce an increase in the immobility time instead of a reduction, as we found. Therefore, the reduction in the immobility found in FST and TST could be assigned to an increased anxiety under these stressing conditions, as previously

reported for FST (Chevallier *et al.*, 2011). It is well known that stress is a risk factor for depression in humans (Alfonso *et al.*, 2005; Palazidou, 2012; Willner *et al.*, 2013). The stress induced by the inescapable situation in rodent models of depression leads to a saving energy strategy that we can measure as immobility. This immobility can be effectively reduced by antidepressants, particularly after repeated treatment. The well-established predictive validity of the models using clinically active antidepressant partially justifies the definition as 'depression-like' models, although currently it is better accepted as models of stress coping (Commons *et al.*, 2017; Molendijk and de Kloet, 2019). Our results on the test day showing an increase in the immobility time after saline treatment, agree with the reported results for sigma-1 KO mice (Sabino *et al.*, 2009; Di *et al.*, 2017). This could mean that sigma-1 KO mice have a better stress coping strategy than WT subjects.

In addition, the differences in the immobility time during the training session in the fluvoxamine experiment could be related to the lower water temperature, as it has been reported in C57BL/6J mice (Bächli et al., 2008). Moreover, in this study, the efficacy of the tricyclic antidepressant desipramine was highly significant when water temperature was 30ºC but not at 25ºC. In our study with fluvoxamine, the temperature of the water, the dose, or the number of subjects used could explain the lack of a statistically significant effect, as we obtained after fluoxetine treatment. Overall, the efficacy of the antidepressant treatment is higher in sigma-1 KO that in WT mice.

Several antidepressants have affinity for sigma-1 receptor (Table 2) (Narita *et al.*, 1996; Ishima *et al.*, 2014), and it has been proposed to play a role in their antidepressant activity (Franchini *et al.*, 2010). Our results on the behaviour under stress conditions further support this role of sigma-1 receptors in depressive-like states.

Table 22. Comparison of the phenotypic profile of KO mice.

	Mouse KO generation and background	**Phenotype**
Langa *et al.*, 2003	Oprs1^{tm1Lmon}/Oprs1^{tm1Lmon} gene targeting 129Sv/CD1	Viable and fertile. Absence of apparent overt phenotypes. Significant lower spontaneous locomotor activity.
Sabino *et al.*, 2009	Oprs1$^{Gt(IRESBetageo)33Lex}$/Oprs1$^{Gt(IRESBetageo)33Lex}$ gene trapping C57BL/6J/129Sv	Viable and fertile with negligible overt phenotype. No differences in locomotor activity. Increased immobility time in the forced swimming test.
Hong *et al.*, 2015	Oprs1$^{Gt(IRESBetageo)33Lex}$/Oprs1$^{Gt(IRESBetageo)33Lex}$ gene trapping C57BL/6J/129Sv	Viable and fertile. Appeared grossly normal. No differences either in locomotor activity or in motor coordination. No differences in anxiety, measured in the open field test.
Chevallier et al., 2011	Oprs1$^{Gt(IRESBetageo)33Lex}$/Oprs1$^{Gt(IRESBetageo)33Lex}$ gene trapping C57BL/6J/129Sv	Viable and fertile with no grossly observable behavioural alteration. HOM have non-significant lower weight than WT. Slight increase in anxiety in anxiety tests. Females showed an impaired performance in memory tests. Higher immobility time in the FST during the training session. No differences in the test session. Increased activity of fluoxetine and sertraline. Not for TCA.
Di et al., 2017	Oprs1$^{Gt(IRESBetageo)33Lex}$/Oprs1$^{Gt(IRESBetageo)33Lex}$ gene trapping C57BL/6J/129Sv	s1R-KO mice showed an increase in the immobility time in the TST or FST. Sigma-1 deficiency in CRF neurons reduces the GR-mediated feedback inhibition of the HPA axis and facilitates the response to stress via the down-regulation of PKC, leading to the long-lasting hyperactivity of HPA axis and the production of depressive-like behaviours.
Welab's research	Oprs1^{tm1Lmon}/Oprs1^{tm1Lmon} gene targeting C57BL/6J/129Sv	Viable and fertile. Absence of apparent overt behavioural alterations. TST: KO mice displayed lower immobility time during the training session. 24 hours later, saline treated animals showed a tendency to increase the immobility time in the TST. SSRIs fluoxetine, sertraline and fluvoxamine reduced immobility time in KO mice but not in WT. IFST: same reduction of immobility during training session, and fluoxetine and fluvoxamine reduced immobility time only in KO mice.

Antidepressant drugs in acute treatment have difficulties demonstrating efficacy in the TST in mice or the FST in mice and rats (Artaiz *et al.*, 2005). Accordingly, in our experiments, fluoxetine, sertraline, and fluvoxamine were ineffective in WT mice at the tested doses. However, in KO mice the antidepressants were more effective reducing the immobility time in a statistically significant manner. This higher efficacy of fluoxetine or sertraline in sigma-1 KO mice was already reported (Chevallier *et al.*, 2011). In this work, both antidepressants, at 40 mg/kg, i.p., induced a higher reduction in the immobility time in KO mice than in WT counterparts, reaching statistical significance for sertraline. Interestingly, the effects of fluoxetine or fluvoxamine on neurite outgrowth were antagonised by sertraline, pointing to a sigma-1 receptor antagonist profile of sertraline (Ishima et al., 2014). Despite this sigma-1 functional profile, the efficacy of fluoxetine, sigma-1 agonist, and sertraline, in WT subjects is similar (Chevallier *et al.*, 2011).

Overall, the results obtained in mice strongly suggest that sigma-1 receptor is involved in stress management, are suggestive of a potential role in the development of depression, and clearly indicates a regulatory role of sigma-1 receptor on the neurochemical pathways involved in SSRI efficacy.

The availability of the sigma-1 KO rats allowed us to extend our research on the involvement of sigma-1 receptor in depression to this species. Although the TST test was adapted for rats (Chermat R 1986), in our experience and for ethical reasons, we consider it is not an option, so we focused our studies on FST, using the homozygous line and WT Wistar rats.

First, we performed an acute administration study in males and females, for assessing the behaviour of KO rats in this condition. The weight, and so the size, of the animals was comparable between WT and KO rats. Although in females a statistically significant difference was obtained, in any case the animals could reach the bottom of the water container with the hind paws, that could jeopardize the results. The studies previously performed in mice showed a reduced immobility time for KO animals in the training session, and a better efficacy of fluoxetine and fluvoxamine treatment. For both treatments, no efficacy was obtained in WT mice. Contrary to mice, we did not find any difference in the immobility time during training in rats, either in males or in females. In

this acute study, the immobility time during the training session was measured by the observer using a stopwatch in the last five min of the 15 min session, when the animal is already 'depressed' and resting, or moving very slowly, most of the time. In mice it was automatically measured by a digital image analysis software (Smart v3.0. Panlab). Therefore, the procedures used could explain the differences in the training session between both species. In relation to the pharmacological treatment, previous results obtained in our laboratory using Wistar rats already showed slight, non-statistically significant activity of venlafaxine 40 mg/kg, i.p. in the FST (data not shown). At this dose, venlafaxine already produced an increase in the average speed, pointing to a side-effect we should avoid. Therefore, we selected 30 mg/kg for both drugs, looking for an improvement of the efficacy in KO rats. On the test day, treatment with fluoxetine or venlafaxine did not reduce the immobility time in WT rats. It has been reported that, after p.o. administration, venlafaxine only reduced the immobility at 60 mg/kg, and fluoxetine was not active even at 120 mg/kg (Artaiz *et al.*, 2005). Therefore, the result in WT animals was expected to some extent. Considering what we had found in mice, we expected to find some improvement in the efficacy of the antidepressant drugs in sigma-1 KO subjects. However, in the acute study no differences between WT and KO rats were found.

The need of repeated administration is a well-known characteristic of antidepressant treatment at clinical level, with a delayed therapeutic onset of several weeks or months (Zanos *et al.*, 2018). Accordingly, at pre-clinical level, several procedures with more than one administration have been used, looking for better efficacy of antidepressants (Detke *et al.*, 1997; Vazquez-Palacios *et al.*, 2004). After the acute study we performed a subacute study administering the compounds for 14 days. In this experiment, the training session was analysed using the SMART image system used in mouse experiments, recording the whole 15 min period. Using this procedure, we found no differences between WT and KO rats on the last five min, as in the acute study, but overall, KO rats showed lower immobility time, as in the mice study. Conversely, the average speed, the time moving fast, and the time moving slow were higher in KO rats. The absence of the sigma-1 receptor seems to induce a resistance to 'despair' in the first exposure to the stress situation. Like in the acute study, after vehicle treatment, no differences were found between WT and KO rats during the test sessions on days 1, 7 and 14, although an

increase in the immobility time over the sessions was shown. In WT rats, neither venlafaxine nor fluvoxamine, both at 10 mg/kg, i.p., induced a reduction in the immobility time. However, in KO rats, both compounds reduced the immobility on day 14 in a statistically significant manner. Fluvoxamine is a SSRI with high affinity for the sigma-1 receptor, but venlafaxine is devoid of this affinity. The fact that both compound improved their efficacy in sigma-1 KO rats, points to a change in SERT, NET or both transporters in these animals. This change could be in the efficacy of the transporter or in the number of transporters available. Preliminary results obtained in gene expression experiments done with tissue samples from rats after 14 days subacute treatment, suggest that the absence of sigma-1 receptor in KO rats induces an up-regulation of SERT and DAT in certain brain areas in comparison to WT rats (data not shown). In this respect, the addition of the sigma-1 agonist SKF-10.047 increased the uptake of SERT in COS-7 cell culture studies. However, the same group demonstrated that knocking-down of sigma-1 receptor did not affect this increase (Asano et al. 2019), pointing a mechanism different from sigma-1 receptor. However, similar increase in SERT uptake was obtained with other sigma-1 receptor ligands, more sigma-1 selective in some cases, and regardless of being agonists or antagonists, like haloperidol, NE-100 or pentazocine. Finally, the uptake activity was not changed in sigma-1 receptor knockdown culture (Asano et al., 2019). Further experimental work is needed for clarifying how sigma-1 receptor and SERT interaction takes place in WT animals, and how the absence of sigma-1 receptor in KO animals influence SERT expression, distribution, and functionality.

In conclusion, in addition to the available literature, our results strongly suggest the involvement of sigma-1 receptor in depression-like states or, at least, in stress management.

Addiction

Sigma-1 receptor was suggested to play a role in the dopaminergic system already in 1976, when Martin and colleagues described the effects of sigma-1 compounds (Martin *et al.*, 1976). The dopaminergic system is a key player in addiction (for a review: Volkow and Morales, 2015; Solinas *et al.*, 2019). Drugs of abuse have dopamine as the common mediator, independently of their initial mechanism of action (Volkow *et al.*, 2019). Moreover, cocaine was found to have sigma-1 affinity and its activity was described to be mediated, at least partially, by this receptor (Sharkey *et al.* 1988; Menkel *et al.*, 1991; Ritz and George 1993). This was further supported by the antagonism of cocaine effects with sigma-1 antisense oligodeoxynucleotides (Matsumoto *et al.* 2001; Matsumoto *et al.* 2002) or sigma-1 antagonists (Menkel *et al.* 1991; Ujike *et al.* 1996; McCracken *et al.*, 1999; Romieu et al, 2000; Lever *et al.* 2014). Initial results also led to the study of sigma-1 receptor involvement in the effects of other psychostimulants, like methamphetamine (Itzhak, 1993; Clissold *et al.* 1993). The relation of sigma-1 receptor with the dopaminergic system has also been revealed by studies on dopamine receptors (Lee *et al.*, 2008; Fu *et al.*, 2010; Navarro *et al.*, 2013) and the dopamine transporter (DAT) (Sambo *et al.*, 2017; Hong *et al.*, 2017).

Previous results obtained in our laboratory using sigma-1 KO mice showed an up-regulation of DAT in several brain areas (unpublished results). A change in the levels of DAT in certain areas could have an impact on the effects of drugs acting on the dopamine pathway. Drugs like the DAT blocker cocaine, the DAT inhibitor and DA releaser d-amphetamine, and the DA agonist apomorphine could behave differentially in sigma-1 KO rats.

d-Amphetamine. The administration of d-amphetamine induced hyperactivity in a dose-dependent manner up to 2 mg/kg. At 3 mg/kg locomotion decreased due to an increase in the stereotyped behaviours (sniffing, licking, head weaving, or gnawing) that block the expression of forward locomotion. This crossed activities have been reported in different rat strains and measuring several behavioural paradigms (Grilly and Loveland, 2001). The mobile time induced by d-amphetamine was higher in KO rats than in WT counterparts

at all doses tested. Conversely, the rearing time was higher in WT than in KO rats. This was the contrary at 3 mg/kg for both parameters, which agrees with the higher efficacy of d-amphetamine in KO rats. Dose-response curves for mobile time showed that there is a significant effect of genotype only in the second half of the reading period, although overall d-amphetamine was more active at all doses tested. This is probably due to the high level of activity induced by the novelty of the experimental cage during the first 30 min. Therefore, for the analysis of the d-amphetamine effects the values obtained in the second half of the reading period a more relevant. Overall, d-amphetamine is more active in KO than in WT rats.

Apomorphine. Systemic administration of apomorphine induce stereotyped behaviour, mainly sniffing and rearing, and an inhibition of forward locomotion (Bury and Schmidt, 1987). However, a dual effect of apomorphine has been described, with an inhibition of several behaviours in an open-field test at lower doses (0.02–0.08 mg/kg, s.c.), whereas at higher doses (0.2 and 0.5 mg/kg, s.c.) stimulation was observed (Nickolson, 1981). In our experiments, apomorphine produced high level of stereotyped behaviour, mainly gnawing and sniffing, during the first half of the experiment. Consequently, mobile time was low during the first half of the session, at 0.5, 0.25, and 0.5 mg/kg. This was found for WT and KO rats. However, at 1 mg/kg, WT rats displayed hyperactivity, with higher mobile time in comparison to vehicle-treated rats, while in KO rats this high dose of apomorphine was still inducing hypolocomotion, like the other doses tested.

Complex relationships among the different DA receptor subtypes have been described, and these relationships may be modulated by other proteins, like sigma-1 receptor. The hypolocomotion could be attributed to a side-effect of the stereotyped behaviour, but also to a direct effect on DA receptors. Several DA receptors have been described (D1, D2, D3, D4, and D5) with additional subtypes for some of them (Missale *et al.*, 1998). The level of expression, the different affinities of DA or the agonist/antagonist used, and the regional and cellular distribution of all the DA subtypes, among other factors, may produce a huge variety of behavioural responses. D2R subtype, due to its role in schizophrenia or in Parkinson's disease, has been one of the most widely studied subtypes. Taking this subtype as example, the agonism on this receptor induce a biphasic

response, with an initial reduction at low doses, and a sustained hyperactivity at higher doses (Eilam *et al*, 1989). This biphasic response has also a temporal pattern, with hypolocomotion within minutes of injection, and hyperlocomotion appearing much later. This pattern has been assigned to an effect on presynaptic auto-receptors, whereas the later were assigned to the activation of less sensitive D2Rs in projection areas (Beaulieu and Gainetdinov, 2011). In our experiments, apomorphine induced a fast hypolocomotion followed by hyperactivity at later reading points, and with the highest dose used inducing hyperactivity from the beginning in WT, but not in KO rats. Whether this was due to a higher efficacy on post-synaptic DA receptors, a lower efficacy on the pre-synaptic auto-receptors, or differences in the expression level of the DA receptors due to the absence of the sigma-1 receptors will require further studies.

Cocaine

Mobile time was increased dose-dependently by cocaine administration but exactly in the same way in WT and KO rats. However, rearing activity was statistically higher in WT rats, at 10 and 20 mg/kg, i.p.. The time-course profiles of rearing activity in WT and KO rats were very similar, despite the difference in the efficacy.

In sigma-1 receptor KO subjects, the absence of the sigma-1 protein may induce changes in DAT that could explain some of the differences reported here.

The involvement of sigma-1 receptor in the regulation of the dopamine pathways has been described (Walker *et al.*, 1990; Gonzalez-Alvear *et al.*, 1994; Hong et al., 2017), and the role of this DA regulation on drug abuse liability or therapeutic potential of sigma-1 ligands for drug addiction reported (Sambo et al., 2018; Robson et al., 2012; Matsumoto, 2009; Katz et al., 2016). Sigma-1 receptor antagonists inhibit cocaine effects, and the agonists potentiate the reinforcing capacity of cocaine (Matsumoto *et al.,* 2001; Hiranita *et al.,* 2010). Moreover, brain levels of the sigma-1 receptor modulate cocaine-induced hyperactivity (Matsumoto *et al.,* 2002). The regulation of DAT by sigma-1 receptor modulation is most likely mediated by protein interaction (Hong *et al.,* 2017). A similar protein-protein interaction has been described for D_1 receptor (Navarro *et al.,* 2010), and D_2 receptor (Aguinaga Andrés *et al.*, 2018; Borroto-Escuela *et al.*, 2017). Moreover, the

dopaminergic system is formed by four differentiated pathways: 1) mesolimbic, 2) mesocortical, 3) nigro-striatal, and 4) tubero-infundibular, and the levels of expression of DA receptors and DAT in these pathways is different (Klein *et al.*, 2019). Therefore, there is a plethora of possibilities for explaining the differences observed between WT and KO rats after stimulant administration.

The results obtained in the drug-induced studies showing differences between WT and KO rats in response to stimulants with different mechanisms of action, points to changes in the receptors and/or transporters involved. Some results obtained in our lab suggest a change in the level of expression of monoamine transporters in several brain areas of KO rodents (data not shown). Prefrontal cortex, striatum, hippocampus, and hypothalamus samples were obtained from rats used in the FST sub-acute studies, and naïve rats. The analysis of all these brain samples should help to shed light on the changes induced by knocking out the sigma-1 receptor on other protein levels.

Overall, the effects induced by d-amphetamine, apomorphine or cocaine point to an interaction of sigma-1 receptor and DA receptors or DAT. The function or the expression levels of these receptors and/or DAT seem to be altered in KO rats, resulting in a differential response in forward locomotion, and rearing activity of rats.

The serotonergic and dopaminergic systems have been described as major players in depression and addiction, respectively. Both systems are interrelated in the control of these two pathological processes, and the noradrenergic system should also be considered as a key player. Considering the different levels of complexity of the neurochemical pathways, summarised in Figure 179, the characterisation and definition of the role of sigma-1 receptor in the regulation of such systems requires further studies far beyond the objectives of this thesis.

Figure 179. A summary of factors influencing the complexity of the 5-HT and DA systems and the relationship with the sigma-1 receptor.

CONCLUSIONS

1. In rats, under normal conditions, the deletion of the gene that encodes the sigma-1 receptor produces a viable phenotype very similar to that of WT rats, in terms of the physiological and behavioural profile.

2. Under environmental or pharmacological stimulation, WT and KO animals show some differences in the behavioural response in models of depression, both in mice and rats.

3. The response to antidepressants after sub-acute treatment is different in WT and KO animals. The antidepressants tested are more efficacious in both sigma-1 KO mice and rats.

4. In sigma-1 KO rats, forward locomotion and rearing activity induced by psychostimulants like d-amphetamine, apomorphine or cocaine are different from the WT rats, pointing to a sigma-1 regulation of the dopaminergic pathways, mainly the nigro-striatal one.

5. The effects observed in the psychostimulant-induced motor effects, suggest a potential role of sigma-1 ligands in the treatment of addiction.

REFERENCES

Aguinaga D, Medrano M, Vega-Quiroga I, Gysling K, Canela EI, Navarro G, Franco R. «Cocaine Effects on Dopaminergic Transmission Depend on a Balance between Sigma-1 and Sigma-2 Receptor Expression.» *Frontiers In Molecular Neuroscience*, 2018: 11: 17.

Albayrak Y, Hashimoto K. «Beneficial effects of the sigma-1 agonist fluvoxamine for tardive dyskinesia in patients with postpsychotic depressive disorder of schizophrenia: report of 5 cases.» Prim Care Companion CNS Disord. 2012;14(6):PCC

Albayrak Y, Hashimoto K. «Sigma-1 Receptor Agonists and Their Clinical Implications in Neuropsychiatric Disorders.» *Adv Exp Med Biol.* , 2017: 153-161.

Alexander, SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, McGrath JC, Catterall WA, Spedding M, Peters JA, and Harmar AJ. «The Concise Guide to PHARMACOLOGY 2013/14: Overview.» *British journal of Pharmacology*, 2013: 1449-1458.

Alon A, Schmidt HR, Wood MD, Sahn JJ, Martin SF, Kruse AC. «Identification of the gene that codes for the σ2 receptor.» *Proc Natl Acad Sci U S A*, 2017: 114:7160-7165.

Alonso G, Phan VL, Guillemain I, Saunier M, Legrand A, Anoal M, Maurice T. «Immunocytochemical localization of the sigma1 receptor in the adult rat central nervous system.» *Neuroscience* , 2000: 97:155-170.

Asano, M, Motoike, S, Yokota, C, Usuki, N, Yamamoto, H, Urabe, T, Katarao, K, Hide, I, Tanaka, S, Kawamoto, M, Irifune, M, Sakai, No. «SKF-10047, a Prototype Sigma-1 Receptor Agonist, Augmented the Membrane Trafficking and Uptake Activity of the Serotonin Transporter and Its and Its C-Terminus-Deleted Mutant via a Sigma-1 Receptor-Independent Mechanism.» *Journal of pharmacological science*, 2019: 139: 29-36.

Aydar E, Palmer CP, Klyachko VA, Jackson MB. «The sigma receptor as a ligand-regulated auxiliary potassium channel subunit.» *Neuron*, 2002: 34:399-410.

Bächli H, Steiner MA, Habersetzer U, Wotjak CT. «Increased water temperature renders single-housed C57BL/6J mice susceptible to antidepressant treatment in the forced swim test.» *Behavioural Brain Research*, 2008: 67–71.

Beaulieu JM, Gainetdinov RR. «The physiology, signaling, and pharmacology of dopamine receptors.» *Pharmacol Rev*, 2011: 182-217.

Bermack JE, Debonnel G. «Modulation of serotonergic neurotransmission by short- and long-term treatments with sigma ligands.» *Br J Pharmacol*, 2001: 134: 691-699.

Boehning D, Patterson RL, Sedaghat L, Glebova NO, Kurosaki T, Snyder SH. «Cytochrome c binds to inositol (1,4,5) trisphosphate receptors, amplifying calcium-dependent apoptosis.» *Nature cell biology*, 2003: 5:1051-1061.

Borroto-Escuela DO, Narváez M, Wydra K, Pintsuk J, Pinton L, Jimenez-Beristain A,Di Palma M, Jastrzębska J, Filip M, Kjell Fuxe K. «Cocaine self-administration specifically increases A2AR-D2R and D2R-sigma1R heteroreceptor complexes in the rat nucleus accumbens shell. Relevance for cocaine use disorder.» *Pharmacology Biochemistry and Behavior*, 2017: 24-31.

Bradford, M.M. «A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.» *Analytical Biochemistry*, 1976: 72, 248-54.

Brady, K.T., Balster, R.L., May, E.L. «Stereoisomers of N-allylnormetazocine: phencyclidine-like behavioral effects in squirrel monkeys and rats. .» *Science* , 1982: 215:178-80.

Brammer, Matthew K, Deborah L Gilmore, and Rae R Matsumoto. «Interactions Between 3,4-Methylenedioxymethamphetamine and Σ1 Receptors.» *European journal of pharmacology*, 2006: 141–145.

Bravo-Caparrós I, Perazzoli G, Yeste S, Cikes D, Baeyens JM, Cobos EJ, Nieto FR. «Sigma-1 Receptor Inhibition Reduces Neuropathic Pain Induced by Partial Sciatic Nerve Transection in Mice by Opioid-Dependent and -Independent Mechanisms.» *Front Pharmacol.*, 2019: 613.

Bruna J, Velasco R. «Sigma-1 receptor: a new player in neuroprotection against chemotherapy-induced peripheral neuropathy.» *Neural Regen Res.* , 2018: 775-778.

Cagnotto, A, Bastone A, Mennini T. «[3H](+)-Pentazocine binding to rat brain σ1 receptors.» *Eur. J. of Pharmacol.*, 1994: 266, 131-138.

Castany S, Gris G, Vela JM, Verdú E, Boadas-Vaello P. «Critical role of sigma-1 receptors in central neuropathic pain-related behaviours after mild spinal cord injury in mice.» *Sci Rep.* , 2018: 3873.

Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. «Quantitative assessment of tactile allodynia in the rat paw.» *J Neurosci Methods*, 1994: 55-63.

Chermat R, Thierry B, Mico JA, Steru L, Simon P. «Adaptation of the tail suspension test to the rat.» *J Pharmacol*, 1986: 348-350.

Chien CC, Pasternak GW. «(-)-Pentazocine analgesia in mice: interactions with a sigma receptor system.» *Eur J Pharmacol.*, 1995: 303-308.

Clissold DB, Pontecorvo MJ, Jones BE, Abreu ME, Karbon EW, Erikson RH, Natalie Jr, KJ, Borosky S, Hartman T, Mansbach RS, Balster RL, Ferkany JW, Enna SJ. «NPC 16377 a potent and seletctive -ligand II Behavioral and neuroprotective profile.» *J Pharmacol Exp Ther*, 1993: 876-886.

Collina, S, Gaggeri R, Marra A, Bassi A, Negrinotti S, Negri F, and Rossi D. «Sigma Receptor Modulators: a Patent Review.» *Expert opinion on therapeutic patents*, 2013: 597–613.

Couly S, Khalil B, Viguier V, Roussel J, Maurice T, Liévens JC. «Sigma-1 receptor is a key genetic modulator in amyotrophic lateral sclerosis.» *Hum Mol Genet.*, 2020: 529-540.

Crouzier L, Couly S, Roques C, Peter C, Belkhiter R, Arguel Jacquemin M, Bonetto A, Delprat B, Maurice T. «Sigma-1 (σ(1)) receptor activity is necessary for physiological brain plasticity in mice.» *Eur Neuropsychopharmacol.*, 2020: 29-45.

Cunningham, Michael L. «A Mouse Is Not a Rat Is Not a Human: Species Differences Exist.» *Toxicological Sciences*, 2002: 157–158.

Curzon P, Zhang M, Radek RJ, Fox GB. «The Behavioral Assessment of Sensorimotor Processes in the Mouse: Acoustic Startle, Sensory Gating, Locomotor Activity, Rotarod, and Beam Walking.» En *Methods of Behavior Analysis in Neuroscience*, de Buccafusco JJ, Chapter 8. Curzon P, Zhang M, Radek RJ, Fox GB. The Behavioral Assessment of Sensorimotor Processes in the Mouse: Acoustic Startle, Sensory Gating, Locomotor Activity, Rotarod, and Beam Walking. In: Buccafusco JJ, editor., 2009.

D'Amour, FE and Smith, DL. «A method for determining loss of pain sensation.» *J Pharmacol Exp Ther*, 1941: 74-78.

DeHaven-Hudkins DL, Fleissner LC. «Competitive interactions at [3H]-13-(2-tolyl)guanidine (DTG)-defined sigma recognition sites in guinea pig brain.» *Life Sci*, 1992: PL65-PL70.

DeHaven-Hudkins DL, Fleissner LC, Ford-Rice FY. «Characterization of the binding of [3H](+)-pentazocine to sigma recognition sites in guinea pig brain.» *Eur J Pharmacol.*, 1992: 371-378.

Dhir, A and Kulkarni, SK. «Antidepressant-like effect of 17β-estradiol: involvement of dopaminergic, serotonergic, and (or) sigma-1 receptor systems.» *Canadian Journal of Physiology and Pharmacology*, 2008: 726-735.

Di T, Zhang S, Hong J, Zhang T, Chen L. «Di T, Zhang S, Hong J, Zhang T, Chen L. Hyperactivity of Hypothalamic-Pituitary-Adrenal Axis Due to Dysfunction of the Hypothalamic Glucocorticoid Receptor in Sigma-1 Receptor Knockout Mice.» *Front Mol Neurosci*, 2017: 1-14.

Doudna JA, Charpentier E. «Genome editing. The new frontier of genome engineering with CRISPR-Cas9.» *Science*, 2014: 1258096.

Dubuisson D, Dennis SG. «The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats.» *Dubuisson D, Dennis SG. The formalin test: a quantitative study of the analgesic effects of Pain*, 1977: 161-174.

Duman, R.S., S. Deyama, y M.V. Fogaca. «Role of BDNF in the pathophysiology and treatment of depression: Activity-dependent effects distinguish rapid-acting antidepressants.» *Eur. J. Neurosci.*, 2019.

Dunham NW, Miya TS. «A note on a simple apparatus for detecting neurological deficit in rats and mice.» *J Am Pharm Assoc Am Pharm Assoc*, 1957: 208-209.

Eilam D, Golani I, Szechtman H. «D2-agonist quinpirole induces perseveration of routes and hyperactivity but no perseveration of movements.» *Brain Res*, 1989: 255-267.

Entrena, José M, Enrique J Cobos, Francisco R Nieto, Cruz M Cendán, José M Baeyens, and Esperanza Del Pozo. «Antagonism by Haloperidol and Its Metabolites of Mechanical Hypersensitivity Induced by Intraplantar Capsaicin in Mice: Role of Sigma-1 Receptors.» *Psychopharmacology*, 2009: 21-33.

Geyer MA, Swerdlow NR, Mansbach RS, Braff DL. «Startle response models of sensorimotor gating and habituation deficits in schizophrenia.» *Brain Res Bull*, 1990: 485-498.

Gonzalez-Alvear, G. M., and Werling, L. L. «Regulation of [3H]dopamine release from rat striatal slices by sigma receptor ligands.» *J. Pharmacol. Exp. Thera.*, 1994: 212–219.

Grilly DM, Loveland A. «What is a "low dose" of d-amphetamine for inducing behavioral effects in laboratory rats?» *Psychopharmacology (Berl)*, 2001: 155-169.

Gromek, Katarzyna A, Fabian P Suchy, Hannah R Meddaugh, Russell L Wrobel, Loren M LaPointe, Uyen B Chu, John G Primm, Arnold E Ruoho, Alessandro Senes, and

Brian G Fo. «The Oligomeric States of the Purified Sigma-1 Receptor Are Stabilized by Ligands.» *Journal of biological chemistry*, 2014: 289: 20333–20344.

Gundlach AL, Largent BL, Snyder SH. «Autoradiographic localization of sigma receptor binding sites in guinea pig and rat central nervous system with (+)3H-3-(3-hydroxyphenyl)-N-(1-propyl)-piperidine.» *J Neurosci* , 1986: 6:642-647.

Guo, Yan, y otros. «Receptor Ligands Improves Ventricular Repolarization-Related Ion Remodeling in Rats with Major Depression Disorder.» *Psychopharmacology*, 2020: 487–499.

Guzmán-Lenis M-S, Navarro X, Casas C. «Selective sigma receptor agonist 2-(4-morpholinethyl)1-phenylcyclohexanecarboxylate (PRE084) promotes neuroprotection and neurite elongation through protein kinase C (PKC) signaling on motoneurons.» *Neuroscience*, 2009: 31-38.

Haertzen, C.aA. «Subjective effects of narcotic antagonists cyclazocine and nalorphine on the addiction research center inventory (ARCI).» *Psychopharmacologia*, 1970: 18:366-377.

Hanner M, Moebius FF, Flandorfer A, Knaus HG, Striessnig J, Kempner E, Glossmann H. «Purification molecular cloning and expression of the mammalian sigma1–binding site.» *Proc Natl Acad Sci USA*, 1996: 93:8072-8077.

Hayashi T, Su TP. «Intracellular and body distributions of sigma-1 receptors .» *Intramural Research Program, the National Insti- Drug Abuse, NIH. Bethesda, MD*, 2002: (Data on file).

Hayashi T, Su TP. «Sigma-1 receptor ligands: potential in the treatment of neuropsychiatric disorders.» *CNS Drugs*, 2004: 269-284.

Hayashi T, Su TP. «Sigma-1 receptor chaperones at the ER-mitochondrion interface regulate Ca(2+) signaling and cell survival.» *Cell*, 2007: 131:596-610.

Hayashi T, Su TP. «An update on the development of drugs for neuropsychiatric disorders: focusing on the sigma 1 receptor ligand.» *Expert Opin Ther Targets*, 2008: 45-58.

Hellewell SB, Bowen WD. «A sigma-like binding site in rat pheochromocytoma (PC12) cells: decreased affinity for (+)-benzomorphans and lower molecular weight suggest a different sigma receptor form from that in guinea pig brain.» *Brain Res*, 1990: 244-253.

Hellewell SB, Bruce A, Feinstein G, Orringer J, Williams W, Bowen WD. «Rat liver and kidney contain high densities of sigma-1 and sigma-2 receptors: characterization

by ligand binding and photoaffinity labeling.» *Eur J Pharmacol Mol Pharmacol*, 1994: 268: 9-18.

Herrando-Grabulosa M, Gaja-Capdevila N, Vela JM, Navarro X. «Sigma 1 receptor as a therapeutic target for amyotrophic lateral sclerosis.» *Br J Pharmacol.*, 2021: 1336-1352.

Hiranita T, Soto PL, Tanda G, Katz JL. «Reinforcing effects of sigma-receptor agonists in rats trained to self-administer cocaine.» *J Pharmacol Exp Ther*, 2010: 515–524.

Hirst WD, Abrahamsen B, Blaney FE, Calver AR, Aloj L, Price GW, Medhurst AD. «Differences in the central nervous system distribution and pharmacology of the mouse 5-hydroxytryptamine-6 receptor compared with rat and human receptors investigated by radioligand binding, site-directed mutagenesis, and molecular modeling.» *Mol Pharmacol.*, 2003: 1295-1308.

Hjørnevik T, Cipriano PW, Shen B, Park JH, Gulaka P, Holley D, Gandhi H, Yoon D, Mittra ES, Zaharchuk G, Gambhir SS, McCurdy CR, Chin FT, Biswal S. «Biodistribution and Radiation Dosimetry of 18F-FTC-146 in Humans.» *J Nucl Med.*, 2017: 2004-2009.

Hok V, Poucet B, Duvelle É, Save É, Sargolini F. «Spatial cognition in mice and rats: similarities and differences in brain and behavior.» *Wiley Interdiscip Rev Cogn Sci.*, 2016: 406-421.

Hong WC, Yano H, Hiranita T, Chin FT, McCurdy CR, Su TP, Amara SG, Katz JL. «The Sigma-1 Receptor Modulates Dopamine Transporter Conformation and Cocaine Binding and May Thereby Potentiate Cocaine Self-Administration in Rats.» *The Journal of biological chemistry* , 2017: 292: 11250–11261.

Ikeda M, Takahashi A, Kamatani Y, Okahisa Y, Kunugi H, Mori N, Sasaki T, Ohmori T, Okamoto Y, Kawasaki H, Shimodera S, Kato T, Yoneda H, Yoshimura R, Iyo M, Matsuda K, Akiyama M, Ashikawa K, Kashiwase K, Tokunaga K, Kondo K, Saito T, Shimasaki A, et al. «A genome-wide association study identifies two novel susceptibility loci and trans population polygenicity associated with bipolar disorder.» *Mol Psychiatry*, 2018: 639-647.

Irwin, Samuel. «Drug Screening and Evaluative Procedures: Current approaches do not provide the information needed for properly predicting drug effects in man.» *Science*, 1962: 123-128.

Ishima T, Fujita Y, Hashimoto K. «Interaction of new antidepressants with sigma-1 receptor chaperones and their potentiation of neurite outgrowth in PC12 cells.» *Eur J Pharmacol.*, 2014: 167-173.

Itzhak, Y. «Repeated methamphetamine-treatment alters brain sigma receptors.» *Eur J Pharmacol*, 1993: 243-244.

Iwamoto, ET «Locomotor activity and antinociception after putative mu, kappa and sigma opioid receptor agonists in the rat: influence of dopaminergic agonists and antagonists.» *J Pharmacol Exp Ther* , 1981: 217:451-60.

The Neurotrophic Hypothesis of Depression Revisited: New Insights and Therapeutic Implications

Jaggar M, Fanibunda SE, Ghosh S, Duman RS, and Vaidya VA. «The Neurotrophic Hypothesis of Depression Revisited: New Insights and Therapeutic Implications.» In: *Neurobiology of Depression*, Carvalho J, Zarate AF, Elsevier Inc., 2019: 43-62.

Jansen KL, Elliot M, Leslie RA. «Sigma receptors in rat brain and testes show similar reductions in response to chronic haloperidol.» *Eur J Pharmacol.*, 1992: 281-283.

Jia H, Zhang Y, Huang Y. «Imaging sigma receptors in the brain: New opportunities for diagnosis of Alzheimer's disease and therapeutic development.» *Neurosci Lett*, 2019: 3-10.

Jin JL, Fang M, Zhao YX, Liu XY. «Roles of sigma-1 receptors in Alzheimer's disease.» *Int J Clin Exp Med.*, 2015: 4808-4820.

Craviso GL, Musacchio JM «High affinity dextromethorphan binding sites in guinea pig. II. Competition ex.» *Mol Pharmacol*, 1983: 629-640.

Johnston AL, File SE. «Sex differences in animal tests of anxiety.» *Physiol Behav*, 1991: 245-250.

Katz JL, Hong WC, Hiranita T, Su TP. «A role for sigma receptors in stimulant self-administration and addiction. .» *Behav Pharmacol.* , 2016: 100-115.

Kekuda R, Prasad PD, Fei YJ, Leibach FH, Ganapathy V. «Cloning and functional expression of the human type 1 sigma receptor (hSigmaR 1).» *Biochem Biophys Res Commun*, 1996: 229:553-558.

Kim FJ, and Pasternak GW. «Sigma Proteins.» *Springer International Publishing AG*, 2017.

Kishi T, Yoshimura R, Okochi T, Fukuo Y, Kitajima T, Okumura T, Tsunoka T, Kawashima K, Yamanouchi Y, Kinoshita Y, Umene-Nakano W, Naitoh H, Nakamura J, Ozaki N, Iwata N. «Association Analysis of SIGMAR1 with Major Depressive Disorder and SSRI Response.» *Neuropharmacology*, 2010: 1168–1173.

Klein MD, Musacchio JM. «High affinity dextromethorphan binding sites in guinea pig brain Effect of sigma ligands and other agents.» *J Pharmacol Exp Ther* , 1989: 207-215.

Klein MO, Battagello DS, Cardoso AR, Hauser DN, Bittencourt JC, Correa RG. «Dopamine: Functions, Signaling, and Association with Neurological Diseases.» *Cell Mol Neurobiol*, 2019: 31-59.

Langa F, Codony X, Tovar V, Lavado A, Giménez E, Cozar P, Cantero M, Dordal A, Hernández E, Pérez R, Monroy X, Zamanillo D, Guitart X, Montoliu L. «Generation and phenotypic analysis of sigma receptor type I (sigma 1) knockout mice.» *Eur J Neurosci.*, 2003: 2188-2196.

Largent BL, Gundlach AL, Snyder SH. «Pharmacological and autoradiographic discrimination of sigma and phencyclidine receptor binding sites in brain with (+)-[3H]SKF10,047, (+)-[3H]-3-[3-hydroxyphenyl]-N-(1-propyl)piperidine, and [3H]-1-[1-(2-thienyl)cyclohexyl]-piperidine.» *J Pharmacol Exp Ther*, 1986: 238: 739-748.

LePage KT, Ishmael JE, Low CM, Traynelis SF, Murray TF. «Differential binding properties of [3H]dextrorphan and [3H]MK-801 in heterologously expressed NMDA receptors.» *Neuropharmacology*, 2005: 1-16.

Liu DY, Chi TY, Ji XF, Liu P, Qi XX, Zhu L, Wang ZQ, Li L, Chen L, Zou LB. «Sigma-1 receptor activation alleviates blood-brain barrier dysfunction in vascular dementia mice.» *Exp Neurol.*, 2018: 90-99.

López-Estévez S, Gris G, de la Puente B, Carceller A, Martínez V. «Intestinal inflammation-associated hypersensitivity is attenuated in a DSS model of colitis in Sigma-1 knockout C57BL/6 mice.» *Biomed Pharmacother.* , 2021: 112126.

Mancuso R, Oliván S, Rando A, Casas C, Osta R, Navarro X. «Sigma-1R agonist improves motor function and motoneuron survival in ALS mice.» *Neurotherapeutics*, 2012: 814-826.

Mandelli L, Wang SM, Han C, Lee SJ, Patkar AA, Masand PS, Pae CU, Serretti A. «The Impact of a Single Nucleotide Polymorphism in SIGMAR1 on Depressive Symptoms in Major Depressive Disorder and Bipolar Disorder.» *Adv Ther.*, 2017: 713-724.

Martin, WR, Eades, CE, Thompson, JA, Huppler, RE «The effects of morphine and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dog.» *J Pharmacol Exp Ther* , 1976: 517-532.

Mathiasen JR, Moser VC. «The Irwin Test and Functional Observational Battery (FOB) for Assessing the Effects of Compounds on Behavior, Physiology, and Safety Pharmacology in Rodents.» *Curr Protoc Pharmacol*, 2018: e43.

Matsumoto RR, Pouw B. «Correlation between neuroleptic binding to sigma(1) and sigma(2) receptors and acute dystonic reactions.» *Eur J Pharmacol*, 2000: 155-160.

Matsumoto RR, McCracken KA, Friedman MJ, Pouw B, De Costa BR, Bowen WD. «Conformationally restricted analogs of BD1008 and an antisense oligodeoxynucleotide targeting sigma1 receptors produce anti-cocaine effects in mice.» *Eur J Pharmacol.*, 2001: 163-174.

Matsumoto, Rae R, Kari A McCracken, Buddy Pouw, Jeffrey Miller, Wayne D Bowen, Wanda Williams, and Brian R De Costa. «N-Alkyl Substituted Analogs of the σ Receptor Ligand BD1008 and Traditional σ Receptor Ligands Affect Cocaine-Induced Convulsions and Lethality in Mic.» *European journal of pharmacology*, 2001: 261–273.

Matsumoto RR, McCracken KA, Pouw B, Zhang Y, Bowen WD. «Involvement of sigma receptors in the behavioral effects of cocaine: evidence from novel ligands and antisense oligodeoxynucleotides.» *Neuropharmacology*, 2002: 1043-1055.

Matsumoto RR. «Targeting sigma receptors: novel medication development for drug abuse and addiction.» *Expert Rev Clin Pharmacol*, 2009: 351-358.

Matsuno K, Kobayashi T, Tanaka MK, Mita S. «Sigma 1 receptor subtype is involved in the relief of behavioral despair in the mouse forced swimming test.» *Eur J Pharmacol.*, 1996: 267-271.

Maurice T, Goguadze N. «Role of σ1 Receptors in Learning and Memory and Alzheimer's Disease-Type Dementia.» *Adv Exp Med Biol.*, 2017: 213-233.

Maurice T, Grégoire C, Espallergues J. «Neuro(active)steroids actions at the neuromodulatory sigma1 (sigma1) receptor: biochemical and physiological evidences, consequences in neuroprotection.» *Pharmacol Biochem Behav*, 2006: 581-597.

Maurice T, Roman FJ, Privat A. «Modulation by neurosteroids of the in vivo (+)-[3H]SKF 10,047 binding to sigma1 receptors in the mouse forebrain.» *J Neurosci Res* , 1996: 46:734-743.

Maurice T, Su TP, Privat A. «Sigma1 (sigma 1) receptor agonists and neurosteroids attenuate B25-35-amyloid peptide-induced amnesia in mice through a common mechanism.» *Neuroscience.*, 1998: 413-428.

Maurice, T, and Su TP. «The Pharmacology of Sigma-1 Receptors.» *Pharmacology & therapeutics (Oxford)*, 2009: 195–206.

Maurice, T, Meunier J, Feng B, Ieni J, and Monaghan DT. «Interaction with Sigma(1) Protein, but Not N-Methyl-D-Aspartate Receptor, Is Involved in the Pharmacological Activity of Donepezil.» *The Journal of pharmacology and experimental therapeutics*, 2006: 606-614.

Mavlyutov TA, Guo LW, Epstein ML, Ruoho AE. «Role of the Sigma-1 receptor in Amyotrophic Lateral Sclerosis (ALS).» *J Pharmacol Sci.*, 2015: 127:10-16.

Mazor A, Matar MA, Kaplan Z, Kozlovsky N, Zohar J, Cohen H. «Gender-related qualitative differences in baseline and post-stress anxiety responses are not reflected in the incidence of criterion-based PTSD-like behaviour patterns.» *World J Biol Psychiatry*, 2009: 856-869.

McCann DJ, Su TP. «Solubilization and characterization of haloperidol-sensitive (+)-[3H]SKF 10,047 binding sites (sigma sites) from rat liver membranes.» *J Pharmacol Exp Ther*, 1991: 547-554.

McCann DJ, Weissman AD, Su TP. «Sigma-1 and sigma-2 sites in rat brain: comparison of regional ontogenic and subcellular patterns.» *Synapse*, 1994: 17:182-189.

McCracken KA, Bowen WD, Matsumoto RR. «Novel sigma receptor ligands attenuate the locomotor stimulatory effects of cocaine.» *Eur J Pharmacol*, 1999: 35-38.

McLean S, Weber E. «Autoradiographic visualization of haloperidol-sensitive sigma receptors in guinea-pig brain.» *Neuroscience* , 1988: 25:259-269.

Mei J, Pasternak GW. «Molecular cloning and pharmacological characterization of the rat sigma1 receptor.» *Biochem Pharmacol* , 2001: 62:349-355.

Mendelsohn, L.G., Kalra, V., Johnson, B.G., Kerchner, G.A. «Sigma opioid receptor: characterization and co-identity with the phencyclidine receptor. .» *J Pharmacol Exp Ther* , 1985: 233:597-602 .

Menkel M, Terry P, Pontecorvo M, Katz JL, Witkin JM. «Selective sigma ligands block stimulant effects of cocaine.» *Eur J Pharmacol* , 1991: 251-252.

Merlos M, Romero L, Zamanillo D, Plata-Salamán C, Vela JM. «Sigma-1 Receptor and Pain.» *Handb Exp Pharmacol.* , 2017: 131-161.

Minabe, Yoshio, Kiyoshi Matsuno, and Charles R Ashby Jr. «Acute and Chronic Administration of the Selective Sigma1 Receptor Agonist SA4503 Significantly

Alters the Activity of Midbrain Dopamine Neurons in Rats: An in Vivo Electrophysiological Study.» *Synapse*, 1999: 33: 129–140.

Mishra, Ashish K, Timur Mavlyutov, Deo R Singh, Gabriel Biener, Jay Yang, Julie A Oliver, Arnold Ruoho, and Valerică Raicu. «The Sigma-1 Receptors Are Present in Monomeric and Oligomeric Forms in Living Cells in the Presence and Absence of Ligands.» *Biochemical journal*, 2015: 466: 263–271.

Missale C, Nash SR, Robinson SW, Jaber M, Caron MG. «Dopamine receptors: from structure to function.» *Physiol Rev*, 1998: 189-225.

Moebius FF, Striessnig J, Glossmann H. «The mysteries of sigma receptors: new family members reveal a role in cholesterol synthesis.» Trends Pharmacol Sci. 1997: 67-70

Mori T, Hayashi T, Hayashi E, Su TP. «Sigma-1 receptor chaperone at the ER-mitochondrion interface mediates the mitochondrion-ER-nucleus signaling for cellular survival.» *PLoS One*, 2013: 8:e76941.

Narita N, Hashimoto K, Tomitaka S, Minabe Y. «Interactions of selective serotonin reuptake inhibitors with subtypes of sigma receptors in rat brain.» *Eur J Pharmacol*, 1996: 117-119.

Navarro G, Moreno E, Bonaventura J, Brugarolas M, Farré D, Aguinaga D, Mallol J, Cortés A, Casadó V, Lluís C, Ferre S, Franco R, Canela E, McCormick PJ. «Cocaine Inhibits Dopamine D2 Receptor Signaling via Sigma-1-D2 Receptor Heteromers.» *PLoS One*, 2013: e61245.

Neal R. Swerdlow, Zoe A. Martinez, Faith M. Hanlon, Amanda Platten, Marjan Farid, Pamela Auerbach, David L. Braff, and Mark A. Geyer. «Neal R. Swerdlow, Zoe A. Martinez, Faith M. Hanlon, Amanda Platten, Marjan Farid, Pamela Auerbach, David L. Braff, and Mark A. Geyer Toward Understanding the Biology of a Complex Phenotype: Rat Strain and Substrain Differences in the Sensorimotor Gating-D.» s.f.

Netser S, Meyer A, Magalnik H, Zylbertal A, de la Zerda SH, Briller M, Bizer A, Grinevich V, Wagner S. «Distinct dynamics of social motivation drive differential social behavior in laboratory rat and mouse strains.» *Nat Commun.* , 2020: 5908.

Nguyen, E. C., McCracken, K. A., Liu, Y., Pouw, B. & Matsumoto, R. R. «Involvement of sigma (sigma) receptors in the acute actions of methamphetamine: receptor binding and behavioral studies.» *Neuropharmacology*, 2005: 638-645.

Nickolson, VJ. «Detailed analysis of the effects of apomorphine and d-amphetamine on spontaneous locomotor behaviour of rats as measured in a TV-based, automated open-field system.» *European Journal of Pharmacology*, 1981: 45-56.

Nieto FR, Cendán CM, Sánchez-Fernández C, Cobos EJ, Entrena JM, Tejada MA, Zamanillo D, Vela JM, Baeyens JM. «Role of sigma-1 receptors in paclitaxel-induced neuropathic pain in mice.» *J Pain.*, 2012: 1107-1121.

Nishimura T, Ishima T, Iyo M, Hashimoto K. «Potentiation of Nerve Growth Factor-Induced Neurite Outgrowth by Fluvoxamine: Role of Sigma-1 Receptors, IP.sub.3 Receptors and Cellular Signaling Pathways.» *PloS one*, 2008: e2558.

Okuyama S, Chaki S, Yae T, Nakazato A, Muramatsu M. «Autoradiographic characterization of binding sites for [3H]NE-100 in guinea pig brain.» *Life Sci* , 1995: 57:PL333-PL337.

Oshiro Y, Sakurai Y, Sato S, Kurahashi N, Tanaka T, Kikuchi T, Tottori K, Uwahodo Y, Miwa T, Nishi T. «3,4-dihydro-2(1H)-quinolinone as a novel antidepressant drug: synthesis and pharmacology of 1-[3-[4-(3-chlorophenyl)-1-piperazinyl]propyl]-3,4- dihydro-5-methoxy-2(1H)-quinolinone and its derivatives.» *J Med Chem*, 2000: 177-189.

Pan YX, Mei JF, Xu J, Wan BL, Zuckerman A, Pasternak GW. «Cloning and characterization of a sigma-1 receptor.» *J Neurochem* , 1998: 70:2279-2285.

Peña Y, Prunell M, Dimitsantos V, Nadal R, Escorihuela RM. «Environmental enrichment effects in social investigation in rats are gender dependent. Behav Brain Res.» *Behav Brain Res* , 2006: 181-187.

Phan, VL, Urani, A, Romieu, P, Maurice T. «Strain differences in σ1 receptor-mediated behaviours are related to neurosteroid levels.» *Eur J Neurosci*, 2002: 15: 1523-1534.

Porsolt RD, Bertin A, Jalfre M. «Behavioral despair in mice: a primary screening test for antidepressants.» *Arch Int Pharmacodyn Ther*, 1977: 327-336.

Porsolt RD, Anton G, Blavet N, Jalfre M. «Behavioural despair in rats: a new model sensitive to antidepressant treatments.» *Eur J Pharmacol*, 1978: 379-391.

Prasad PD, Li HW, Fei YJ, Ganapathy ME, Fujita T, Plumley LH, Yang-Feng TL, Leibach FH, Ganapathy V. «Exon-intron structure analysis of promoter region and chromosomal localization of the human type 1 sigma receptor gene.» *J Neurochem*, 1998: 70:443-451.

Quirion, R., Bowen, W.D., Itzhak, Y., Junien, J.L., Musacchio, J.M., Rothman, R.B., Su, T.P., Tam, S.W., Taylor, D.P. «A proposal for the classification of sigma binding sites. .» *Trends Pharm Sci*, 1992: 13:85-86.

Ramos A, Berton O, Mormède P, Chaouloff F. «A multiple-test study of anxiety-related behaviours in six inbred rat strain.» *Behavioural Brain Research*, 1997: 57-69.

Randall LO, Selitto JJ. «A method for measurement of analgesic activity on inflamed tissue.» *Arch Int Pharmacodyn Ther*, 1957: 409-419.

Redfern WS, Dymond A, Strang I, Storey S, Grant C, Marks L, Barnard C, Heys C, Moyser K, Greenwood K, Cobey D, Moore N, Karp NA, Prior H. «The functional observational battery and modified Irwin test as global neurobehavioral assessments in the rat: Pharmacological validation data and a comparison of methods.» *Pharmacol Toxicol Methods*, 2019: 106591.

Ritz MC, George FR. «Cocaine-induced seizures and lethality appear to be associated with distinct central nervous system binding sites.» *J Pharmacol Exp Ther.*, 1993: 1333-1343.

Robson MJ, Noorbakhsh B, Seminerio MJ, Matsumoto RR. «Sigma-1 receptors: potential targets for the treatment of substance abuse.» *Curr Pharm Des*, 2012: 902-919.

Rodríguez-Muñoz M, Sánchez-Blázquez P, Herrero-Labrador R, Martínez-Murillo R, Merlos M, Vela JM, Garzón J. «The σ1 receptor engages the redox-regulated HINT1 protein to bring opioid analgesia under NMDA receptor negative control.» *Antioxid Redox Signal*, 2015: 22:799-818.

Romieu P, Martin-Fardon R, Maurice T. «Involvement of the sigma 1 receptor in the cocaine-induced conditioned place preference.» *Neuroreport*, 2000: 2885-2888.

Ruscher K, and Wieloch T. «The involvement of the sigma-1 receptor in neurodegeneration and neurorestoration.» *J Pharmacol Sci*, 2015: 30-35.

Sałaciak, K, and Pytka, K. «Revisiting the sigma-1 receptor as a biological target to treat affective.» *Neuroscience and Biobehavioral Reviews*, 2021: 1114-1136.

Sambo DO, Lebowitz JJ, Khoshbouei H. «The sigma-1 receptor as a regulator of dopamine neurotransmission: A potential therapeutic target for methamphetamine addiction.» *Pharmacol Ther*, 2018: 152-167.

Sambo, DO, Lebowitz JJ, and Khoshbouei H. «The Sigma-1 Receptor as a Regulator of Dopamine Neurotransmission: A Potential Therapeutic Target for Methamphetamine Addiction.» *Pharmacology & therapeutics (Oxford)* , 2018: 152–167.

Samovilova NN, Nagornaya LV, Vinogradov VA. «(+)-[3H]SKF 10,047 binding sites in rat liver.» *Eur J Pharmacol* , 1988: 147:259-264.

Samovilova NN, Vinogradov VA. «Subcellular distribution of (+)-[3H]SKF 10,047 binding sites in rat liver.» *Eur J Pharmacol.*, 1992: 69-74.

Sanacora G, Treccani G, Popoli M. «Towards a glutamate hypothesis of depression: an emerging frontier of neuropsychopharmacology for mood disorders.» *Neuropharmacology*, 2012: 63-77.

Schmidt HR, Zheng S, Gurpinar E, Koehl A, Manglik A, Kruse AC. «Crystal structure of the human σ1 receptor.» *Nature*, 2016: 532:527-530.

Seth P, Fei YJ, Huang W, Leibach FH, Ganapathy, V. «Cloning and functional characterization of a sigma receptor from rat brain.» *J Neurochem*, 1998.

Sharkey J, Glen KA, Wolfe S, Kuhar MJ. «Cocaine binding at sigma receptors.» *Eur J Pharmacol.*, 1988: 171-174.

Shin EJ, Nah SY, Chae JS, Bing G, Shin SW, Yen TP, Baek IH, Kim WK, Maurice T, Nabeshima T, Kim HC. «Dextromethorphan attenuates trimethyltin-induced neurotoxicity via sigma1 receptor activation in rats.» *Neurochem Int.*, 2007: 791-799.

Shin SM, Wang F, Qiu C, Itson-Zoske B, Hogan QH, Yu H. «Sigma-1 receptor activity in primary sensory neurons is a critical driver of neuropathic pain.» *Gene Ther.*, 2022: 1-15.

Snyder MA, McCann K, Lalande MJ, Thivierge JP, Bergeron R. «Sigma receptor type 1 knockout mice show a mild deficit in plasticity but no significant change in synaptic transmission in the CA1 region of the hippocampus.» *J Neurochem.* , 2016: 700-709.

Sowa-Kucma, M., Styczen, K., Siwek, M., Misztak, P., Nowak, R.J., Dudek, D., Rybakowski, J.K., Nowak, G., Maes, M. «Lipid Peroxidation and Immune Biomarkers Are Associated with Major Depression and Its Phenotypes, Including Treatment-Resistant Depression and Melacholia.» *Neurotox. Res*, 2018448-460.

Steru L, Chermat R, Thierry B, Simon P. «The tail suspension test: a new method for screening antidepressants in mice.» *Psychopharmacology (Berl)*, 1985: 367-370.

Su TP, Su TC, Nakamura Y, Tsai SY. «The Sigma-1 Receptor as a Pluripotent Modulator in Living Systems.» *Trends Pharmacol Sci.*, 2016: 37:262–278.

Sugimoto Y, Tagawa N, Kobayashi Y, Mitsui-Saito K, Hotta Y, Yamada J. «Involvement of the sigma1 receptor in the antidepressant-like effects of fluvoxamine in the

forced swimming test in comparison with the effects elicited by paroxetine.» *Eur J Pharmacol.* , 2012: 96-100.

Swerdlow NR, Caine SB, Braff DL, Geyer MA. «Neural substrates of sensorimotor gating of the startlereflex: A review of recent findings and their implications.» *J Psychopharmacol*, 1992: 176-190.

Swerdlow NR, Martinez ZA, Hanlon FM, Platten A , Farid M,Auerbach P, Braff DL, Geyer MA. «Toward Understanding the Biology of a Complex Phenotype: Rat Strain and Substrain Differences in the Sensorimotor Gating-Disruptive Effects of Dopamine Agonists.» *The Journal of Neuroscience*, 2000: 4325–4336.

Tagashira H, Bhuiyan MS, Shioda N, Fukunaga K. «Fluvoxamine rescues mitochondrial Ca2+ transport and ATP production through sigma(1)-receptor in hypertrophic cardiomyocytes.» *Life sciences*, 2014: 95:89–100.

Takebayashi M, Hayashi T, Su TP. «A perspective on the new mechanism of antidepressants: neuritogenesis through sigma-1 receptors.» *Pharmacopsychiatry*, 2004: S208-13.

Tam SW. «Naloxone-inaccessible sigma receptor in rat central nervous system.» *Proceedings of the National Academy of Sciences of the United States of America*, 1983: 80: 6703–6707.

Tam SW, Mitchell KN. «Neuropeptide Y and peptide YY do not bind to brain sigma and phencyclidine binding sites.» *Eur J Pharmacol*, 1991: 121-122.

Tang AH, Himes CS. «Apomorphine produced more yawning in Sprague-Dawley rats than in F344 rats: a pharmacological study.» *European Journal of Pharmacology*, 1995: 13-18.

Tottori K, Miwa T, Uwahodo Y, Yamada S, Nakai M, Oshiro Y, Kikuchi T, Altar CA. «Antidepressant-like responses to the combined sigma and 5-HT1A receptor agonist OPC-14523.» *Neuropharmacology*, 2001: 976-988.

Tsai SY, Chuang JY, Tsai MS, Wang XF, Xi ZX, Hung JJ, Chang WC, Bonci A, Su TP. «Sigma-1 receptor mediates cocaine-induced transcriptional regulation by recruiting chromatin-remodeling factors at the nuclear envelope.» *Proceedings of the National Academy of Sciences of the United States of America*, 2015: 12:E6562-70.

Ujike H, Kanzaki A, Okumura K, Akiyama K, Otsuki S. «Sigma antagonist BMY 14802 prevents methamphetamine-induced sensitization.» *Life Sci*, 1992: PL129-PL134.

Ujike H, Kuroda S, Otsuki S. «Sigma receptor antagonists block the development of sensitization to cocaine.» *Eur J Pharmacol*, 1996: 123-128.

Ukai M, Maeda H, Nanya Y, Kameyama T, Matsuno K. «Beneficial effects of acute and repeated administrations of sigma receptor agonists on behavioral despair in mice exposed to tail suspension.» *Pharmacol Biochem Behav*, 1998: 247-252.

Urani A, Roman FJ, Phan VL, Su TP, Maurice T. «The antidepressant-like effect induced by sigma(1)-receptor agonists and neuroactive steroids in mice submitted to the forced swimming test.» *J Pharmacol Exp Ther.*, 2001: 1269-1279.

Urani A, Romieu P, Portales-Casamar E, Roman FJ, Maurice T. «The antidepressant-like effect induced by the sigma(1) (sigma(1)) receptor agonist igmesine involves modulation of intracellular calcium mobilization.» *Psychopharmacology (Berl)*, 2002: 26-35.

Valenza M, DiLeo A, Steardo L, Cottone P, Sabino V. «Ethanol-related behaviors in mice lacking the sigma-1 receptor.» *Behav Brain Res.*, 2016: 196-203.

van Broekhoven F, Verkes RJ. «Neurosteroids in depression: a review.» *Psychopharmacology (Berl)*, 2003: 97-110.

Vaupel, D.B. «Naltrexone fails to antagonize the sigma effects of PCP and SKF 10,047 in the dog. Eur J Pharmacol .» *Eur J Pharmacol* , 1983: 92:269-274.

Vavers, Edijs, Liga Zvejniece, Tangui Maurice, and Maija Dambrova. «Allosteric Modulators of Sigma-1 Receptor: A Review.» *Frontiers in pharmacology*, 2019: 223–223.

Vela JM, Merlos M, Almansa C. «Investigational sigma-1 receptor antagonists for the treatment of pain.» *Expert Opin Investig Drugs.*, 2015: 883-896.

Villard V, Meunier J, Chevallier N, Maurice T. «Pharmacological interaction with the sigma1 (σ1)-receptor in the acute behavioral effects of antidepressants.» *J Pharmacol Sci.*, 2011: 279-292.

Vilner BJ, Bowen WD. «Modulation of cellular calcium by sigma-2 receptors: release from intracellular stores in human SK-N-SH neuroblastoma cells.» *J Pharmacol Exp Ther*, 2000: 900-911.

Walker, J. M., Bowen, W. D., Walker, F. O., Matsumoto, R. R., De Costa, B.,. «Sigma receptors: biology and function.» *Pharmacol. Rev.*, 1990: 355–402.

Wang J, Shanmugam A, Markand S, Zorrilla E, Ganapathy V, Smith SB. «Sigma 1 receptor regulates the oxidative stress response in primary retinal Muller glial cells via NRF2 signaling and system x, the Na-independent glutamate-cystine exchanger.» *Free radical biology & medicine*, 2015: 86:25–36.

Wang Y, Gao C, Gao T, Zhao L, Zhu S, Guo L. «Plasma exosomes from depression ameliorate inflammation-induced depressive-like behaviors via sigma-1 receptor delivery.» *Brain Behav Immun.* , 2021: 225-234.

Weber E, Sonders M, Quarum M, McLean S, Pou S, Keana JFW. «1.3-di(2-[5-3H]tolyl)guanidine : a selective ligand that labels sigma-type receptors for psychotomimetic opiates and antipsychotic drugs.» *Proc Natl Acad Sci USA*, 1986: 8784-8788.

Weiland NG. «Estradiol selectively regulates agonist binding sites on the N-methyl-D-aspartate receptor complex in the CA1 region of the hippocampus.» *Endocrinology*, 1992: 662-668.

Wolfe SA, Jr. «Differential localization of three distinct binding sites for sigma receptor ligands in rat spleen.» *Journal of Neuroimmunology* , 1997: 72: 45-58.

Yang K, Wang C, Sun T. «The Roles of Intracellular Chaperone Proteins, Sigma Receptors, in Parkinson's Disease (PD) and Major Depressive Disorder (MDD).» *Front Pharmacol.*, 2019: 528.

Yano, H., Liu, L., Naing, S., & Shi, L. «The Effects of Terminal Tagging on Homomeric Interactions of the Sigma 1 Receptor.» *Frontiers in neuroscience*, 2019: 13: 1356.

Zamanillo D, Romero L, Merlos M, Vela JM. «Sigma 1 receptor: a new therapeutic target for pain. Eur J Pharmacol. 2013 Sep 15;716(1-3):78-93.» *Eur J Pharmacol*, 2013: 78-93.

Zhang, L., Zhang, J., You, Z. «Switching of the Microglial Activation Phenotype Is a Possible Treatment for Depression Disorder.» *Handb.Front. Cell. Neurosci.*, 2018: 306.

Zimmermann, Manfred. «Ethical guidelines for investigations of experimental pain in conscious animals.» *Pain*, 1983: 109-110.

Zukin, S.R., Brady, K.T., Slifer, B.L., Balster, R.L. «Behavioral and biochemical stereoselectivity of sigma opiate/PCP receptors. .» *Brain Res* , 1984: 294:174-177.

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ABBREVIATIONS

ALB	albumin
ALT	alanine aminotransferase
ALP	alkaline Phosphatase
ANOVA	one-way analysis of variance
Approx.	approximately
AST	aspartate transaminase
AUC	area under the curve
BASO	basophil
BiP	binding immunoglobulin protein
BUN	blood urea nitrogen
Ca	calcium
Cas9	CRISPR associated protein 9
CHOL	cholesterol
CL^-	chlorite
cm	centimetres
CRE	creatinine
Cont.	continuation
CRISPR	clustered Regularly Interspaced Short Palindromic Repeats
DAT	dopamine transporter
EOS	eosinophil
ER	endoplasmic reticulum
GLOB	globulin
GLU	glucose
GRP78	glucose-regulated protein 78
H.C.T.	haematocrit
H.G.B.	haemoglobin
HPMC	hydroxypropyl methylcellulose
i.p.	intraperitoneal

K^+	potassium
kDa	kilodalton
KO	knock-out
LYM	lymphocyte
MAM	mitochondria-associated endoplasmic reticulum
M.C.H.	mean corpuscular haemoglobin
M.C.H.C.	mean corpuscular haemoglobin concentration
M.C.V.	mean corpuscular volume
MON	monocyte
M.P.V.	mean platelet volume
NEU	neutrophil
Na^+	sodium
NET	norepinephrine transporter
PCP	phencyclidine
PLT	platelet
p.o.	per oralis (orally)
R.B.C.	red blood cell
R.D.W.	red blood cell volume distribution
s.c.	subcutaneous
sec	seconds
s.e.m.	standard error of the mean
SERT	serotonin transporter
sgRNA	single guide RNA
TBIL	bilirubin
tCO2	carbon dioxide
TG	triglycerides
TMEM97	endoplasmic reticulum-resident transmembrane protein 97
TP	total protein
W.B.C.	white blood cell
WT	wild type