UNIVERSITAT DE BARCELONA

Stress influence in neurodegeneration

Unravelling the mechanisms underlying stress response in brain ageing by 11ß-HSD1 inhibition

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		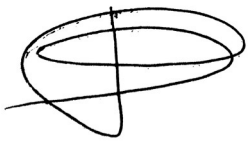
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“Don't let anyone rob you of your imagination, your creativity, or your curiosity.
It's your place in the world; it's your life.
Go on and do all you can with it,
and make it the life you want to live.”
Mae Jemison

“Life is not easy for any of us. But what of that?
We must have perseverance and above all confidence in ourselves.
We must believe that we are gifted for something and that this thing must be attained.”
Marie Sklodowska Curie

STATEMENT OF ORIGINALITY

I declare that this PhD thesis is my own original work and includes material from three articles that have been previously published in international peer-reviewed journals. One other article is currently pending to submit.

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ABSTRACT

The world is facing an unprecedented situation: soon the number of people over 60 years old will exceed the number of children, and more people at extreme old age than ever before. This is due to medical advances that have increased life expectancy and with it, the number of elderly. The latest data published by the WHO predicts that by 2050 the world's population aged 60 years and older is expected to nearly double from 12% to 22% achieving a total 2 billion, up from 900 million in 2015. A longer life brings with it opportunities, however, there is little evidence to suggest that older people today are experiencing their later years in better health than their parents. While rates of severe disability have declined over the past 30 years, there has been no significant change in mild to moderate disability over the same period. If this added years are dominated by declines in physical and mental capacity, the implications for older people and for society will be more negative. Therefore, in the last years research has focused on the biology of ageing with the purpose of achieving better understanding of its mechanisms for preventing the onset and progression of age-related conditions.

Besides, modern society is experiencing an increasingly common stressful lifestyle together to increased rates of metabolic stress caused, in part, by high-fat diet consumption. Several pieces of evidence state that environmental factors are essential in determining the development of different diseases as well as compromising healthy ageing. With upcoming age, the capability to fight against harmful stimuli decreases and the organism becomes more vulnerable to infections and disease. In agreement, stressful experiences have been identified as an important risk factor for cognitive impairment. Therefore, it is important to study the molecular mechanisms underpinning the effects of chronic stress on cognition and its relationship with ageing in order to unveil what challenges we might have to cope with as a society in the not-so-far future.

In parallel with ageing, stress and neurodegenerative diseases, such as Alzheimer's disease (AD), there is impaired glucocorticoid (GC) signalling. Disturbances in the GC-mediated stress response and individual's adaptive abilities appear to increase the vulnerability of elderly to age-related pathologies. In consequence, the present doctoral dissertation has been focused on the study of the mechanisms involved in age-related neurodegeneration modified by GC excess attenuation through the inhibition of the enzyme 11β-HSD1 in an animal model of accelerated ageing, as well as, their response to

chronic mild stress exposure. Last but not least, the present doctoral thesis has been devoted to evaluate the GC-mediated stress response to chronic moderate stressful situations and to metabolic stress underlying neurodegeneration and the potential role of 11β-HSD1 inhibition to restore those detrimental effects induced by stress.

In summary, results obtained pointed out a protective role of the 11β-HSD1 inhibitor tested as improved cognitive and behavioural abilities of aged mice, as well as restored the deleterious effects induced by stressful conditions applied. Additionally, some of the molecular pathways related to ageing and neurodegeneration – particularly AD neurodegeneration – were altered as a consequence of stress, but most of them were re-established after 11β-HSD1 inhibition, such as proteostasis, oxidative stress, neuroinflammation and epigenetics, among others. Overall, GC excess attenuation may become a potential therapeutic strategy for age-related cognitive decline.

RESUM

El món s'està enfrontant una situació sense precedents: aviat el nombre de persones majors de 60 anys superarà el nombre d'infants i, el nombre de persones d'edat avançada extrema és més elevat que mai. Això es deu als avenços mèdics que han incrementat l'esperança de vida i amb ella, el nombre de persones envellides. Les últimes dades publicades per la OMS preveuen que al 2050 la població mundial amb 60 anys o més gairebé es doblarà passant del 12% actual al 22%, estimant arribar a un total de 2 bilions, en comparació dels 900 milions de persones descrites el 2015. Una vida més llarga comporta oportunitats, encara que existeixen poques evidències que suggereixin que les persones envellides actualment visquin els últim dies amb més salut que els seus avantpassats. Si bé és cert que els índex de discapacitat severa han disminuït durant els darrers 30 anys, no hi ha hagut canvis significatius en la discapacitat lleu o moderada durant el mateix període. Si en aquests anys afegits domina el deteriorament de les capacitats físiques i mentals, les implicacions per les persones grans i per la societat seran més negatives. Per tant, en els últims anys la recerca s'ha centrat en la biologia de l'envelliment amb el propòsit d'aconseguir comprendre millor els mecanismes que el determinen per tal de prevenir l'aparició i la progressió de les condicions relacionades amb l'edat.

A més, a la societat moderna cada vegada és més comú un estil de vida estressant, que ha incrementat juntament amb l'estrès metabòlic, causat, en part, pel consum de dieta rica en grassa. Diferents evidències donen suport al fet que els factors ambientals són essencials per determinar el desenvolupament de determinades malalties, comprometent així l'envelliment saludable. A mesura que s'envelleix, la capacitat per lluitar contra estímuls nocius disminueix i l'organisme esdevé més vulnerable a infeccions i malalties. D'acord amb això, s'han identificat les experiències estressants com un factor de risc important pel deteriorament cognitiu. Per tant, és important l'estudi dels mecanismes moleculars que fonamenten els efectes de l'estrès crònic a nivell cognitiu i la seva relació amb l'envelliment, amb l'objectiu de revelar quins reptes podríem afrontar com a societat en un futur no tant llunyà.

En paral·lel amb l'envelliment, l'estrès i les malalties neurodegeneratives, com ara la malaltia d'Alzheimer, es situa el deteriorament de la senyalització dels glucocorticoides (GCs). Les alteracions en la resposta a l'estrès pròpia dels GCs i per tant, en les capacitats adaptatives dels individus semblen augmentar la vulnerabilitat de la gent gran a patologies

relacionades amb l'edat. En conseqüència, la present tesi doctoral s'ha centrat en l'estudi dels mecanismes implicats en la neurodegeneració relacionada amb l'edat modificats per l'atenuació de l'excés de GCs mitjançant la inhibició de l'enzim 11β-HSD1 en un model animal d'envelliment accelerat, així com la seva resposta a l'exposició a estrès crònic lleu. Per últim, però no per això menys important, la present tesi doctoral s'ha dedicat a avaluar la resposta a l'estrès regulada pels GCs, tant per l'exposició a estímuls estressants crònics com per estrès metabòlic, que participen en el desenvolupament de la neurodegeneració i el potencial paper de la inhibició de l'enzim 11β-HSD1 per restaurar els efectes perjudicials induïts per l'estrès.

En resum, els resultats obtinguts assenyalen un paper protector de l'inhibidor 11β-HSD1 estudiat, ja que millora les habilitats cognitives i conductuals en ratolins envellits, com també evita els efectes nocius induïts per les condicions estressants utilitzades. Addicionalment, algunes de les vies moleculars relacionades amb l'envelliment i la neurodegeneració – en particular la neurodegeneració característica de la malaltia d'Alzheimer – es van alterar a conseqüència de l'estrès, però la majoria d'elles es van tornar a re-establir després de la inhibició de l'enzim 11β-HSD1, com ara la proteostàsia, l'estrès oxidatiu, la neuroinflamació i els canvis epigenètics, entre d'altres. En general, l'atenuació de l'excés de GCs és una estratègia terapèutica potencial per al tractament del deteriorament cognitiu relacionat amb l'edat.

RESUMEN

El mundo está enfrentándose a una situación sin precedentes: pronto el número de personas mayores de 60 años superará el número de niños y, el número de personas de edad avanzada extrema es el más elevado jamás registrado. Eso se debe a que los avances médicos han incrementado la esperanza de vida y con ella, el número de personas envejecidas. Los últimos datos publicados por la OMS prevén que al 2050 la población mundial con 60 años o más prácticamente se doblará pasando del 12% actual al 22%, estimándose alcanzar un total de 2 billones, en comparación con los 900 millones de personas contabilizadas en 2015. Una vida más larga conlleva oportunidades, aunque existen pocas evidencias que, actualmente, las personas envejecidas vivan los últimos días con una salud mejor de la que lo hicieron sus antepasados. Si bien es cierto que los índices de discapacidad severa han disminuido durante los últimos 30 años, no ha habido cambios significativos en la discapacidad leve o moderada durante el mismo período. Si en estos años añadidos predomina el declive de las capacidades físicas y mentales, las implicaciones para les personas mayores y para la sociedad serán más negativas. Por tanto, en los últimos años la investigación se ha centrado en la biología del envejecimiento con el propósito de conseguir comprender mejor los mecanismos que lo determinan con la finalidad de prevenir la aparición y la progresión de las condiciones relacionadas con la edad.

Además, en la sociedad moderna cada vez es más común un estilo de vida estresante, que se ha incrementado juntamente con el estrés metabólico, causado en parte, por el consumo de una dieta rica en grasas. Diferentes evidencias indican que los factores ambientales son esenciales para determinar el desarrollo de determinadas enfermedades, comprometiendo así el envejecimiento saludable. A medida que se envejece, la capacidad para luchar contra estímulos nocivos disminuye y el organismo se hace más vulnerable a infecciones y enfermedades. De hecho, se han identificado las experiencias estresantes como un factor de riesgo importante para el deterioro cognitivo. Por lo tanto, es importante el estudio de los mecanismos moleculares que fundamentan los efectos del estrés crónico a nivel cognitivo y su relación con el envejecimiento, con el fin de revelar qué retos podríamos afrontar como sociedad en un futuro no tan lejano.

En paralelo al envejecimiento, el estrés y las enfermedades neurodegeneratives, como la enfermedad de Alzheimer, se encuentra el deterioro en la señalización de los

glucocorticoides (GCs). Los cambios en la respuesta al estrés propia de los GCs y por tanto, en las capacidades adaptativas de los individuos parecen aumentar la vulnerabilidad de las personas mayores a patologías relacionadas con la edad. En consecuencia, la presente tesis doctoral se ha centrado en el estudio de los mecanismos implicados en la neurodegeneración relacionada con la edad, modificados por la atenuación del exceso de GCs mediante la inhibición de la enzima 11β-HSD1 en un modelo animal de envejecimiento acelerado, así como su respuesta a la exposición a estrés crónico leve. Por último, pero no por ello menos importante, la presente tesis doctoral se ha centrado a evaluar la respuesta al estrés regulada por los GCs, tanto por la exposición a estímulos estresantes crónicos como por estrés metabólico, que participan en el desarrollo de la neurodegeneración y el potencial papel de la inhibición de la enzima 11β-HSD1 para restaurar los efectos perjudiciales inducidos por el estrés.

En resumen, los resultados obtenidos señalan el papel protector del inhibidor 11β-HSD1 estudiado, ya que mejora las habilidades cognitivas y conductuales en ratones envejecidos, como también previene los efectos nocivos inducidos por las condiciones estresantes utilizadas. Además, algunas de las vías moleculares relacionadas con el envejecimiento y la neurodegeneración - particularmente la neurodegeneración ligada a la enfermedad de Alzheimer - se alteraron a consecuencia del estrés, pero la mayoría de ellas se volvieron a restablecer tras la inhibición de la enzima 11β-HSD1, como la proteostasis, el estrés oxidativo, la neuroinflamación y los cambios epigenéticos, entre otros. En general, la atenuación del exceso de GCs es una estrategia terapéutica potencial para el tratamiento del deterioro cognitivo relacionado con la edad.

THESIS STRUCTURE

The present doctoral dissertation is presented in several sections as follows: firstly, a general **Introduction** places the reader in the context of the thesis, providing a comprehensive overview of the topic in which it is framed, in this case, stress, ageing and neurodegeneration, and more specifically, the relationship between attenuating glucocorticoids excess and the improvement of age-related cognitive decline. Afterwards, the section **Objectives** collects the principal issues that were aimed to be addressed at the beginning of each investigation. Following, **Methods and Results** section is divided into four chapters defining the beneficial effects resulting from treatment with RL-118, a drug that reduces glucocorticoid activity, both on cognition and molecular pathways in aged mice. In addition, it is determined that the drug is able to improve cognition and related molecular mechanisms, even in a situation of metabolic stress and chronic moderate stress in adult mice, which mimic situations similar to ageing. Thereafter, a general **Discussion** compares and contextualizes the results achieved with the evidence already described in the literature, always trying to clarify the likely existing discrepancies. Finally, the thesis ends with the **Conclusions** derived from the whole study and the **Bibliography** comprises all the scientific literature consulted and cites during the thesis development, which, in addition, helped to further discuss the results.

Four scientific papers are the result of the present dissertation, three of them already published, and the another pending to submit:

Chapter 1

Puigoriol-Illamola, D., Griñán-Ferré, C., Vasilopoulou, F., Leiva, R., Vázquez, S., Pallàs, M. (2018). 11β-HSD1 inhibition by RL-118 promotes autophagy and correlates with reduced oxidative stress and inflammation, enhancing cognitive performance in SAMP8 mouse model. *Molecular Neurobiology, 55*:8904-8915. *doi:10.1007/s12035-018-1026-8.*

Chapter 2

Puigoriol-Illamola, D., Martínez-Damas, M., Griñán-Ferré, C., Pallàs, M. (2020). Chronic Mild Stress Modified Epigenetic Mechanisms Leading to Accelerated Senescence and Impaired Cognitive Performance in Mice. *International Journal of Molecular Sciences, 21*(3):1154. *doi:10.3390/ijms21031154.*

Chapter 3

Puigoriol-Illamola, D., Companys-Alemany, J., Homer, N., Leiva, R., Vázquez, S., Mole, D., Griñán-Ferré, C., Pallàs, M. Chronic Mild Stress Modified Epigenetic Mechanisms Leading to Accelerated Senescence and Impaired Cognitive Performance in Mice *[pending to submit]*.

Chapter 4

Puigoriol-Illamola, D., Leiva, R., Vázquez-Carrera, M., Vázquez, S., Griñán-Ferré, C., Pallàs, M. (2020). 11β-HSD1 Inhibition Rescues SAMP8 Cognitive Impairment Induced by Metabolic Stress. *Molecular Neurobiology, 57*(1):551-565. *doi:10.1007/s12035-019-01708-4*. Epub 2019 Aug 09.

Importantly, the third arises from the author's short-term stay at the Queen's Medical Research Institute – The University of Edinburgh (Edinburgh, United Kingdom) during the last year of the thesis preparation period.

Additionally, seven extra scientific papers have been published during the time period of the thesis as a result of the author's participation in other projects carried out by members of the same research group. Nevertheless, the content of these publications has not been included in the core thesis since they are beyond its scope and/or the author contributed as a collaborator instead of leading the investigation.

Palomera-Ávalos, V., Griñán-Ferré, C., **Puigoriol-Illamola, D.**, Camins, A., Sanfeliu, C., Canudas, A.M., Pallàs, M. (2016). Resveratrol protects SAMP8 brain under metabolic stress: focus on mitochondrial function and Wnt pathway. *Molecular Neurobiology, 54*:1661-1676. *doi:10.10007/s12035-016-9770-0.*

Griñán-Ferré, C., Sarroca, S., Ivanova, A., **Puigoriol-Illamola, D.**, Aguado, F., Camins, A., Sanfeliu, C., Pallàs, M. (2016). Epigenetic mechanisms underlying cognitive impairment and Alzheimer's disease hallmarks in 5XFAD mice. *Aging, 8*:664-684. *doi:10.18632/aging.100906.*

Griñán-Ferré, C., Palomera-Ávalos, V., **Puigoriol-Illamola, D.**, Camins, A., Porquet, D., Plá, V., Aguado, F., Pallàs, M. (2016). Behaviour and cognitive changes correlated with hippocampal neuroinflammaging and neuronal markers in female SAMP8, a model of accelerated senescence. *Experimental Gerontology, 80*:57-69. *doi:10.1016/j.exger.2016.03.014.*

Griñán-Ferré, C., **Puigoriol-Illamola, D.**, Pérez-Cáceres, D., Palomera-Ávalos, V., Rodrigo, M.T., Pallàs, M. (2016). Environmental enrichment modifies epigenetic mechanisms in SAMP8 reducing oxidative stress and inflammaging and achieving neuroprotection. *Frontiers Aging Neuroscience, 8*:241. *doi:10.3389/fnagi.2016.00241.*

Griñán-Ferré, C., Corpas, R., **Puigoriol-Illamola, D.,** Palomera-Ávalos, V., Sanfeliu, C., Pallàs, M. (2018). Understanding epigenetics in the neurodegeneration of Alzheimer's disease: SAMP8 mouse model. *Journal Alzheimers Disease, 62*:943-963. *doi:10.3233/JAD-170664.*

Griñán-Ferré, C., Izquierdo, V., Otero, E., **Puigoriol-Illamola, D.**, Corpas, R., Sanfeliu, C., Ortuño-Sahagún, D., Pallàs, M. (2018). Environmental enrichment improves cognitive deficits, AD Hallmarks and epigenetic alterations presented in 5xFAD mouse model. *Frontiers Cell Neuroscience, 12*:224. *doi:10.3389/fncel.2018.00224.*

Pérez-Areales, F.J., Garrido, M., Aso, E., Bartolini, M., De Simone, A., Espargaro, A., Ginex, T., Sabaté, R., Pérez, B., Andrisano, V., **Puigoriol-Illamola, D.**, Pallàs , M., Luque, F.J., Ferrer, I., Ciruela, F., Messeguer, A., Muñoz-Torrero, D. (2020). Centrally Active Multitarget Anti-Alzheimer Agents Derived from the Antioxidant Lead CR-6. Journal of Medicinal Chemistry *[submitted]*.

The scientific outputs of the thesis and the extra contributions were shared with the scientific community in different national and international congresses as poster presentations, as well as oral presentation in a research meeting:

Poster presentations

Griñán-Ferré, C., Palomera-Ávalos, V., **Puigoriol- Illamola, D.**, Alvarez-López, M.J., Cosín-Tomás, M., Camins, A., Kaliman, P., Pallàs, M. Could microRNA modulation overcome senescence process in SAMP8 strain? *V Chromatin and epigenetics annual meeting SCB*. 10 March 2015. Barcelona (Spain).

Griñán-Ferré, C., Palomera-Ávalos, V., **Puigoriol-Illamola, D.**, Camins, A., Pallàs, M. Time course screening of miRNAs in female SAMP8: implications in senescence process. E*MBO Conference of Chromatin and Epigenetics.* 6-10 May 2015. Heidelberg (Germany).

Palomera-Ávalos, V., Griñán-Ferré, C., Camins, A., Amaro-Umbert, N., **Puigoriol-Illamola, D.**, Sanfeliu, C., Canudas, A.M., Pallàs, M. Reframing the role of resveratrol in neurodegeneration: oxidative stress, mitocondrial function and Wnt-pathway modulation in the brain of metabolically stressed SAMP8 mice. *Society for Neuroscience 45thanual meeting.* 17-21 October 2015. Chicago (USA).

Griñán-Ferré, C., Palomera-Ávalos, V., **Puigoriol-Illamola, D.**, Camins, A., Ortuño-Sahagún, D., Pallàs, M. Epigenetic changes mediated by miRNAs as a cause of rapidly aging and cognitive impairments in female SAMP8 mouse model. *Society for Neuroscience 45th annual meeting.* 17-21 October 2015. Chicago (USA).

Pallàs, M., **Puigoriol-Illamola, D.**, Palomera-Ávalos, V., Camins, A., Griñán-Ferré, C. Cognition and behaviour impairment: is oxidative stress the earliest change commanding senescence? Lessons from senescence accelerated P8 mice. *Society for Neuroscience 45th anual meeting.*

17-21 October 2015. Chicago (USA).

Griñán-Ferré, C., **Puigoriol-Illamola, D.**, Pérez-Cáceres, D., Palomera-Ávalos, V., Rodrigo, M.T., Pallàs, M. Environmental enrichment modifies epigenetic mechanisms in SAMP8 reducing oxidative stress and inflammaging and achieving neuroprotection. *VI Chromatin and Epigenetics annual meeting SCB.* 8 April 2016. Barcelona (Spain). As well as in the *10th FENS Forum of Neuroscience.* 2-6 July 2016. Copenhagen (Denmark).

Puigoriol-Illamola, D., Griñán-Ferré, C., Vasilopoulou, F., Leiva, R., Webster, S.P., Vázquez, S., Pallàs M. Neuroprotective effect of 11β-HSD1 inhibition through autophagy activation in SAMP8 mouse model. *X Neurobiology Symposium of the SCB*. 6-7 October 2016. Barcelona (Spain). As well as in the *I PhD Workshop of the Institute of Neuroscience of the UB*. 15 December 2016. Barcelona (Spain).

Puigoriol-Illamola, D., Griñán-Ferré, C., Companys-Alemany, J., Leiva, R., Otero, E., Vázquez, S., Pallàs, M. 11β-HSD1 inhibition improves cognitive decline modifying epigenetic marks in SAMP8 mice under chronic mild stress exposure. *17th meeting of the Spanish Society for Neuroscience*. 27-30 September 2017. Alicante (Spain). As well as in the *II PhD Workshop of the Institute of Neuroscience of the UB*. 30 November – 1 December 2017. Barcelona (Spain).

Puigoriol-Illamola, D., Griñán-Ferré, C., Companys-Alemany, J., Leiva, R., Vázquez, S., Vázquez, M., Pallàs, M. 11β-HSD1 inhibition improved cognitive decline associated with highfat diet in female SAMP8. *11th FENS Forum of Neuroscience*. 7-11 July 2018. Berlin (Germany). As well as in the *III PhD Workshop of the Institute of Neuroscience of the UB.* 16-17 October 2018. Barcelona (Spain).

Puigoriol-Illamola, D., Griñán-Ferré, C., Companys-Alemany, J., Leiva, R., Vázquez, S., Vázquez, M., Pallàs, M. Beneficial effects of 11β-HSD1 inhibition on cognitive performance in metabolic stressed SAMP8 female. *XI Neurobiology Symposium of the SCB.* 12-13 November 2018. Barcelona (Spain).

Puigoriol-Illamola, D., Griñán-Ferré, C., Pallàs, M. Epigenetic changes after unpredictable chronic mild stress in female SAMR1 and SAMP8: Effects on behaviour. *49th annual meeting of the Society for Neuroscience*. 19-23 October 2019. Chicago (USA). As well as in the *IV PhD Workshop of the Institute of Neurosciences of the UB*. 29 November 2019. Barcelona (Spain).

Oral communication

Puigoriol-Illamola, D. Beneficial effects of 11β-HSD1 inhibition on cognitive performance in a mouse model of Alzheimer's disease. Doctoral Journey in Pharmacology of the Universitat Autònoma de Barcelona (UAB) and Universitat de Barcelona (UB). Organized by the Academy of Medical and Health Sciences of Catalonia and the Balearic Islands, Catalan Society of Pharmacology. 19 June 2018. Barcelona (Spain).

INDEX

ABBREVIATIONS

5-mC	5-methylcytosine
5-hmC	5-hydroxymethylcytosine
11β-HSD1	11beta-hydroxysteroid dehydrogenase type 1
11β-HSD2	11beta-hydroxysteroid dehydrogenase type 2
Aβ	Amyloid beta
ACTH	Adrenocorticotropic hormone
AD	Alzheimer's disease
ADAM10	A disintegrin and metalloproteinase domain-containing protein 10
ADE	Aβ-degrading enzymes
Aldh2	Aldehyde dehydrogenase 2
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AOX1	Aldehyde oxidase 1
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
ARE	Antioxidant response element
ATF4	Activating transcription factor 4
ATP	Adenosine triphosphate
AUC	Area under the curve
AVP	Arginine vasopressin
BACE1	Beta-site APP cleaving enzyme 1
BBB	Blood-brain barrier
BCL2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
BIP/GRP78	Binding immunoglobulin protein
CA1	*Cornu Ammonis*
CAT	Catalase
CCL3	C-C-motif ligand 3
CDK5	Cell division protein kinase 5
CHOP	C/EBP homologous protein
CMS	Chronic mild stress
CNS	Central nervous system
CREB	cAMP response element-binding

CRH	Corticotropin-releasing hormone
CTF	Carboxy-terminal fragment
CXCL2	Chemokine (C-X-C motif) ligand 2
DI	Discrimination index
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
eIF2-α	Eukaryotic initiation factor 2 alpha
EPM	Elevated plus maze
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
fAD	Familial Alzheimer's disease
FGF-21	Fibroblast growth factor 21
FOXO	Forkhead box O
GAS	General adaptation syndrome
GC	Glucocorticoid
GFAP	Glial fibrillar acidic protein
GPX	Glutathione peroxidase
GR	Glucocorticoid receptor
GSH	Glutathione
GSK3	Glycogen-synthase kinase 3
H_2O_2	Hydrogen peroxide
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDM	Histone demethylase
HEK	Human embryonic kidney
HFD	High-fat diet
HLM	Human liver microsomes
Hmox1	Heme oxygenase 1
HMT	Histone methyltransferase
HPA	Hypothalamic-pituitary-adrenal
IBA1	Ionized calcium-binding adapter molecule 1
IDE	Insulin-degrading enzyme
IGF-1	Insulin-like growth factor 1
IIS	Insulin and insulin-like growth factor 1 signalling
IL	Interleukin

iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneally
JNK	c-Jun N-terminal protein kinase
LC3	Microtubule-associated protein light chain 3
LDL	Low-density lipoprotein
Leu	Leucine
LKB1	Liver kinase B1
Lys	Lysine
MAPK	Mitogen-activated protein kinase
miRNA	MicroRNA
MnSOD	Manganese SOD
MPO	Myeloperoxidase
MR	Mineralocorticoid receptor
MS	Mass spectrometry
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
MWM	Morris water maze
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light chain enhancer of activated B cells
NFT	Neurofibrillary tangles
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NORT	Novel object recognition test
NOS	Nitric oxide synthase
NRF2	Nuclear factor erythroid-derived 2
NSAID	Non-steroid anti-inflammatory drug
O_2	Oxygen
O_2^-	Superoxide
OFT	Open field test
OH^-	Hydroxyl ion
OLT	Object location test
ONO_2^-	Nitric peroxide
OS	Oxidative stress
PERK	Serine/threonine kinase RNA-like ER kinase

PGC1-α	Peroxisome proliferator-activated receptor gamma coactivator alpha
PSD95	Postsynaptic density protein 95
PSEN	Presenilin
PTM	Post-translational modifications
PVN	Paraventricular nucleus
RNA	Ribonucleic acid
RONS	Reactive oxygen and nitrogen species
ROS	Reactive oxygen species
sAD	Sporadic Alzheimer's disease
SAM	Senescence-accelerated mice
SAMP	Senescence-accelerated mice prone
SAMR	Senescence-accelerated mice resistant
sAPPα	Soluble APP alpha
Ser	Serine
SIRT	Sirtuins
SOD	Superoxide dismutase
SNAP	Synaptosomal-nerve associated protein 25
SNP	Single-nucleotide polymorphism
SNS	Sympathetic nervous system
T2DM	Type 2 diabetes mellitus
TAPS	Toxicity-affinity-permeability-selectivity
TCT	Three-chamber test
TET	Ten-eleven translocation
TGF-β	Transforming growth factor beta
Thr	Threonine
TNF-α	Tumour necrosis factor alpha
TREM2	Triggering receptor expressed on myeloid cells 2
UPR	Unfolded protein response
WHO	World Health Organization

1. INTRODUCTION

1.1 STRESS

Considering the changes in our society in the last few years, the life rhythm has changed becoming more and more intense and demanding. Apart from all the benefits and advantages that technological advances have supposed to us, they have contributed to increasing our need for immediacy and, in consequence, our feeling of stress. In fact, the World Health Organization (WHO, 2010) has named stress as the "Health Epidemic of the 21st Century", affecting more than 40 million individuals across the European Union. Thus in such scenario, it is essential to study stress and the mechanisms underlying stress response. So, what is stress?

Stress is defined as a physical or emotional factor that causes great bodily or mental tension, anxiety, discomfort and difficulty in adjustment. The stress source (i.e. the stressor) can be psychogenic (psychologically-based disturbance) and systemic (including external – from the environment or social situations – and internal – illness or inflammation) (Fink, 2017). However, an issue to consider in terms of stressors is the subjectivity, as usually a stressful event is not perceived in the same way among different individuals. Therefore, stress and stress response consist of a complex interplay between physiological, psychological and behavioural processes that varies across situations (Thiel & Dretsch, 2011). Nevertheless, other authors introduce a three-component definition, accounting that stress requires heightened excitability, aversive experience perception and lack of control. The last component is the variable that ultimately determines the magnitude of the stress experience and the susceptibility of the individual to develop stress-induced behavioural and physiological sequelae (Fink, 2017).

To appreciate the function of stress in a given situation, it is important to consider the stressor, the magnitude (high or mild/moderate stress) and the duration of the stressful events (acute or chronic stress). Chronic stress is characterized by prolonged exposure to a given stress condition, broadly defined as stresses lasting from four to six hours and it is supposed to cause harmful effects (Eisenmann et al., 2016; Reineke & Neilson, 2019). Causes of chronic stress can differ from a wide range of issues, such as job pressure, poor working conditions, financial difficulties, health, unfulfilling or conflicting intimate relationships, nutrition, media overload or sleep deprivation, among others (Hammen et al., 2009).

1.1.1 History

Despite the high presence of stress in the modern society, it has always been of interest to different civilizations. Initially, the firsts to be aware of stress were Aristotle, Hippocrates and other Ancients (Brehm, 2014). However, the cellular mechanisms to fight against stress were not described until the second half of the 19th century by Claude Bernard. He reported that cells were surrounded by an internal medium that minimizes changes around biologically determined set points, thereby providing a steady state. Fifty years later, Walter Bradford Cannon helped to designate homeostasis – from the Greek *homoios* (similar) and *stasis* (position) – to describe this process, by postulating that stress disturbs equilibrium and that the autonomic response to a threat restores one's internal processes to steady-state levels necessary for health and survival in the face of challenge (Cannon, 1932). This response was later recognized as one of the whole multiple changes in the body that constitute the universal stress response among vertebrates and other organisms, postulated by Hans Selye (Fink et al., 2009).

Selye, who is considered the father of stress, expanded upon Cannons' work by investigating the other primary systems involved in stress: the endocrine system, involving glucocorticoids (GCs). He observed that patients with a variety of illnesses had many of the same "non-specific" symptoms that were a common response to stressful stimuli. Hence he asserted that prolonged exposure to stressful circumstances caused gastro-duodenal ulcers, enlarged adrenal glands and high blood pressure, as well as major effects on the immune system and joined all these signs into the General Adaptation Syndrome (GAS), which represents a reliable pattern of physiological reactions that correspond to the body's attempt to mediate resistance to a threat (Fink, 2017; Selye, 1956). GAS consists of three stages: alarm (i.e., physiological activation of autonomous and endocrine systems), resistance (i.e., the period following the initial reaction whereby the body mediates on-going stress and attempts to return to steady-state levels) and exhaustion (i.e., when a prolonged stress response overexerts the body's defence systems, thus leading to finally death).

Selye also proposed that not all states of stress or threatened homeostasis are deleterious, terming healthy stress "eustress" and "distress" as pathogenic form. In an attempt to address what happens when the body is continually trying to restabilize, a novel concept called allostasis was introduced to the stress field (McEwen, 1998). It refers to the

ability of the body to achieve and maintain stability through change and includes active physiological and behavioural responses to a specific threat with the aim of re-establishing homeostasis.

To this end, allostasis is useful for illustrating the importance of adaptation to promote and maintain survival mechanisms. However, the cost that these responses exert over time is named allostatic load, which can result from either too much stress output or inefficient stress response (McEwen, 2000). The chronic impact of stress is named allostatic overload, and could manifest as metabolic syndrome, anxiety and neuropsychological disorders, among others (Weger & Sandi, 2018).

1.1.2 Stress response

Although stress experiences are often perceived in a negative light, they create actually an instrumental and highly adaptive response. The experience of too much stress over time can have adverse consequences on health and behaviour, but never experiencing any stress would result in inactivity, boredom and an inability to adequately respond to internal and external demands (Stults-Kolehmainen & Sinha, 2014).

The physiological response to stress engages a concerted action of different brain systems, along with the activation of the sympathetic nervous (SN) and endocrine systems in order to coordinate all organic systems to help the organism overcoming challenging situations, by stimulating metabolic and neurobiological changes. These changes, typically, provide the organism with additional energy, simultaneously inhibit body functions that are nonessential for immediate survival and coordinate brain responses to organize behavioural adaptation (Eisenmann et al., 2016). The stress response is essential for living, but its cessation is pivotal to prevent damage.

The SNS triggers the release of mediator molecules named neurotransmitters, while the endocrine system regulates body functions through hormones. A hormone – from the Greek *hormé* (setting in motion) –is any member of a class of signalling molecules produced by glands that are released to interstitial liquid and transported by the circulatory system to target distant organs in order to regulate physiology and behaviour (Shuster, 2012). Both neurotransmitters and hormones exert their effects by binding to their receptors or target enzymes, although there are some differences in the way they act (Table 1).

Table 1. *Comparison of the control exerted by nervous and endocrine systems.*

Characteristics	Nervous system	Endocrine system
Mediating molecules	Neurotransmitters released locally in response to nervous impulses	Hormones distributed to the whole body through bloodstream
Targets of mediator	Close from the release place; binding to a postsynaptic membrane receptor	Far from the release place (usually); binding to the inner or outer cellular receptor
Types of cellular targets	Muscular cells, glandular cells and neurons	Whole body
Time to start the action	Milliseconds (ms)	From seconds (s) to hours (h) or days
Duration of the action	Short (ms)	Long (from s to days)

Activation of the SNS results in the local release of catecholamines (i.e., noradrenaline) onto target organs, and stimulates additional catecholamine (i.e., both adrenaline and noradrenaline) release from the adrenal medulla (Thiel & Dretsch, 2011). This constitutes the first response to acute stress, which is generally known as the fight-or-flight response. The collective result of catecholamine release is a cascade of physiological effects including increased respiration and heart rate, dilation of skeletal muscle blood vessels, glycogen to glucose conversion and vasoconstriction of digestive and reproductive organ blood vessels (Figure 1) (McCarty, 2000).

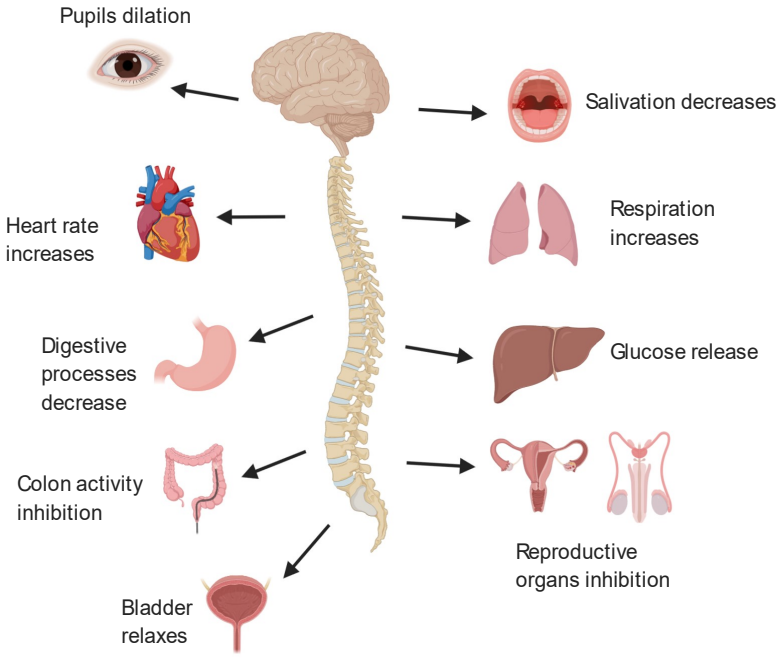

Figure 1. *Representation of the SNS stress response with several of its targets.*

On the other hand, the hallmark of neuroendocrine system response to stress involves activation of the hypothalamic – pituitary – adrenal (HPA) axis, which administer

hormone secretion. As far as the synthesis is concerned, three types of mechanisms regulate hormone secretion: other hormones, chemical changes in the bloodstream and nervous system signalling. HPA axis encompasses a complex set of direct influences and feedback interactions among the hypothalamus, the hypophysis and the adrenal glands.

Firstly, painful experiences, stressful events and emotional situations cause changes in hypothalamic activity and activate the secretion of different hormones in the paraventricular nucleus (PVN), such as corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) and release them into the portal blood system. The hypothalamus is a small brain region under thalamus that comprises the connection between nervous and endocrine systems. It receives afferent signals from the limbic system, cerebral cortex, thalamus and reticular activator system, as well as sensorial signals from internal organs and the retina. Besides, this cerebral unit regulates body temperature, food and thirst sensations, sexual conduct, fear, anger and the autonomous nervous system. Overall the hypothalamus is both, an essential nervous system regulatory centre as well as a crucial endocrine gland.

Downstream the portal blood system, the pituitary gland or hypophysis – from the Greek *hypophysis* (growing from below) – responds to hypothalamic releasing or inhibitory hormones that stimulate or inhibit pituitary activity, respectively. CRH and AVP, although predominantly CRH, specifically target the synthesis and release of adrenocorticotropic hormone (ACTH) from pituitary corticotrophs cells located within the anterior pituitary gland to the circulating bloodstream. Importantly, AVP synergistically potentiates CRH-elicited ACTH secretion, but it is typically relegated to promote maintenance of basal ACTH production (Thiel & Dretsch, 2011). Indeed under chronic stress conditions, there is a marked shift in hypothalamic signal in favour of AVP as CRH receptors in the anterior pituitary gland become down-regulated (Scott & Dinan, 1998). The pituitary gland rests upon the hypophysial fossa of the sphenoid bone in the centre of the middle cranial fossa and surrounded by a small bone cavity, named sella turcica (Figure 2). It is connected to the hypothalamus by the pituitary stalk or infundibulum and has two anatomically and functionally separate lobes:

- The anterior lobe: also called the **adenohypophysis**. It constitutes about 75% of the total weight of the gland and it secretes hormones. The anterior lobe consists of two parts in the adult:
    - **Pars distalis**: representing the most substantial portion

- **Pars tuberalis**: forming a pod around the infundibulum.

- The posterior lobe: or **neurohypophysis**, which is also constituted by two parts:
  - **Pars nervosa**
  - **Infundibulum**
- Pars intermedia: is another pituitary region, atrophied during human fetal development, so that does not exist as a separate lobe in adulthood.

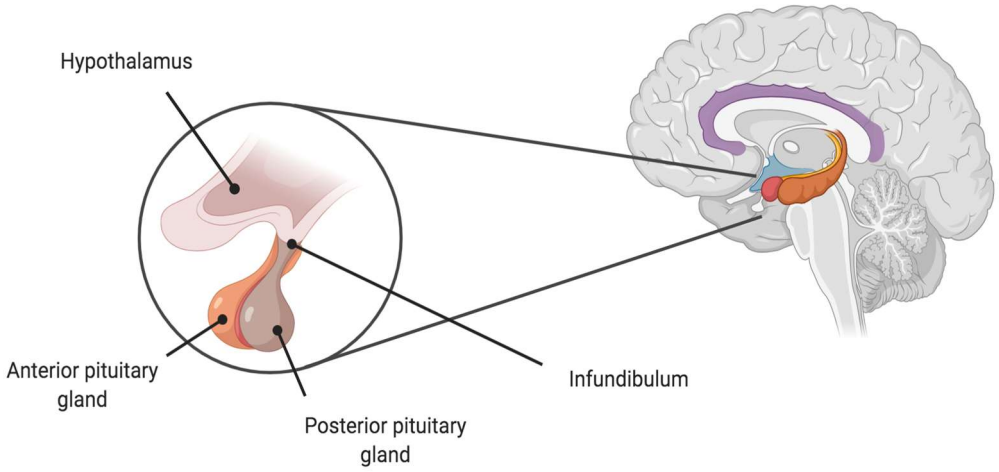

Figure 2. *Representation of hypothalamus and pituitary gland locations.*

ACTH travels to the adrenal glands, whereby it binds to cells located in the *zona fasciculata* of the adrenal cortex and leads to a cascade of intracellular enzymatic events that convert free cholesterol into GCs via a steroidogenic pathway (Thiel & Dretsch, 2011). Adrenal glands are located above kidneys and composed of the adrenal medulla as well as the adrenal cortex (consists of the 80-90% of the gland). The medulla synthesizes catecholamines, such as noradrenaline, adrenaline and small quantities of dopamine. On the other hand, the adrenal cortex is divided into three major anatomic zones: *zona glomerulosa*, which produces aldosterone; and the *zona fasciculata* and *reticularis*, which together produce corticosteroids and adrenal androgens, like cortisol, 11-deoxycortisol, aldosterone, corticosterone and 11-deoxycorticosterone (Holst et al., 2004).

GC secretion is under a typical negative feedback system control (Figure 3). Accurate regulation of the HPA axis is essential as GC metabolism alterations result in decreased GC secretion. In contrast, increased GC metabolism and clearance are offset by increased HPA drive and GC secretion is increased to maintain circulating concentrations (Gathercole et al., 2013).

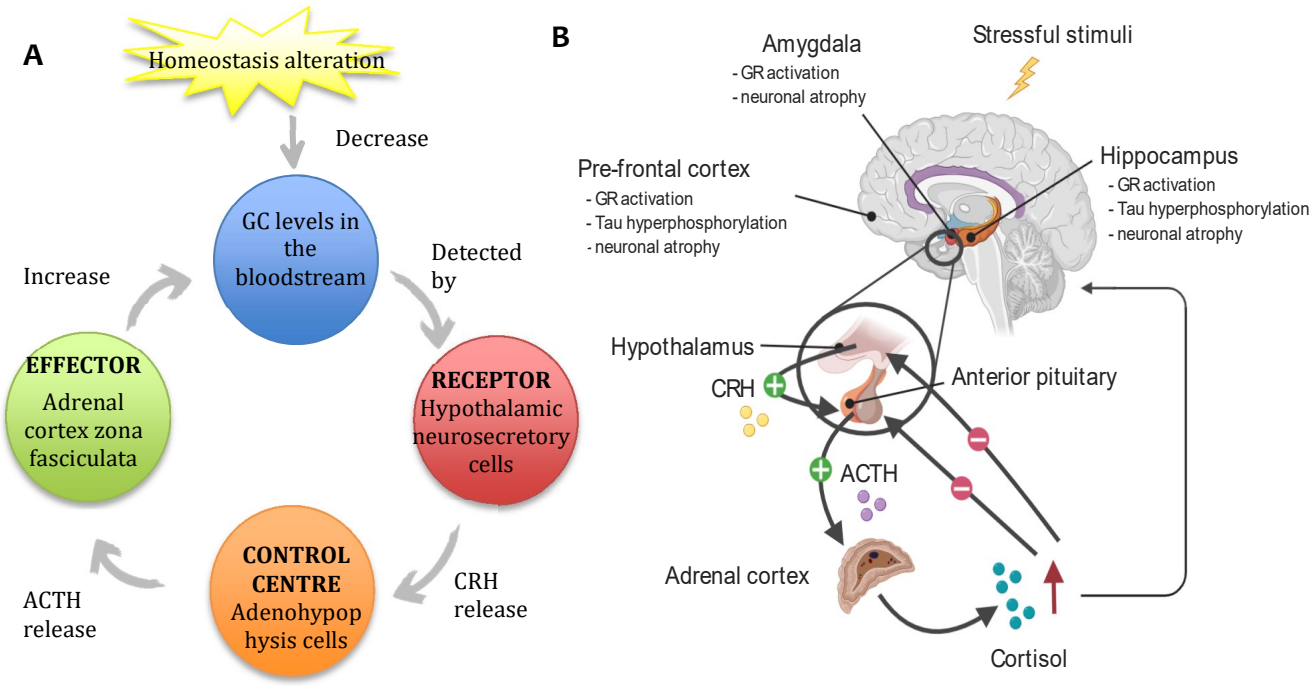

Figure 3. *Schematic representation of HPA axis in stress response (A) and its regulatory system (B). Adapted from Sotiropoulous et al., 2020.*

1.1.3 Glucocorticoids

The name "glucocorticoid" is composed of its role in the regulation of glucose homeostasis, synthesis in the adrenal cortex and its steroidal structure, so glucose + cortex + steroid. The main GCs are cortisol (hydrocortisone), which account for the 95% of GC activity (Holst et al., 2004), and cortisone that are equivalent to corticosterone and 11-dehydrocorticosterone in rodents.

Under normal conditions, GC secretion follows a robust circadian oscillation with a peak around the onset of the active period of a day, i.e. about 1 hour before arising (Son et al., 2018). This "basal" level of GC secretion is important in exerting tonic effects upon metabolic, immune and neuronal pathways (Chapman & Seckl, 2008). As mentioned, other hormonal signals from the adrenal medulla, cytokine stimulation and direct neuronal control via SNS innervation also mediate GC release, although ACTH is the predominant regulator.

Once synthesized, GCs diffuse away from the cell and are released into circulation. As they are highly lipophilic, GCs bind reversibly to corticosteroid-binding globulin, also named transcortin, and serum albumin where they remain inactive while transported throughout the body. Approximately 90% of circulating cortisol is bound and only 4% is free, so biologically active; similarly to cortisone. Circulating half-life of cortisol has been estimated

to vary between 70 and 120 minutes and it is cleared through several pathways (Giannini & Mohn, 2015).

GCs serve essential functions related to stress response. In one respect, GCs play a permissive role as stimulate gluconeogenesis (especially in those organs that require high-energy demand like central nervous system (CNS)), increase protein degradation and lipolysis for energy production. Also, aid the catabolic processes mediated by catecholamines, prime neural regions involved in sensory processing, attention and adaptive responding, as well as regulate immune and inflammatory response mediators accounting for a protective role via immunosuppressive and anti-inflammatory effects (Sapolsky et al., 2000; Thiel & Dretsch, 2011; Tortora & Derrickson, 2010). In addition, GCs affect critical brain regions such as the hippocampus and amygdala to regulate mood and activate learning and memory processes that promote adaptive behaviours in the future in response to a particular stressor (Chapman & Seckl, 2008; Korte et al., 2001). In conclusion, GCs are vital to facilitate stress response and reduce it once the response is no longer necessary, as well as preparing the organism for future threats.

Its activity occurs through two types of receptors located in the brain, primarily the hippocampus: mineralocorticoid and glucocorticoid receptors (MR and GR, respectively) (Reul & De Kloet, 1985). A differential affinity profile characterises each receptor for its endogenous ligands. Despite the terminology, MRs possess heightened and relatively equal affinity for both GCs and aldosterone. In contrast, GRs have a much lower affinity for GCs compared to MRs, but they are more selective for GCs over aldosterone. Accordingly, it has been demonstrated that corticosterone shows higher affinity for MR (K_d=0.1-0.5 nM) than GR (K_d=2-5 nM) (Mifsud & Reul, 2016). Therefore, it has been estimated that more than 80% of MRs are occupied with the endogenous GC under resting conditions, whereas GRs become substantially occupied during elevated GC levels periods, such as after stressful stimuli and at the circadian peak of GC secretion. These data suggests that MRs exert tonic actions on brain, while GRs mediate the negative feedback, and long-term cognitive changes evoked by GCs (Mifsud & Reul, 2016). GRs are located within the cell cytoplasm and are translocated into the nucleus upon binding with a GC that enters the cell via passive diffusion. Once in the nucleus, they function as transcription factors to regulate gene expression. However, GCs are able to produce faster actions whereby they rapidly hyperpolarize and inhibit neuron firing within regions such as the hippocampus and hypothalamus (Gathercole et al., 2013).

1.1.4 11β-hydroxysteroid dehydrogenase

Tissue GC action is determined not only by circulating cortisol levels and cell density of intracellular receptors, but also by enzymes that metabolize GCs and thus "gate" their access to receptors (MacLullich et al., 2012). This tissue-specific metabolism of GC is catalysed by two microsomal enzymes: 11β-hydroxysteroid dehydrogenases type 1 (11β-HSD1) and type 2 (11β-HSD2). They modulate the intracellular conversion of non-binding cortisone to physiologically active cortisol in humans and 11-dehydrocorticosterone to corticosterone in rodents (Figure 4) (Peng et al., 2016).

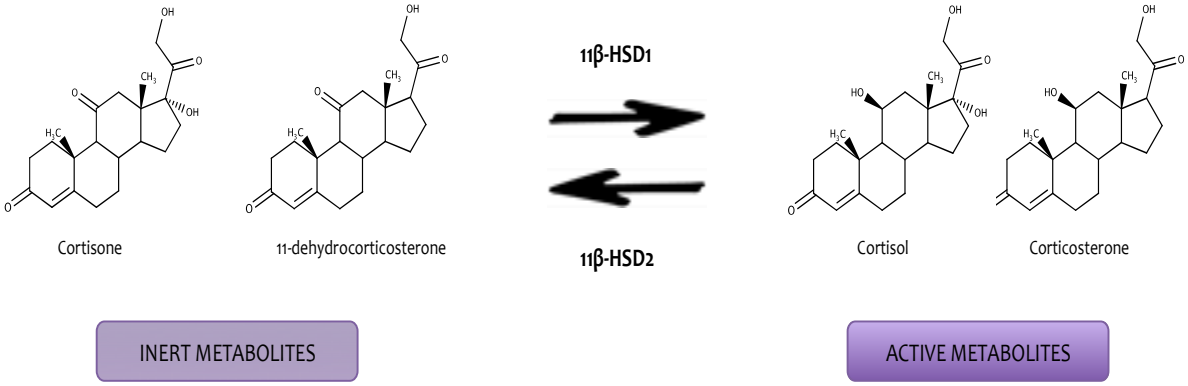

Figure 4. *Simplified scheme of 11β-HSD activities.*

11β-HSD1 is a nicotinamide adenine dinucleotide (NAD) phosphate (NADPH)-dependent enzyme and predominantly functioning as a reductase converting inert cortisone to active cortisol, therefore amplifying local GC action within the cells (MacLullich et al., 2012). Pre-receptor metabolism of GCs by 11β-HSD1 amplifies its activity. Given its involvement in regulating GC levels, many studies have addressed whether it is by GCs themselves, and effectively results showed that GCs increase *11β-HSD1* expression in many cell types but not all (Sun & Myatt, 2003). Nevertheless, the potential to further amplify intracellular GC levels provides a further mechanism to fine-tune cellular GC action in a tissue-specific, and indeed possibly, cell-specific manner (Chapman & Seckl, 2008). 11β-HSD1 is present in peripheral tissues, notably the liver, adipose tissue and vasculature, and in multiple regions of the brain like anterior pituitary, hypothalamic PVN and the hippocampus (Holmes et al., 2010; Peng et al., 2016). It is anchored in the endoplasmic reticulum (ER) membrane and there is a high sequence homology between species, particularly within the cofactor-binding region and the catalytic site (Gathercole et al., 2013). Designated HSD11B1, the human gene is located at chromosome 1 and is over 30 Kb in length and consists of 6 exons and 5 introns (Tannin et al., 1991). Referring to protein, rodent 11β-HSD1 was purified in 1980, while the human enzyme in 2002. It exists in three variants, although the first is the

predominant. It consists of 292 amino acids, with a molecular weight of 32401 Da and its quaternary structure is a homodimer (NCBI GenBank nucleotide, 2020).

In contrast, 11β-HSD2 is a NAD-dependent dehydrogenase that catalyses the conversion of cortisol into inactive metabolites in the kidney, thus reducing local GC action. It is distributed mainly in the kidney, colon, placenta and discrete areas of the brain involved in salt regulation (Peng et al., 2016; MacLullich et al., 2012). Notably in the kidney and colon, 11β-HSD2 activity protect MRs from inappropriate activation by cortisol and allow aldosterone to act as the ligand, whereas in the placenta protects the fetus from maternal GCs (Chapman & Seckl, 2008; Gathercole et al., 2013). The brain is nearly devoid of 11β-HSD2 (Thiel & Dretsch, 2011).

1.1.5 Stress effects on the development of several disorders

Different studies have provided evidence that the HPA axis can show abnormalities during ageing and disease (Bloss et al., 2011). Despite the fact that GCs play a pivotal role in orchestrating adaptive responses to stressful challenges to maintain health and wellbeing, aberrant GC secretion as a result of chronic stress damages the whole body, negatively affects cognition and increases the susceptibility to mental diseases, such as major depression, anxiety and posttraumatic stress disorder (Mifsud & Reul, 2016). In particular, prolonged exposure to heightened GCs levels has been associated with the deployment of immunosuppression; osteoporosis and osteonecrosis; increment in fat tissue and redistribution to the abdomen, shoulders and face; skin thinning with dermal atrophy leading to bruising, purpura and livid striae; the appearance of acne; hirsutism; irregular menstruation and reproductive failure (Sato et al., 2018; Sotiropoulous et al., 2008). Not only that but also stress precipitates physical health outcomes leading to muscle atrophy, proximal myopathy, diabetes, obesity, metabolic disturbances, hypertension and cardiovascular disease (Eisenmann et al., 2016; Harman & Martín, 2019; You et al., 2020). Altogether, signs and symptoms converged into Cushing's syndrome. In the last decade, the role of 11β-HSD1 enzyme in the causation of metabolic alterations of GCs overexposure has been extensively documented an all converge in that GC-induced insulin resistance was dependent on 11β-HSD1 (Peng et al.,2016; Qi et al., 2005). Accordingly, lower 11β-HSD1 levels in the brain correlate with higher body mass index (BMI), which is indicative of obesity (Bini et al., 2020).

Also, numerous mechanistic studies in animal models have determined that low birth weight was associated with high fasting cortisol levels, both in rodents and humans. Interestingly, birth weight is negatively correlated with high circulating levels of maternal cortisol during pregnancy, supporting that GC excess can by-pass the placental barrier and affect fetal development (Reynolds, 2013).

1.1.6 Stress effects on cognition and cognitive decline

Cognition is a broad concept involving a variety of processes that deal with information and manipulate representations in the brain to produce a suitable response. They range from perception and attention to various types of memory, language and executive control processes (Sandi, 2013).

Nowadays, there is great consensus in the literature that stress is a potent modulator of cognitive function, and more precisely, of learning and memory processes (Sandi & Pinelo-Nava, 2007). Although the cognitive effects of stress are frequently assumed to be detrimental, there are many instances in which cognitive functions are not impaired by stress, or on the contrary, are even improved (Shields et al., 2016). Considering stress intensity effects on memory, it is believed to follow an inverted-U-shape, meaning that low and high stress levels impair memory, whereas intermediate stress facilitates them. Several studies, including hippocampal-dependent tasks have successfully substantiated the inverted-U-shape relationship between hormonal levels and both learning and synaptic plasticity (Sandi, 2013). In other words, implicit memory (non-conscious learning) follows a linear relation but explicit memory (conscious memory) an inverted-U-shape (Figure 5). In addition, chronic stress effects on memory are overall consistent with those observed under high-acute stress conditions (Luethi et al., 2009).

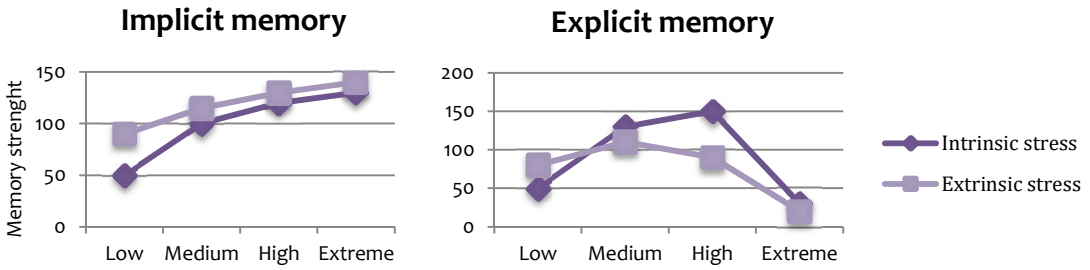

Figure 5. *Scheme showing the effects of intrinsic and extrinsic stresses in different memory types depending on the intensity of the stressful event.*

In conclusion, mild stress tends to facilitate cognitive function, particularly in implicit memory or simple declarative tasks, or when the cognitive load is not excessive. However,

high or very-high stress exposure, rather acutely or chronically, is associated with impairment of explicit memories formation and, more generally, of those that require complex and flexible reasoning (as typically observed for hippocampal-related functions), while improving the performance of implicit memory (Sandi, 2013).

Nevertheless, it is important to consider that individual differences in the cognitive impact of stress exist. In fact, considering how an individual responds to threats, we could differ between "vulnerable" and "resilient" individuals (Weger & Sandi, 2018). Vulnerability to stress is generally accepted to result from both genetic factors and exposure to environmental adversity, in particular, adversity that occurs during early life. In addition, these traits seem to be propagated across generations via epigenetic mechanisms, highlighting the overall complexity of the underlying factors involved in stress response (Weger & Sandi, 2018).

Of note, GCs play an important role in motivational behaviours and regulate mood and cognition may be due to GC-induced changes in brain structure and function, involving neuronal loss, deleterious neurotransmission, dendritic atrophy, electrophysiological activity impairment and altered neuronal cellular signalling (Sandi, 2013; Sooy et al., 2015; Sotiropoulous et al., 2008). Within the brain, the hippocampus is particularly sensitive to maladaptive responses, and the damaging effects of chronic GC excess on neuronal structure and function become more marked with ageing (Chapman & Seckl, 2008). How stress and GCs may contribute to hippocampal ageing was described with the GC cascade hypothesis (Sapolsky et al., 1986) based on that GCs secreted during periods of stress desensitize the hippocampus to further GC exposure, by down-regulating GRs. However, the GC hypersecretion continues and at some point, hippocampal cell loss occurs. The loss of neurons is irreversible, and this permanent hippocampal damage was proposed to make the hippocampus forever insensitive to further GC elevations, creating a feed-forward cycle of elevated GCs and hippocampal destruction as ageing. However, this hypothesis has some inconsistencies and has been modified to GC vulnerability hypothesis, which states that cumulative stress causes heightened GC levels and may make the hippocampus vulnerable to disruption (Conrad et al., 2011).

Chronic stress in midlife exerts persisting effects leading to cognitive and affective dysfunctions in old age via mechanisms that depend, at least in part, on brain GCs generated locally by 11β-HSD1 (Wheelan et al., 2018). Thus, 11β-HSD1 may be an important

factor in the regulation of the HPA axis and may itself be relevant to age-related diseases susceptibility, severity or outcome (Carter et al., 2009). Evidence to date has suggested that sustained stress exposure mimics the effects of age on multiple neurobiological measures of neuronal structure and function (Bloss et al., 2011). In line with this, it has been described that elderly who exhibit learning and memory impairments showed high GC levels and correlated with greater hippocampal atrophy (Lara et al., 2013). In fact, cortisol has been postulated as a potential biomarker for neurodegenerative disorders (Dhama et al., 2019).

Notwithstanding, it has been suggested a causal role of stress in the onset and progression of age-related cognitive decline and neurodegenerative disorders like Alzheimer's disease (AD), as sustained GC overexposure has been found to increase amyloid β (Aβ) formation and hyperphosphorylated Tau accumulation, hallmarks of human AD; and adversely affect behaviour (Bini et al., 2020; Bisht et al., 2018; Green et al., 2006; MacLullich et al., 2012). Accordingly, clinical evidence has shown that elevated cortisol may predict a faster progression of AD as cortisol levels were inversely correlated with cognitive performance and hippocampal volume (Bloss et al., 2011). Furthermore, a rare single-nucleotide polymorphism (SNP) in the 11β-HSD1 gene has been reported to increase the risk of developing sporadic AD (de Quervain et al., 2004), supporting that local tissue levels of GCs may be a significant risk factor for AD.

Overall, these data provide compelling evidence that chronic stressors modulate ageing and suggest that enhancing a healthy lifestyle might result in resilience to ageing and age-related pathologies. On the other hand, the maintenance of GC sensitivity during ageing might be neuroprotective, as rodent and human studies have shown that successful ageing is associated with neuroendocrine responses similar to younger subjects (Bloss et al., 2011; Yau et al., 2015).

1.1.7 11β-HSD1 inhibition

As mentioned, 11β-HSD1 participates in the development of different features related to metabolic and cognitive disturbances. On the one hand, its overexpression in rodents exerts increased corticosterone levels and displays a phenotype mimicking human metabolic syndrome, which is prevented by its inhibition, proposing that intracellular metabolism of GCs by 11β-HSD1 is critical to the development of insulin resistance rather than the circulating GCs (Bujalska et al., 2006; Peng et al., 2016; Schnackenberg et al., 2013).

On the other hand, available literature demonstrates that this enzyme is implied in age-related cognitive decline. For instance, a recent clinic study published a positive correlation between increased brain *11β-HSD1* expression with advancing age (Bini et al., 2020). Not only in humans, but also in rodents *11β-HSD1* expression was increased with ageing and its overexpression accelerated age-related cognitive decline, while 11β-HSD1-knockout mice resisted age-dependent cognitive loss (Caughey et al., 2017; Mohler et al., 2011; Yau et al., 2015). Consistent with this, aged 11β-HSD1-knockout mice performed better than aged wild-type mice in hippocampal-dependent behavioural tests, similarly to young wild-type mice, despite elevated plasma GCs, thus agreeing with that tissue-specific control may be more important than systemic levels (Yau et al., 2007).

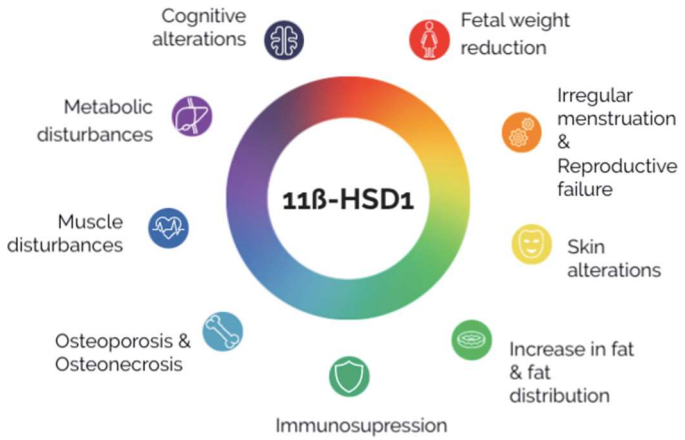

Figure 6. *Scheme of 11β-HSD1 effects on the body.*

Observing all the detrimental effects of excessive GC and the crucial role of 11β-HSD1 mediating them (Figure 6), 11β-HSD1 inhibitors have been identified and developed. Among naturally occurring inhibitors, there are progesterone metabolites, flavanone, bile acids and liquorice derivate glycyrrhetinic acid (Gathercole et al., 2013). However, most of these compounds inhibit both 11β-HSD1 and -2, thus ensuing adverse effects such as hypertension and hypokalaemia. Further research identified carbenoxolone as an 11β-HSD1 inhibitor with reported therapeutic metabolic consequences. Despite its poor selectivity, it helped to validate 11β-HSD1 as a therapeutic target in metabolic disease and up to now, several inhibitors have been studied with over than 40 United States and 90 European patents filed (Gathercole et al., 2013). To sum up, different pharmaceutical companies have developed a wide range of compounds to modify metabolic parameters and some have reached clinical trials, like AZD4017 that is in phase II (Tomlinson & Othonos, 2020) and INCB13739, which added to metformin therapy has been proved effective and well tolerated (Rosenstock et al., 2010). Despite that, other drugs have failed clinical studies, for instance ASP3662

(Astellas), PF915275 (Pfizer) and AMG-221 (Amgen) due to futility analysis, formulation issues and lack of efficacy, respectively (Astellas Pharma Global Development Inc., 2019; Harno & White, 2010).

Although there has been a focus of attention upon targeted 11β-HSD1 inhibition in the context of metabolic disease including obesity, insulin resistance and type 2 diabetes mellitus (T2DM), in the recent years its interest for neurodegenerative diseases treatment has increased (Gathercole et al., 2013). In fact, early clinical studies demonstrated that an 11β-HSD1 inhibitor (UE2343) is well tolerated and is, therefore, a suitable candidate to improve memory in patients with AD (Webster et al., 2017). In view of these results, Leiva et al. (2017) synthesized a new family of potent 11β-HSD1 inhibitors, featuring unexplored pyrrolidine-based polycyclic substituents. The most potent compounds were characterized in terms of cellular potency, isoenzyme selectivity, human metabolic stability and predicted brain penetration to select a candidate. The candidate was named RL-118 and it was selected for *in vivo* experiments in an animal model of ageing (Table 2), allowing the deployment of this thesis.

Table 2. *Biological profile of RL-118 drug. Percentage inhibition was determined relative to a non-inhibitor control. HEK293 cells stably transfected with the full-length gene coding for human either 11 β-HSD1/2 or 11β-HSD2 were used. The microsomal stability was determined using human liver microsomes and CNS+ predicted positive brain penetration.*

Characteristic	RL-118
HEK human HSD1 inhibition at 10 μM (%)	100
Human HSD1 IC_{50} (μM)	0.03
Human HSD2 IC_{50} (μM)	<0.1
HLM parent (%)	94
PAMPA-BBB $P_e(10^{-6}cm\ s^{-1})$	>30 (CNS+)

1.2 AGEING

The global increase of life expectancy has focused on the biology of ageing research to achieve a better understanding of its mechanisms for preventing the onset and progression of age-related conditions. Biological ageing is defined as the collection of morphological and physiological modifications causing a decrease in the adaptive capacity of an organism and eventually leading to functional impairment and increased vulnerability to death (López-Otín et al., 2013). Age-related loss of physiological integrity in the brain

boosts a slow deterioration in cerebral function, which results in decreased cognitive abilities, memory loss and movement disorders (Donmez, 2013). Individuals, and even different organ systems within the same person, age at varying rates depending on various lifestyle and environmental factors (Roza & Rughwani, 2019). The time-dependent accumulation of cellular damage is widely considered the general cause of ageing (Almeida & Manolagas, 2020; Gems & Partridge, 2013). However, its origin and the interconnection between different types of damage and their compensatory responses to delay ageing, still remain unknown.

Nonetheless, several cellular and molecular hallmarks of ageing have been identified (Kennedy et al., 2014; López-Otín et al., 2013). In particular, nine tentative hallmarks that are generally considered to contribute to the ageing process in different organisms as well as to determine the ageing phenotype have been described (Figure 7) (López-Otín et al., 2013). Each hallmark should converge, at some degree, to the following criteria:

1. It should manifest during normal ageing
2. Its experimental aggravation should accelerate ageing
3. Its experimental amelioration should retard the normal ageing process and hence increase healthy lifespan

The nine hallmarks described by López-Otín et al. (2013) ageing can be categorized into primary, antagonistic and integrative hallmarks. The former includes all harmful components of ageing, while the next involves those marks that exert opposite effects depending on their intensity. The last category comprises the marks that directly affect homeostasis and tissue functions.

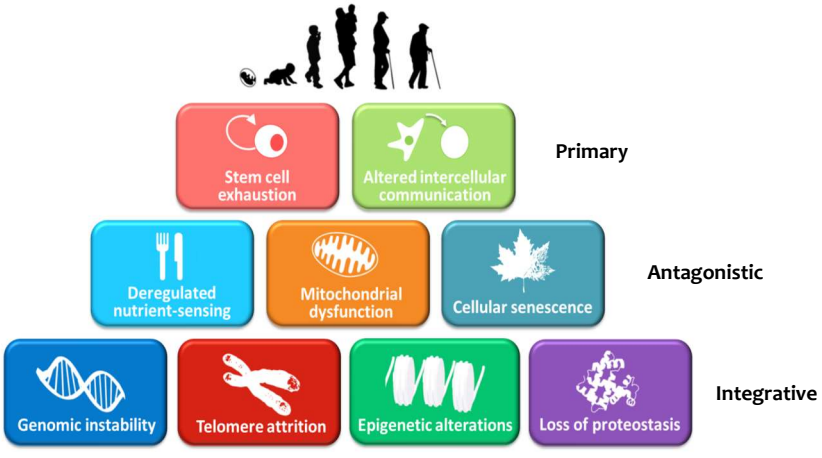

Figure 7. *Cellular and molecular mechanisms of ageing (Adapted from López-Otín et al., 2013).*

- **Genomic instability**

One common denominator of ageing is the accumulation of genetic damage throughout life (Moskalev et al., 2012), which involve exogenous physical, chemical and biological agents, as well as endogenous threats, including DNA replication errors, DNA mutations, spontaneous hydrolytic reactions and reactive oxygen species (ROS) accumulation (Niedernhofer et al., 2018; Vigj & Montagna, 2017). Additionally, exposure to genotoxics accelerates the ageing process (Niedernhofer, et al., 2018). Therefore, targeting DNA repair mechanisms has been proposed as a strategy to alleviate age-associated dysfunctions (Petr et al., 2020).

- **Telomere attrition**

Telomeres are a chromosomal region particularly susceptible to age-related deterioration. Replicative DNA polymerases lack the capacity to replicate completely the terminal ends of linear DNA molecules, a function of telomerase. However, most mammalian somatic cells do not express it, and this leads to progressive and cumulative loss of telomere-protective sequences from chromosome ends, inducing senescence and/or apoptosis (Fumagalli et al., 2012; Rossi & Gorospe, 2020). Accordingly, pathological telomere dysfunction accelerates ageing in mice and humans, whereas experimental telomerase stimulation delays ageing.

- **Epigenetic alterations**

Epigenetic changes involve alterations in DNA methylation patterns, histone post-translational modifications (PTM) and chromatin remodelling, through DNA methyltransferases (DNMTs), histone acetyltransferases (HATs), deacetylases (HDACs), methyltransferases (HMTs) and demethylases (HDMs); as well as protein complexes. For instance, deletion of histone methylation extends longevity (Greer et al., 2010; Molina-Serrano et al., 2019), whereas global DNA hypomethylation has been associated with ageing (Salas-Pérez et al., 2019). Of note, unlike DNA mutations, epigenetic modifications are reversible, hence offering opportunities for the design of novel anti-ageing treatments.

- **Loss of proteostasis**

Proteostasis (protein homeostasis) involves mechanisms for the stabilization of incorrectly folded proteins – most prominently, the heat shock family of proteins – and mechanisms for the degradation of protein by the ubiquitin-proteasome system or the autophagy-lysosomal system. Moreover, there are other regulators of age-related proteotoxicity that

act through an alternative pathway distinct from molecular chaperones and proteases (Njomen & Tepe, 2019; van Ham et al., 2010). Overall, all theses systems coordinate to restore the structure of misfolded polypeptides or to remove and degrade them entirely to prevent the accumulation of damaged components and to assure the continuous renewal of intracellular proteins. Although these efforts to re-establish proteostasis, it becomes altered with ageing and this contributes to the development of some age-related pathologies, such as AD and Parkinson's disease.

- **Deregulated nutrient sensing**

Evidence asserts that deregulation in molecular pathways related to nutrients absorption, as well as excessive and prolonged anabolic signalling, accelerate ageing (Farr & Almeida, 2018). However, dietary restriction is associated with increased longevity or healthspan (i.e. the life period free of chronic diseases). Remarkably, among the intracellular signalling pathway of insulin and insulin-like growth factor 1 (IGF-1) known as the "insulin and IGF-1 signalling" (IIS) targets, there are the forkhead box O (FOXO) and the mammalian target of rapamycin (mTOR) complexes, which are involved in regulating glucose sensing and ageing (Farr & Almeida, 2018). In addition, three related and interconnected nutrient sensing systems have been identified. Particularly, mTOR for the sensing of high amino acid concentrations; AMP-activated protein kinase (AMPK), which detect low-energy states by high AMP levels; and Sirtuins (SIRT), which sense low-energy states by detecting high NAD^+ levels.

- **Mitochondrial dysfunction**

As cells and organisms age, the efficacy of the respiratory chain located in the mitochondria tends to diminish, thus increasing electron leakage and reducing ATP generation (Green et al., 2011). In fact, one of the ageing theories states that it causes progressive mitochondrial dysfunction leading to increased ROS production, which in turn causes further mitochondrial deterioration and global cellular damage. Indeed, ROS accumulation damages the cell, although mounting evidence suggests its role in triggering proliferation and survival in response to physiological signals and stress conditions (Kong & Chandel, 2020; Sena & Chandel, 2012).

- **Cellular senescence**

Cellular senescence is a dynamic process whereby a cell loses its proliferative potential, undergoing an essentially irreversible growth arrest that occurs in response to various

stressors, triggering activation of several pathways that induce senescence. This process involves increased cell size, extensive gene expression changes, telomere shortening and chromatin modifications (Farr & Almeida, 2018). Conceivably, the accumulation of senescent cells with ageing may be due to increased rate of senescence and/or decreased rate of clearance, maybe as a consequence of an attenuated immune response.

- **Stem cell exhaustion**

One of the most obvious characteristics of ageing is the decline in the regenerative capacity of tissues. With ageing haematopoiesis deteriorates, resulting in diminished adaptive immune cells production, and increased incidence of anaemia and myeloid malignancies (Shaw et al., 2010). Although the deficient proliferation of stem and progenitor cells is obviously detrimental, an excessive proliferation of these cells can also be deleterious as accelerates the exhaustion of stem cell niches.

- **Altered intercellular communication**

Compelling evidence indicates that beyond cell-autonomous alterations, age-related dysfunction in one cell can spread and cause age-related deterioration systemically in other cells located in various tissues throughout an organism (Farr & Almeida, 2018). Ageing alters intercellular communication at different levels, including elevated inflammation, exacerbation of oxidative stress (OS), as well as deregulated endocrine and neuronal signalling.

Noteworthy, the differentiation between physiological and pathological ageing, is often subtle but fundamental (Figure 8). Ageing should naturally be a physiological process occurring prior to death. Thus, physiological ageing features a slow process of balanced functional decline across various organs in a coordinated manner, manifesting healthy or successful ageing and longevity. In contrast, pathological ageing originates from premature ageing often in a tissue-specific manner, resulting in impairment of adaptive responses and finally, chronic diseases (Liu, 2017). Up to now, the mechanisms that evolve physiological to pathological ageing remain unclear. However, it is currently thought that mTOR operates as a thermostat switching in between physiological and pathological ageing, with inhibition of mTOR robustly lengthening animal lifespans due to all the mediator effects commented above (Liu, 2017).

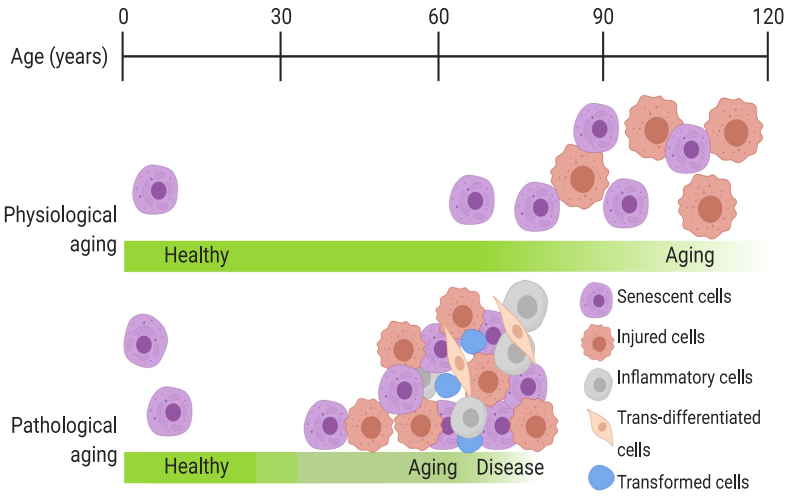

Figure 8. *Scheme of different cellular profiles in human ageing leading to longevity and chronic diseases.*

1.2.1 Theories of ageing

In an attempt to address the origin of ageing, several theories have been proposed and comprised into two categories:

1. Stochastic or environmental theories: based on the loss of functions during ageing due to the accumulation of unpredictable lesions, in part, caused by environmental factors.
2. Non-stochastic or deterministic theories: hold the hypothesis that ageing is caused by those mechanisms that describe a high number of concrete and known variables, which are innate and programmed in the individual genome.

Trevisan et al. (2019) reviewed ageing theories and described the following subgroups of theories (Table 3 and 4). Notwithstanding, none of them has been able to fully explain this process, as it may not exist a single responsible mechanism.

Table 3. *Stochastic theories of ageing.*

Theory	Description	Reference
Theory of somatic mutations	It states that radiation exposure during life increases the probability of acquiring diseases and diminishes life expectancy due to the destruction of biomolecules, mainly the DNA of somatic cells.	Szilard, 1959
Theory of error-catastrophe	It is associated with the perpetuation of protein synthesis error, creating aberrant proteins and consequently cellular death and ageing.	Orgel, 1963
Theory of DNA	It claims that the number of DNA replications	Hart & Setlow,

reparation	determine the lifespan and could generate more probability of mutations within the DNA itself, therefore impairing proteins through the process of transcription.	1974
Theory of the breaking of biomolecular bonds	The increase of chemical bonds in DNA causes protein modifications that could generate the functional failure of cell metabolism, resulting in physiological processes decline during ageing.	Cristofalo et al., 1994
Theory of advanced glycosylation	High concentration of glucose in the blood and tissues due to impaired nutrient sensing leads to protein glycosylation, perturbing biological functions.	Cerami, 1985
Free radical theory	Based on the reactions oxidizing biomolecules, thus destructing them, and causing many degenerative alterations. It evolved to the mitochondrial theory of ageing when mitochondria were implicated as the primary source of ROS (Barja, 2013).	Harman, 1991
Theory of adaptive homeostasis	It is the most recent theory and considers anti-stress as a form of protection that maintains homeostasis within the organism.	Pomatto & Davies, 2018

Table 4. *Non-stochastic theories of ageing.*

Theory	Description	Reference
Theory of telomeres	Considers the cell repositioning diminishment linked to telomeres modification, which is responsible for chromosome integrity, affecting cellular functions.	Blackburn, 1999
Theory of intrinsic mutagenesis	The longevity depends on the reliability of genetic material in its replication and maintenance of the restorative mechanisms.	Burnet, 1974
Neuroendocrine theory	It proposes that ageing results from functional perturbations, both in neuronal control and in endocrine output, of the HPA axis, thereby causing dysfunction of various endocrine glands and their target organs.	Sonntag et al., 1999
Immunological theory	Immunological responses decrease with ageing, thus generating low resistance to infections and diseases.	Walford, 1969
Theory of programmed senescence	Ageing is based on genetic programming that controls cell development and determines the lifespan of each of the cells.	Hayflic & Moorhead, 1961

1.2.2 Ageing and neurodegeneration

Ageing is a process regulated by different molecular and genetic mechanisms. Three pathways modifying longevity have been described: increase of AMPK and reduction of mTOR, reduction of insulin signalling and reduction of SIRT. Relevantly, mTOR complex (mTORC) 1 inhibition by rapamycin can postpone ageing by affecting energy sensing, maintenance of proteostasis, improving mitochondrial dysfunction and cellular senescence, but also modulating stem cell functions in different tissues (Papadopoli et al., 2019). Recently, AMPK signalling activation with age has been identified as a critical factor determining age-related decline of hippocampal neurogenesis and thus, increased susceptibility to age-associated neurological diseases (B. Z. Wang et al., 2019). The later is a family of HDACs, which activation has been associated with neuroprotective effects.

Extended lifespan is associated with an increase in the prevalence of age-related diseases. Indeed, ageing is a major risk factor for several chronic pathologies, including osteoporosis, cardiovascular and metabolic disorders, as well as neurodegenerative diseases, such as AD (Almeida & Manolagas, 2020; Salas-Pérez et al., 2019; Trevisan et al., 2019).

Referring to brain ageing, it produces changes in neuronal and cognitive abilities, like mental speed and executive function. In the hippocampus, a brain area that comprises the spatial and episodic learning, memory and is predisposed to the detrimental effects of ageing, long-term potentiation results impaired at synapses and immediate early genes for the synaptic plasticity are down-regulated when ageing (Liu, 2017). However, human umbilical cord plasma treatment, which is abundant of plasticity-enhancing proteins, revitalizes the hippocampus and improves cognitive function in aged mice (Castellano et al., 2017).

1.3 EPIGENETICS AND NEURODEGENERATION

During ageing, biological, environmental, and lifestyle factors drive epigenetic modifications resulting in phenotypic differences. Environmental stressors interact with the genome leading to stable changes in DNA structure, gene expression and behaviour. In turn, these changes may underlie and contribute to the development and progression of mental disorders, including AD (Park et al., 2019).

Epigenetics refers to potentially heritable and environmentally modifiable changes in gene expression that are mediated via non-DNA-encoded mechanisms (Sun et al., 2013). The three main epigenetic marks are DNA-methylation, histone PTM and non-coding RNA regulation. All these mechanisms play a key role in regulating transcriptional activity, modulating chromatin structure through recruitment, assembly or retention of chromatin-associated factors (Harman & Martín, 2019; Nair et al., 2017).

1.3.1 DNA methylation and hydroxymethylation

DNA methylation consists in the addition of methyl groups to the DNA, which alters the DNA segment transcriptional activity without altering its sequence (Park et al., 2019). DNMT enzymes – DNMT1, DNMT3A and DNMT3B – catalyse the transference of a methyl group at the fifth position of cytosine in order to generate 5-methylcytosine (5-mC). DNMT1 is responsible for maintaining the methyl groups by copying the methylation pattern of the parent DNA strand during cell division so that afterwards methylation is inherited (Hermann et al., 2004). On the other hand, DNMT3A and DNMT3B catalyse the de novo methylation, adding methyl groups to non-methylated CpG cytosine dinucleotide. Methylation on gene promoter regions, particularly at CpG islands, is the canonical mechanism for cell-specific gene silencing during development, thus plays a critical role in neural plasticity (Miller et al., 2010). According to the primary role of DNA methylation in brain function, DNMT1 and DNMT3A deletion in mouse prefrontal cortex leads to deficits in neuronal morphology, synaptic plasticity, learning and memory (Harman & Martín, 2019). Although initially it was thought that this epigenetic mark was irreversible, recent research has demonstrated that DNA may experience fast and reversible methylation changes and may also undergo demethylation (Figure 9 A) (Miller & Sweatt, 2007).

On the other hand, demethylation that consists in removing methyl groups is performed by ten-eleven translocation (TET) enzymes. TET1, 2 and 3 oxidize 5-mC to 5-hydroxymethylcytosine (5-hmC). It is more labile and can rapidly generate demethylated cytosines. Although 5-hmC and 5-mC appear to be negatively related, according to Chen et al. (2012) increased global 5-hmC in the hippocampus of aged mice do not correlate with decreased 5-mC neither to increased TETs, suggesting that 5-hmC could act as an epigenetic marker itself and not only as an intermediary in DNA demethylation. TET1 and TET2 participate in controlling gene expression and cell death and are associated with ageing and

neurodegeneration, while TET3 is related to early embryonic development (Fetahu et al., 2019; Ficz et al., 2011).

1.3.2 Post-translational histone modifications

DNA is wound around histone proteins conforming nucleosomes. It consists of a core histone octamer (H3, H4, H2A and H2B) and the N-terminal tail of each extends and contains sites that can expect more than 100 PTM, involving acetylation, methylation, ubiquitinylation and phosphorylation (Harman & Martín, 2019). Multiple PTM can be added to a single histone molecule. These epigenetic marks modulate chromatin compaction, allowing or denying the transcriptional machinery access to the regulatory regions of genes. Highly compacted chromatin, also known as heterochromatin, is generally associated with low transcription, while less compacted regions of chromatin, named euchromatin, are associated with active transcription (Taylor et al., 2019).

Among the histone modifications known to be involved in longevity and ageing, the most widely described are acetylation and methylation of lysine (Lys) residues (Figure 9 B) (Pal & Tyler, 2016). Acetylation removes histone positive charge, thereby increasing transcriptional activity of the acetylated segment, while methylation generally the contrary.

HATs acetylate Lys residues, and conversely HDACs catalyse histone deacetylation and function as transcription repressors (Harman & Martín, 2019). The expression of HATs and HDACs has been reported to be altered in the aged brain and could be linked to age-related altered gene transcription (Barter & Foster, 2018). Studies using pharmacological and transgenic approaches demonstrated that HDAC2 is the most linked to negatively regulate cognition, as its levels increased in the hippocampus of aged mice and its inhibition enhanced synaptic plasticity, the number of dendritic spines and memory (Harman & Martín, 2019). In line with this, increased HDAC2 activity correlated with diminished Lys 9 and Lys 14 acetylated H3 (H3K9 and H3K14) levels (Singh & Thakur, 2018).

Considering histone methylation, it has been linked to either transcriptional activation or repression depending on the particular methylated residue and extent of methylation (mono, di or trimethylation), although ageing has been related to increased histone methylation and repressed transcriptional activity (Pal & Tyler, 2016). Compelling

evidence proposed that methylation of H3K9 promotes DNA methylation maintenance in mammals and subsequent gene silencing (Zhao et al., 2016).

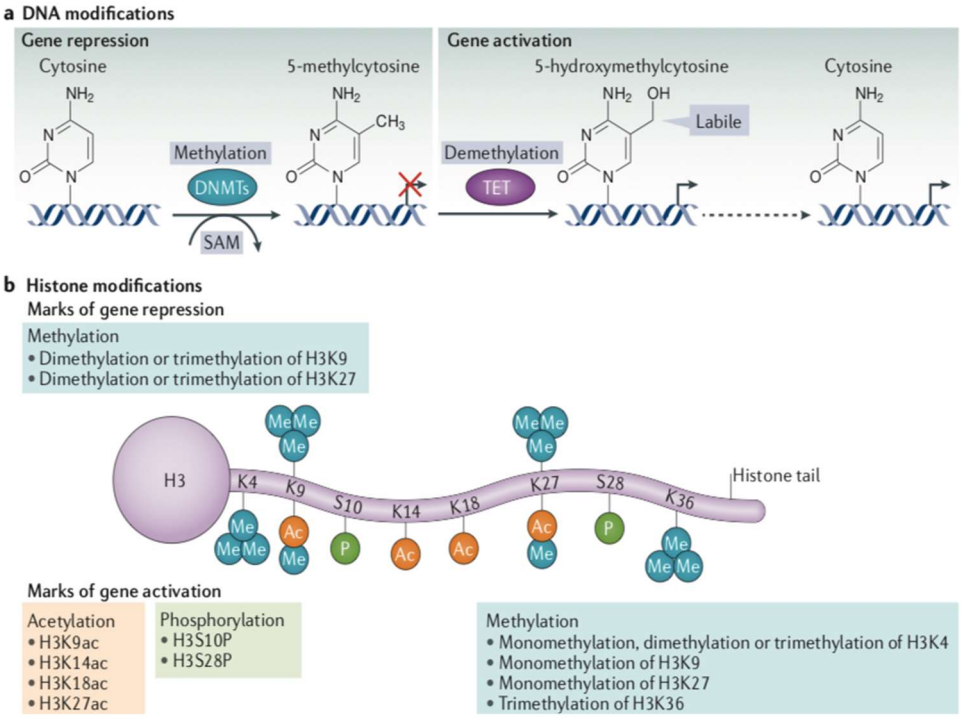

Figure 9. *Epigenetic modifications. A. DNA modifications. B. Histone modifications (Hwang et al., 2017).*

1.3.3 non-coding RNAs

MicroRNAs (miRNAs) are small non-coding RNA molecules containing about 18-25 nucleotides involved in gene expression regulation. miRNAs bind to complementary sites on 3' untranslated regions of target mRNA molecules and direct their degradation or inhibit their translation (Harman & Martín, 2019). One of the most abundantly expressed miRNAs in the aged brain is *let-7* family, but on the contrary, *miR-101* and *-433* were down-regulated, indicating miRNAs changes might be implicated in cognitive decline (Inukai et al., 2012). Other miRNA have been implicated in AD pathogenesis (Table 5), such as *miR-107* as it targets β-site amyloid precursor protein (APP) cleaving enzyme 1 (BACE1) (Wang et al., 2008) and *miR-106b* (Hébert et al., 2009).

Table 5. *Summary of miRNA modulating Amyloid-β plaques formation (M. Wang et al., 2019).*

Target mRNA	miRNA	Main effect	Reference
APP	*miR-106a/b, -520c, -20a, -17-5p, -101, -16, -153, -200b*	Overexpression results in translational repression of *APP* mRNA	Patel et al., 2008; Hébert et al., 2009; Vilardo et al. & Liu et al., 2010; Long et al., 2012; Liu et al., 2014

BACE1	*miR-29a/b/c, -107, -195, -124, -135a/b, -339-5p, -186, -298, -328*	Reduction correlated with higher levels of BACE1	Hébert et al., 2008; Lei et al., 2015; Wang et al., 2008; Zhu et al. & Fang et al., 2012; Liu et al. & Long et al., 2014; Zhang et al., 2016; Kim et al., 2016; Boissonneault et al., 2009

1.4 PROTEOSTASIS AND NEURODEGENERATION

The term proteostasis reflects the fine-tuned balance of cellular protein levels, which require strict control of protein folding, PTM and degradation. Due to the complex interactions and intersection of proteostasis pathways, exposure to stress conditions may lead to a disruption of the entire network (Kurtishi et al., 2019). Ageing, oxidative and environmental stresses and protein dysfunction can alter cellular proteostasis; all risk factors for neurodegenerative diseases like AD.

As mentioned, protein misfolding and aggregation are features of many neurodegenerative diseases. Incorrect protein folding and/or modifications during protein synthesis result in inactive or toxic proteins, which may overload degradation mechanisms. Although misfolded proteins are generally inactive, their accumulation can activate stress responses in cells and organelles (Kurtishi et al., 2019). Indeed, the ER is a key organelle in maintaining proteostasis. The accumulation of misfolded proteins disrupts ER functioning and evolves to ER stress. Under stress, the unfolded protein response (UPR) signalling pathways, which include ER stress response, autophagy and the ubiquitin-proteasome system, activate to restore cell homeostasis (Figure 10). However when stress is continued it may promote cell damage and death (Karna et al., 2019). ER stress may be due to a variety of physiological conditions, including disturbances in calcium homeostasis, glucose/energy deprivation, redox changes, ischemia and mutations that eventually disrupt protein folding. The UPR accounts for the following protective actions:

- Promotes protein folding by increasing chaperone expression, such as the binding immunoglobulin protein (BIP/GRP78).
- Reduces ER protein levels by translation mitigation, proteasomal degradation and autophagy (Hetz et al., 2012).

Protein translational attenuation is mediated by serine (Ser)/threonine (Thr) kinase RNA-like ER kinase (PERK), which phosphorylates the eukaryotic initiation factor 2 α (eIF2α) in Ser 51 causing general protein synthesis reduction and promoting cell survival. Moreover, PERK activation has been shown to modulate the activity of nuclear factor erythroid-derived 2 (NRF2) and FOXO proteins, linking this pathway to the antioxidant response, insulin responsiveness and autophagy (Martínez et al., 2017). In particular, PERK phosphorylates NRF2 in Thr 80 in order to activate it and therefore translocate to the nucleus where it binds to the antioxidant response element (ARE) inducing an increase in glutathione (GSH) levels and a reduction of ROS levels, promoting cell survival (Cullinan & Diehl, 2004; Ma et al., 2013). Additionally, eIF2-α is activated by AD hallmarks, inflammation and OS pathways, and nutrient imbalance. By contrast, its reduction in mice has been linked to enhanced synapse plasticity and cognition (Ma et al., 2013). As misfolded proteins accumulate during neurodegeneration, ER capability becomes overrun and eventually proves insufficient, leading to proteotoxicity and protein aggregation (Kurtishi et al., 2019). Accordingly, evidence for canonical UPR activation in AD neurons has determined that increased BIP/GRP78 protein levels in the hippocampus and prefrontal cortex of AD animal models, as well as the higher presence of p-PERK and p-eIF2α in the neurons of AD patients (Chang et al., 2002; Scheper & Hoozemans, 2015). These markers appeared in morphologically healthy neurons or neurons with an abnormally phosphorylated Tau protein, indicating that the UPR is involved in the early stages of AD pathology (Scheper & Hoozemans, 2015). In accordance, induction of ER stress leads to neuronal metabolic stress, AD hallmarks and cognitive impairment in mice (Lourenco et al., 2013; van der Harg et al., 2014; Yoon et al., 2012).

On another note, the UPR signalling also activates autophagy. However, ER stress response and autophagy are linked in different ways. Among others, it has been described that PERK reduces nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation, which promotes the induction of autophagy. Also, PERK-mediated activating transcription factor 4 (ATF4) activation is required for gene expression of various autophagy genes. Autophagy degrades protein aggregates that cannot be processed by the proteasome (Blasiak et al., 2019) to promote cell survival. For instance, the autophagosome can degrade and recycle entire damaged organelles. Autophagy means "self-eating" and is defined as a catabolic process in eukaryotic cells in which intracellular and extracellular altered components are delivered into lysosomes, where their degradation occurs (Boya et al., 2013). Of interest, autophagy declines with ageing; so that

it may contribute to the deleterious accumulation of unnecessary or/and harmful components and damaged organelles observed in aged cells, as well as worsens age-related diseases (Gouras et al., 2019). Its dysregulation has been related to a diversity of pathological processes such as neurodegenerative, infectious and metabolic disorders, as well as cancer (Barbosa et al., 2018). In fact, many of the alterations mentioned during ageing, such as nutrient sensing, mitochondrial damage, cellular senescence, and stem cell exhaustion, among others; are linked to changes in autophagic flow (Ma et al., 2011; Plaza-Zabala et al., 2017). In the brain particularly, it has been observed that aged rodents showed increased mTORC1 activity, indicative of autophagy function reduction (Plaza-Zabala et al., 2017). Interestingly, mTORC1 was found to regulate not only lysosomal protein degradation, but also proteasomal proteolysis, suggesting a common regulation of both proteolytic systems by nutrient-sensing (Zhao et al., 2015).

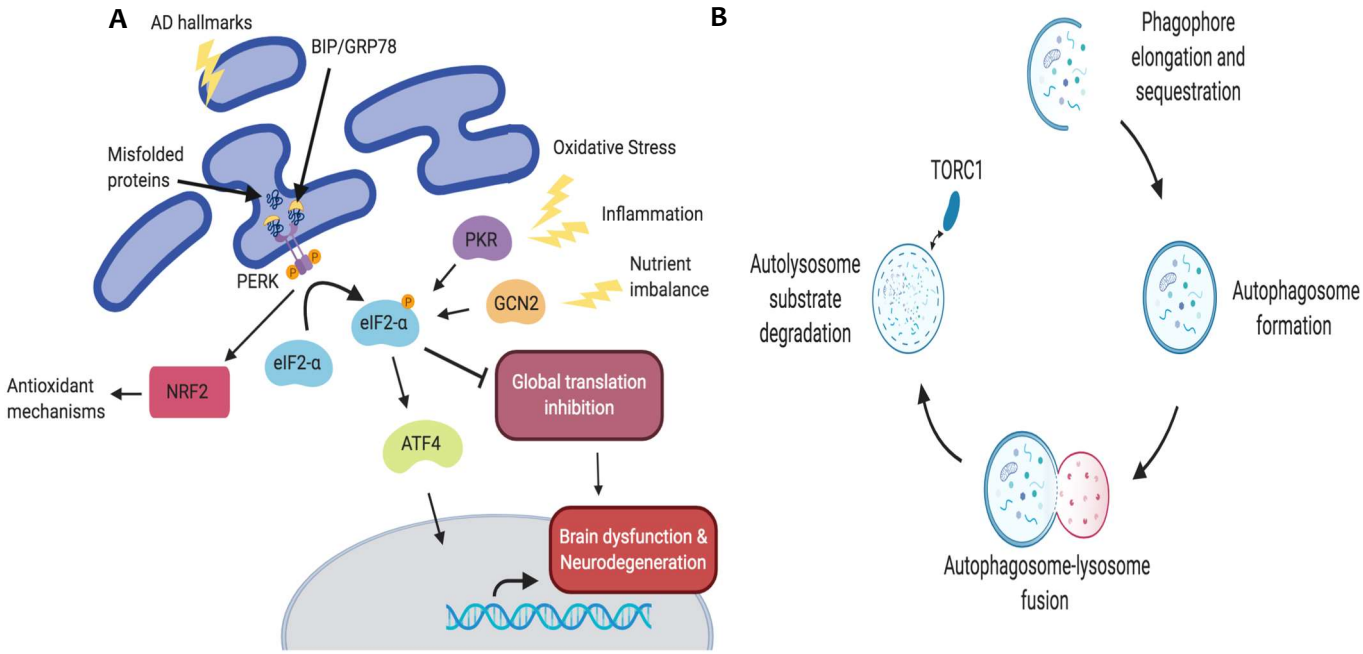

Figure 10. *Representation of the molecular pathways triggered by the UPR activation. A. ER stress response inhibiting translational activity and promoting protein folding and antioxidant mechanisms. B. Autophagy process, in which misfolded proteins and disturbed organelles are surrounded by a phagophore that is transformed to an autophagosome and fusioned with a lysosome to form an autolysosome, and finally digested by hydrolytic enzymes, yielding basic metabolites that are released into the cytoplasm for new synthesis or as sources of energy.*

Further, autophagy and ER degradation pathway disruption may result in additional cellular stress, which could ultimately cease to cell death. However, the UPR may orchestrate repairing processes of the nervous system by controlling the expression of neurotrophins such as brain-derived neurotrophic factor (BDNF), and activating different pro-inflammatory cytokine secretion pathways (Martínez et al., 2017). Mainly, the UPR

activates NF-κB, mitogen-activated protein kinase (MAPK), c-Jun N-terminal protein kinase (JNK) and p38 (Brown et al., 2016; Sprenkle et al., 2017). Chronic ER dysfunction has been associated with memory and cognitive deficits observed in AD patients (Duran-Aniotz et al., 2017; Sprenkle et al., 2017).

1.5 OXIDATIVE STRESS AND NEURODEGENERATION

OS is the redox state resulting from an imbalance between reactive oxygen and nitrogen species (RONS) generation and detoxification. RONS are highly reactive molecules with one or more unpaired electrons that behave as oxidants or reductants and antioxidant defences neutralize their harmful effects.

On the one hand, RONS production is not only limited to exert deleterious effects, but also involved in energy production and immune defence (Liguori et al., 2018). The main RONS studied are superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl ion (OH^-), nitric peroxide (ONO_2^-) and nitric oxide (NO), the majority (95-98%) of which come from the by-products of the mitochondrial electron transport chain. However, there are other sources of RONS:

- Endogenous: include NADPH-oxidase, myeloperoxidase (MPO), lipoxygenase and angiotensin II. The first is the prevalent source of the O_2^-, which is formed during cellular respiration and, mostly, it is converted into H_2O_2 by superoxide dismutase (SOD) (Liguori et al., 2018). It, in turn, can form the highly reactive ROS OH^- that is extremely reactive, especially with phospholipids in cell membranes and proteins, thus contributing to increase oxidative damage. H_2O_2 in turn, is metabolised by catalase (CAT) to water and oxygen. NO is produced by NO synthase (NOS) and combined with oxygen (O_2) react to form another relatively reactive molecule, ONO_2^- (Genestra, 2007).

- Exogenous: involve air and water pollution, tobacco, alcohol, heavy or transition metals, drugs, industrial solvents and radiation (Liguori et al., 2018).

RONS overproduction, together to mitochondrial dysfunction, produce oxidative damage to cells, embracing protein oxidation and nitration, glycoloxidation and lipid peroxidation. Altogether cause alterations in cellular biochemical and physiological

functions leading to apoptosis and, therefore, to neurodegenerative pathologies (Fivenson et al., 2017; Peterson & Smith, 2016).

On the other hand, antioxidant defence protects biological systems from RONS toxicity and involves both endogenous and exogenous molecules:

- Endogenous antioxidants: contain enzymatic and non-enzymatic pathways.
    - **Antioxidant enzymes:** the primary antioxidant enzymes are SOD, CAT and glutathione peroxidase (GPX). As mentioned above, SOD dismutase O_2^- to H_2O_2, which is decomposed to water and O_2 by CAT, preventing OH^- production. In addition, GPX converts peroxides and OH^- into nontoxic forms by the oxidation of reduced GSH into glutathione disulphide and then reduced to GSH by glutathione reductase. Other antioxidant enzymes are glutathione-S-transferase and glucose-6-phosphate dehydrogenase (Liguori et al., 2018).
    - **Non-enzymatic antioxidants:** they are molecules that interact with RONS terminating the free radical chain reactions, such as bilirubin, α-tocopherol (vitamin E), β-carotene, albumin and uric acid (Wu et al., 2013).

- Exogenous antioxidants: include ascorbic acid (vitamin C), which scavenges OH^- and O_2^-; α-tocopherol, which is involved against lipid peroxidation of cell membranes; and phenolic antioxidants like stilbene derivatives (resveratrol and flavonoids), oil lecithins, selenium, zinc and drugs such as acetylcysteine (Liguori et al., 2018; Pisoschi & Pop, 2015).

Despite ageing is the leading cause of cognitive impairment; patients with high GPX activity showed slower progression of cognitive deficits, while on the contrary, low GPX activity was related to cognitive decline acceleration (Revel et al., 2015). In addition, it has been demonstrated that increased OS biomarkers correlated with raised levels of inflammatory cytokines and both were associated with poor cognitive performance in institutionalized elderly people (Baierle et al., 2015). The brain is especially exposed to RONS as it is a high energy-demanding organ, i.e. requires more oxygen than other tissues – approximately 20% of the basal body oxygen – so undergoes mitochondrial respiration, which increases ROS production (Du et al., 2018). Mitochondrial abnormalities, initially caused by gradual oxidative disturbances, are major contributors to OS (Tadokoro et al., 2020). Indeed, perturbed mitochondrial operations result in impaired metabolic capacity

(Unzeta et al., 2016). This abnormality creates a vicious positive-feedback cycle where RONS accumulation achieves a threshold and activates unavoidably a compensatory mechanism in order to control the cellular environment. This hyper-defensive state, intended to prolong cell life, raises vulnerability to additional insults such as AD hallmarks (Cheignon et al., 2018; Zhu et al., 2007), which in consequence, leads to neuroinflammation, mitochondrial damage and further RONS generation (Unzeta et al., 2016). Also, altered antioxidant enzymes levels were found in specific AD brain regions (Sultana et al., 2011). Similarly, RONS could be involved in OS influencing AD, as it has been described that affect the activity of the N-methyl-D-aspartate (NMDA) receptors triggering a cascade of events that increase RONS production, inflammation, AD hallmarks, lipid peroxidation, and ultimately, leading to synaptic dysfunction responsible for AD (Ligouri et al., 2018).

1.6 NEUROINFLAMMATION AND NEURODEGENERATION

Neuroinflammation is defined as a cerebral physiological response to injury, infection or disease in order to prevent potential damage, neuronal destruction or to eliminate the damaging agent. To date, several alterations of innate and acquired immunity have been observed in the elderly, like diminished adaptive immune cells production. Altogether are comprised in the immunosenescence term. It refers to increased susceptibility of aged people to infection and may be explained in terms of molecular and cellular mechanisms responsible for inflammatory age-associated disorders (Fard & Stough, 2019).

One feature of immunosenescence is a chronic subclinical elevated production of pro-inflammatory mediators typical of elderly as a consequence of cumulative lifetime exposure to antigenic load due to infections and non-infective antigens (Baylis et al., 2013; Blagosklonny, 2010). In response to an insult, inflammatory pathways activate and several cytokines are released from glial cells, lymphocytes, monocytes and macrophages (Figure 11). As mentioned, stress can also increase pro-inflammatory cytokines levels. In fact, it has been described that stress raises tumour necrosis factor-alpha (*Tnf-α*), interleukin (*Il*) 1-β and *Il-6* expression, as well as reduce anti-inflammatory cytokines expression like *Il-10*, *Il-4* and transforming growth factor β (*Tgf-β*). In addition, cytokines are potent regulators of the HPA axis and subsequent eliciting GC secretion, so they can alter local GC availability and GR function, therefore affecting an individual's GC sensitivity (Marques et al., 2009; Thiel & Dretsch, 2011). Besides, stress contributes to increase ROS production and in

consequence, altogether impair neurogenesis and promote cellular death, leading to neurodegeneration (Fajemiroye et al., 2018). In line with this, compelling evidence emphasizes the importance of cytokines in cognitive processes at the molecular level, such as in synaptic plasticity, neurogenesis and neuromodulation, which may subserve learning and memory, among others (Fard & Stough, 2019). Another cellular phenomena relevant to immunosenescence is cellular senescence, which is mediated through a large increase of factors mediating intercellular signalling and stimulating inflammatory pathways, like IL-8, IL-6 and IL-1β, among others (Chinta et al., 2015).

Besides during the inflammatory process, microglia and astrocytes are the main active immunological cells in the CNS, which regulate both the increment as well as the reduction of inflammatory signalling pathways, through cytokine synthesis and up- or down-regulation of different receptors. On the one hand, the origin of microglia and its self-propelling regeneration, causing telomere shortening, has resulted in the hypothesis that microglia ages and develop the age-dependent cellular dystrophy and consequently become senescent. Microglial senescence has been linked to functional changes that contribute to age-dependent increase of microglia-mediated neuroinflammatory responses, which are believed to further threaten aged neurons and, thus, drive the progression of age-related neurodegenerative pathologies, such as AD (Spittau, 2017). Reactive gliosis and neuroinflammation are deeply involved in AD pathogenesis. Evidence considers microglia-related pathways to be central to AD risk and pathogenesis. Microglia, the complement system and the triggering receptor expressed on myeloid cells 2 (TREM2) are responsible for synaptic pruning; the fundamental cellular process underpinning learning and memory (Chen et al., 2017).

On the other hand, astrocytes acquire different phenotypes in response to numerous pathological stimuli, such as neurodegenerative disorders, tumours, infection and ageing. Although it is recognized that astrocytes gain or loss functions at the site of the insult, it is not clear whether this is beneficial or damaging to their surrounding. Apart from the protective roles that can be enhanced by astrocyte's activation, such as the production of anti-inflammatory factors, these cells can also produce cytokines that exacerbate the injury, increase Aβ production or become atrophied and loose their neuroprotective functions in AD (Matias et al., 2019).

Nevertheless, chronic neuroinflammation stimulates microglia activation, hence promotes microglia and astrocytes turnover and exacerbates the process of brain cellular ageing (Ma et al., 2013). In addition, reactive microglia and astrocytes surround AD hallmarks and secrete numerous pro-inflammatory cytokines and inflammatory mediators (Du et al., 2018). However, it is still debated whether inflammation is the cause or effect of the neurodegenerative process.

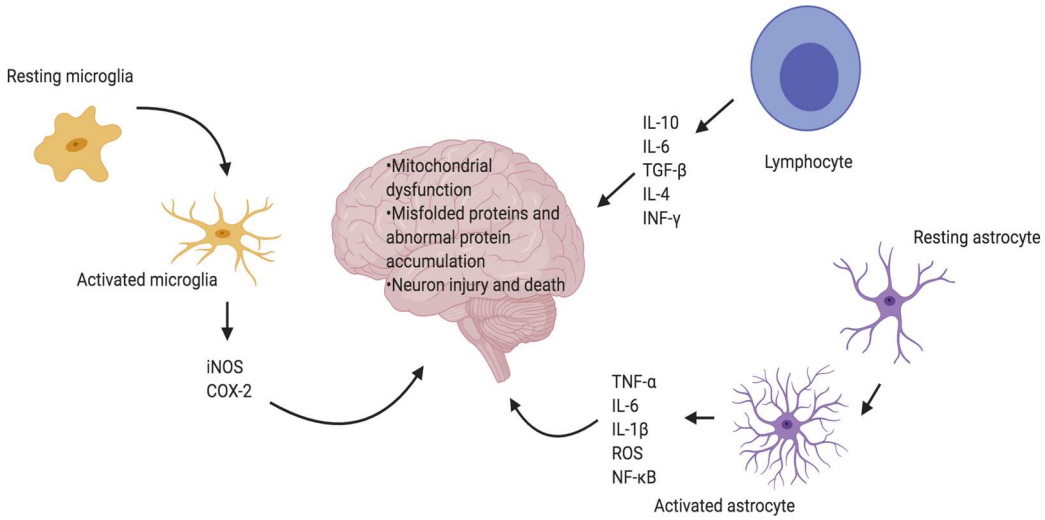

Figure 11. *Representation of neuroinflammatory pathways.*

1.7 METABOLIC STRESS AS AN ACCELERATOR OF COGNITIVE DECLINE

In recent years, interest in the nutrition and health relationship has grown. In particular, obesity constitutes a public health problem as it is a risk factor for many disorders, such as diabetes, hypertension, cerebrovascular accident, heart diseases, AD and cognitive impairment (Anstey et al., 2014; Beydoun et al., 2008; Haslam et al., 2006). Obesity and diabetes mellitus have been regarded as AD risk factors, and ample evidence provides both clinical and experimental evidence into how these disorders may course together (De Felice & Lourenco, 2015; Palomera-Ávalos et al., 2016). Thus, different pieces of literature have focused on the study of cognitive decline and neurodegeneration through metabolic stress induced by a high-fat diet (HFD) intervention (Wei et al., 2018). It has been demonstrated that HFD contributes to obesity and increases insulin resistance; signs related to pre-diabetes, cardiovascular pathologies, depressive-like behaviour and mental health problems (Cai & Liu, 2012). Additionally, it has been reported that HFD consumption has a profound impact on brain function, which involves the acceleration of cognitive impairment due to obesity-associated OS, neuronal insulin resistance, microglial

activation and neuroinflammation, which are considered important risk factors for neurodegeneration (Hahm et al., 2020).

Diversely, obesity has been linked to abnormalities in myelin, white and grey matter (Mueller et al., 2014) and cerebral atrophy (Brooks et al., 2013). Particularly, the hippocampus and frontal cortex are very sensitive to those disturbances as Debette et al. (2011) related obesity to altered memory and executive function.

Metabolic derangements, including inflammation, insulin resistance and ER stress are known to underlie glucose intolerance and T2DM, which are present in AD brains (De Felice et al., 2014; Freeman et al., 2014). In line with this, recent data has demonstrated that Aβ actions stimulate pro-inflammatory mechanisms to impair neuronal insulin signalling and trigger stress kinase activation, resulting in synapse and memory deteriorations (De Felice & Lourenco, 2015). Although classical alterations in glucose metabolism have been observed in AD cerebral tissue, metabolic stress also comprises disturbances in proteostasis and activation of signalling pathways that mediate cellular stress (De Felice & Lourenco, 2015). In fact, stress modulates insulin signalling. Initially, catecholamines inhibit insulin but increase glucagon secretion, while later GCs also cause global hyperinsulinemia, which may further fuel insulin resistance. However, it has been demonstrated tissue-specific regulation of insulin signalling by GCs (Gathercole et al., 2011). Interestingly, a recent study demonstrates insulin-signalling potentiation after chronic stress exposure and prevention from fructose diet-induced attenuation of the insulin metabolic pathway (Romic et al., 2020).

1.8 ALZHEIMER'S DISEASE

AD has been a focus of recent research as it represents between 60-80% of total cases of dementia over the world, with the prevalence continuing to grow in part because of the ageing world population. It is one of the most common neurodegenerative diseases and consists in an irreversible, progressive cognitive disorder characterized by a progressive decline in two or more cognitive domains, including memory, language, executive and visuospatial function, movement, personality and behaviour, which in turn causes loss of abilities to perform instrumental and/or basic activities of daily living (Weller & Budson, 2018). The other most common forms of dementia include vascular dementia, Lewy body

dementia and Parkinson's disease with dementia, frontotemporal lobar degeneration and normal pressure hydrocephalus (DeTure & Dickson, 2019).

1.8.1 History

AD was the term coined by Dr. Alois Alzheimer in 1906 when he observed a patient with a striking cluster of symptoms including reduced comprehension and memory, as well as aphasia (difficulty to comprehend or formulate language), disorientation, unpredictable behaviour, paranoia, auditory hallucinations and pronounced psychosocial impairment (Maurer et al., 1997).

Regarding his scientific outcomes, Alzheimer anticipated dementia a century before its discovery in arteriosclerotic patients. Particularly, he could differ between pre-senile dementia and dementia. This was key in the diagnosis of August D, whose case was studied by Alzheimer in Frankfurt from 1901 until she died on 9 April 1906. Auguste D manifested progressive cognitive impairment, focal symptoms, hallucinations, amnestic writing, anxiety, delusions and psychosocial incompetence (Maurer et al., 1997). At necropsy, he detected atrophy of the cerebral cortex accompanied by the loss of cells and a great excrescence of the glia. Moreover, numerous plaques throughout the cortex were associated with excrescences of the vessels (arteriosclerotic changes). This pattern, which could hardly be described as atypical for a patient of 70 to 80 years of age, was present in Auguste D, who was only aged 51 years when she died. He presented these results in a meeting and published them in 1907 under the title "A characteristic serious disease of the cerebral cortex". Despite that, nobody attached importance to Alzheimer's results until 1909 and in 1911 the first case of Alzheimer's disease was described by Gonzalo Lafora, a histopathologist from Madrid and co-worker of Ramon y Cajal. Lafora associated William CF pathology to AD in an article titled "Contribution to the knowledge of Alzheimer's disease or pre-senile dementia with focal symptoms" revised by Alois Alzheimer and published in the Neurology and Psychiatry Journal (Tagarelli et al., 2006).

1.8.2 Risk factors

Given the high personal and social burden, it is imperative to identify a comprehensive list of risk factors (Table 6).

Table 6: *Risk Factors that influenced the onset and development of AD. In bold, those studied in the present thesis work.*

Risk factors				
Physiological	**Genetics**	**Pathologies**	**Environment**	**Lifestyle**
-Age	*-ApoE4* gene	**-T2DM**	-Air pollution	-Low physical activity
-Female gender	*-TREM2* gene	**-**Immune dysfunction	-Noisy environment	-Smoking
-Mild Cognitive Impairment	-Down syndrome	-Cardiovascular disease	-Access to green space	-Alcohol
		-Traumatic brain injury		-Diet
		-Sleep disturbances		**-Stress**
		-Depression		
		-Epilepsy		

1.8.3 Neuropathology

1.8.3.1 Macroscopic features

Cerebral cortical atrophy can be detected, involving the frontotemporal and posterior cortex, and limbic lobe structures (Figure 12) (Rami et al., 2012). Moreover, there is significant atrophy of medial temporal lobe affecting the amygdala and the hippocampus, usually accompanied by temporal horn enlargement (DeTure & Dickson, 2019; Perl, 2010). In addition, another macroscopic trait commonly observed in AD is the loss of neuromelanin pigmentation in the locus coeruleus (Serrano-Pozo et al., 2011). Despite that, none of the macroscopic characteristics is specific to AD as older people may have moderate cortical atrophy, especially affecting frontal lobes, with volume loss mostly affecting white matter (DeTure and Dickson, 2019).

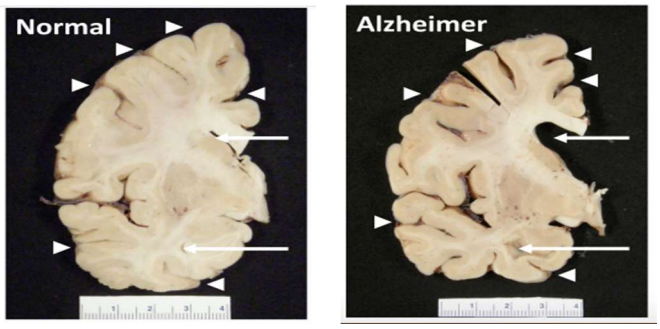

Figure 12. *Anatomy of AD patient's brain (DeTure and Dickson, 2019).*

1.8.3.2 Microscopic features

AD neuropathology is, classically, characterized by two hallmark lesions: the neurofibrillary tangles (NFTs) and the senile plaques. Notwithstanding, other neuropathologic lesions are encountered, like granulovacuolar degeneration and synaptic loss (Perl, 2010).

- **Neurofibrillary tangles**

Neurofibrillary tangles consist of abnormal accumulations of altered phosphorylated Tau protein within the perikaryal cytoplasm of pyramidal neurons (Perl, 2010). In the hippocampus, NFTs appear as parallel, thickened fibril surrounding the nucleus and extending toward the apical dendrite. The primary constituent of the NFT is the microtubule-associated protein Tau, which within NFTs becomes hyperphosphorylated and abnormally folded, so that loose its normal ability to bind and stabilize microtubules in the axon (Figure 13) (DeTure & Dickson, 2019). Tau protein localization is usually the axons of neurons, although it can be found to a lesser extent in dendrites (Ittner & Götz, 2011).

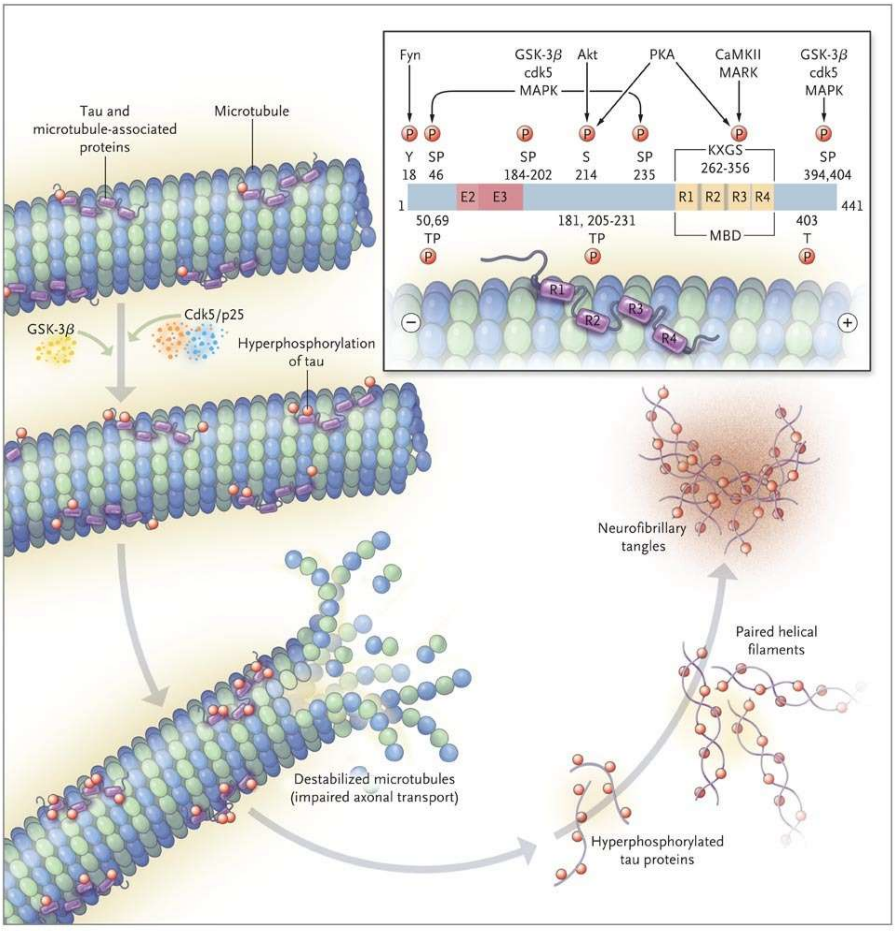

Figure 13. *Phosphorylation sites of Tau protein by the main proline-directed and non-proline directed protein kinases (Querfurth & LaFerla, 2010).*

84 Tau's possible phosphorylation sites have been described, among which 45 are Ser, 35 Thr and 4 tyrosine (Brion et al., 1993). Tau phosphorylation is due to different enzymes divided into two groups:

- Proline-directed protein kinases, which include glycogen-synthase kinase 3 (GSK3) (Ishiguro et al., 1993), among others. It has been described that GSK3-β

activity increases in AD brains as well as hyperphosphorylated Tau (Hernandez et al., 2013).

- Non-proline directed protein kinases.

Several studies have demonstrated a correlation between NFT extent and distribution and the degree of dementia. Although NFTs are a cardinal hallmark of AD, this neuropathologic feature may also be encountered in other disorders, like type C Niemann-Pick disease, post-traumatic dementia and amyotrophic lateral sclerosis (Perl, 2010).

- **Senile plaques**

Senile or neuritic plaques are formed by the extracellular nonvascular accumulation of Aβ40 and Aβ42 peptides, surrounded by abnormally configured neuronal processes or neurites (either dendrites or axons) (Perl, 2010). Additional Aβ peptides containing between 38 and 43 amino acids are also detected, but Aβ42 is the most fibrillogenic, neurotoxic and the predominant component of amyloid plaques in AD, as it can form monomers, oligomers, protofibrils and fibrils (DeTure & Dickson, 2019). Monomers are not pathologic, although oligomers and protofibrils are associated with favoured Tau hyperphosphorylation, proteasome disruption, mitochondrial dysfunction, calcium homeostasis dysregulation, pro-inflammatory cytokines release, synaptic damage and cognitive decline (Hughes et al., 2020; Pákáski & Kálmán, 2008). Importantly, post-mortem evidence asserts that a significant proportion of individuals with brain amyloidopathy did not express disease when alive (Dubois et al., 2016).

Aβ peptides result from abnormal processing of APP, a highly conserved transmembrane glycoprotein that can be catalysed through two mechanisms: non-amyloidogenic and amyloidogenic pathways (Figure 14). The main APP processing pathway is the non-amyloidogenic, which comprises an α-secretase that cleavages the peptide between Lys 16 and Leu 17 releasing a soluble peptide named sAPPα and staying a 10 kDa C-terminal fragment (p3CTF) inside the membrane (Pákáski & Kálmán, 2008). sAPPα may possess neuroprotective actions. Diversely in the amyloidogenic pathway, the β- and γ-secretases act and generate Aβ, an insoluble peptide that aggregates and constitutes senile plaques. Notwithstanding, they may be a consequence of an imbalance in the production and clearance pathways.

As far as Aβ clearance is concerned, in the brain, it is accomplished by several mechanisms that involve non-enzymatic and enzymatic pathways. The first includes interstitial fluid drainage, uptake by microglial phagocytosis and transport across the blood vessels walls into the circulation. Diversely, multiple Aβ-degrading enzymes (ADE) have been identified like neprilysin, insulin-degrading enzyme (IDE), matrix metalloproteinase-9, glutamate carboxypeptidase II and others (Yoon & Jo, 2012). Besides, the cell possesses other mechanisms to eliminate unfolded aberrant proteins, which include autophagy. Recent pieces of evidence demonstrate that defective Aβ autophagy vacuoles clearance creates favourable conditions for Aβ accumulation and increased autophagy flux reduced amyloid burden (Ries & Sastre, 2016). Likewise, astrocytes from AD animal models showed strong expression of microtubule-associated protein light chain 3 (LC3) involved in autophagy, providing a link between this process and inflammation (Pomilio et al., 2016).

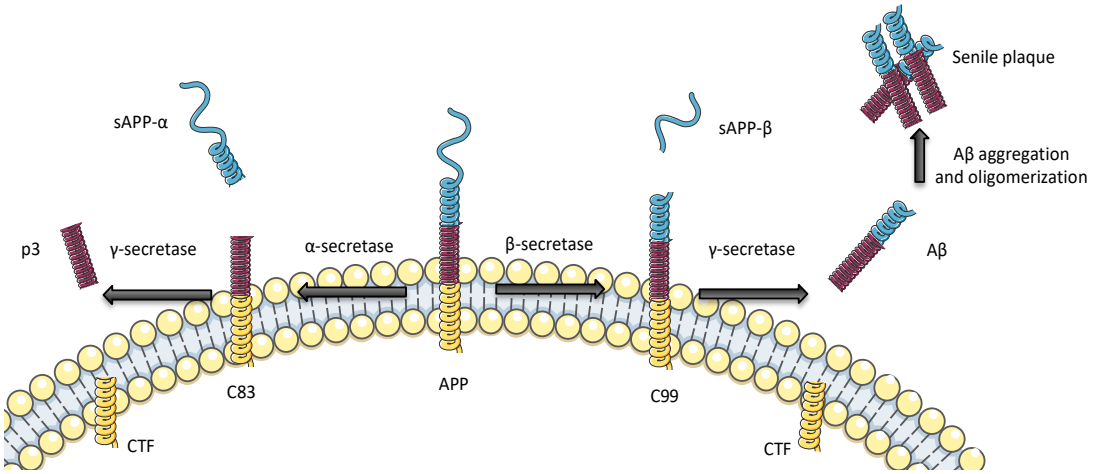

Figure 14. *Scheme of APP processing through non-amyloidogenic pathway represented to the left and to the right, amyloidogenic pathway leading to Aβ accumulation and formation of extracellular senile plaques.*

1.8.4 Classification

Depending on the age at onset, two subtypes of AD have been described:

- **Familial AD**

Familial AD (fAD) represents only between 1 and 5% of AD cases and is caused by dominant autosomal mutations in APP, presenilin 1 (PSEN1) or PSEN2 genes, located in chromosome 21, 14 and 1, respectively (Pimenova & Goate, 2020). FAD cases can appear as early as 20 years, with the average age of onset at 46.2 years (DeTure & Dickson, 2019). Although clinically it does not differ from sporadic AD, it is associated with an aggressive course.

- **Sporadic AD**

Sporadic AD (sAD) accounts for nearly 99% of AD patients, and is caused by a combination of genetic factors and environmental risk factors without documented familial history of AD (Zhang et al., 2020). It courses with deposition of Aβ, Tau hyperphosphorylation, OS, neuroinflammation, cholinergic neuron degeneration, gut microbiota disorders, lipid metabolism abnormalities, autophagy dysfunction, insulin resistance, synapse dysfunction and metal ions disorders (Zhang et al., 2020; DeTure & Dickson, 2019). The onset is typically at 65 years or more.

1.8.5 Hypothesis

Many factors are involved in the pathogenesis of AD. Due to the complexity of human brains and the lack of reasonable animal models, the detailed AD pathogenesis is still unclear (Du et al., 2018). So far, several hypotheses have been described and consequently, many efforts have been made to develop drugs based on these hypotheses.

- **Aβ cascade hypothesis**

Some of the strategies studied consisted of targeting β- and γ-secretase; however, undesirable side effects were the overriding problem. Another approach was targeting Aβ clearance. To sum up, all the Aβ modulator drugs have failed in clinical trials, which insight doubts on this theory (Karran et al., 2011). However, a phase Ib trial of aducanumab (Biogen) showed a positive correlation between brain Aβ levels and disease exacerbation (Sevigny et al., 2017) and fortunately, in early 2020 aducanumab entered phase IIIb. Although it has been planned to apply for regulatory approval in the USA in 2020, its clinical trials are projected to run through September 2023 (Alzforum, 2020).

- **Tau hypothesis**

Strategies developed to target Tau involve blocking its aggregation, utilizing Tau vaccinations, stabilizing microtubules, manipulating kinases and phosphatases that govern Tau modifications. However, most of these efforts have failed in clinical trials, although some drugs are currently under study (Li & Götz, 2017).

- **Oxidative stress hypothesis**

The presence of OS markers in early AD stages indicate that it precedes other hallmarks, although other studies identify OS as a result of brain damage and as a contributor to AD

progression (Unzeta et al., 2016). In consequence, up to date, disease-modifying antioxidant approaches are part of combined treatments (Du et al., 2018).

- **Neuroinflammation hypothesis**

The first strategy consisted of using non-steroid anti-inflammatory drugs (NSAIDs), but they did not show enough benefits in the clinic stage, as the immune response can be either deleterious or beneficial depending on the context (Du et al., 2018). It was hypothesized that such usage might only be beneficial in the very early stages of AD pathogenesis, coincident with initial Aβ deposition, microglial activation and release of pro-inflammatory cytokines. Instead, NSAIDs may produce a detrimental effect owing to their inhibitory activity on already activated microglial cells once Aβ deposition has started (Ali et al., 2019). Of note, the recent advances in understanding the mechanism underlying microglia dysfunction are opening possibilities for new AD therapeutic interventions (Jetvic et al., 2017). For instance, Vorinostat, an HDAC inhibitor, has been yielded efficiency in attenuating human microglial-mediated immune responses, and it is currently under clinical trial (Esposito & Sherr, 2019).

- **Glucocorticoid hypothesis**

Although it is not an AD hypothesis but considering that directly targeting AD hallmarks have not yet been proved effective to redress the insidious shift from physiological to pathological actions along the course of AD, several pieces of evidence have pointed the central role played by the HPA axis, GCs and GR in the aetiology of AD. It has been reported that life events like chronic stress or stress-related disorders may increase the probability to develop AD (Canet et al., 2018). Several strategies directly targeting GR or inhibiting the 11β-HSD1 enzyme were tested for neutralizing the HPA axis dysregulation and GC overproduction (Lesuis et al., 2018; Webster et al., 2017).

1.8.6 Treatment

At present, there is no cure for AD. Few drugs have been approved for the treatment of AD, none of which are modifying or slowing down the disease progression; however, they can relieve some of the symptoms (Oudin, 2020). Available treatments include cholinesterase inhibitors like donepezil for patients with any stage of AD dementia, and memantine for people with moderate-to-severe AD dementia, which has activity as both a non-competitive NMDA receptor antagonist and a dopamine agonist.

1.9 AGEING ANIMAL MODELS – SENESCENCE ACCELERATED MOUSE

The use of humans in ageing research is complicated by many factors, including ethical issues, environmental and social factors, and perhaps most importantly, their long natural lifespan. Diversely, although cellular models of human disease provide valuable mechanistic information, they are limited in that they may not replicate the *in vivo* biology (Mitchell et al., 2015). In consequence, animal models become a useful tool for studying ageing.

Senescence accelerated mice (SAM) are one of the accelerated senescence strains that spontaneously evolved from AKR/J strain inbreeds at Kyoto University (Takeda et al., 1981). After endogamous inbreeding for colony maintenance, it was observed that various litters displayed phenotypic characteristics indicative of rapid ageing, including loss of hair, lordokyphosis, peri-ophthalmic injuries, locomotor activity reduction and lower lifespan, among others. The selection of this phenotype and through siblings' breeding along various generations led to the development of several sublines of SAM prone (SAMP) and SAM resistant (SAMR) mice by 1991 (Figure 15) (Takeda et al., 1991). There are 12 lines; SAMP inbred strains include SAMP1-11, while SAMR inbred strains are SAMR1,4,5. The median survival time of SAMP and SAMR was 9.7 months and 16.3 months, respectively (Takeda et al., 1994).

Figure 15. *SAMR1 and SAMP8 mice at 6 months of age respectively (Delerue et al., 2013).*

SAMP strain has a relatively strain-specific pathologic phenotype, which is characteristic enough to distinguish them. In particular, SAMP8 exhibit significant brain atrophy and age-related deteriorations in memory and learning abilities (Akiguchi et al.,

2017; Pallàs, 2012). Although no specific pathological hallmarks such as senile plaques or NFTs have been found, a variety of age-associated pathomorphological alterations and blood-brain barrier (BBB) dysfunction involving neurons, glia and vessels have been identified in SAMP8 mice brains (Akiguchi et al., 2017; Virgili et al., 2018). Accordingly, some specific reactions of microglia and astrocytes have been found (Kawamata et al., 1998). Thus, the morphological alterations with abnormal glial responses, age-related increases in phosphorylated Tau and early Aβ accumulation in the hippocampus of SAMP8 mice brains may result in impaired memory and behavioural and learning disturbances (Pallàs, 2012).

In line with the ageing phenotype, SAMP8 mice show emotional disorders and abnormal circadian rhythms together to auditory, epigenetic, neuropathologic and neurochemical alterations (Griñán-Ferré et al., 2018; Morley et al., 2012).

1.9.1 APP processing and Aβ accumulation

Apart from Aβ and APP overproduction, SAMP8 mice show increased cortical and hippocampal APP mRNA expression (Morley et al., 2000). Other genes related to AD pathology have been reported to be altered in SAMP8 mice, like PSEN-1 and -2, whose mutations result in an increase of the amyloidogenic APP processing pathway (Kumar et al., 2009). Furthermore, ApoE gene expression has been found to be less expressed in SAMP8 than SAMR1. On the other hand, gene expression reduction of genes involved in Aβ clearance and OS signalling has also been described in SAMP8 compared to SAMR1 (Pallàs, 2012). In addition, several studies have been performed on the regulation of APP in SAMP8 to improve cognitive and behavioural deficits, with different strategies, like the use of specific oligonucleotides to negatively regulate *APP* expression and therefore improve cerebral senescence parameters typical of SAMP8 (Kumar et al., 2000). Other consisted of using miRNAs, post-transcriptional regulators, particularly *miR-16, -31, -144, -135* and *-383* (Griñán-Ferré et al., 2018; Liu et al., 2010; Zhang et al., 2016); or administrating a monoclonal antibody targeting Aβ oligomers (Zhang et al., 2011).

Overall, these results indicate that SAMP8 is a valuable animal model for understanding mechanisms involved in Aβ alteration of AD and that age-related cognitive impairment observed in SAMP8 is dependent of APP processing.

1.9.2 Tau hyperphosphorylation

Hyperphosphorylated Tau protein was found in the hippocampus, cortex and striatum of SAMP8 mice in comparison to SAMR1 mice as early as 5 months of age (Álvarez-García et al., 2006; Canudas et al., 2005). Also, kinases such as GSK3β and cell division protein kinase 5 (CDK5), were highly expressed in SAMP8 than SAMR1 mice in an age-dependent manner (Canudas et al., 2005; Casadesús et al., 2012).

1.9.3 Neuroinflammation

Astrocytic clusters have been identified in SAM mice hippocampus and cortex, although to a greater extent in SAMP8 compared to SAMR1 (Cristòfol et al., 2012). Consistent with this, astrocyte hypertrophy and astrogliosis were observed in SAMP8 mice brains (Han et al., 2010; Morley et al., 2012). In addition, the proliferation of microglia, characteristic of ageing, was observed in cerebral cortex and hippocampus (Kawamata et al., 1998), and activated microglia produces abundant amounts of toxic substances, including free radicals and glutamate (Akiguchi et al., 2017).

1.9.4 Oxidative stress

As discussed earlier, OS is the result of ROS generation exceeding the cellular antioxidant capacity, causing neuronal death and neurodegeneration in the brain. It has been demonstrated that SAMP8 of 4- and 8- weeks showed higher lipid peroxidation and protein carbonilation levels in the cerebral cortex than SAMR1 (Albasanz et al., 2011; Sato et al., 1996). Moreover, excessive oxidation of cortical proteins was found in the brains of aged SAMP8 mice but not of aged SAMR1 (Butterfield et al., 1997). However, literature related to OS in SAMP8 is not always conclusive. For instance, decreased expression of detoxifying enzymes such as manganese SOD (MnSOD), CAT or GPX were found in SAMP8 compared to age-matched SAMR1 mice (Álvarez-García et al., 2006; Schmitt et al., 2012). By contrast, Griñán-Ferré et al. (2016) described higher protein levels in SAMP8 in comparison with SAMR1 mice; those discrepancies can be explained by the age when determinations were performed.

1.9.5 Glucose metabolism

Studies have demonstrated that glucose metabolism is decreased in SAMP8 aged 2 months (Cuesta et al., 2013). Additionally, it has been described that SAMP8 mice brain

tissue shows a transient enhancement of anaerobic glycolysis in 2 months-old mice and a subsequent decline in mitochondrial function in comparison with SAMR1 mice (Omata et al., 2001). Moreover, another study revealed a correlation between glucose metabolism impairment and the severity of the learning deficits (Akiguchi et al., 2017; Mehla et al., 2014).

2. OBJECTIVES

Ageing, stress and obesity are three factors that define modern society. Modern society has to cope with higher levels of stress than past societies. Despite not having to worry about hunting and whether we will be attacked for an animal, humans are subjected to various stressors; not only psychological but also physical, environmental and social. For instance, high workloads, economic difficulties, relationships, nutrition, media overload, sleep deprivation and social pressure to boost our careers, among others. The continued presence of these stressful situations influences our health and also predicts our ageing. Additionally, although life expectancy has increased, age-related pathologies, such as degenerative diseases and in particular AD, have become a problem for the health sector, as it has been estimated that people over the age of 60 will increase from 12 to 22 % in the world in the following years. Consequently, finding strategies that entail therapeutic benefits for patients and reduction of the impact of ageing on health, particularly brain ageing, is of paramount importance and constitute an exciting challenge. In this sense, the use of drugs that modify the main pathways activated by stress and, therefore increase individuals' adaptive ability arises as an optimal strategy for promoting healthy ageing.

In this context, as ageing is the major risk factor for age-related neurodegenerative disorders, the general aim of the present doctoral thesis has been to study the mechanisms involved in stress-induced neurodegeneration and evaluate the neuroprotective effect of RL-118 drug, an 11β-HSD1 inhibitor, in a senescence animal model.

In order to pursue this aim, several specific objectives have been set up:

1. Assess the protective effect of RL-118 on cognition in SAMP8 mice aged 12 months and reveal which underlying mechanisms might modulate it.

    - To evaluate cognitive function and behavioural alterations.
    - To investigate the protective effect of RL-118 in the process of autophagy.
    - To study the changes induced by RL-118 treatment in APP processing and tau hyperphosphorylation.
    - To elucidate the changes in antioxidant enzymes and cerebral inflammatory markers.

- To determine the correlation between autophagy markers and cognitive parameters, as well as between autophagy, pro-inflammatory and OS markers.

2. Investigate the effects on brain function of a stressful lifestyle, by chronic mild stress, in animal models prone and resistant to accelerated senescence, SAMP8 and SAMR1 respectively.

    - To study different epigenetic marks related to learning and memory.
    - To analyse the cerebral OS state and antioxidant enzymes changes.
    - To assess the effects of CMS on neuroinflammation and autophagy.
    - To determine the effects of CMS on different pathways implicated in neurodegeneration.
    - To evaluate cognitive performance and behavioural alterations.

3. Determine the target engagement between RL-118 and 11β-HSD1 and evaluate whether 11β-HSD1 inhibition by RL-118 is able to face the detrimental effects of CMS in SAMP8 mice aged 6 months.

    - To investigate RL-118 target engagement.
    - To study different epigenetic marks related to learning and memory.
    - To assess the effects of CMS and RL-118 treatment on neuroinflammatory pathways.
    - To elucidate cellular OS state and determine antioxidant mechanisms changes after CMS exposure and 11β-HSD1 inhibition.
    - To describe the effects of CMS and RL-118 treatment on autophagy and synaptic plasticity markers.
    - To analyse different pathways altered implicated in neurodegeneration by CMS and potential reversal by 11β-HSD1 inhibition.
    - To evaluate cognitive performance and behavioural alterations.

4. Study the detrimental effect of metabolic stress by HFD and the beneficial effect of 11β-HSD1 inhibition in SAMP8 mice aged 6 months.

    - To elucidate the inhibitory effect of RL-118 by determining and comparing corticosterone levels in blood and brain tissue.
    - To determine the glucose tolerance and triglyceride levels following HFD feeding and RL-118 treatment.
    - To analyse the impact of HFD-induced metabolic stress and RL-118 treatment on different pathways implicated in neurodegeneration and energy metabolism.
        - To investigate the effects of HFD and 11β-HSD1 inhibition on cerebral OS state and antioxidant mechanisms.
        - To evaluate different neuroinflammatory pathways activated after HFD-induced metabolic stress and 11β-HSD1 inhibition effects.
        - To analyse the molecular changes produced by metabolic stress and RL-118 treatment regarding ER stress response and autophagy.
        - To evaluate the deleterious effect of HFD on cognitive abilities and behavioural performance and potential reversal by RL-118 treatment.

3. METHODS AND RESULTS

3.1 11β-HSD1 Inhibition by RL-118 Promotes Autophagy and Correlates with Reduced Oxidative Stress and Inflammation, Enhancing Cognitive Performance in SAMP8 Mouse Model

Adapted from: **Puigoriol-Illamola, D.**[1,2], Griñán-Ferré, C.[1,2], Vasilopoulou, F.[1,2], Leiva, R.[3,4], Vázquez, S.[3,4], Pallàs, M[1,2]. 11β-HSD1 inhibition by RL-118 promotes autophagy and correlates with reduced oxidative stress and inflammation, enhancing cognitive performance in SAMP8 mouse model

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3.1.1 Abstract

With the increase in life expectancy, the study of age-related diseases and the development of different strategies to deal with them become mandatory. Among the features of ageing, disturbances in the GC stress response and individual's adaptive abilities appear to increase the vulnerability of the elderly to age-related pathologies. In fact, elevated GC exposure is widely accepted as a key factor in age-related cognitive decline in rodents and humans. Of note, 11β-HSD1 is a key enzyme controlling GC action as it catalyses the conversion of 11β-dehydrocorticosterone to corticosterone in mice (i.e. from inactive to active GC forms). Compelling evidence has demonstrated prolonged GC excess implication in neurodegenerative processes and cognitive impairment. Therefore, the study of the effects on age-related alterations due to GC attenuation by 11β-HSD1 inhibition results essential. The study aimed to assess the potential protective effect of RL-118, an 11β-HSD1 inhibitor, on cognitive performance and which molecular mechanisms were modified in an ageing animal model.

Here, we determined the effect of RL-118 administered to 12-month-old SAMP8 mice that display neuropathologic Alzheimer's disease-like alterations and are widely used as a rodent model of cognitive dysfunction. Behavioural tests were conducted – particularly the open field and object location tests – and neurodegenerative molecular markers were studied.

RL-118 treatment increased locomotor activity and improved cognitive performance. Likewise, 11β-HSD1 inhibition by RL-118 induced changes in hippocampal autophagy markers such as Beclin1, LC3B, AMPKα, and mTOR, indicating enhancement of the intracellular degradation system. In line with the autophagic increase, a diminution in phosphorylated tau species (Ser 396 and Ser 404) jointly with an increase in non-amyloidogenic APP processing pathway mediators suggested that the increase of the removal of abnormal proteins by autophagy might underlie the neuroprotective effect of the 11β-HSD1 inhibitor studied. In addition, gene expression of OS and inflammatory markers, such as *heme oxygenase 1* (*Hmox1*), *aldehyde dehydrogenase 2* (*Aldh2*), *Il-1ß, and C-C motif chemokine ligand 3 (Ccl3)* were reduced in treated mice in comparison to control group. Consistent with this, we further demonstrated a significant direct correlation between autophagy markers and cognitive improvement and significant inverse correlation with autophagy, OS, and neuroinflammatory markers.

We concluded that inhibition of 11β-HSD1 by RL-118 prevented neurodegenerative processes and cognitive decline, acting on the autophagy process, being an additional neuroprotective mechanism involved in 11β-HSD1 inhibition effects not described previously.

3.1.2 Introduction

With the increase in life expectancy, ageing and age-related cognitive impairments are becoming one of the most important challenges for human health. Ageing is a multifaceted process characterized by an intricate and irreversible accumulation of physiological changes and is associated with an increase in transcriptional noise, aberrant protein production, altered intercellular communication, cellular senescence and loss in proteostatic mechanism, among others (López-Otín et al., 2013).

Cellular proteostasis loss has been proposed as one of the primary molecular changes that induce progressive loss of physiological integrity in ageing (López-Otín et al., 2013). Damaged proteins can be degraded through the ubiquitin proteasome system (UPS) or through the autophagy lysosome pathway (Boya et al., 2013; Vílchez et al., 2014). Autophagy is a self-degradative process involved in elimination of proteins and organelles, cellular remodelling, and survival during starvation (Levine, 2005). Beclin1, mammalian target of the rapamycin (mTOR), microtubule-associated protein 1 light chain 3B (LC3B) and p62 proteins have a key role in this process (Son et al., 2012). Moreover, autophagy has been postulated to participate in the degradation of extra- and intracellular Aβ deposition and neurodegeneration (Yu et al., 2004; Komatsu et al., 2006).

On the other hand, there is overwhelming evidence that brain tissue is exposed to OS in cognitive decline and age-related neurodegeneration. Evidences are manifested through high levels of oxidized proteins, advanced glycation and lipid peroxidation end products, toxic species formation, such as peroxides, alcohols and aldehydes; and oxidative modifications in nuclear and mitochondrial DNA (Gella & Durany, 2009). Cellular damage by OS is one factor that induces cell senescence promoting protein modification and proteolysis mechanism alterations (Höhn et al., 2016; López-Otín et al., 2013). Moreover, OS is related to inflammaging and development of cognitive impairment and anxious behaviour. Of note, several studies have showed that pro-inflammatory cytokines release is higher when OS increases (Anderson et al., 2013; Casadesús et al., 2002; Terao A, et al.,

2002; Ye et al., 1999). Interestingly, defects in the autophagy machinery led to increased mitochondrial dysfunction and, consequently, increased levels of oxidative stress (Mathew et al., 2009).

Recent studies suggest the excess of GCs exerts deleterious effects on the brain, particularly on the hippocampus, and causes impaired spatial memory, a key feature of age-related cognitive dysfunction (Lara et al., 2013), in rodents and humans, being part of altered intercellular communication described as one of the nine hallmarks of aging (López-Otín et al., 2013). 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) is a key enzyme that mediates the intracellular conversion of cortisone to cortisol in humans (11β-dehydrocorticosterone to corticosterone in mice), the active form of GCs, thus amplifying steroid action. High levels of GCs have been found in elderly who exhibit learning and memory impairments and correlate with higher hippocampal atrophy. Increased circulating cortisol levels are detected in patients with Alzheimer's disease (AD) causing faster disease progression (De Quervain et al., 2004). AD is the most common form of dementia and represents a progressive brain disorder characterized by loss of cognitive functions (i.e. memory impairments) and behavioural abilities. AD presents three representative pathological hallmarks: synapse loss, extracellular senile plaques (SPs) and intracellular neurofibrillary tangles (NFTs). Furthermore, it has been demonstrated that aged mice with cognitive deficits showed increased gene expression of 11β-HSD1 in the hippocampus and parietal cortex (Holmes et al., 2010). Likewise, overexpression of this enzyme displays a similar premature memory decline (Yau et al., 2001). In line with this, some preclinical studies demonstrate that 11β-HSD1 inhibition improved cognition and AD hallmarks suggesting a neuroprotective effect (Yau et al., 2001, 2007; Mohler et al., 2011).

Design and chemical structure of the compound RL-118 [(4-azatetracyclo[5.3.2.$0^{2,6}$.$0^{8,10}$]dodec-4yl) (cyclohexyl)methanone], one of the first pyrrolidine-based 11β-HSD1 inhibitor, was rationally developed using the X-ray structure of human enzyme 4BB6 (Goldberg et al., 2012; Leiva et al., 2017). Its potency against the human and murine enzyme (IC_{50} values of 29 and 81 nM, respectively), its microsomal stability (94% and 93% in HLM and MLM, respectively) and its proven in vivo efficacy (Leiva et al., 2017) makes RL-118 an interesting drug to further study its potential neuroprotective effects on an age-related cognitive decline mice model.

To explore the possible RL-118 neuroprotective role by 11β-HSD1 inhibition, we employed a senescence accelerated mice-prone strain, SAMP8, a well-characterized model for studying neurodegeneration and cognitive deficits related to aging, as a model of early AD (Morley et al., 2012; Nomura et al., 1999; Pallàs et al., 2008). Therefore, the present study sought to assess how the reduction of active GCs regeneration, through the inhibition of 11β-HSD1 by RL-118, leads to neuroprotective effects and which underlying mechanisms might modulate the reduction of AD hallmarks, opening new ways to face challenges in AD prevention and treatment.

3.1.3 Material and methods

Animals

Female SAMP8 mice (n=14) (12-months-old) were used to carry out cognitive and molecular analyses. We divided these animals into two groups: Control (SP8 Ct, n=6) and treated with RL-118, an 11β-HSD1 inhibitor (SP8 treated, n=8). RL-118, 21 mg/kg was administered through drinking water for 4 weeks. Animals had free access to food and water and were kept under standard temperature conditions (22±2°C) and 12h:12h light-dark cycles (300 lx/0 lx).

Studies and procedures involving mice brain dissection and subcellular fractionation were performed in accordance with the institutional guidelines for the care and use of laboratory animals established by the Ethical Committee for Animal Experimentation at the University of Barcelona.

Behavioural tests

Open Field Test

The Open Field Test (OFT) is an experiment used to assay general locomotor activity and anxiety in rodents. The test is based on the fear and anxiety of rodents to be in opened and luminous spaces. The OFT apparatus was a white polywood box (50x50x25 cm). The floor was divided in two areas defined as centre zone and periphery zone (15 cm between centre zone and the wall). Behaviour was scored with SMART® ver.3.0 software and each trial was recorded for later analysis, utilizing a camera situated above the apparatus. Mice were placed at the centre and allowed to explore the box for 5 min. Afterwards, mice were

returned to their home cages and the OFT apparatus was cleaned with 70% ethanol. The parameters scored included centre staying duration, number of rears, defecations and the distance travelled, calculated as the sum of total distance travelled in 5 min.

Object Location Test

The Object Location Test (OLT) assesses cognition deficits and evaluates novel chemical entities by their effects on cognition, specifically spatial memory and discrimination (Murai et al., 2007). This task involves exploiting rodent's ability to recognize when an object has been relocated, and they are inherently stressless. The test was performed during 4 days in a wooden box (50x50x25 cm), in which three walls were white but one black. The first day, the box was empty and the animals just habituated to the open field cage for 10 min. The second day, two objects were placed in front of the black wall, equidistant from each other and from the wall. Objects were 10 cm high and had different shapes, but were composed of similar materials. The animals were placed in the open field cage and allowed to explore both, objects and surroundings, for 10 min. Afterwards, mice were returned to their home cages and the OLT apparatus was cleaned with 70% ethanol. The third day, one object was moved in front of the white wall to test spatial memory. Trials were recorded using a camera mounted above the working area and the total exploration time was determined by scoring the amount of time (s) spent sniffing the two objects in the new (novel) and old (familiar) locations. In order to analyse the cognitive performance, a location index (%) was calculated as follows: (Tnovel x 100)/(Tnovel + Tfamiliar), where the Tnovel is the time spent exploring the objects in the new location and Tfamiliar is the time spent exploring the object in the old location.

Brain processing

Mice were euthanized one day after the behavioural test finished by cervical dislocation. Brains were immediately removed from the skull. Hippocampus were then isolated and frozen on powdered dry ice. They were maintained at -80°C for further use. Tissue samples were homogenized in lysis buffer containing phosphatase and protease inhibitors (Cocktail II, Sigma). Total protein levels were obtained and protein concentration was determined by the method of Bradford.

Protein levels determination by Western blotting

For Western Blotting (WB), aliquots of 15 µg of hippocampal protein extracted with lysis buffer composed by Tris HCl pH7.4 50mM, NaCl 150mM, EDTA 5mM and 1X-Triton X-100 were used. Protein samples were separated by SDS-PAGE (8-12%) and transferred onto PVDF membranes (Millipore). Afterwards, membranes were blocked in 5% non-fat milk in 0,1% Tween20 TBS (TBS-T) for 1 h at room temperature, followed by an overnight incubation at 4°C with the primary antibodies listed in Table S1.

Afterwards, membranes were washed and incubated with secondary antibodies for 1 h at room temperature. Immunoreactive proteins were observed with a chemiluminescence-based detection kit, following the manufacturer's protocol (ECL Kit, Millipore) and digital images were acquired using a ChemiDoc XRS+ System (BioRad). Semi-quantitative analyses were carried out using ImageLab software (BioRad) and results were expressed in Arbitrary Units (AU), considering control protein levels as 100%. Protein loading was routinely monitored by immunodetection of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-tubulin.

RNA extraction and gene expression determination

Total RNA isolation was carried out by means of TRIzol reagent following the manufacturer's instructions. RNA content in the samples was measured at 260 nm, and the purity of the samples was determined by the A260/280 and A260/230 ratios in a NanoDrop™ ND-1000 (Thermo Scientific). Reverse transcription-polymerase chain reaction (RT-PCR) was performed as follows: 2 µg of messenger RNA (mRNA) was reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time quantitative PCR (qPCR) was used to quantify the mRNA expression of inflammatory genes such as interleukin 1β (*Il-1β*), C-C-motif ligand 3 (*Ccl3*), heme oxygenase 1 *(Hmox1*) and aldehyde dehydrogenase 2 (*Aldh2*) (Table S2). SYBER Green real-time PCR was performed on the Step One Plus Detection System (AppliedBiosystems) employing the SYBR Green PCR Master Mix (AppliedBiosystems). Each reaction mixture contained 7.5 µL of complementary DNA (cDNA), whose concentration was 2 µg, 0.75 µL of each primer (whose concentration was 100 nM), and 7.5 µL of SYBR Green PCR Master Mix (2X) and for TaqMan gene expression assays (Applied Biosystems), each 20 µL of TaqMan reaction, 9 µL cDNA (25 ng) was mixed with 1 µL 20x probe of TaqMan Gene Expression Assays and 10 µL of 2x TaqMan Universal PCR Master Mix.

Data was analysed using the comparative Cycle threshold (Ct) method (ΔΔCt) where the actin transcript level was used to normalize differences in sample loading and preparation. Each sample (n = 4-5) was analysed in duplicate, and the results represented the n-fold difference of transcript levels among different samples.

Data analysis

Data is expressed as the mean ± Standard error of the mean (SEM) from at least six to eight samples. Data analysis was conducted using GraphPad Prism ver. 6 statistical software. Means were compared with the unpaired Student's *t*-test for independent samples. Statistical significance was considered when p values were <0.05. Statistical outliers were detected through Grubss' test and were removed from analysis.

3.1.4 Results

Effect of the 11β-HSD1 inhibitor, RL-118, in spontaneous activity, anxiety and object location memory in female SAMP8

The OFT was used to determine anxious behaviour in mice. Results obtained, indicate that the 11β-HSD1 inhibitor, RL-118, treated group exhibited a significant increase in locomotor activity as well as spent more time in the central zone compared to control group. In addition, the treated group showed significant higher number of rears and freezing behaviour but reduced defecation events compared to SP8 Ct group (Figs. 1 A-D). All results and statistical scores obtained in the OFT are listed in Table S3. Results for the RL-118-treated group showed a clear and significant behaviour change in comparison with the SP8 Ct group, indicating that this compound reduced the stress feeling and the anxiety, increasing locomotor activity.

Referring to cognitive performance, the OLT demonstrated that RL-118-treated group exhibited an improved spatial memory in reference to control group, because the location index (%) was significantly higher in the treated animals in comparison with control group, showing that treated group distinguish the location of the objects (Fig. 1 E).

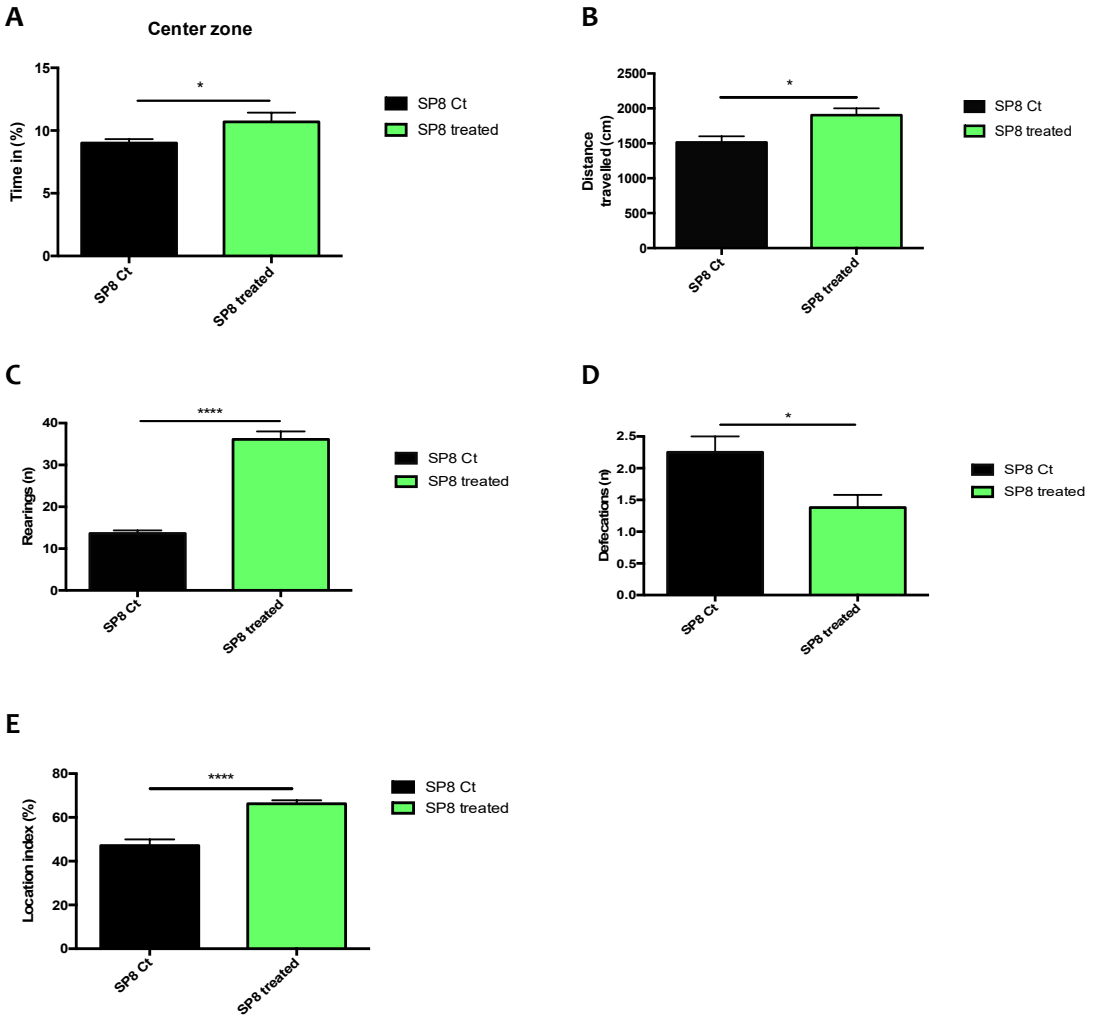


Figure 1. Results of Open Field Test (OFT) in female mice with 12 months old, control SAMP8 (SP8 Ct) and SAMP8 treated with RL-118 (SP8 treated). *Percentage of time spent in Center zone (A), Distance travelled (B), Rearing (C), Defecations (D).* ***Results of Object Location Test (OLT) (E) in female mice with 12 months old (SP8 Ct, SP8 treated).*** *Data represented as observed mean ± SEM; SP8 Ct n = 6, SP8 treated n = 8. Statistics: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.*

Autophagy process modification by 11β-HSD1 inhibition in the hippocampus of female SAMP8

Beclin1, LC3B, p62 and mTOR protein levels, as well as AMP-activated protein kinase α (AMPK-α) activation were determined by Western blotting as indicators of the autophagic flux. 11β-HSD1 inhibitor, RL-118 treatment significantly increased Beclin1 protein levels (Figs. 2 A, B), whereas p62 protein levels were higher although did not reach statistical significance (Figs. 2 A-D). Moreover, the pharmacological treatment increased LC3-I and II ratio compared to control group (Figs. 2 A, C). There were not differences in

pAMPKα/AMPKα ratio (Figs. 2 A, E) but we found a reduction in mTOR protein levels in the treated animals (Figs. 2 A, F).

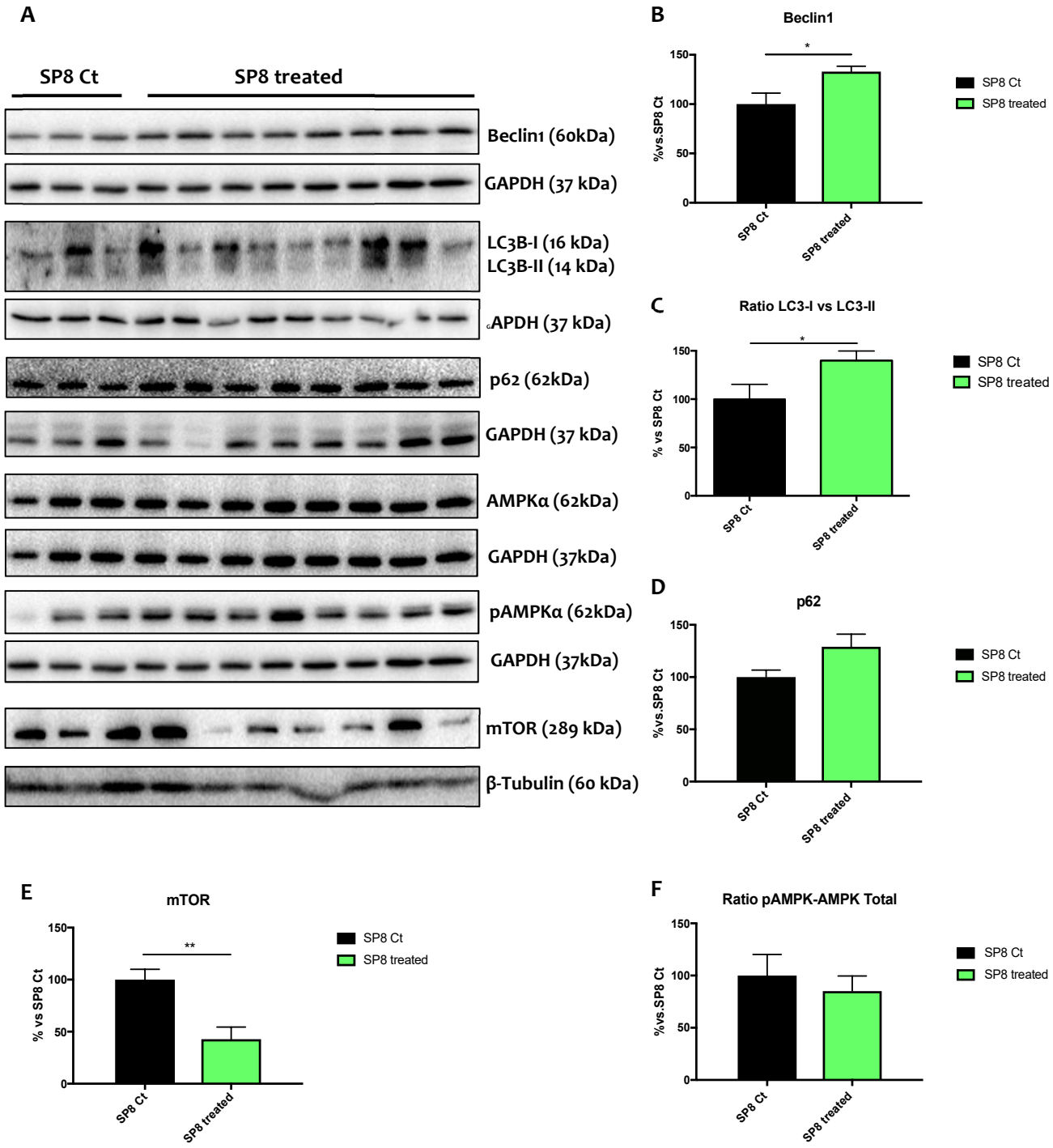


Figure 2. Autophagy markers in female mice with 12 months old, control SAMP8 (SP8 Ct) and SAMP8 treated with RL-118 (SP8 treated). *Representative Western blot for Beclin1, LC3, p62, AMPKα and mTOR protein levels (A) and their respective quantification (B, C D, E, F). Values in bar graphs are adjusted to 100% for protein of control SAMP8 (SP8 Ct). Values are mean ± SEM; SP8 Ct n = 6, SP8 treated n = 8. Statistics: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs SP8 Ct.*

Effect of RL-118, 11β-HSD1 inhibitor, in neurodegenerative, oxidative stress and neuroinflammation markers in the hippocampus of female SAMP8

11β-HSD1 inhibition by RL-118 increased a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) and soluble APPα (sAPPα) protein levels compared to SP8 Ct (Figs. 3 A-C). A significant decrease in tau hyperphosphorylation in Ser 396 and Ser 404 in SP8 treated compared to SP8 Ct, was found (Figs. 3 D-F).

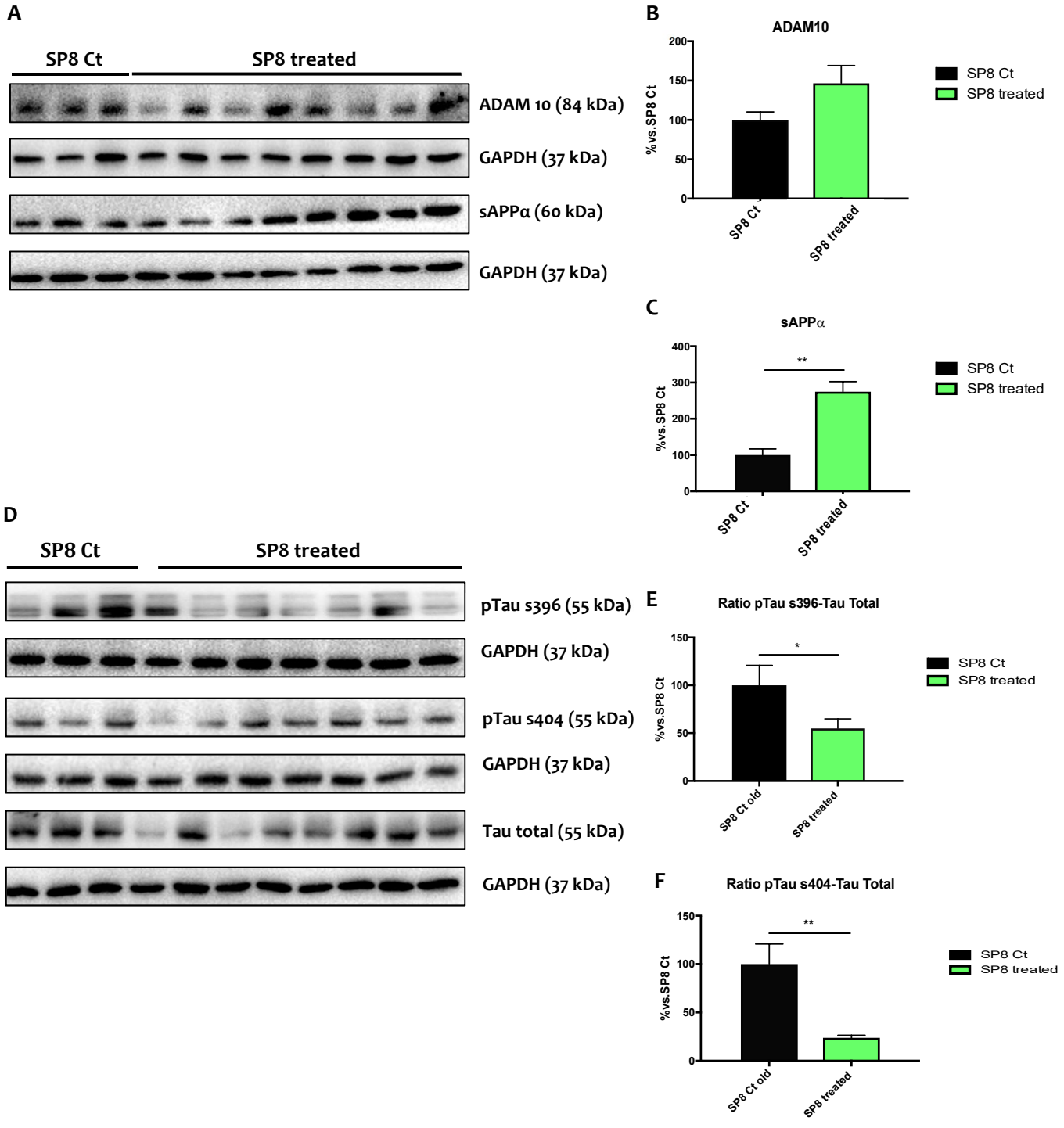


Figure 3. Alzheimer's Disease markers in female mice with 12 months-old control, SAMP8 (SP8 Ct) and SAMP8 treated with RL-118 (SP8 treated). *Representative Western blot for ADAM10 and sAPPα protein levels (A) and quantification (B, C), Tau total protein levels and pTau ser396 and pTau ser404 protein levels (D) and quantification (E, F). Values in bar graphs are adjusted to 100% for protein of control SAMP8 (SP8 Ct). Values are the mean ± SEM; SP8 Ct n = 6, SP8 treated n = 8. Statistics: *p<0.05; **p<0.01 vs SP8 Ct.*

Neuroimmunological responsive parameters were determined by gene expression of *Il-1β* and *Ccl3*, and also p65 as active form of NF-κβ protein expression. There was a significant increase in p65 content in SP8 treated group (Figs. 4 A-B). Moreover, SP8 treated group showed a significant lower gene expression of *Il-1β* and *Ccl3* in hippocampus (Figs. 4 C, D). Response to OS was determined by *Hmox1* and *Aldh2* gene expression. SP8 treated group showed lower gene expression levels of *Hmox1* and *Aldh2*, suggesting a lower OS state than in SP8 Ct group (Figs. 4 E, F).

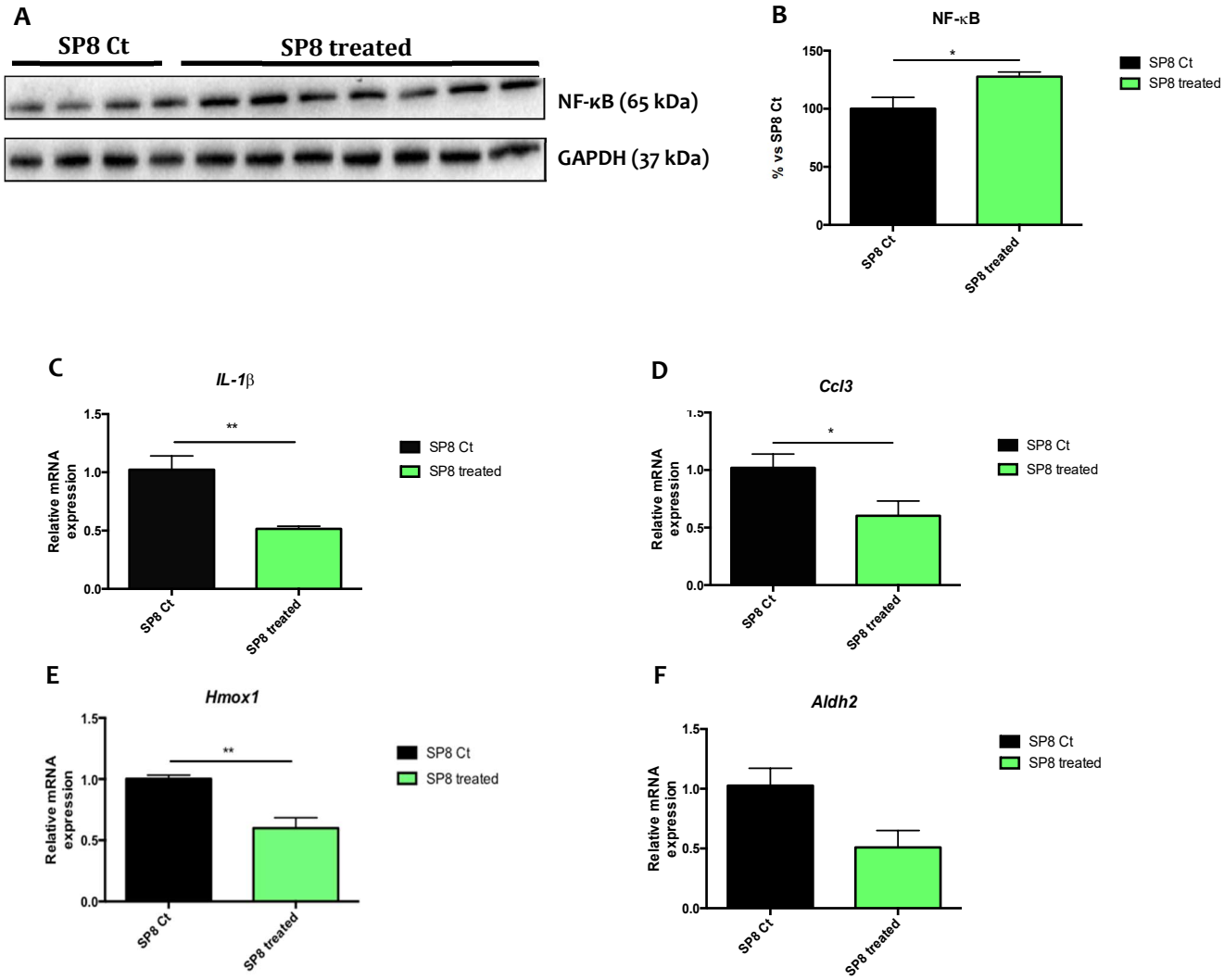


Figure 4. Inflammatory and OS markers in female mice with 12 months-old control SAMP8 (SP8 Ct) and SAMP8 treated with RL-118 (SP8 treated). *Representative Western Blot for NF-kβ protein levels (A) and quantification (B). Values in bar graphs are adjusted to 100% for protein levels of control SAMP8 (SP8 Ct). Representative gene expression for Il-1β (C), Ccl3 (D), Hmox1 (E), Aldh2 (F). Gene expression levels were determined by real-time PCR. Values are mean ± SEM; SP8 Ct n = 6, SP8 treated n = 8. Statistics: *p<0.05; **p<0.01; ***p<0.001 vs SP8 Ct.*

Relationship between autophagy and cognitive performance

There was a significant positive correlation between Beclin1 protein levels, LC3B ratio and Location index (%) during the OLT [r^2 = 0.7459, p = 0.0084], [r^2 = 0.7347, p = 0.0155], respectively, indicating that higher levels of Beclin1 and LC3B ratio in the hippocampus are associated with best location memory. Moreover, a significant negative correlation between mTOR protein levels and Location index (%) during the OLT [r^2 = -0.6938, p = 0.05] was found (Figs. 5 A-C). In contrast, we did not find a significant correlation between Beclin1 levels and centre zone (%) during OFT [r^2=0.4636, p = 0.1509] (Fig. 5D).

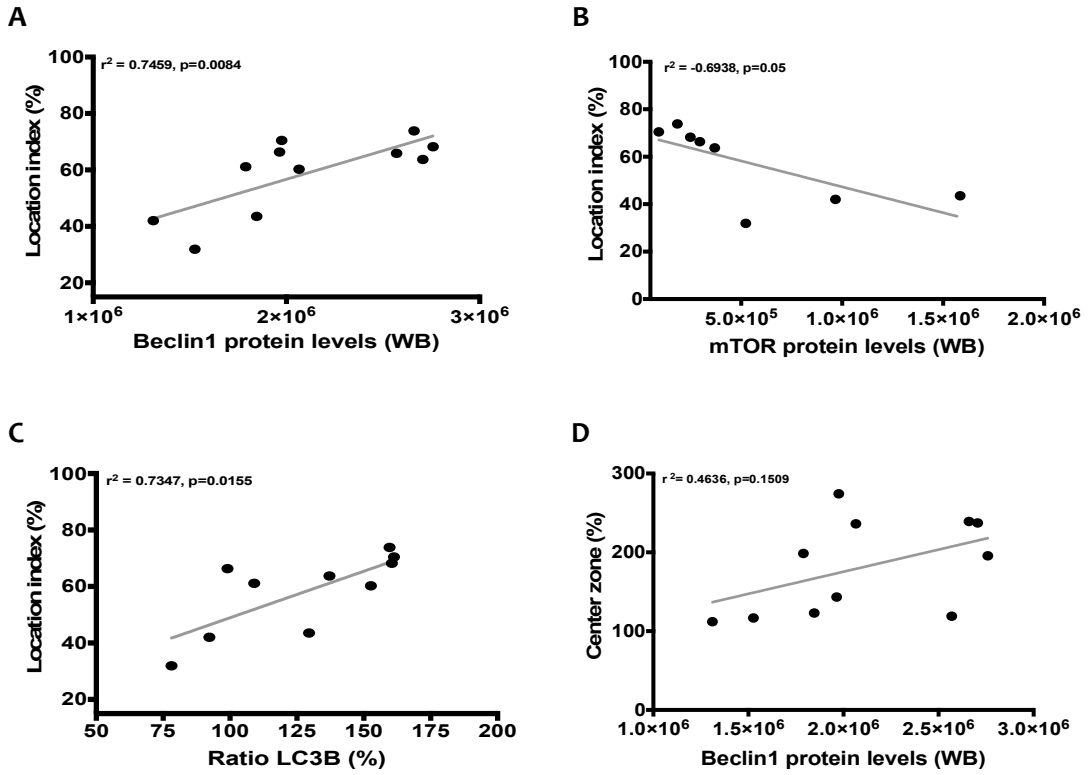
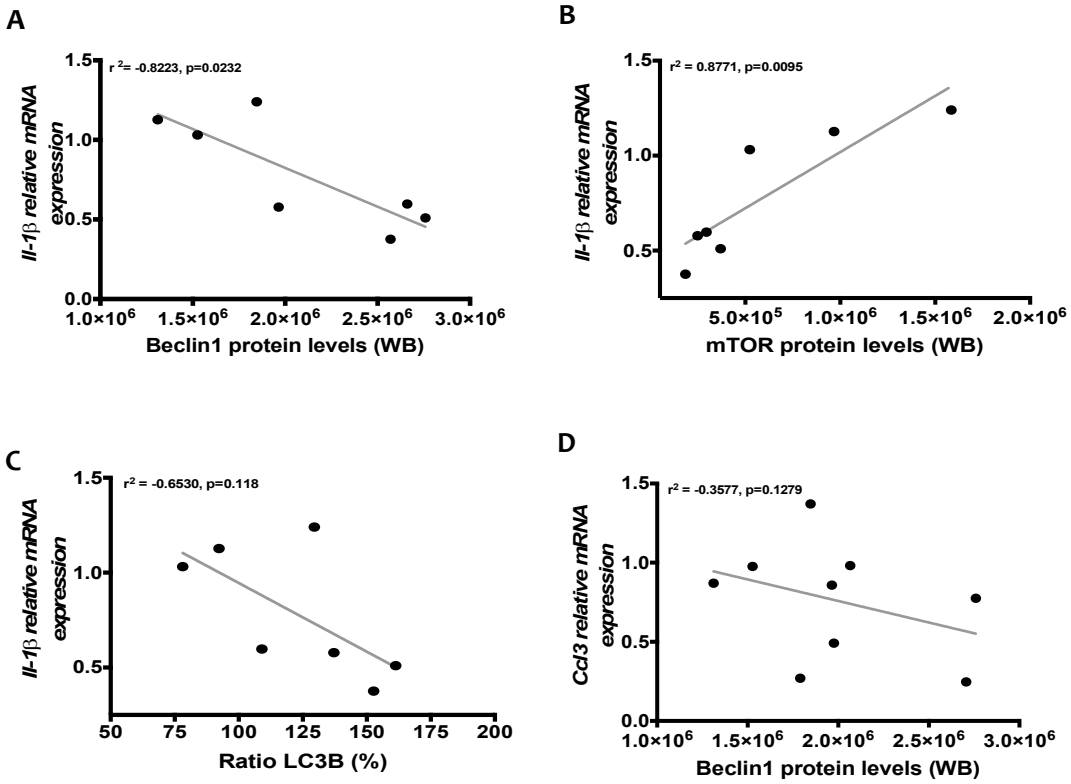


Figure 5. Correlations between Beclin1 (A), mTOR (B) and LC3B (C) ratio with Location index (%) in female SAMP8 with 12-months old. Correlation between Beclin1 and Centre zone (%) (D). *Pearson correlations were performed between protein levels of autophagy markers in*

hippocampus and behavioural test parameters in both groups (n = 14). r^2 and p values were indicated on graphs. The levels of significance were p<0.05.

Relationship between autophagy and neuroinflammation

There was a significant negative correlation between Beclin1 protein levels and *Il-1β* gene expression [r^2 = -0.8223, p = 0.0232]. However, we also did not find a significant negative correlation between LC3B ratio and *Il-1β* gene expression [r^2 = -0.6530, p = 0.118], indicating that higher levels of Beclin1, although not LC3B ratio, in the hippocampus is associated with reduced gene expression of the inflammatory marker studied. Moreover, a significant positive correlation between mTOR protein levels and *Il-1β* gene expression [r^2 = 0.8771, p = 0.0095] was found (Figs. 6 A-C). Interestingly, we found a significant positive correlation between mTOR protein levels and *Ccl3* gene expression [r^2 = 0.6581, p = 0.05], indicating that reduced mTOR protein levels in the hippocampus are associated with reduced gene expression of this inflammatory marker (Fig. 6 E). In contrast, no significant correlations between Beclin1 protein levels, LC3B ratio and *Ccl3* gene expression respectively, were found (Figs. 6 D, F).



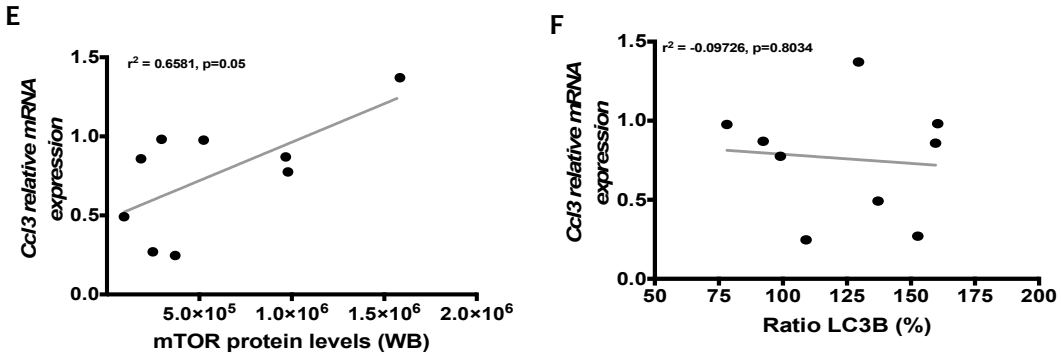
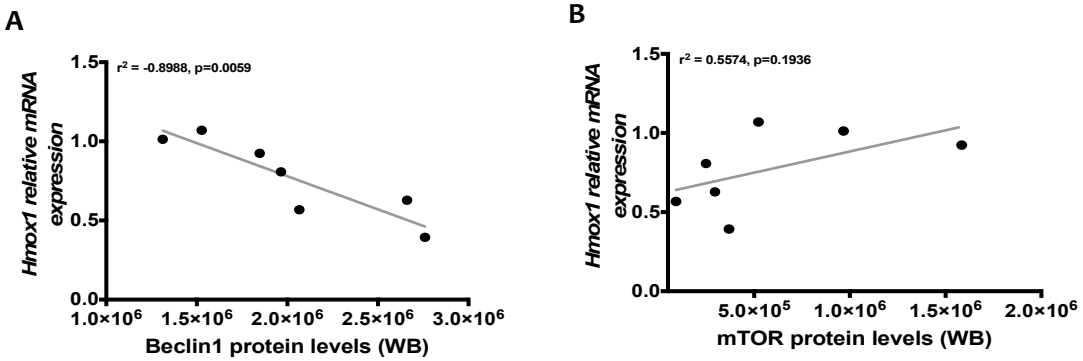


Figure 6. Correlations between Beclin1 (A), mTOR (B) and LC3B ratio (C) with Il-1β gene expression. Correlations between Beclin1 (D), mTOR (E) and LC3B ratio (F) with Ccl3 gene expression in female SAMP8 with 12-months old. *Pearson correlations were performed between protein levels of autophagy markers and inflammatory gene expression in hippocampus in both groups (n = 14). r^2 and p values were indicated on graphs. The levels of significance was p<0.05.*

Relationship between autophagy and oxidative stress

There were significant negative correlations between Beclin1 protein levels, LC3B ratio and *Hmox1* gene expression [r^2 = -0.8988, p = 0.0059], [r^2 = -0.8870, p = 0.078], indicating that higher levels of Beclin1 and LC3B ratio in the hippocampus are associated with reduced gene expression of OS markers (Figs. 7 A, C). In addition, we found a significant positive correlation between mTOR protein levels and *Aldh2* gene expression [r^2 = 0.7172, p = 0.0296], indicating that reduced protein levels of mTOR in the hippocampus are associated with reduced gene expression of the OS marker studied (Fig. 7 E). No significant correlations were found between mTOR protein levels and *Hmox1*, neither between Beclin1 protein levels, LC3B ratio and *Aldh2* (Figs. 7 B, D, F).



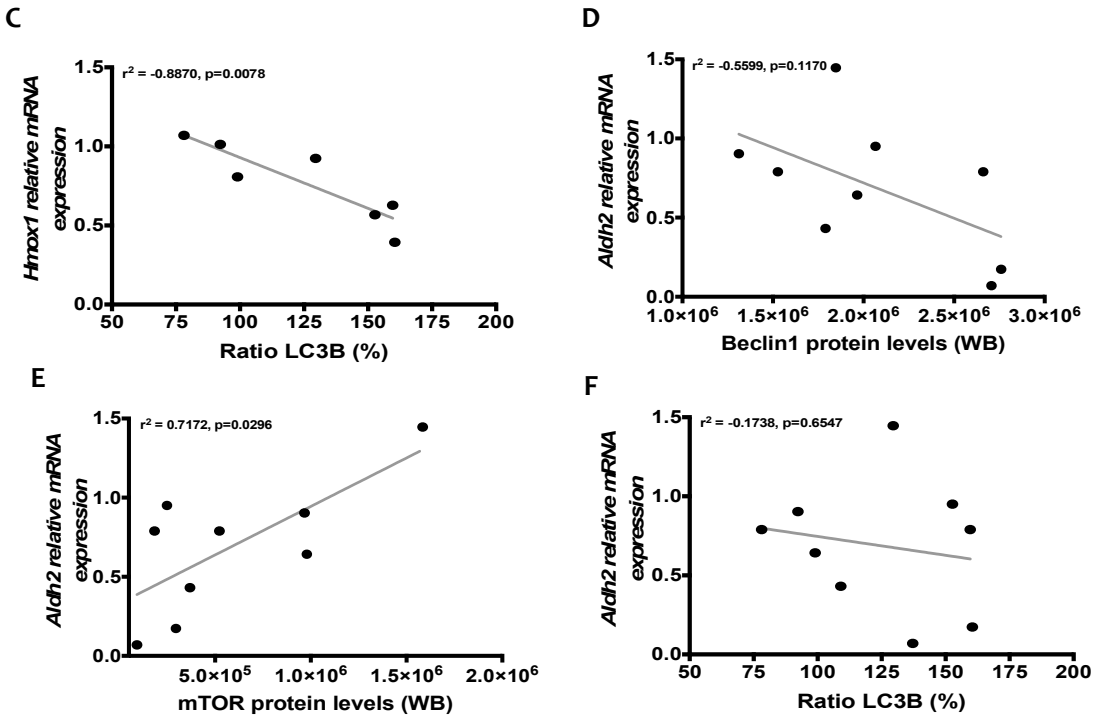


Figure 7. Correlations between Beclin1 (A), mTOR (B) and LC3B ratio (C) with Hmox1 gene expression. Correlations between Beclin1 (D), mTOR (E) and LC3B ratio (F) with Aldh2 gene expression in female SAMP8 with 12-months old. *Pearson correlations were performed between protein levels of autophagy markers and oxidative stress gene expression in hippocampus in both groups (n = 14). r^2 and p values were indicated on graphs. The levels of significance was p<0.05.*

3.1.5 Discussion

In the present work, we demonstrated the neuroprotective effect of a potent 11β-HSD1 inhibitor, RL-118, in old female SAMP8. Moreover, to our knowledge, autophagy appears, for the first time, as a molecular mechanism gated to neuroprotection mediated by 11β-HSD1 inhibition.

Neurodegeneration and emotional/cognitive disturbances present in SAMP8 are directly related, at least in the brain, to an oxidant environment and lead to changes in molecular markers of inflammation, APP processing dysfunction, tau hyperphosphorylation and autophagy (Griñán-Ferré et al., 2016). Besides, previous works in our lab demonstrated that SAMP8 mice presented anxiety and unquiet behaviour accompanied by locomotor activity diminution (Griñán-Ferré et al., 2016).

According to previous reports (Holmes et al., 2010; Mohler et al., 2011; Sooy et al., 2010; Yau et al., 2001, 2007), modulation of 11β-HSD1 or pharmacological treatment with 11β-HSD1 inhibitors leads to ameliorations in memory abilities and beneficial effects on behaviour, reducing fear sensation and increasing locomotor activity. Also, it has been published that cognitive improvement delivered by RL-118 occurs jointly with synaptic plasticity recovery (assessed by PSD95 marker) and reduction in amyloidogenic APP processing pathway, tau hyperphosphorylation and inflammatory markers (Leiva et al., 2017). Altogether indicate a putative therapeutic role for 11β-HSD1 in neurodegenerative disorders like AD.

There is evidence that higher plasma GC levels due to stress influence hippocampal atrophy (Mah et al., 2015). Besides, patients with mild cognitive impairment (MCI) and mood disorders have neuropathological AD-like hallmarks in the cerebrospinal fluid, supporting GCs role in cognitive impairment (Ramakers et al., 2013). Moreover, GCs are physiological immunomodulatory hormones that not only regulate immune cell development, but also decrease autophagy process and trigger apoptosis (El Zaoui et al., 2015; Harr et al., 2010).

The two major systems responsible for the digestion of most cytosolic and aggregated or misfolded protein in brain cells are UPS and the autophagy-lysosome pathway. The decline in autophagy function could determine cell and individual lifespan (Guo et al., 2017; Maiese, 2016; Triplett et al., 2017). Thus, autophagy impairment has been related to several neurodegenerative disorders characterized by accumulation of intracellular protein aggregates in specific brain regions, such as AD, Parkinson's and Huntington's disease (Koyuncu et al., 2017; Rahman & Rhim, 2017). Specifically in AD, autophagy dysfunction leads to β-amyloid aggregation, accumulation, therefore favouring the formation of SPs (Funderbunk et al., 2010). Compounds improving autophagy-lysosome pathway have been reported to be neuroprotective in experimental models of AD (Caballero & Coto-Montes, 2012; Friedman et al., 2015).

Herein, increased levels of Beclin1 and p65 were found in RL-118 treated mice. It is well known that Beclin1 regulates autophagic flux as it orchestrates autophagosome formation (Kang et al., 2011; Wirawan et al., 2012). On the other hand, p65 directly binds to Beclin1 promoter and up-regulates gene expression of proteins involved in autophagy, leading to its activation. In turn, p62 protein recognizes toxic cellular waste and ubiquitin-

containing aggregates, ensuing the subsequent turnover of potentially harmful proteins by autophagy (Rusten et al., 2010).

To complete autophagic process, autophagosome formation is needed and LC3B is one of the mediators. LC3B-I is the cytoplasm active form and it conjugates with phosphatydilethanolamine through two consecutive ubiquitylation-like reactions to become LC3B-II that is recruited to autophagosome membrane (Klionsky et al., 2008; Tanida et al., 2008). 11β-HSD1 inhibition by R-118 promoted LC3B-II levels in detriment to LC3B-I indicating autophagosome formation and enhanced autophagy. In addition, upstream in this process there are AMPKα and mTOR, which modulate autophagic flux. On one hand, AMPKα activation acts as an autophagy activator signal, whereas mTOR pathway is one of the main cellular autophagy inhibitors. The mTOR is a serine/threonine protein kinase involved in the process of cell growth, regulation of energy homeostasis and metabolism after nutrient ingestion. The importance and complexity of mTOR signalling is evident, as it is linked to several neurodegenerative diseases via its role in autophagy regulation. We found an increase in the ratio pAMPKα/AMPKα induced by 11β-HSD1 inhibitor, demonstrating that RL-118 renders a better autophagic flux process in SAMP8 brain. Autophagy promotion by RL-118 should force the removal of aberrant proteins in the hippocampus and, in consequence, improve cognitive and behavioural disturbances. Moreover, correlations between the autophagy protein levels studied and behavioural parameters suggest that the cognitive amelioration found after RL-118 treatment is related to an autophagy potentiation. Of note, RL-118 induced a diminution in hyperphosphorylated tau protein at Ser396 and Ser404 residues indicating, once more, an improvement in autophagy.

Molecular, biochemical and cellular changes implicated in cellular senescence are intrinsically gated to inflammation and OS (López-Otín et al., 2013). Aged population show a low chronic inflammatory state, which worsens in AD patients. In fact, AD pathology is characterized by a chronic elevation of OS and increased gliosis and neurodegeneration (Ginaldi et al., 2001; Williams et al., 2015).

Because altered inflammatory and OS markers levels are already described in SAMP8, here we screened gene expression of some of the most important proteins implicated in those processes. The results obtained demonstrated a lower gene expression of *Il-1β and Hmox1* in SP8 treated mice and an increase in the active fraction of NF-κB indicating that

neuroinflammation and oxidative environment were reduced when 11β-HSD1 was inhibited. Significant correlations found among inflammation and OS versus autophagy markers suggested that increased autophagy was linked to decreased inflammation and OS.

In conclusion, we showed an improvement in cognition induced by 11β-HSD1 inhibition by RL-118 in female SAMP8. These beneficial effects were related to a modification in APP processing, tilting it to the non-amyloidogenic pathway; a reduction of tau hyperphosphorylation, inflammatory and OS markers (Fig. 8). Finally, autophagy process was enhanced and strongly correlated with the reduction of neuroinflammation and OS after RL-118 treatment. Of note, autophagy appears as a novel cellular function accounted by 11β-HSD1 inhibition. Thus, these results obtained with RL-118 treatment initiate the path for a new family of compounds that by inhibiting 11β-HSD1 could cope with cellular processes implied in age-related cognitive impairment or neurodegenerative diseases, such as AD.

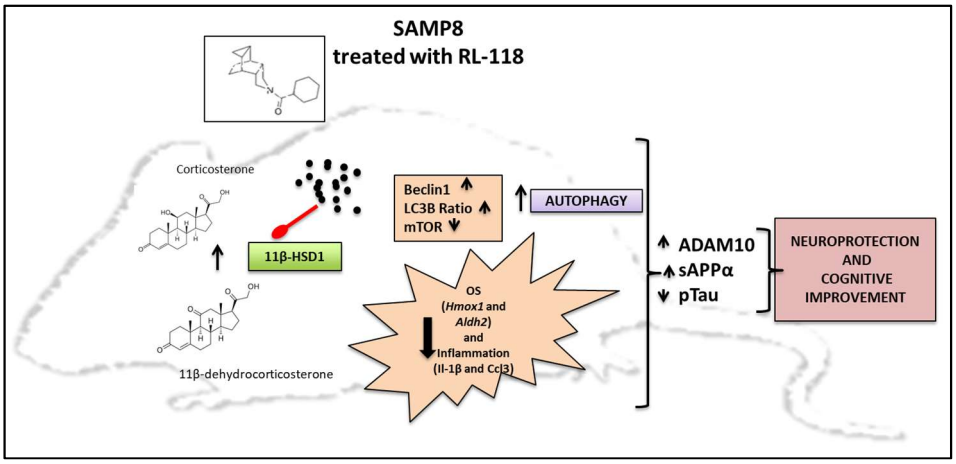


Figure 8. Schematic representation of the results obtained in the present study.

3.1.6 Acknowledgements

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3.1.8 Supplementary material

The online version of this article (https://doi.org/10.1007/s12035-018-1026-8) contains electronic supplementary material, which is available to authorized users.

3.2 Chronic Mild Stress Modified Epigenetic Mechanisms Leading to Accelerated Senescence and Impaired Cognitive Performance in Mice

Adapted from: **Puigoriol-Illamola, D.**[1,3], Martínez-Damas, M.[2], Griñán-Ferré, C.[1,3], Pallàs, M[1,3]. Chronic Mild Stress Modified Epigenetic Mechanisms Leading to Accelerated Senescence and Impaired Cognitive Performance in Mice

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3.2.1 Abstract

Cognitive and behavioural disturbances are growing public healthcare issue for modern society, which is experiencing an increasingly common stressful lifestyle. Besides, several pieces of evidence state that the environment is crucial in the development of several diseases as well as compromising healthy ageing. With upcoming age, the capability to fight against harmful stimuli decreases and the organism becomes more vulnerable to infections and disease. In agreement, stressful experiences have been identified as an important risk factor for cognitive impairment. Therefore, it is important to study the molecular mechanisms underpinning the effects of chronic stress on cognition and its relationship with ageing in order to unveil what challenges we might have to cope with as a society in the not-so-far future. The study hypothesised that chronic stress would modulate a large constellation of cellular mechanisms implied in ageing and age-related neurodegenerative pathologies. Thus, the aim was to determine which molecular mechanisms were altered after chronic stress situations in an ageing animal model that could be responsible of cognitive decline.

To address these queries, Chronic Mild Stress (CMS) paradigm was used in the SAMP8 and SAMR1. On the one hand, we determined the changes produced in the three main epigenetic marks after 4 weeks of CMS treatment, such as a reduction in histone PTM and DNA methylation, and up- or down-regulation of several miRNAs involved in different cellular processes in mice. In addition, CMS treatment induced ROS damage accumulation and loss of antioxidant defence mechanisms like NRF2, GPX1, SOD1 and CAT, as well as inflammatory signalling activation through NF-κB pathway and astrogliosis markers, like *Il-6*, *Tnf-α* and glial fibrillar acidic protein (*Gfap*). Remarkably, CMS altered mTORC1 signalling in both strains, decreasing autophagy only in SAMR1 mice.

We found a decrease in GSK-3β inactivation, hyperphosphorylation of Tau and an increase in soluble amyloid precursor protein β (sAPPβ) protein levels in mice under CMS. Moreover, the non-amyloidogenic secretase, a disintegrin and metalloproteinase-domain containing protein 10 (ADAM10) protein levels were reduced in SAMR1 CMS group. Consequently, detrimental effects on behaviour and cognitive performance were detected in CMS treated mice, affecting mainly SAMR1 mice, promoting a turning to SAMP8 phenotype. In conclusion, CMS is a feasible intervention to understand the influence of stress on epigenetic mechanisms underlying cognition and accelerating senescence.

3.2.2 Introduction

Aging is a multifactorial process characterized by a progressive loss of physiological integrity, resulting in an impaired function and increased vulnerability to death. Physiological brain aging involves cognitive impairment, which includes decreased learning and memory abilities and slower responses to different stimulus (Cristòfol et al., 2012). Indeed, normal aging differs from pathological aging and this might be explained by the lifestyle. Environmental factors drive epigenetic modifications resulting in phenotypic differences, which alter almost all tissues and organs. Although the brain is one of the most affected structures, such modifications lead to a progressive decline in the cognitive function and create a favourable context for the development of neurodegenerative diseases (Harman & Martín, 2019). Aging is the most significant risk factor for most chronic diseases, such as age-related cognitive decline and Alzheimer's disease (AD) (Guerreiro & Bras, 2015). In fact, AD is the most prevalent dementia and its progression is influenced by both genetic and environmental factors (Griñán-Ferré et al., 2016).

Several studies define that a good environment is essential to enhance learning and cognitive abilities, besides the continued presence of stressors is related to the opposite effects. Epigenetics refers to the potentially heritable and environmentally modifiable changes in gene expression mediated via non-encoded DNA mechanisms (Sun et al., 2013). Recent evidence has demonstrated significant associations between epigenetic alterations and stress (Harman & Martín, 2019; Turecki & Meaney, 2016). Potential threats cause a course of action releasing numerous transmitters and hormones throughout our body, particularly catecholamines and glucocorticoids. The interaction of glucocorticoids and adrenergic systems in specific brain regions has proved an essential mediating mechanism for a wide variety of actions displayed by stress on cognition (Sandi, 2013). Firstly, stress response allows body adaptation. However, when stress is prolonged it has been shown that decreases synaptic plasticity (Wang et al., 2019), alters hippocampal volume (Borcel et al., 2008; Rahman et al., 2016) and neurotransmitters (Jett et al., 2017) and may lead to Alzheimer's disease (Justice, 2018). In particular, chronic stress is associated with increased Aβ deposits and hyperphosphorylated Tau (Bisht et al., 2018).

Oxidative stress (OS) and inflammation are deeply involved in age-related deleterious disorders. Along with the aging process, several factors, such as a naturally decreased capacity of the antioxidant enzymes system, create an imbalance between antioxidant

mechanisms and reactive oxygen system (ROS)-production equilibrium, accumulating ROS beyond the detoxifying capacity of the antioxidant system resulting in OS, eventually causing cellular damage that can no longer be repaired by internal mechanisms, finally causing the dysfunction of the system (Griñán-Ferré et al., 2018). In addition, one of the major changes while aging is the dysregulation of the immune response, leading to chronic systemic inflammatory state (Chung et al., 2019). Overall, OS imbalance, mitochondrial dysfunction and the inflammatory response have been linked to accelerate aging and fasten progression of neurodegenerative diseases (Griñán-Ferré et al., 2018; Petersen & Smith, 2016). Moreover, accumulation of dysfunctional and damaged cellular proteins and organelles occurs during aging, resulting in a disruption of cellular homeostasis and progressive degeneration and increasing the risk of cell death (Escobar et al., 2019). Autophagy is particularly important in the maintenance of homeostasis, as it participates in the elimination of disrupted proteins and molecules and helps to maintain a clean cellular environment. It has been widely described that there are alterations in autophagy while aging as well as in age-related cognitive decline, AD and other dementias (Gouras, 2019; Martinez-Lopez, 2015; Puigoriol-Illamola et al., 2018). Moderating all these detrimental components is key in the promotion of cell survival and longevity.

The senescence-accelerated mouse (SAM) models include senescence-accelerated mice prone (SAMP) and resistant (SAMR) mice (Takeda et al., 1991). SAMP8 strain has been widely used as an aging model for the study of brain aging and age-related pathologies. Its cognitive impairment is linked with alterations in both hippocampal structure and activity, causing OS imbalance and driving to AD neuropathology, such as tau- and amyloid-related alterations (Cristòfol et al., 2012; Wang et al., 2016). On the contrary, SAMR1 strain manifests a normal aging process.

Understanding the epigenetic modifications that stressful environment triggers in neurodegeneration mechanisms is essential to develop novel therapeutics for age-related cognitive decline. This study aims to determine the effects on brain function of a stressful lifestyle in an animal model with accelerated senescence, SAMP8, and in the resistant senescence strain, SAMR1.

3.2.3 Materials and methods

Animals and Chronic Mild Stress Procedure

Females SAMP8 mice (n=56) were used to perform behavioural, cognitive and molecular analyses. We divided these animals into four groups: SAMR1 (SAMR1 control, n=14), SAMR1 treated with CMS (SAMR1 CMS, n=14), SAMP8 (SAMP8 control, n=14) and SAMP8 treated with CMS (SAMP8 CMS, n=14). Animals had free access to food and water and were kept under standard temperature conditions (22±2°C) and 12h: 12h light-dark cycles (300lux/0 lux). Starting at 4 months of age, CMS groups received the Chronic Mild Stress (CMS) treatment. CMS procedure that was used in the present study had previously been validated in SAMR1 and SAMP8 mice, with some modifications (Wang et al., 2016). Mice were exposed to various randomly scheduled, low-intensity environmental stressors every day for 4 weeks. Different stressful stimuli were applied every day and the sequence of the stressors was altered every week to guarantee the degree of unpredictability. Among others, CMS stimuli were 24h of water deprivation, 24h of food deprivation, 2h of physical restraint, 24h of sawdust removal, 24h of wet bedding, overnight illumination and 1min of tail nipping at 1cm from the tip of the tail. Before the performance of the cognitive tests, the glucose tolerance test was conducted.

All experimental procedures involving animals were performed followed by standard ethical guidelines European Communities Council Directive 86/609/EEC and by the Institutional Animal Care and Use Committee of the University of Barcelona (670/14/8102, approved at 11/14/2014) and by Generalitat de Catalunya (10291, approved 1/28/2018). All efforts were made to minimize the number of mice used and their suffering.

Glucose tolerance test

Intraperitoneal (i.p.) glucose tolerance test was performed following 4 weeks of CMS treatment, as described previously. In brief, mice fasted overnight for 12h. The test was performed in a quiet room and 2g/kg i.p. glucose injection was administered (diluted in H_2O) and blood glucose levels were measured at 0, 5, 10, 15, 30, 60 and 120 min after the injection with the Accu-Chek® Aviva blood glucose meter (Accu-Chek® Aviva, Roche, Barcelona, Spain).

Behavioural and cognitive test

Open Field Test (OFT)

The OFT was performed as previously described in Griñán-Ferré et al. (2015). It evaluates anxiety-like behaviour. Mice were placed at the centre of a white polywood box (50x50x25cm) and allowed to explore it for 5 minutes. Behaviour was scored with SMART® ver.3.0 software and each trial was recorded for later analysis. The parameters scored included centre staying duration, rearing, grooming and the distance travelled.

Novel Object Recognition Test (NORT)

The Novel Object Recognition Test (NORT) protocol employed was as described in Puigoriol-Illamola et al. (Puigoriol-Illamola et al., 2020). In brief, mice were placed in a 90°, two-arms, 25cm-long, 20cm-high, 5cm-wide black maze. Before performing the test, the mice were individually habituated to the apparatus for 10min for 3 days. On day 4, the animals were submitted to a 10min acquisition trial (first trial), during which they were placed in the maze in the presence of two identical, novel objects at the end of each arm. After a delay (2h and 24h), the animal was exposed to two objects one old object and one novel object. The Time that mice explored the Novel object (TN) and Time that mice explored the Old object (TO) were measured. A Discrimination Index (DI) was defined as (TN−TO)/(TN+TO). To avoid object preference biases, objects were counterbalanced. The maze, the surface, and the objects were cleaned with 70% ethanol between the animals' trials to eliminate olfactory cues.

Morris Water Maze (MWM)

This test evaluates both learning and spatial memory (Vorhees et al., 2006). An open circular pool (100 cm in diameter, 50 cm in height) filled with water was used. Water was painted white with latex in order to make it opaque and its temperature was 22± 1°C. Two main perpendicular axes were established (North-South and East-West), thus configuring four equal quadrants (NE, NW, SE, and SW). Four visual clues (N, S, E, W) were placed on the walls of the tank so that the animal could orientate and could fulfil the objective. The test consists of training a mouse to find a submerged platform (Learning phase) and assess whether the animal has learned and remembered where the platform was the day that it is removed (Test). The training lasts five consecutive days and every day five trials are

performed, which have a different starting point (NE, E, SE, S, and SW), with the aim that the animal recognizes the visual clues and learns how to locate the platform, avoiding learning the same path. At each trial, the mouse was placed gently into the water, facing the wall of the pool, allowed to swim for 60 seconds and there was not a resting time between trials. If the animal was not able to locate the platform, the investigator guided it to the platform and was allowed to rest and orientate for 30 seconds. The platform was placed approximately in the middle of one of the quadrants, 1.5 cm below the water level. Above the pool there was a camera that recorded the animals' swimming paths and the data was analysed with the statistical program SMART® ver.3.0. During the learning phase, a learning curve was drawn, in which is represented the latency to find the platform every training day. On the day test, more parameters were measured, such as the target crossings and the swum distance in the platform zone.

Immunodetection experiments

Brain processing

Three days after the behavioural and cognitive tests, animals were euthanized for protein extraction, RNA and DNA isolation. Brains were immediately removed, and the hippocampus was isolated, frozen on powdered dry ice and maintained at -80°C until procedures.

Western blotting

Tissue samples were homogenized in lysis buffer (Tris HCl pH 7.4 50mM, NaCl 150mM, EDTA 5mM and 1X-Triton X-100) containing phosphatase and protease inhibitors (Cocktail II, Sigma-Aldrich) to obtain total protein homogenates. Aliquots of 15 µg of hippocampal protein extraction per sample were used. Protein samples were separated by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (8-14%) and transferred onto Polyvinylidene difluoride (PVDF) membranes (Millipore). Afterwards, membranes were blocked in 5% non-fat milk in Tris-buffered saline (TBS) solution containing 0.1% Tween 20 TBS (TBS-T) for 1 hour at room temperature, followed by overnight incubation at a 4°C with the primary antibodies listed in (Table S1). Then, the membranes were washed and incubated with secondary antibodies listed in (Table S1) for 1 hour at room temperature. Immunoreactive proteins were viewed with the chemiluminescence-based ChemiLucent™ detection kit, following the manufacturer's protocol (ECL Kit, Millipore), and digital images

were acquired using ChemiDoc XRS+System (BioRad). Semi-quantitative analyses were done using ImageLab software (BioRad) and results were expressed in Arbitrary Units (AU), considering control protein levels as 100%. Protein loading was routinely monitored by immunodetection of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-tubulin.

RNA extraction and gene expression determination by q-PCR

Total RNA isolation was carried out using TRIsure™ reagent according to the manufacturer's instructions (Bioline Reagent, UK). The yield, purity, and quality of RNA were determined spectrophotometrically with a NanoDrop™ ND-1000 (Thermo Scientific) apparatus and an Agilent 2100B Bioanalyzer (Agilent Technologies). RNAs with 260/280 ratios and RIN higher than 1.9 and 7.5, respectively, were selected. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed as follows: 2 μg of messenger RNA (mRNA) was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time quantitative PCR (qPCR) was used to quantify mRNA expression of OS and inflammatory genes listed in (Table S2). SYBR® Green real-time PCR was performed in a Step One Plus Detection System (Applied-Biosystems) employing SYBR® Green PCR Master Mix (Applied-Biosystems). Each reaction mixture contained 6.75 μL of complementary DNA (cDNA) (which concentration was 2 μg), 0.75 μL of each primer (which concentration was 100 nM), and 6.75 μL of SYBR® Green PCR Master Mix (2X).

Data was analysed utilizing the comparative Cycle threshold (Ct) method (ΔΔCt), where the housekeeping gene level was used to normalize differences in sample loading and preparation (Cosín-Tomás et al., 2014). Normalization of expression levels was performed with β-actin for SYBR® Green-based real-time PCR results. Each sample was analysed in duplicate, and the results represent the n-fold difference of the transcript levels among different groups.

Global DNA Methylation and Hydroxymethylation Determination

Isolation of genomic DNA was conducted using the FitAmp™ Blood and Cultured Cell DNA Extraction Kit (EpiGentek, Farmingdale, NY, USA) according to the manufacturer's instructions. Following this, Methylflash Methylated DNA Quantification Kit (Epigentek, Farmingdale, NY, USA) and MethylFlash HydroxyMethylated DNA Quantification Kit were used in order to detect methylated and hydroxymethylated DNA. Briefly, these kits are

based on specific antibody detection of 5-mC and 5-hmC residues, which trigger an ELISA-like reaction that allows colorimetric quantification at 450 nm.

miRNA Expression Array and Validation by Single Real-Time PCR

For microRNA (miRNA) expression array, total RNA and miRNA were extracted employing the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The yield, purity and quality of the samples were determined by the A260/280 ratio in a NanoDrop® ND-1000 apparatus (Thermo Scientific). RNA samples from 24 females (n=6 per group) were converted into cDNA through a Reverse Transcription (RT) reaction using the miRCURY™ LNA™ miRNA RT Kit (Exiqon) according to the manufacturer's instructions. The expression of 22 mature miRNAs was then analysed using Mouse Pick&Mix miRNA PCR panel (Exiqon) (Table S3 and S4). miRNA expression was measured in a StepOnePlus Real-Time PCR system (Applied Biosystems).

SYBR Green-based real-time PCR was performed on the detection system StepOnePlus (Applied Biosystems) for miRNA expression. In compliance with the miRCURY LNA™ SYBR Green PCR Kit Protocol (Qiagen), each reaction mixture contained 4,5µL of SYBR Green PCR Master Mix (2X), 0,5µL of ROX, 3µL of product from RT reaction diluted 1:60, 1µL of nuclease-free water and 1µL of miRNA probes (Exiqon) (Table S2). Data were analysed using the comparative cycle threshold (Ct) method in which the SNORD68 small non-coding RNA transcript level was employed to normalize differences, since it presented similar expression level between groups. Each sample was analysed in duplicate and results represent the n-fold difference of the transcript levels among different groups.

Oxidative Stress Determination

Hydrogen peroxide was measured in hippocampus protein homogenates as an indicator of OS, and it was quantified using the Hydrogen Peroxide Assay Kit (Sigma-Aldrich, St. Louis, MI) according to the manufacturer's instructions.

Data analysis

Data analysis was conducted using GraphPad Prism ver. 7 statistical software. Data is expressed as the mean ± standard error of the mean (SEM) of at least 6 samples per group. Diet and treatment effects were assessed by the Two-Way ANOVA analysis of variance,

followed by Tukey post-hoc analysis or two-tail Student's t-test when it was necessary. Statistical significance was considered when *p*-values were <0.05. The statistical outliers were determined with Grubbs' test and subsequently removed from the analysis when necessary.

3.2.4 Results

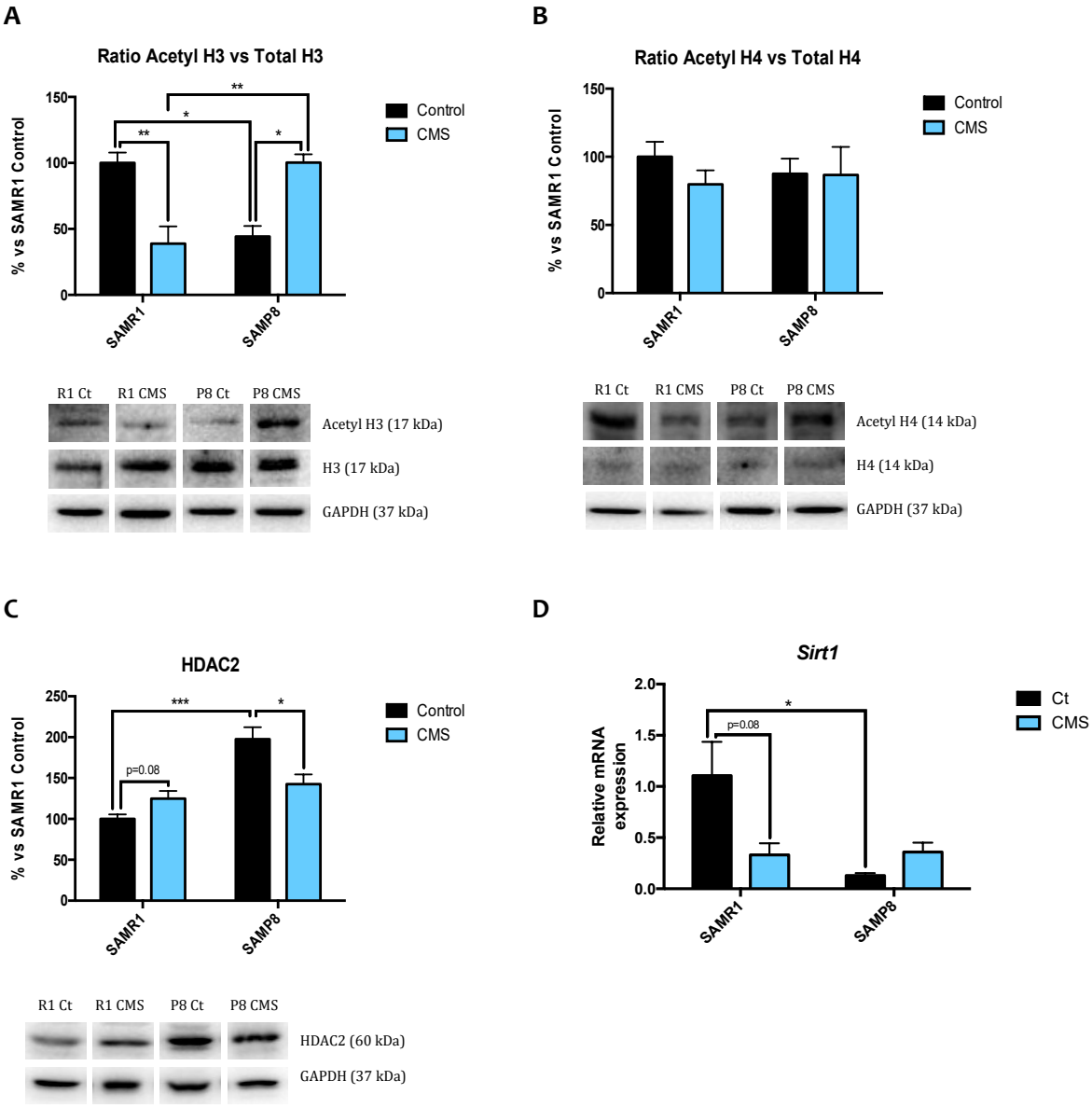
Epigenetic modulation triggers compacted chromatin after Chronic Mild Stress

In attempting to systematically address which are the epigenetic modifications caused by chronic mild stressful stimuli, we studied the major epigenetic marks that include histone modifications, DNA methylation and non-coding RNAs.

Regarding histone modifications, we studied global histone 3 (H3) and histone 4 (H4) acetylation, phosphorylation of histone 2A (H2A.X) protein levels, methylation of H3 acetylated at Lys9 protein levels, as well as, several deacetylases gene expression and/or protein levels, such as HDAC2, Sirt1, Sirt2 and Sirt6. CMS decreased H3 acetylation in senescence-resistant mice, similarly to SAMP8 control group, and increased in SAMP8 mice (Fig. 1 A). Considering H4, no statistically significant differences were determined, although a tendency to diminish in SAMR1 was observed (Fig. 1 B). On one hand, in accordance to H3 acetylation levels, there was a huge difference in histone deacetylase 2 (HDAC2) protein levels between SAMR1 and SAMP8 control groups, being higher in SAMP8 (Fig. 1 C). On the other hand, CMS modified HDAC2 protein levels, particularly decreased in SAMP8 and increased in SAMR1 compared to their littermates. Other deacetylases enzymes are sirtuins (Sirt) family. Not only gene expression (Figs. 1 D-E), but also protein levels of Sirt1, Sirt2 and Sirt6 were clearly decreased in SAMP8 mice compared to their control strain (SAMR1). In addition, CMS reduced *Sirt1* (Fig. 1 D) and *Sirt2* (Fig. S1) gene expression and protein levels (Fig. S1) in SAMR1 mice, but not in SAMP8. Moreover, histones can suffer different modifications such as phosphorylation or methylation, among others. In our hands, p-H2A-X protein levels did not differ between SAMR1 and SAMP8 control groups. Methylation of H3K9 was also higher in SAMP8 (Fig. 1 G). Of note, CMS increased phosphorylated form of histones p-H2A-X and methylation of H3K9 in SAMP8 in reference to their control littermates, as well as, compared to stressed SAMR1 (Figs. 1 F-G).

Considering DNA methylation, global 5-mC DNA was slightly lower in SAMP8 compared to SAMR1. CMS significantly reduced global methylation both in SAMP8 and

SAMR1 (Fig. 1 H). DNA methylation occurs through DNA MethylTransferase (DNMT) enzymes, of which DNMT1, DNMT3A and DNMT3B are the best characterized. As described, DNMTs were significantly high expressed in SAMP8 than SAMR1 control group in CMS animals (Fig. 2 B for *Dnmt3*, Data not shown for *Dnmt1* and *Dnmt2*). 5-mC can be further oxidized to 5-hydroxymethyl-cytosine (5-hmC) due to ten-eleven translocase (TET) enzymes. SAMP8 mice had lower 5-hmC levels than SAMR1 mice (Fig. 1 I). Accordingly, TET2 protein levels were lower in SAMR1 than in SAMP8. CMS reduced TET2 protein levels in SAMR1 up to levels observed in SAMP8 control group (Fig. 1 J).



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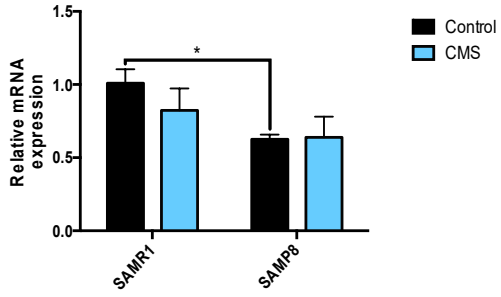
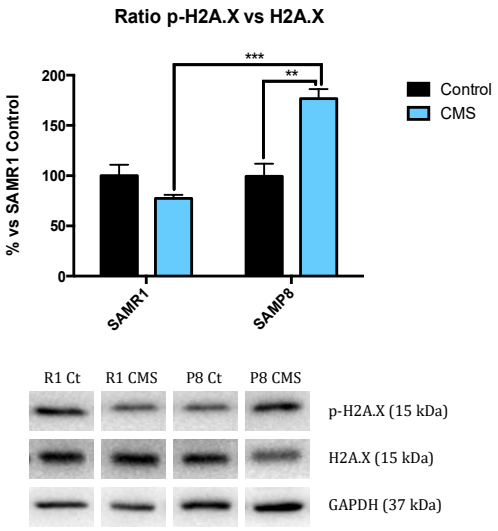
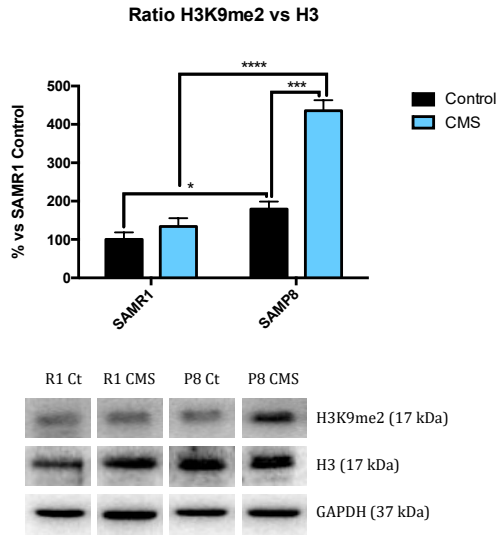

Sirt6
Relative mRNA expression
1.5
1.0
0.5
0.0
*
SAMR1
SAMP8
Control
CMS

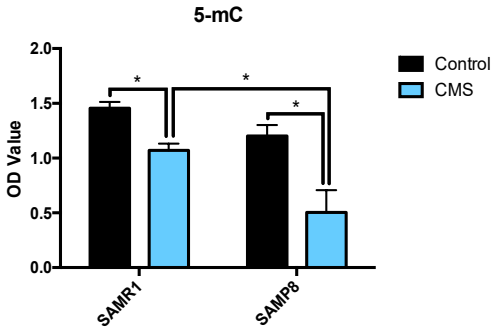
F


Ratio p-H2A.X vs H2A.X
% vs SAMR1 Control
200
150
100
50
0
***
**
SAMR1
SAMP8
Control
CMS
R1 Ct
R1 CMS
P8 Ct
P8 CMS
p-H2A.X (15 kDa)
H2A.X (15 kDa)
GAPDH (37 kDa)

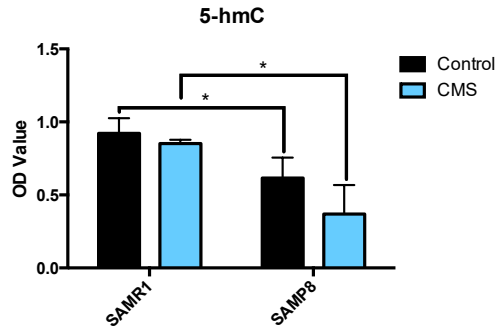
G


Ratio H3K9me2 vs H3
% vs SAMR1 Control
500
400
300
200
100
0
****
***
*
SAMR1
SAMP8
Control
CMS
R1 Ct
R1 CMS
P8 Ct
P8 CMS
H3K9me2 (17 kDa)
H3 (17 kDa)
GAPDH (37 kDa)

H


5-mC
OD Value
2.0
1.5
1.0
0.5
0.0
*
*
*
SAMR1
SAMP8
Control
CMS

I


5-hmC
OD Value
1.5
1.0
0.5
0.0
*
*
SAMR1
SAMP8
Control
CMS

J

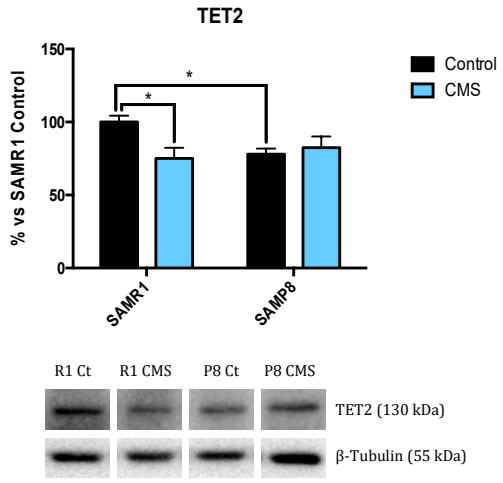
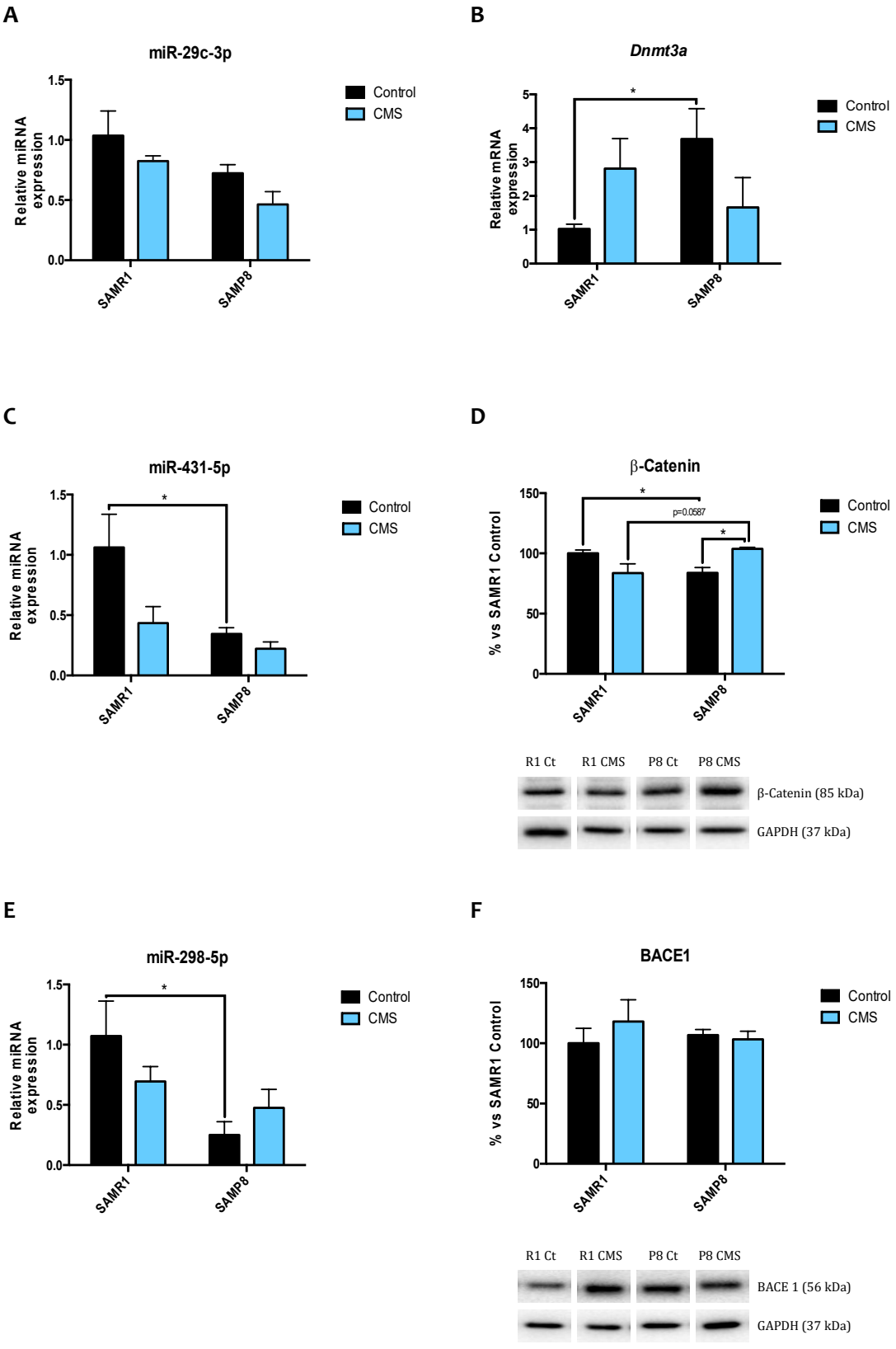

TET2
% vs SAMR1 Control
150
100
50
0
*
*
SAMR1
SAMP8
Control
CMS
R1 Ct
R1 CMS
P8 Ct
P8 CMS
TET2 (130 kDa)
β-Tubulin (55 kDa)

Figure 1. Epigenetic markers changes after CMS. *Representative Western Blot for the ratio of Acetylated at Lys9 H3 protein levels and quantification (A), the ratio of Acetylated at Lys 12 H4 protein levels and quantification (B), HDAC2 protein levels and quantification (C), the ratio of p-H2A.X protein levels and quantification (F), the ratio of H3K9me2 protein levels and quantification (G) and TET2 protein levels and quantification (J). Relative gene expression of Sirt1 (D) and Sirt6 (E). Global 5-methylated cytosine (H) and 5-hydroxymethylated cytosine levels (I). Gene expression levels were determined by real-time PCR. Values in bar graphs are adjusted to 100% for protein levels of SAMR1 control (R1 Ct). Values are mean ± Standard error of the mean (SEM); (n = 4 for each group). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.*

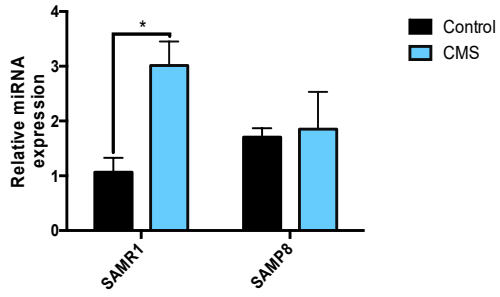
miRNA also play an important role in regulating gene expression. Thereby we decided to perform a microarray with 84 miRNAs related with neurodegeneration and aging in order to evaluate the influence of our experimental conditions (strain and CMS) those miRNA expression. Considering these results (Table S3 and S4), we proceeded to validate the relative expression of those modified miRNA in a significant manner regarding strain and CMS (Fig. S2).

miR-29c is involved in neural proliferation regulation by Dnmt3. Although no statistical differences were seen, it seems that SAMP8 have higher relative expression than SAMR1 and CMS contributed to increase it (Figs. 2 A-B). Those changes correlate with the tendencies seen in *miR-29c-3p* relative expression. *miR-431-5p, miR-298-5p, miR-98-5p* and *miR-140-5p* expression were lower in SAMP8 than in SAMR1 (Figs. 2 C,E,G,I). Those miRNAs are related to inhibition of neurodegenerative pathways, its expression diminution correlated with changes in effector protein expression as β-catenin, β-site APP Cleaving Enzyme 1 (BACE1), soluble amyloid precursor protein-β (sAPPβ) and A Disintegrin and Metalloproteinase Domain-containing protein 10 (ADAM10) (Figs. 2 D,F,H,J). Furthermore, CMS seemed to reduce *miR-431-5p* expression in SAMR1 group, although not statistical differences were observed (Figs. 2 C-D). *miR-181a-5p* expression was higher in SAMP8 than in SAMR1 (Fig. 2 K). Mammalian Target Of Rapamycin Complex 1 (mTORC1) is targeted by this miRNA, regulating cell growth, proliferation and survival. Likewise, mTORC1 protein levels were significant higher in SAMP8 than in SAMR1 mice (Fig. 2 L). Stressful stimuli only increased *miR-181a-5p* relative expression in SAMR1 mice compared to their control littermates but not in SAMP8 (Fig. 2 K). Correlating with miRNA expression, SAMR1 stressed group showed higher protein levels than SAMR1 control (Fig. 2 L). BCL2 protein levels are controlled by *miR-106b-5p*. SAMP8 mice showed higher relative expression in comparison

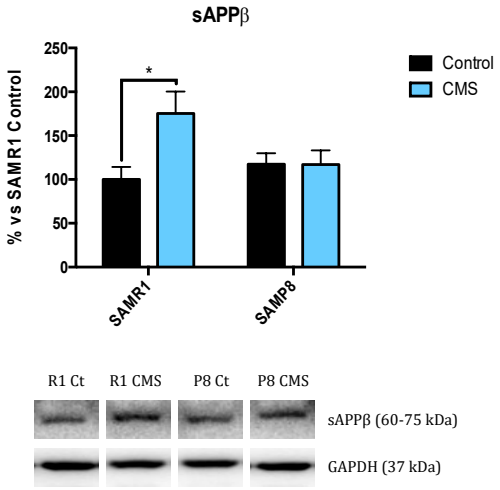
with SAMR1 animals and CMS reduced its expression only in SAMP8 strain (Fig. 2 M), which correlated with BCL2 protein levels (Fig. 2 N).



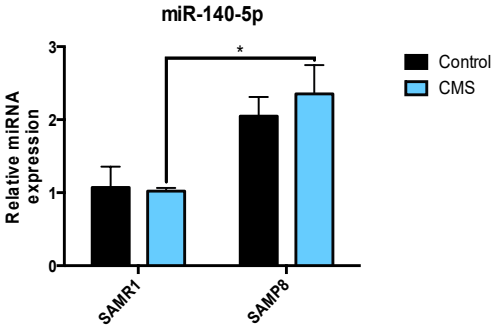
G


miR-98-5p
Relative miRNA expression
Control
CMS
SAMR1
SAMP8

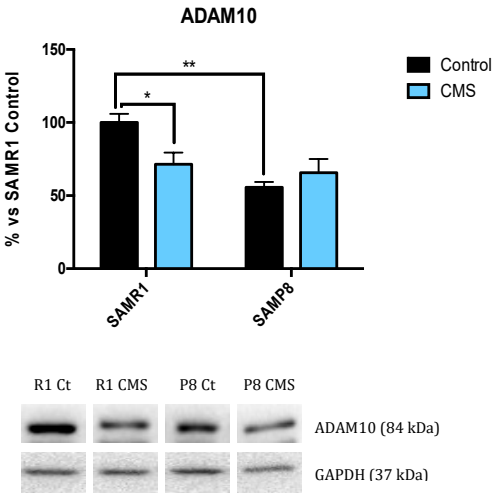
H


sAPPβ
% vs SAMR1 Control
Control
CMS
SAMR1
SAMP8
R1 Ct
R1 CMS
P8 Ct
P8 CMS
sAPPβ (60-75 kDa)
GAPDH (37 kDa)

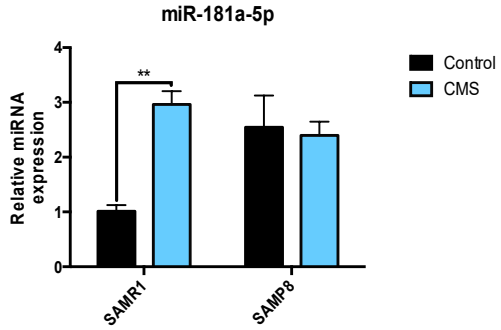
I


miR-140-5p
Relative miRNA expression
Control
CMS
SAMR1
SAMP8

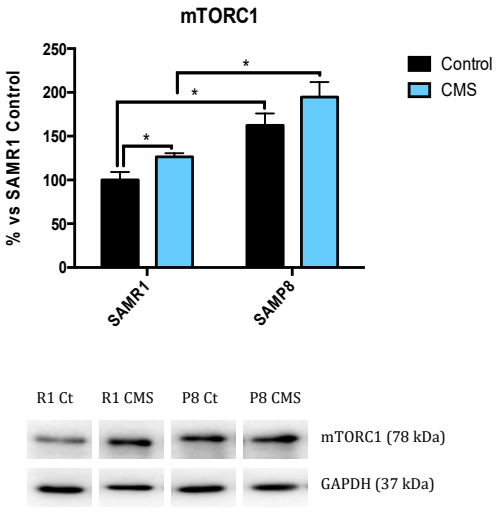
J


ADAM10
% vs SAMR1 Control
Control
CMS
SAMR1
SAMP8
R1 Ct
R1 CMS
P8 Ct
P8 CMS
ADAM10 (84 kDa)
GAPDH (37 kDa)

K


miR-181a-5p
Relative miRNA expression
Control
CMS
SAMR1
SAMP8

L


mTORC1
% vs SAMR1 Control
Control
CMS
SAMR1
SAMP8
R1 Ct
R1 CMS
P8 Ct
P8 CMS
mTORC1 (78 kDa)
GAPDH (37 kDa)

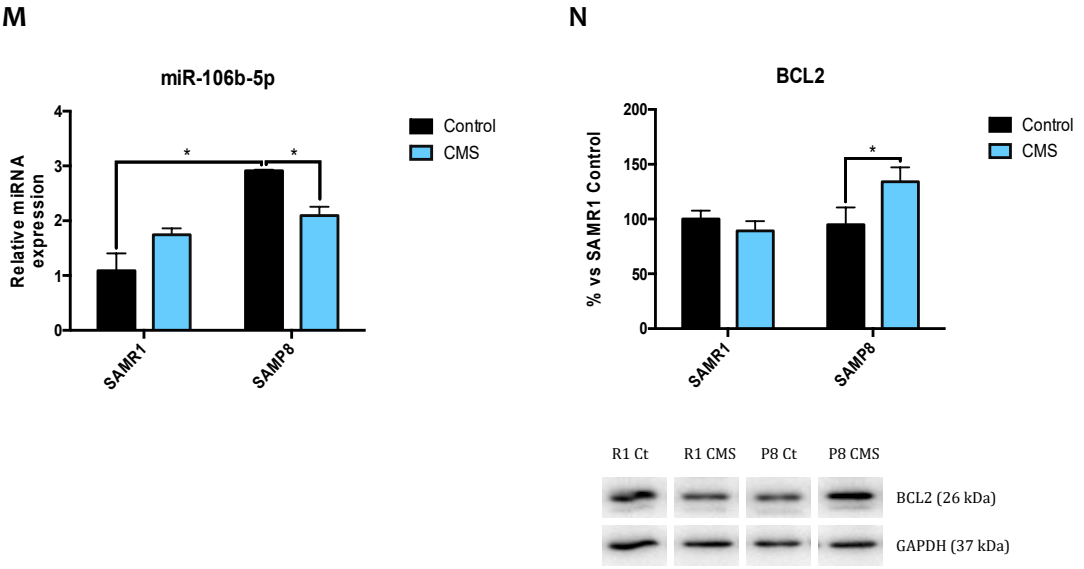


Figure 2. Validation of a representative subset of miRNA involved in the brain aging, neurodegeneration and autophagy. *Relative expression of miR-29c-3p, miR-431-5p, miR-298-5p, miR-98-5p, miR-140-5p, miR-181a-5p and miR-106b-5p in the hippocampus of SAMR1 and SAMP8 mice (A, C, E, G, I, K, M). Relative gene expression of Dnmt3a (B) and representative Western Blot for β-Catenin protein levels and quantification (D), BACE1 protein levels and quantification (F), sAPPβ protein levels and quantification (H), ADAM10 protein levels and quantification (J), mTORC1 protein levels and quantification (L) and BCL2 protein levels and quantification (N). Gene expression levels were determined by real-time PCR. Values in bar graphs are adjusted to 100% for protein levels of SAMR1 control (R1 Ct). Values are mean ± Standard error of the mean (SEM); (n = 6 for each group in miRNAs validation; n=4 for each group in WB and real-time PCR studies). *p<0.05; **p<0.01.*

Chronic Mild Stress attenuates antioxidant defence mechanisms

As mentioned before, CMS modulated cell OS mechanisms favouring an oxidative state. SAMP8 animals showed higher H_2O_2 levels in the hippocampus than SAMR1 groups, as well as reduced protein levels of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX1), suggesting impaired mechanisms to fight against OS (Figs. 3 A-E). CMS induced a diminution in Nuclear factor erythroid 2-related factor 2 (NRF2) protein levels only in SAMR1, but reduced antioxidant enzymes studied in both strains. In addition, CMS produced a slight but not significant tendency to increase aldehyde oxidase 1 (*Aox1*) gene expression, a pro-oxidant enzyme, in both strains (Fig. 3 F).

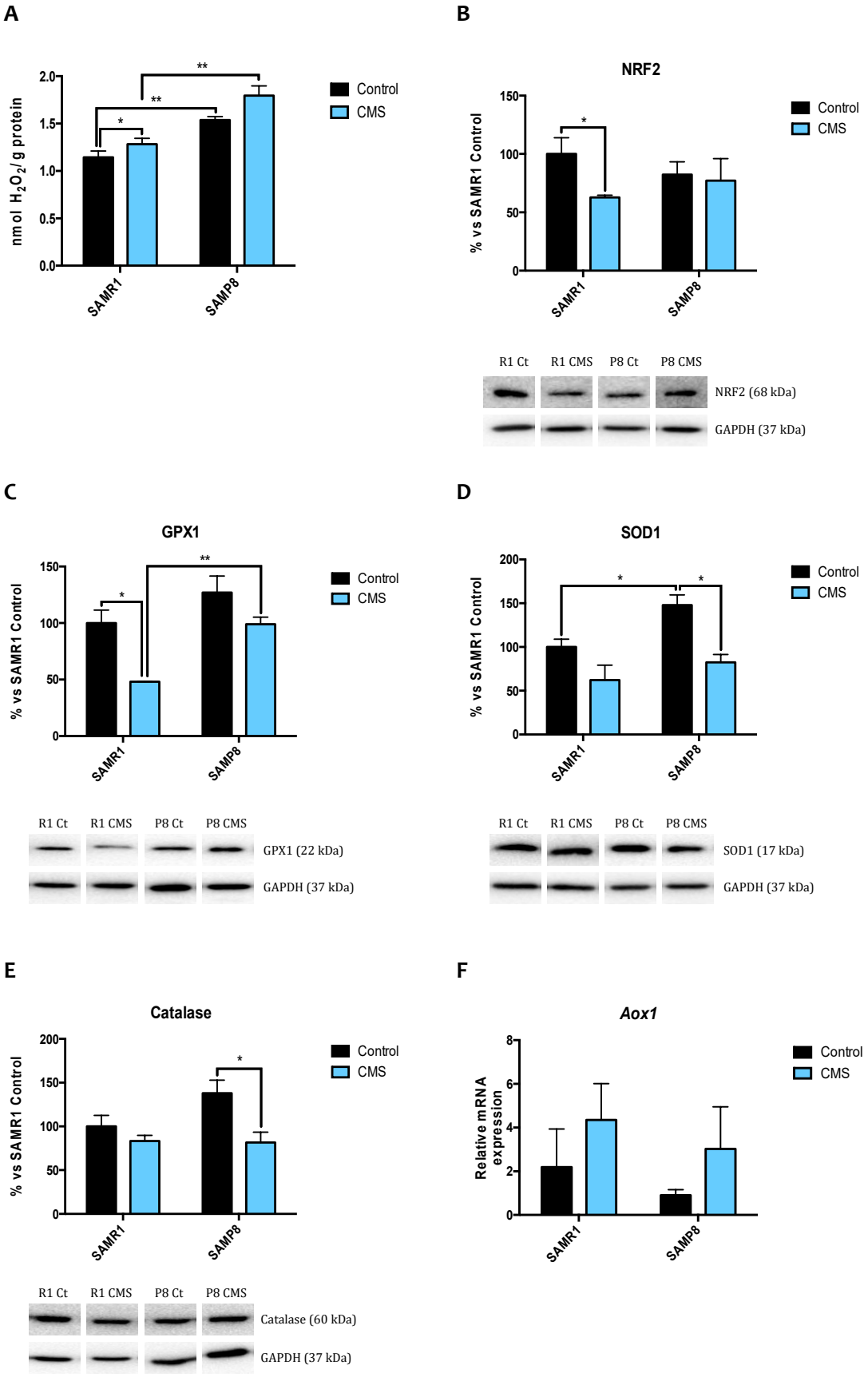


Figure 3. Changes in cellular oxidative stress mechanisms after CMS. *Representative OS measured as hydrogen peroxide concentration in homogenates of hippocampus tissue (A).*

*Representative Western Blot for NRF2 protein levels and quantification (B), GPX1 protein levels and quantification (C), SOD1 protein levels and quantification (D), and Catalase protein levels and quantification (E). Relative gene expression of Aox1 (F). Values in bar graphs are adjusted to 100% for protein levels of SAMR1 control (R1 Ct). Gene expression levels were determined by real-time PCR. Values are mean ± Standard error of the mean (SEM); (n=4 for each group). *p<0.05; **p<0.01.*

Inflammatory activation is induced after application of Chronic Mild Stress

SAMP8 mice show higher protein levels of NF-κB compared to SAMR1 control group. CMS promoted higher cytokines expression in SAMR1 than in SAMP8, as showed in the ratio *Interleukin(Il)-6/Il-10* and *tumor necrosis factor (Tnf) α* levels (Figs. 4 A-C), although not statistical differences were found. In accordance, *Glial fibrillar acidic protein (Gfap)* gene expression increased in both CMS groups in comparison to respective controls (Fig. 4 D).

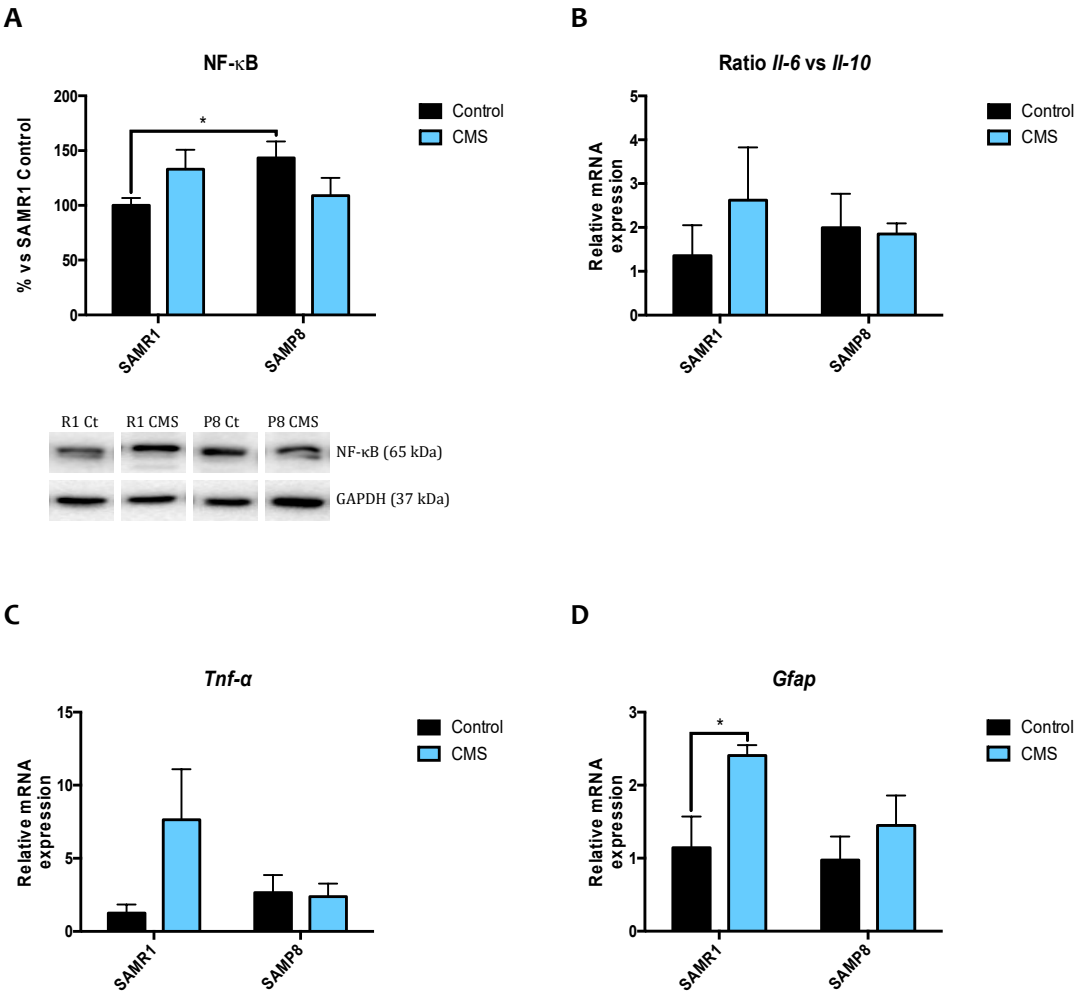


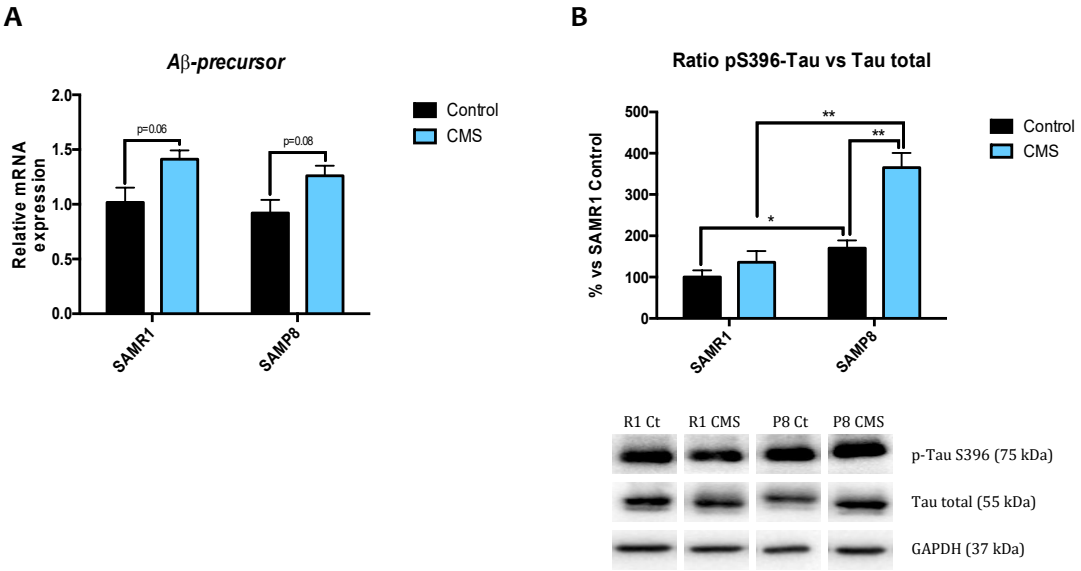
Figure 4. CMS induced changes in inflammatory markers. *Representative Western Blot for NF-*

*κB protein levels and quantification (A). Relative gene expression of Il-6/Il-10 ratio, Tnf-α and Gfap (B-D). Values in bar graphs are adjusted to 100% for protein levels of SAMR1 control (R1 Ct). Gene expression levels were determined by real-time PCR. Values are mean ± Standard error of the mean (SEM); (n=4 for each group). *p<0.05.*

Chronic Mild Stress modulates APP processing as well as alters microtubule stabilization through hyperphosphorylation of Tau protein

Observing the results, SAMP8 showed lower ADAM10 protein levels than SAMR1 animals. CMS promoted β-amyloid amyloidogenic pathway in SAMR1 mice, increased sAPPβ protein levels, increased Aβ-precursor gene expression and BACE1 protein levels as well as reduced ADAM10 protein levels (Figs. 2 F, H, J; 5 A).

SAMP8 had higher Ser396 p-Tau levels (Figs. 5 B-C) than SAMR1. Similarly, GSK-3β phosphorylation at Ser9 protein level was higher in SAMP8 in reference to SAMR1 (Fig. 5 D). CMS increased tau hyperphosphorylation at Ser396 and Ser404 compared to control groups; surprisingly, CMS reduced GSK-3β protein levels (Fig. 5 D).



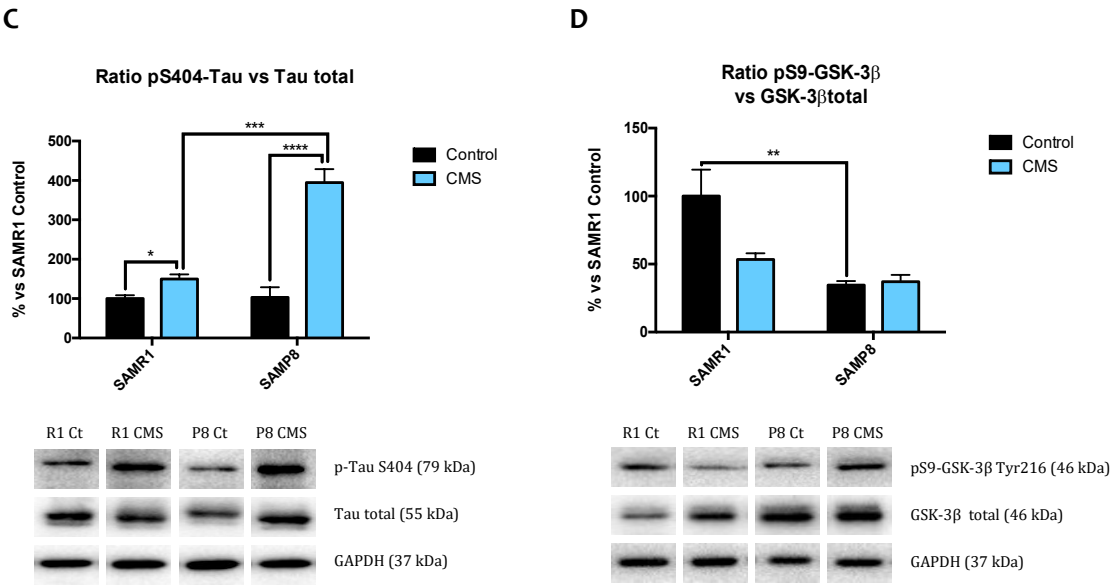


Figure 5. AD hallmarks changes after CMS. *Relative gene expression of Aß-precursor (A). Representative Western Blot for the ratio of pS396-Tau protein levels and quantification (B), the ratio of pS404-Tau protein levels and quantification (C) and the ratio of pS9-GSK-3ß protein levels and quantification (D). Gene expression levels were determined by real-time PCR. Values in bar graphs are adjusted to 100% for protein levels of SAMR1 control (R1 Ct). Values are mean ± Standard error of the mean (SEM); (n=4 for each group). *p<0.05; **p<0.01.*

Chronic Mild Stress promotes cell clearance through autophagy activation

Because autophagy has a key role in neurodegeneration, we evaluated Beclin 1, microtubule-associated protein 1A/1B-Light Chain 3 (LC3B), B-Cell Lymphoma 2 (BCL2) and mammalian Target Of Rapamycin (mTOR). Beclin 1 and LC3B protein levels were lower in SAMP8 than in SAMR1, whereas mTOR activation, measured by p-mTOR/mTOR ratio was higher in SAMP8 mice. CMS decreased pro-autophagic protein levels studied (Beclin 1 and LC3B) in SAMR1 mice, as well as there was a slight increase in mTOR activation. By contrast, in SAMP8 animals, stressful stimuli increased autophagy markers evaluated (Figs. 6 A-C; 2 N).

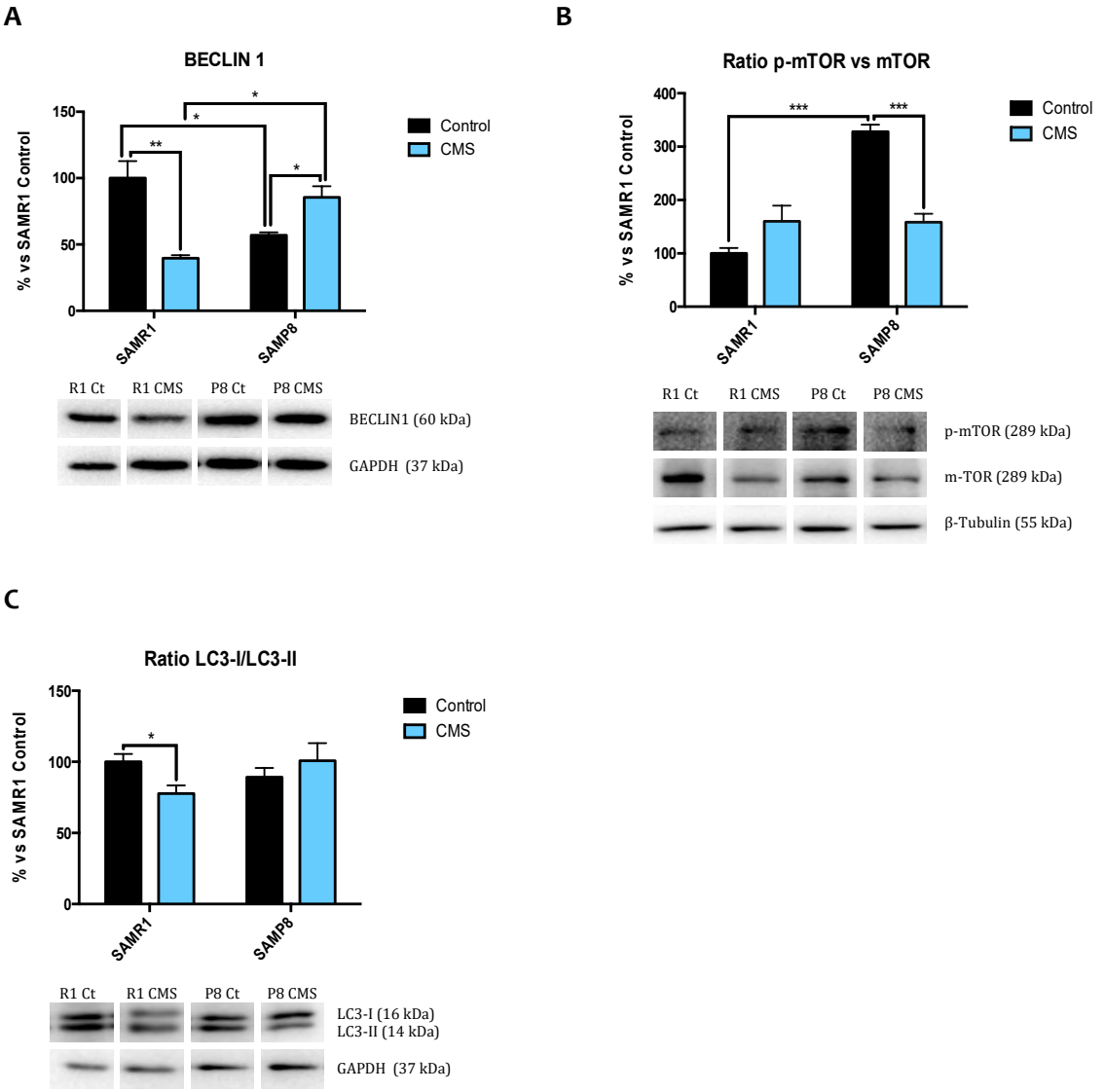


Figure 6. CMS induced autophagy flow modifications in SAMR1 and SAMP8. *Representative Western Blot for Beclin 1 protein levels and quantification (A), the ratio of p-mTOR protein levels and quantification (B) and the ratio of LC3 protein levels and quantification (C). Values in bar graphs are adjusted to 100% for protein levels of SAMR1 control (R1 Ct). Values are mean ± Standard error of the mean (SEM); (n=4 for each group). *p<0.05; **p<0.01; ***p<0.001.*

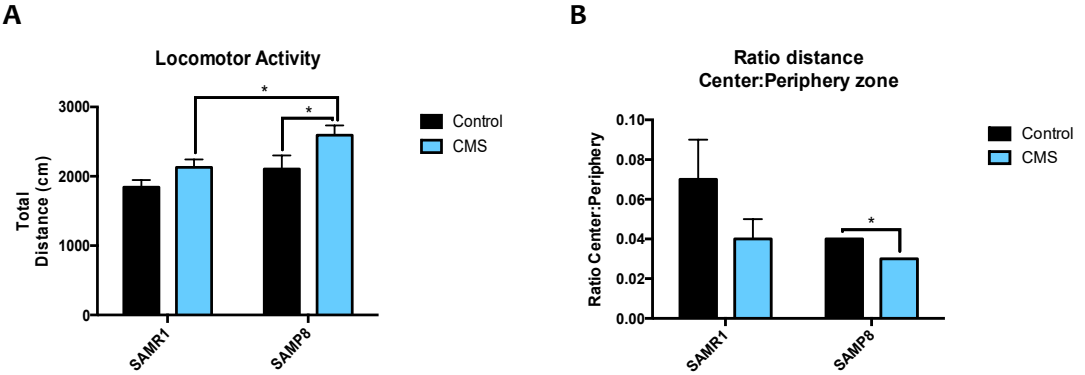
Anxiety-like behaviour, memory decline and poorer cognitive abilities after Chronic Mild Stress

As mentioned, stress produced behavioural changes in animals. In particular, the CMS treatment applied to animals contributed to increase locomotor activity in both mice strains, indicating that anxiety sensation and escape desire increased (Fig. 7 A). Regarding the other parameters evaluated in the OFT, SAMR1 travelled more distance and stood longer in the centre compared to SAMP8, although there were no statistically significant

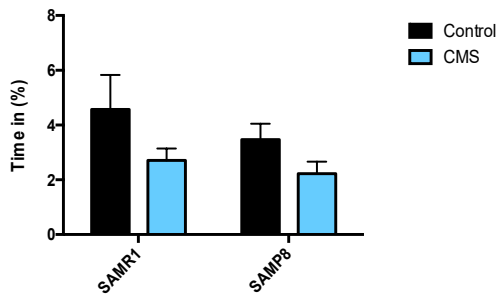
differences. In addition, CMS reduced those parameters in both strains compared to control animals (Figs. 7 B-D). Among these parameters indicating anxious behaviour were grooming and rearing. CMS treated groups showed higher number of rears and lower number of grooms than their controls (Figs. 7 E-F).

Referring to memory, we evaluated the recognition and spatial memory as well as learning abilities, through MWM and NORT. On one hand, there was a clear difference between strains because SAMP8 groups showed lower recognition memory than SAMR1 mice. On the other hand, MWM learning curves demonstrated that all mice learned where the platform was during the training days, as the time to reach the platform and the distance travelled was lower than the first day (Figs. 7 I-J). CMS treatment reduced recognition memory at both, short- and long-term (2h and 24h) (Figs. 7 G-H). Considering results obtained in MWM, SAMR1 animals crossed more times the platform zone and its quadrant than SAMP8 mice, as well as they stood and swum more distance in the platform zone (Figs 7. K-M) indicating a significantly better performance in this maze from SAMR1 than SAMP8. CMS decreased the value of those parameters in SAMR1 bringing them closer to SAMP8 values, although did not reach significance. Overall, results obtained in MWM and in accordance to OFT results indicated that CMS increased the total distance travelled during the training in comparison with control groups.

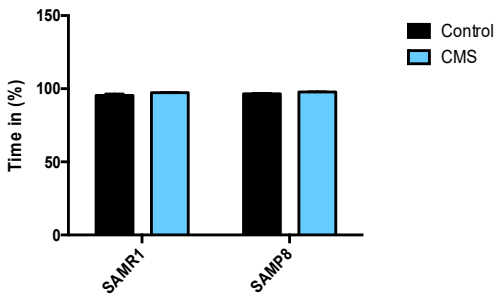
Lastly, glucose metabolism was evaluated through glucose tolerance test. SAMP8 mice showed higher glucose area under the curve than SAMR1 mice, although this relation was upside down between animals that received CMS (Fig. 7 N), suggesting that stressed SAMR1 mice develop a sugar metabolism similar to SAMP8 animals.



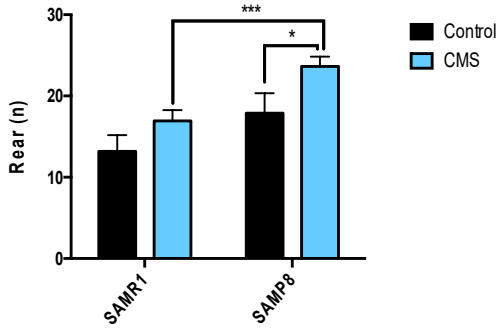
C


Center
Time in (%)
8
6
4
2
0
SAMR1
SAMP8
Control
CMS

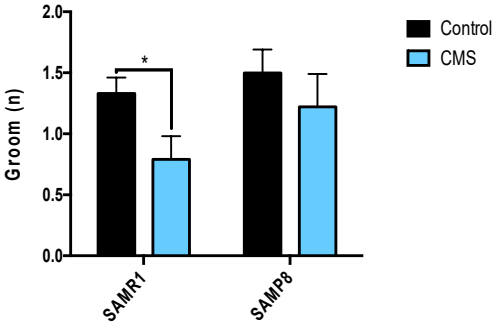
D


Periphery
Time in (%)
150
100
50
0
SAMR1
SAMP8
Control
CMS

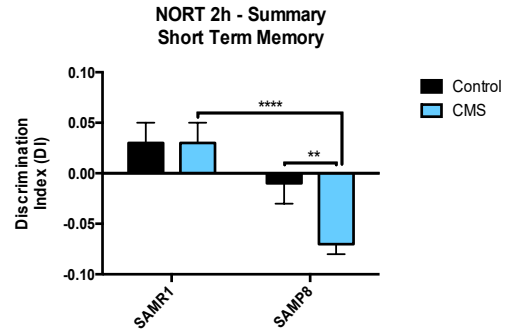
E


Rear (n)
30
20
10
0
***
*
SAMR1
SAMP8
Control
CMS

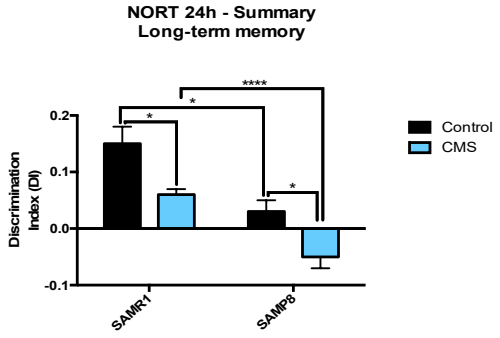
F


Groom (n)
2.0
1.5
1.0
0.5
0.0
*
SAMR1
SAMP8
Control
CMS

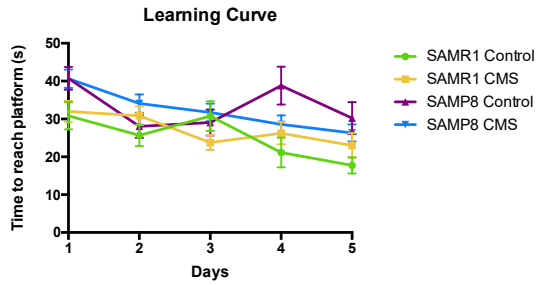
G


NORT 2h - Summary
Short Term Memory
Discrimination Index (DI)
0.10
0.05
0.00
-0.05
-0.10
****
**
SAMR1
SAMP8
Control
CMS

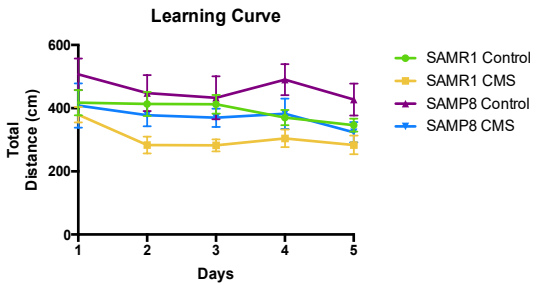
H


NORT 24h - Summary
Long-term memory
Discrimination Index (DI)
0.2
0.1
0.0
-0.1
****
*
*
*
SAMR1
SAMP8
Control
CMS

I


Learning Curve
Time to reach platform (s)
50
40
30
20
10
0
1
2
3
4
5
Days
SAMR1 Control
SAMR1 CMS
SAMP8 Control
SAMP8 CMS

J


Learning Curve
Total Distance (cm)
600
400
200
0
1
2
3
4
5
Days
SAMR1 Control
SAMR1 CMS
SAMP8 Control
SAMP8 CMS

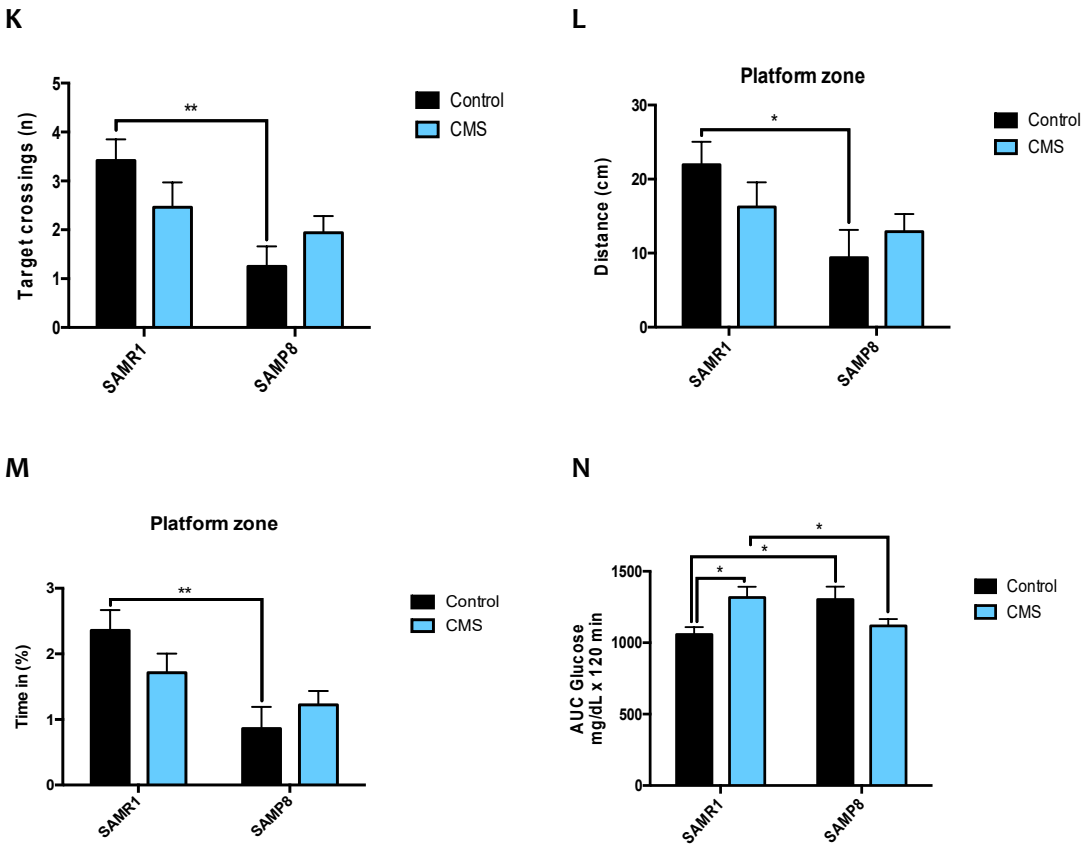


Figure 7. Behavioural and cognitive parameters after CMS in SAMR1 and SAMP8 mice. *Results of OFT for all mice groups. Locomotor Activity (A), Ratio of the distance travelled in the Center/Periphery zone (B), Time in the Center (C) and in the Periphery (D), number of Rearing (E) and number of Grooming (F). Results of NORT for all mice groups. Summary of DI from 2 and 24 hours after familiarization phase (G-H). Results of MWM for all mice groups. Learning curves of MWM during the spatial acquisition phase (I-J), number of Entries (K), Distance travelled (L) and Time (M) in platform zone during the test. Plasma levels of glucose 2g/kg intraperitoneal (i.p.) administration (N). Values are mean ± Standard error of the mean (SEM) (n=14 for each group). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.*

3.2.5 Discussion

Understanding the mechanisms that define aging and differentiate what determines whether it is pathologic or healthy is one of the challenges that science has faced in recent years. Life expectancy has increased and so the number of older people. In addition, the life rhythm has changed becoming increasingly stressful. It has been shown that the environment is extremely important in the development of several diseases, compromising healthy aging; specifically, stressful lifestyle has been identified as an important risk factor for cognitive decline. Therefore, it is crucial to study the effects of stress on cognition and

its relationship with aging in order to unveil what challenges we might have to cope with as a society in a not so far future. We hypothesize that chronic stress would modulate a large constellation of cellular mechanisms implied in aging and age-related neurodegenerative pathologies.

During aging, there are several epigenetic mechanisms altered, which include DNA methylation, histone modifications, nucleosome remodelling and miRNA-mediated gene regulation (Harman & Martín, 2019; Foster et al., 2017). In the present study, we evaluated the three main epigenetic marks, which modulate chromatin structure and act as platforms for recruitment, assembly or retention of chromatin-associated factors: histone posttranslational modifications, DNA methylation and miRNA-mediated gene regulation. One of the posttranslational modifications of histones is acetylation. This process removes histone positive charge; thereby the condensed chromatin (heterochromatin) is transformed into a more relaxed structure (euchromatin) that is associated with greater levels of gene transcription (Turner, 2000; Watson et al., 2014). However, chromatin condensation depends on different processes including methylation and histone deacetylation. This last process is due to histone deacetylases (HDAC) enzymes. Deregulation of histone acetylation has been related to increase the risk of age-dependent memory impairment in mice (Cuadrado-Tejedor et al., 2019; Griñán-Ferré et al., 2016; Peleg et al., 2010). Specifically, lysine 12 histone H4 acetylation alterations cause impaired memory consolidation as well as its restoration reinstates the expression of learning-induced genes and in consequence, cognitive abilities (Peleg et al., 2010). In accordance to Cosín-Tomás (Cosín-Tomás et al., 2014), we found that CMS increased HDAC2 protein levels only in SAMR1 females, similarly to SAMP8 control mice, which leads us to hypothesize that acetylated histone protein levels were diminished. This was confirmed evaluating acetylated H3 and H4 protein levels. However, while stress produced changes in H3 acetylation, these changes were not observed in H4. Nevertheless, the decrease in H3 acetylation in SAMR1 mice under CMS, similarly to not stressed SAMP8 was correlated with changes on cognition. Other deacetylase enzymes are the Sirt family; however, it is worth noting that several members of this family do not have deacetylase activity (Herskovits & Guarente, 2014). Sirt have been linked to aging as they modulate genomic stability, stress resistance and energy metabolism. Activation of Sirt1 enables the deacetylation of a variety of proteins, resulting in a robust, protective cellular response, as it regulates processes such as cell death, metabolism or neurodegeneration (Pallàs, 2012); while Sirt2 has been reported to regulate OS, genome integrity and myelination and its dysfunction is found in

most of age-related neurodegenerative disorders such as AD, Parkinson's disease or Amyotrophic Lateral Sclerosis, as well as in physiological aging (Fourcade et al., 2018). Accumulated evidence indicates that *Sirt6* gene expression is lower in the hippocampus and cerebral cortex of aged mice (Fourcade et al., 2018; Jesko et al., 2017) and that it is concerned with acetylated lysine 9 histone 3 (H3K9) (Khan et al., 2018). In reference to this family of deacetylases our results demonstrate decreased *Sirt1, Sirt2* and *Sirt6* gene expression in SAMP8 mice compared to SAMR1 and to SAMR1 under CMS. Compelling evidence has proposed that methylation of H3K9 promotes DNA methylation maintenance in mammals and is a hallmark of heterochromatin formation and subsequent gene silencing (Harman and Martín, 2019; Palmer et al., 2008; Zhao et al., 2016). As with histone phosphorylation, CMS treatment increased H3K9 methylation in SAMP8 animals but not in SAMR1 suggesting that even they showed more H3 acetylation, also resulted in more methylation. Therefore, we demonstrate that CMS favoured chromatin condensation and in consequence, promoted gene silencing.

In reference to other modifications, histone phosphorylation belongs to the cellular response to DNA damage, as phosphorylated histone H2A.X demarcates large chromatin domains around the site of DNA breakage (Harman and Martín, 2019). Additionally, multiple studies have also shown that histone phosphorylation plays crucial roles in other nuclear processes, such as DNA replication because of apoptosis or DNA damage (Rossetto et al., 2012). Here CMS raised phosphorylated H2A.X protein level in senescence-accelerated but not in senescence-resistant mice, suggesting that stressful stimuli activate repair/survival mechanisms rather than apoptotic response in SAMP8 mice but not in SAMR1. As two major mechanisms for epigenetic regulation, DNA methylation and histone modifications must act coordinately (Zhao et al., 2016). It is well known that DNA methylation at the fifth position of cytosine (5-mC) plays an important role in neuronal gene expression and neural development. Several studies support that dysregulated DNA methylation/demethylation is linked to many neuronal disorders, including AD onset and progression; however, the relationship between AD and altered 5-mC levels is not known (Fetahu et al., 2019). 5-mC can be further oxidized to 5-hmC, among others, by the TET family of dioxygenases. 5-mC and 5-hmC exert opposite effects on gene expression; the former is general associated with gene silencing, whereas the latter is mainly involved in up-regulation of gene expression (Sherwani and Khan, 2015). In this study, stressful environment produced lower 5-mC in both mice strains and differences between strains were observed in 5-hmC marker. Accordingly, TET2 protein levels differed between SAMR1 and SAMP8 animals. Despite our

previous results, conversely 5-mC and 5-hmC results appear to contradict the transcriptional access to DNA, but as is known, the term global DNA methylation describes this process across the entire genome and does not represent a precise landscape for specific transcriptional activity; however, global methylation determination is useful as it provides an over-arching picture of methylation status, it is misleading which genes show altered DNA methylation and which do not (Yokoyama et al., 2017).

Furthermore, we evaluated expression changes of miRNAs related to OS, AD neurophatological hallmarks, autophagy and neurodegeneration. Of 22 miRNA evaluated, those that presented statistically significant changes under experimental conditions were further validated and target genes studied (Fig. S2). Firstly, we studied miR-29c relative expression, which is involved in neural proliferation regulation through Dnmt3a. Dnmt3a expression was higher in SAMP8 compared to SAMR1 mice correlating with miR-29c-3p gene expression. In our hands CMS slightly contributed to change *Dnmt3a* expression in mice, being increased in SAMR1. Because, recent studies demonstrate that DNMT inhibitors provided neuroprotection in cellular cultures (Chestnut et al., 2011; Hernandez et al., 2011), the increase in *Dnmt3a* induced by CMS could mean a deleterious effect on SAMR1 health.

Considering AD neuropathology, we studied *miR-431-5p, miR-298-5p, miR-98-5p* and *miR-140-5p*. It has been described that *miR-431-5p* cooperates with DKK1 to inhibit Wnt/β-catenin pathway. SAMP8 showed lower relative expression of this miRNA in comparison to SAMR1 mice; changes in β-catenin protein levels were in line with *miR-431-5p*. Furthermore, CMS reduced mildly *miR-431-5p* gene expression and β-catenin protein levels in SAMR1. β-catenin is regulated by GSK-3β activity, depending on its phosphorylation. As reported, GSK3-β inactive form (Ser9 phosphorylated) protein levels were lower in SAMP8 in reference to SAMR1 (Pallàs, 2012), and also CMS decreased them, especially in SAMP8. It is known that GSK3-β activity is associated with AD neuropathology as it exacerbates cognitive impairment (Llorens-Martín et al., 2014). In fact, higher activity of this kinase has been found in AD patients and its inhibition restores spatial memory deficits, reduces Tau hyperphosphorylation and decreases reactive gliosis and neuronal death in rodents (Llorens-Martín et al., 2014). Furthermore, it has been described that GSK3-β inhibition reduces BACE1-mediated cleavage of APP through NF-κB signalling-mediated mechanism, so that it reduces β-amyloid pathology (Ly et al., 2013). Accordingly, we found that CMS increased Ser396 and Ser404 Tau hyperphosphorylation, most pronounced in SAMP8, and promoted amyloid precursor protein (APP) gene expression in both mice strains. In fact,

APP processing is regulated by *miR-298-5p*, which in turn regulates BACE1. *miR-298-5p* expression differed between SAMR1 and SAMP8 animals, in contrast with BACE1 protein levels. It is reported that BACE1, sAPPβ and β-Carboxiterminal fragments protein levels were increased when *miR-98-5p* up-regulated. CMS increased *miR-98-5p* relative expression and sAPPβ in SAMR1 compared to the control group, but not in SAMP8. However as mentioned, BACE1 was not modulated under CMS. Taking into account that BACE1 can be up- or down-regulated by different miRNAs, discrepancies can be explained by compensatory responses among different signals. ADAM10 protein levels are controlled by *miR-140-5p*. Again, huge differences in *miR-140-5p* gene expression and ADAM10 protein levels were observed between strains. Differences were between mice under CMS in SAMR1 but not in SAMP8.

miR-181a-5p is involved in regulation of cell growth, proliferation and survival through mTOR complex 1 (mTORC1) and downstream pathway, which protein levels were increased in SAMP8 in comparison to SAMR1 mice. In addition, stressful stimuli increased *miR-181a-5p* relative expression in SAMR1 mice compared to their control littermates and no changes were observed between SAMP8 animals. By contrast, CMS groups in both strains showed higher mTORC1 protein levels than respective control littermates. Lastly, BCL2 protein levels were controlled by *miR-106b-5p*. SAMP8 mice had higher *miR-106b-5p* compared to SAMR1, but no changes were found in BCL2 protein levels. CMS reduced *miR-106b-5p* gene expression, increasing BCL2 protein levels in SAMP8, without affecting SAMR1 strain.

Autophagy declines during aging, so that it may contribute to the deleterious accumulation of aberrant proteins observed in aged cells. Interestingly, failure of this process has been reported to worsen aging-associated diseases, such as neurodegeneration or cancer (Gouras, 2019). Pro autophagic proteins such as Beclin1 and LC3B became decreased after CMS treatment and in concordance p-mTOR ratio was increased. Moreover, we demonstrated huge differences between SAMR1 and SAMP8 control groups, indicating a reduced autophagic flux in senescent mice. CMS induced impairment in autophagy was more robust and consistent in SAMR1 than in SAMP8, supporting the hypothesis that CMS accelerates the senescence process.

Following the same line about overall processes linked to senescence, it has been widely described that OS possesses a pre-eminent role in pathological senescence and the pathogenesis of AD. In general, cells possess antioxidant mechanisms to cope with OS,

such as GPX, Catalase and SOD, among others; which in turn are regulated by NRF2 transcriptional pathway. Herein we found that mice under CMS had lower NRF2 protein levels than control groups and this in turn could explain the lowest protein levels of antioxidant defence as GPX1, SOD1 and Catalase. Consistent with this, CMS promoted an increase in the pro-oxidant enzyme *Aox1* gene expression and higher accumulation of ROS. Noteworthy, as described, differences between strains were found in most of the evaluated markers (Griñán-Ferré et al., 2016).

As far as aging and AD are concerned, dysregulation of inflammatory mediators and astrogliosis are major culprits in the development of chronic inflammation and the immunosenescence process, as well as are related to cognitive decline and progression of neurodegenerative diseases (Chung et al., 2019). For instance, our results demonstrate significant increase in protein levels for NF-κB, transcription factor regulating pro-inflammatory signals, in SAMP8 mice in comparison with SAMR1. In accordance to Chung et al. (Chung et al., 2019), *Il-6* and *Tnf-α* gene expression were up regulated in SAMP8 mice. Interestingly CMS increased proinflammatory pathways in SAMR1 but not in SAMP8. Moreover, we found an increase in *Gfap* gene expression in SAMR1 CMS compared to SAMR1 control group. In accordance, it has been recently described that modulating astrogliosis enhances AD pathology in mice (Reichenbach et al., 2019).

It is widely accepted the detrimental effect of stress on psychological well-being and cognitive functioning, emphasizing the relationship between stress and memory. It is noteworthy that chronic stress signs underlie some of the characteristics described in cognitive decline, either in aging or in neurodegenerative diseases, such as AD (Puigoriol-Illamola et al., 2018; Turner & McCarthy, 2017). It has been stated that epigenetic machinery is essential for cognitive function (Harman & Martín, 2019; Peleg et al., 2010; Stilling & Fischer, 2011). Likewise, DNA methylation influences hippocampal memory formation and growing evidence suggests that the modulation of epigenetic processes by stress, environmental enrichment and/or hormones is key in regulating memory function.

In line with the molecular results presented, behavioural changes induced by CMS were explored in SAMP8 and SAMR1 mice. Results obtained pointed out that not only a stressful environment triggers anxiety-like behaviour, but also it mitigates cognitive performance. Locomotor activity, the distance travelled in the central zone and other parameters indicating well-being/discomfort were altered in mice under CMS. Regarding

recognition memory, clear differences were found between the different strains, although we can also assert a detrimental effect caused by CMS, especially on long-term memory. In agreement with these results, learning abilities and spatial memory in SAMR1 and SAMP8 at 6 months of age were significantly different and were negatively affected by the presence of chronic stressors. While stressful situations, glucose levels increase in order to have enough energy available to cope with stress. Aberrant glucose metabolism potentiates the aging phenotype and contributes to early stage central nervous system pathology (Currais et al., 2012). According to previous works, we found differences on blood glucose levels due to stress in SAMR1 mice, exhibiting similar glucose tolerance to SAMP8 control mice.

3.2.6 Conclusions

Overall, CMS treatment produces detrimental effects in female SAM mice, such as inflammatory signalling activation, loss of antioxidant defence mechanisms, changes in behaviour and reduced cognitive abilities (Fig. 8). Interestingly, CMS promoted significant epigenetic and biochemical changes in SAMR1 animals, a normal mice strain, driving them to an aging-specific phenotype represented by SAMP8 mice, a senescence mice model, in which the negative effects of CMS are probably limited because its senescence level is already elevated.

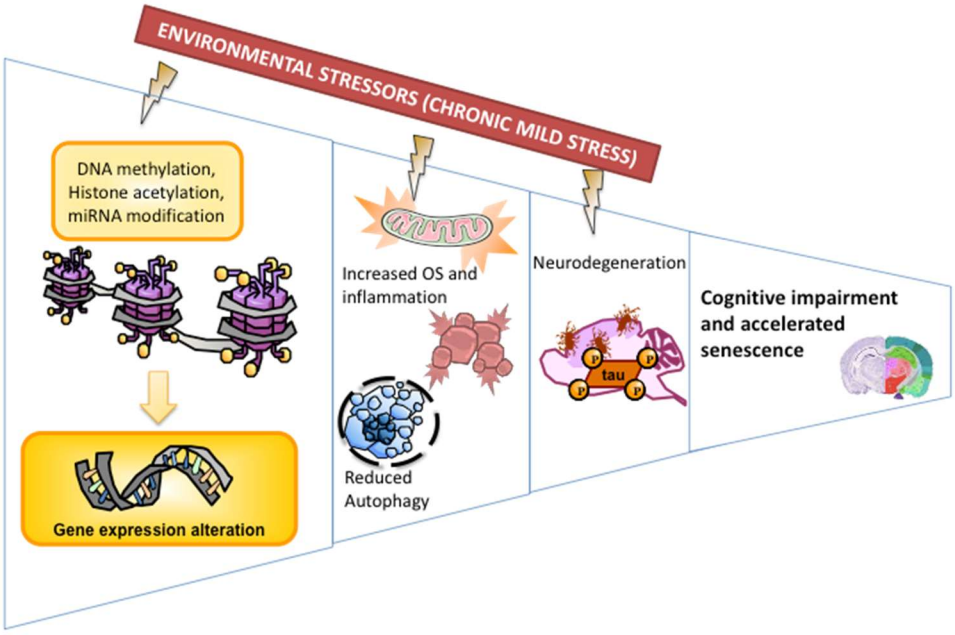


Figure 8. Representative scheme of molecular pathways altered after Chronic Mild Stress.

3.2.7 Acknowledgements

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3.2.9 Supplementary material

Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/3/1154/s1.

3.3 RL-118 and 11β-HSD1 target engagement through TAPS assay: behaviour and molecular analysis

Puigoriol-Illamola, D.[1,2], Companys-Alemany, J.[1,2], Homer, N.[3], Leiva, R.[4], Vázquez, S.[4], Mole, D.[5], Griñán-Ferré, C.[1,2], Pallàs, M[1,2]. RL-118 and 11β-HSD1 target engagement through TAPS assay: behaviour and molecular analysis

[pending to submit]

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***** *Chapter 3 is adapted from a paper pending to be submitted in near future. Therefore, it has been included and structured as a full article, but it may be modified as new results are obtained prior to being sent for peer review. On date of its final edition, it constitutes the most updated version.*

3.3.1 Abstract

Taking into consideration the convergence of ageing, stress and neurodegenerative diseases, such as AD, there is impaired GC signalling. Therefore, the study of GC-mediated stress response to chronic moderate stressful situations, as account in daily life, becomes of huge interest to design pharmacological strategies to prevent neurodegeneration.

To address this issue, SAMP8 were exposed for 4 weeks to the CMS paradigm and treated with RL-118, an 11β-HSD1 inhibitor. In fact, several pieces of evidence link CMS exposure to reduced cognitive performance, while inhibition of 11β-HSD1 has been associated with reduction of GC activity and cognitive improvement. The aim of this project was to assess whether RL-118 treatment could restore the deleterious effects of CMS on cognition and behavioural abilities, but also on molecular mechanisms that compromise healthy ageing in SAMP8 mice.

On the one hand, we determined the target engagement between RL-118 and 11β-HSD1. Therefore all the beneficial effects previously described in SAMP8 treated with the drug can undoubtedly be attributed to the inhibition of this enzyme. Besides, herein we observed decreased DNA methylation, hydroxymethylation and histone phosphorylation induced by CMS but, on the contrary, increased after RL-118 treatment. In addition, CMS exposure produced ROS damage accumulation, and increments of pro-oxidant enzymes as well as pro-inflammatory mediators through NF-κB pathway and astrogliosis markers, like *Gfap*. Of note, those modifications were recovered by 11β-HSD1 inhibition. Remarkably, although CMS altered mTORC1 signalling, autophagy was increased in SAMP8 treated with RL-118 mice. Also, we found that amyloidogenic APP processing pathway was favoured and decreased synaptic plasticity and neuronal remodelling markers in mice under CMS, but changed after RL-118 treatment. In consequence, detrimental effects on behaviour and cognitive performance were detected in CMS exposed mice, although recovered after concomitant 11β-HSD1 inhibition by RL-118.

Overall, CMS is a feasible intervention to understand the influence of stress on epigenetic mechanisms underlying cognition and accelerating senescence. However and most important, 11β-HSD1 inhibition through RL-118 turned up to restore the majority of these detrimental effects caused by CMS, indicating that GC excess attenuation may become a potential therapeutic strategy for age-related cognitive decline and AD.

3.3.2 Introduction

In the past few years, stress and stress response have been an overwhelming public health issue with a huge amount of literature, and in turn misinformation of the detrimental effects that stress may have in modifying aging, determining healthy or pathological aging of the brain, as well as in the development of several diseases (Catania et al., 2009; Sotiropoulous et al., 2008). Drug discovery studies have become of paramount importance in order to offer different drugs to treat or prevent the detrimental effects on cognition during aging and under aversive conditions. In consequence, diverse tools have recently been developed considering the drug-target binding, as well as cellular permeability, specificity and cytotoxicity, such as the toxicity-affinity-permeability-selectivity (TAPS) assay that is based on overexpressing the protein of interest and treating the cells with the drug, followed by FACS (fluorescence-activated cell sorting) coupled to mass spectrometry (MS) analysis.

Stressful situations activate a neuroendocrine response, which leads to the release of catecholamines at the first stage, and later with glucocorticoids (GCs). Active GCs bind to their receptors promoting slow genomic actions as well as rapid nongenomic effects, such as glucose release, lipolysis, motivation to eat palatable food and up-regulation of the expression of anti-inflammatory cytokines (Pazirandeh et al., 2002; Sandi, 2013). The ability to mount GC release is determined by the quality, intensity and chronicity of the stressful stimulus (Dhabhar, 2018). Primarily, stressful experiences are adaptive, facilitating the restoration of physiological and behavioural homeostasis and necessary for the establishment of enduring memories. However, when stressful situations are persistent in time, memory formation and reasoning become impaired (Tatomir et al., 2014; Wang et al., 2016). In this context it has been suggested that the deleterious effects of stress and GC are transient in nature, because stress-induced hippocampal atrophy and hippocampal-dependent behaviour may be reversed after a stress-free period. Overall, it is proposed that stress and GC can have widespread detrimental effects on mood and cognition, by inducing changes in brain structure (involving the generation and loss of neurons and dendritic atrophy) and function (electrophysiological activity and cellular signalling) (Sotiropoulous et al., 2011).

Several studies suggest that environmental stressors influence hypothalamic-pituitary-adrenal (HPA) axis activity and behaviour by altering the methylation status of key

genes concerned with the regulation of the stress response (Harman & Martín, 2019; Sotiropoulous et al., 2008). Recent evidence has demonstrated significant associations between epigenetic alterations and stress showing that under chronic stressors influence, histone acetylation and DNA methylation are decreased, among others (Harman & Martín, 2019; Puigoriol-Illamola et al., 2020a). Accordingly, it has been demonstrated that chronic mild stress (CMS) paradigm in mice induces epigenetic changes, reduces cognitive abilities and increases anxiety-like behaviour (Puigoriol-Illamola et al., 2020a; Wang et al., 2016).

Moreover, prolonged exposure to GCs has been related to immunosuppression, metabolic syndrome, diabetes, osteoporosis, reproductive failure, hypertension and mood and affective disorders. Maladaptive adjustments to stress may, sequentially, lead to symptoms of depression and Alzheimer's disease (AD). Additionally, recent clinical studies suggest that GCs are implicated in the pathogenesis and/or progression of AD (Canet et al., 2018; Sotiropoulous et al., 2011). In particular, chronic stress has been associated with increased Aβ deposits and hyperphosphorylated Tau (Bisht et al., 2018; Puigoriol-Illamola et al.,2020a). In view of these affections, it is important to note that stressful events can have long-term consequences for the immune system. On one hand, pro-inflammatory cytokines have been implicated in the genesis of AD, becoming part of the amyloid plaques and triggering Aβ production. Additionally, NF-κB appears to be essential as it triggers a feed-forward cycle in which increased cytokine levels result in resistance to the GC immunosuppression, leading to further increases in cytokine release and, therefore, activation of the HPA axis (Irwin & Miller, 2007; Pace et al., 2007). Increasing evidence has reported a link between the HPA axis and Oxidative Stress (OS) in reactive oxygen species (ROS) production (Bonet-Costa et al., 2016; Schiavone et al., 2013), another factor related to AD pathology and progression.

In addition, GCs are also involved in the regulation of cell fate, modulating pro-/anti-apoptotic mechanisms and survival proteins, such BDNF, CREB, Bcl2 and NCAM, so that may exert significant influence over neuroplasticity (Pittenger & Duman, 2008; Sandi, 2004, et al., 2013; Sotiropoulous et al., 2008; Wang et al., 2016). Noteworthy for cell survival and longevity, autophagy activation becomes crucial, as it participates in the elimination of disrupted proteins and is implied in the maintenance of cellular homeostasis (Puigoriol-Illamola et al., 2020a).

Disposition of active GCs is controlled by 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) enzyme and up to now, our group has demonstrated that its inhibition exerts neuroprotective effects (Leiva et al., 2017; Puigoriol-Illamola et al., 2018, 2020b). The aim of this project is to assess RL-118 and 11β-HSD1 target engagement and evaluate whether 11β-HSD1 inhibition is able to face the detrimental effects of CMS.

3.3.3 Material and Methods

Cloning

The E2-Crimson-human HSD11B1 gene (variant 1) was synthesised by GenScript in vector pUC57. The DNA sequence for E2-Crimson was sourced from Clontech. The gene for the fluorescent-HSD11B1 was ligated into vector pcDNA3.1 (Invitrogen) using restriction sites NheI (N-terminus) and NotI (C-terminus) (Fig. S1).

Transient expression of fluorescent target protein

HEK293 cells were passaged in poly-D-Lysine treated plates and incubated overnight in OPTI-MEM medium (Lonza) at 37°C, 5% CO2. The cells were transiently transfected the following day with pcDNA3.1-E1-Crimson-huHSD11B1 DNA using Lipofectamine 2000 (Invitrogen) in OPTI-MEM medium by standard transfection protocol. The transfected cells were maintained at 37°C, 5% CO_2 for 24 h post-transfection before the TAPS assay was performed. Transfection of the cells and compound screening were performed in a 6-well plate format.

TAPS Assay

Compound incubation

RL-118 drug was diluted to a concentration of 20 µM (DMSO<1%) in tissue culture medium (DMEM with 10% FBS, 1% L-Glutamine, 1% penicillin-streptomycin, (Life Technologies)), then incubated with transfected cells for three hours at 37°C, 5% CO_2. 20 µM compound concentration was selected as an appropriate concentration for assay development and screening. Following incubation, the cells were detached from the plate by gentle pipetting and centrifuged for 5 min at 1000 rpm. Culture medium was removed by pipetting and the cell pellet re-suspended in FACS buffer (PBS + 2% FBS) to wash off unbound compound. The cells were centrifuged as above, and the wash buffer removed.

The cells were then re-suspended in FACS buffer and transferred to 5 ml FACS tubes. The tubes were wrapped in foil and placed on ice until sorting.

FACS sorting

Cells were sorted using BD FACS Aria II system fitted with 100 µm nozzle. Data was acquired and processed using BD FACS Diva Software version 8.0.1. Cell fluorescence was detected using the 640 nm laser for E2-Crimson excitation. The filter used was 670/14 nm detecting fluorescence emission of E2-Crimson in the 663-677 nm range. Cells were sorted and collected into four cell populations defined by fluorescence intensity, forward (FSC) and side scatter (SSC) gating was used to exclude dead cells and only live cells were collected. Cells were collected in 5 ml FACS tubes containing 1 ml of DMEM with 10% FBS, 1% L-Glutamine and 1% penicillin-streptomycin. Each population of cells was centrifuged at 1000 rpm for 5 min. Medium was removed and the cell pellet resuspended in 20 mM HEPES pH7.0. The suspension was briefly sonicated to lyse the cells before centrifugation, as before, to pellet cell debris. The lysate (supernatant) was transferred to LC-MS vials and stored at -20°C prior to MS analysis.

Mass Spectrometry detection of the compounds in cell lysates

The chromatographic and mass spectrometer used was the SCIEX Triple Quad 5500+ LC-MS/MS System- QTRAP (Triple Quad™). 10µl injection of the cell lysate was loaded directly onto a HSST3 (150 x 2.1 mm, Thermo Fisher Scientific) column at a high flow rate, causing the proteinaceous material to flow to waste. A series of valve switches led to the elution of the extracted sample from the column directly onto the analytical column. Solvent A was water with 0.1% formic acid and solvent B was methanol with 0.1% of formic acid. Automated tune settings were used to achieve the maximum ion signal for the analyte for initial validation experiments, optimising on tube lens voltage, parent to product transitions and collision energy for transition. Peaks detected in this initial scan were then identified by molecular weight and checked versus their mass-charge ratio. Data was acquired and processed using Sciex OS-MQ software.

Animals

Female SAMP8 mice (n=48) 4 months old were used to carry out behavioural, cognitive and molecular analyses. We divided these animals into four groups: SAMP8

Control (Control, n=12), SAMP8 treated with RL-118 (11β-HSD1i, n=12), SAMP8 under CMS (CMS, n=12) and SAMP8 treated with RL-118 under CMS (SAMP8 11β-HSD1i+CMS, n=12). Animals had free access to food and water and were kept under standard temperature conditions (22±2°C) and 12h: 12h light-dark cycles (300lux/0 lux). RL-118 was administered at 21 mg/kg/day by oral gavage for 4 weeks. CMS consisted of several different stressful stimulus applied to the corresponding animals daily during 4 weeks.

Studies and procedures involving mice brain dissection and subcellular fractionation were performed following the institutional guidelines for the care and use of laboratory animals established by the Ethical Committee for Animal Experimentation at the University of Barcelona.

Chronic Mild Stress Treatment

CMS procedure used in the present study has been previously validated in SAMR1 and SAMP8 mice (Puigoriol-Illamola et al., 2020b). For 4 weeks, mice were daily exposed to various randomly scheduled, low-intensity environmental stressors. The number and the type of stressful stimuli applied changed everyday as well as the sequence of the stressors in order to guarantee the degree of unpredictability (Puigoriol-Illamola et al., 2020a). Stressful stimuli included 2 h of physical restraint, 24 h of sawdust removal, 24 h of food deprivation, 24 h of water deprivation, 24 h of wet bedding, 1 min of tail nipping at 1 cm from the tip of the tail and overnight illumination.

Behavioural and cognitive tests

Elevated Plus Maze (EPM)

The Elevated Plus Maze (EPM) was performed as previously described (Griñán-Ferré et al., 2016). It is based on mice preference for dark enclosed places, therefore it evaluates anxiety-related and risk-taking behaviours. EPM apparatus consists of 2 opened and 2 closed arms elevated 50 cm from the floor. Mice were placed on the central platform, facing opened arms and allowed to explore the apparatus for 5 min. After that, mice were returned to their home cages and EPM arms were cleaned with 70% ethanol in order to avoid any olfactory clues. Behaviour was scored with SMART® ver. 3.0 software and each trial were recorded for later analysis. Parameters evaluated included time spent on opened and closed arms, rearing, defecation and urination.

Novel Object Recognition Test (NORT)

The Novel Object Recognition Test (NORT) protocol employed was as described in Puigoriol-Illamola et al. (2020b). In brief, mice were placed in a 90°, two-arms, 25-cm-long, 20-cm-high, 5-cm-wide black maze. Before performing the test, the mice were individually habituated to the apparatus for 10 min for 3 days. On day 4, the animals were submitted to a 10-min acquisition trial (first trial), during which they were placed in the maze in the presence of two identical, novel objects at the end of each arm. After a delay (2h and 24h), the animal was exposed to two objects one old object and one novel object. The Time that mice explored the Novel object (TN) and Time that mice explored the Old object (TO) were measured. A Discrimination Index (DI) was defined as (TN−TO)/(TN+TO). To avoid object preference biases, objects were counterbalanced. The maze, the surface, and the objects were cleaned with 70% ethanol between the animals' trials to eliminate olfactory cues.

Morris Water Maze (MWM)

This test evaluates both learning and spatial memory (Vorhees, 2006). An open circular pool (100 cm in diameter, 50 cm in height) filled with water was used. Water was painted white with latex in order to make it opaque and its temperature was 22± 1°C. Two main perpendicular axes were established (North-South and East-West), thus configuring four equal quadrants (NE, NW, SE, and SW). Four visual clues (N, S, E, W) were placed on the walls of the tank so that the animal could orientate and could fulfil the objective. The test consists of training a mouse to find a submerged platform (Learning phase) and assesses whether the animal has learned and remembered where the platform was the day that it is removed (Test). The training lasts five consecutive days and every day five trials are performed, which have a different starting point (NE, E, SE, S, and SW), with the aim that the animal recognizes the visual clues and learns how to locate the platform, avoiding learning the same path. At each trial, the mouse was placed gently into the water, facing the wall of the pool, allowed to swim for 60 seconds and there was not a resting time between trials. If the animal was not able to locate the platform, the investigator guided it to the platform and was allowed to rest and orientate for 30 seconds. The platform was placed approximately in the middle of one of the quadrants, 1.5 cm below the water level. Above the pool there was a camera that recorded the animals' swimming paths and the data was analysed with the statistical program SMART® ver.3.0. During the learning phase, a learning curve was drawn, in which the latency to find the platform each training day is

represented. On the day test, more parameters were measured, such as the target crossings and the swum distance in the platform zone.

Brain Processing

Three days after the behavioural and cognitive tests, 12 animals per group were euthanized for protein extraction, RNA and DNA isolation. Brains were immediately removed from the skull and the hippocampus was isolated, frozen on powdered dry ice and maintained at -80°C until procedures.

Western Blotting

Tissue samples were homogenized in lysis buffer (Tris HCl pH 7.4 50 mM, NaCl 150 mM, EDTA 5 mM and 1 X-Triton X-100) containing phosphatase and protease inhibitors (Cocktail II, Sigma-Aldrich) to obtain total protein homogenates. For subcellular fractionation, 150 µL of buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA pH 8, 0.1 mM EGTA pH 8, 1 mM DTT, 1 mM PMSF, protease inhibitors) were added to each sample and incubated on ice for 15 min. After this time, the samples were homogenized with a tissue homogenizer, 12.5 µL Igepal 1% were added, and mixed for 15 s. Following 30 s of full-speed centrifugation at 4°C, supernatants were collected (cytoplasmic fraction); 80 µL of buffer C (20 mM HEPES pH 7.9, 0,4M NaCl, 1 mM EDTA pH 8, 0.1 mM EGTA pH 8, 20% Glycerol 1 mM DTT, 1 mM PMSF, protease inhibitors) were added to each pellet and incubated under agitation at 4°C for 15 min. Subsequently, samples were centrifuged for 10 min at full speed at 4°C. Supernatants were collected (nuclear fraction) and 40 µL of buffer A+HCl (buffer A with 0.2 N HCl) were added to the pellet. After a 30-min incubation on ice, samples were centrifuged, again at full speed, at 4°C for 10 min and the supernatants were collected (the histone fraction). Aliquots of 15 µg of hippocampal protein extraction per sample were used. Protein samples were separated by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (8-14%) and transferred onto Polyvinylidene difluoride (PVDF) membranes (Millipore). Afterwards, membranes were blocked in 5% non-fat milk in Tris-buffered saline (TBS) solution containing 0.1% Tween 20 TBS (TBS-T) for 1 h at room temperature, followed by overnight incubation at 4°C with the primary antibodies listed in (Table S1). Then, the membranes were washed and incubated with secondary antibodies listed in (Table S1) for 1 h at room temperature. Immunoreactive proteins were viewed with the chemiluminescence-based ChemiLucent™ detection kit, following the manufacturer's protocol (ECL Kit, Millipore), and digital images were acquired using

ChemiDoc XRS+System (BioRad). Semi-quantitative analyses were done using ImageLab software (BioRad) and results were expressed in Arbitrary Units (AU), considering control protein levels as 100%. Protein loading was routinely monitored by immunodetection of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-tubulin or TATA-Binding protein (TBP).

RNA extraction and gene expression determination by q-PCR

Total RNA isolation was carried out using TRIsure™ reagent according to the manufacturer's instructions (Bioline Reagent, UK). The yield, purity, and quality of RNA were determined spectrophotometrically with a NanoDrop™ ND-1000 (Thermo Scientific) apparatus and an Agilent 2100B Bioanalyzer (Agilent Technologies). RNAs with 260/280 ratios and RIN higher than 1.9 and 7.5, respectively, were selected. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed as follows: 2 µg of messenger RNA (mRNA) was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time quantitative PCR (qPCR) was used to quantify mRNA expression of oxidative stress and inflammatory genes listed in (Table S2). SYBR® Green real-time PCR was performed in a Step One Plus Detection System (Applied-Biosystems) employing SYBR® Green PCR Master Mix (Applied-Biosystems). Each reaction mixture contained 6.75 µL of complementary DNA (cDNA) (which concentration was 2 µg), 0.75 µL of each primer (which concentration was 100 nM), and 6.75 µL of SYBR® Green PCR Master Mix (2X).

Data was analysed utilizing the comparative Cycle threshold (Ct) method (ΔΔCt), where the housekeeping gene level was used to normalize differences in sample loading and preparation. Normalization of expression levels was performed with β-actin for SYBR® Green-based real-time PCR results. Each sample was analysed in duplicate, and the results represent the n-fold difference of the transcript levels among different groups.

Global DNA Methylation and Hydroxymethylation Determination

Isolation of genomic DNA was conducted using the FitAmp™ Blood and Cultured Cell DNA Extraction Kit (EpiGentek, Farmingdale, NY, USA) according to the manufacturer's instructions. Following this, Methylflash Methylated DNA Quantification Kit (Epigentek, Farmingdale, NY, USA) and MethylFlash HydroxyMethylated DNA Quantification Kit were used in order to detect methylated and hydroxymethylated DNA. Briefly, these kits are

based on specific antibody detection of 5-mC and 5-hmC residues, which trigger an ELISA-like reaction that allows colorimetric quantification at 450 nm.

Oxidative Stress Determination

Hydrogen peroxide was measured in hippocampus protein homogenates as an indicator of OS, and it was quantified using the Hydrogen Peroxide Assay Kit (Sigma-Aldrich, St. Louis, MI) according to the manufacturer's instructions.

Data analysis

Data analysis was conducted using GraphPad Prism ver. 7 statistical software. Data are expressed as the mean ± standard error of the mean (SEM) of at least 6 samples per group. Diet and treatment effects were assessed by the Two-Way ANOVA analysis of variance, followed by Tukey post-hoc analysis or two-tail Student's t-test when it was necessary. Statistical significance was considered when *p*-values were <0.05. The statistical outliers were determined with Grubbs' test and subsequently removed from the analysis.

3.3.4 Results

TAPS Assay

In TAPS assay the RL-118 target engagement was determined. The area of the peak relative to the number of cells was higher in cells that expressed the 11b-HSD1 enzyme (11β-HSD1 positive) compared to those cells that did not express the enzyme but were transfected with this gene (11β-HSD1 negative) (Fig. 1). In addition, differences between cells that expressed 11β-HSD1 positively and the Crimson positive cells were observed, indicating that the RL-118 drug is selective for its target.

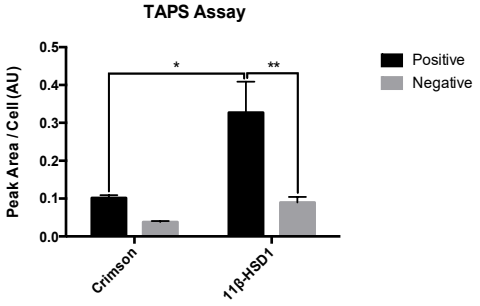
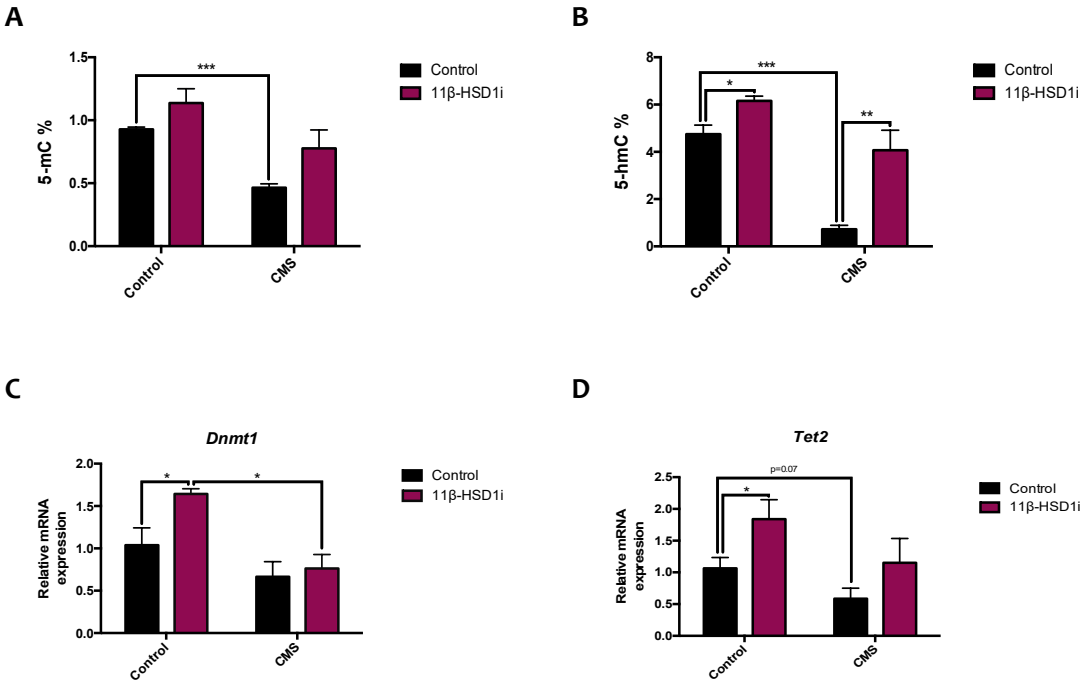


Figure 1. TAPS Assay results showing peak area per cell. *Values are the mean ± Standard error of the mean (SEM) (n=1). *p<0.05; **p<0.01.*

CMS modulated epigenetic marks promoting an increased DNA methylation but reduced histone acetylation, reversed by 11β-HSD1 inhibition

Regarding DNA methylation, it can be observed that CMS reduced the overall DNA methylation and 5-hydroxy methylation compared to control group, although 11β-HSD1 inhibition increased those marks in both treated groups (Figs. 2 A-B). In accordance to the above-mentioned, DNA-methyltransferase 1 (*Dnmt1*) and ten-eleven translocase 2 (*Tet2*) gene expression were lower in CMS treated groups, while increased in control 11β-HSD1i treated group (Figs. 2 C-D).

As far as histone modifications are concerned, histone deacetylase 2 (*Hdac2*) gene expression was diminished by RL-118 treatment in both groups treated, while CMS seemed to increase it compared to control animals (Fig. 2 E). Mice under CMS showed lower lysine 12 acetylated histone 4 (H4K12) protein levels and, accordingly, H4K12 protein levels were higher in 11β-HSD1i control group (Fig. 2 F). H3K9 and its di-methylation protein levels were higher in CMS group. Accounting 11β-HSD1 inhibition effects, mice that also received CMS treatment showed higher H3K9me2 protein levels than the control mice (Figs. 2 G-H).



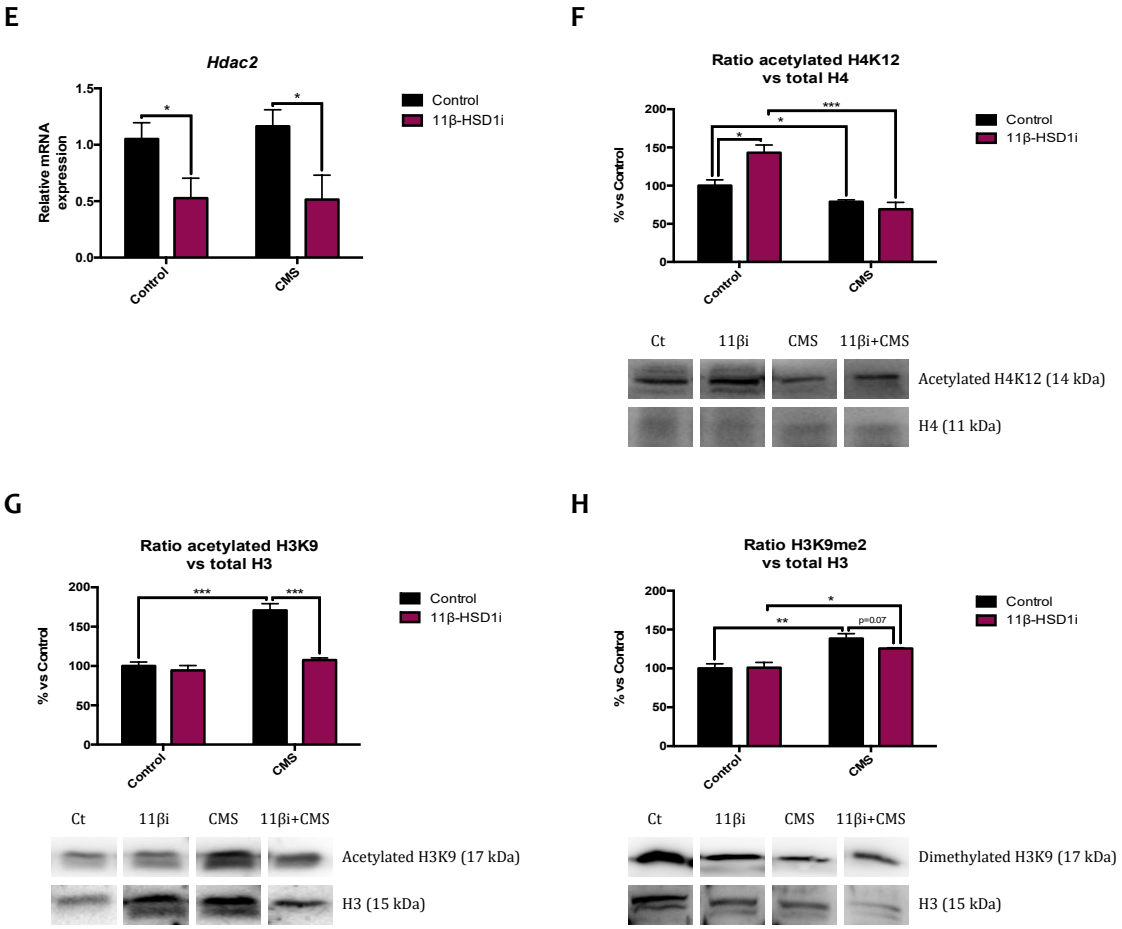


Figure 2. Representative results from epigenetic marks. *Global 5-methylated cytosine (A) and 5-hydroxymethylated cytosine levels (B). Relative gene expression of Dnmt1 (C), Tet2 (D) and Hdac2 (E). Representative Western Blot for the ratio of Lys12 acetylated H4 protein levels and quantification (F), the ratio of Lys9 acetylated H3 protein levels and quantification (G) and the ratio of H3K9me2 protein levels and quantification (H). Gene expression levels were determined by real-time PCR. Western Blot values in bar graphs are adjusted to 100% for protein levels of SAMP8 Control (Control). Values are mean ± Standard error of the mean (SEM) (n = 6 for each group). *p<0.05; **p<0.01; ***p<0.001.*

GC levels attenuation, and conversely CMS application, led to decreased overall cellular OS

CMS increased ROS concentration in both groups compared to control mice. However, RL-118 drug treatment contributed to decrease ROS levels (Fig. 3 A). ROS accumulation is regulated by antioxidant and pro-oxidant enzymes controlled by nuclear erythroid-related factor 2 (NRF2), such as heme oxygenase 1 (Hmox1) and Aldehyde oxidase 1 (Aox1). Hereby, it was found decreased NRF2 protein levels after 11β-HSD1 inhibition, as well as increased protein levels in CMS treated groups (Fig. 3 B). In accordance, *Aox1* gene

expression was diminished in RL-118 treated groups, although increased after CMS treatment (Fig. 3 C). The same gene expression pattern was observed in for inducible nitric oxide synthase (*iNOS*), which is an enzyme implied in the synthesis of pro-oxidant molecules (Fig. 3 D).

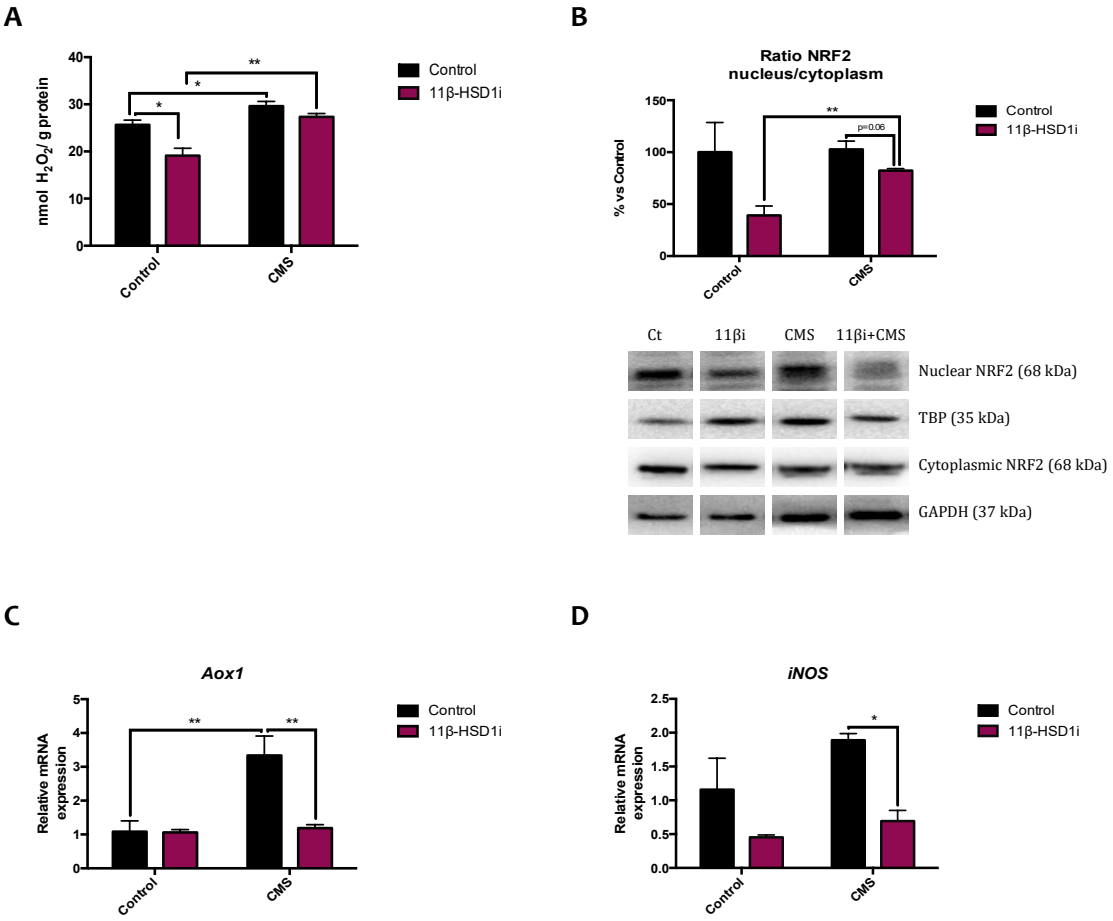


Figure 3. Representative results from pro-oxidant vs antioxidant mechanisms imbalance. *Representative ROS accumulation measured as hydrogen peroxide concentration in homogenates of hippocampus tissue (A). Representative Western Blot for the ratio of nuclear/cytoplasmic NRF2 protein levels and quantification (B). Relative gene expression of Aox1 (C) and iNOS (D). Western Blot values in bar graphs are adjusted to 100% for protein levels of SAMP8 Control (Control). Gene expression levels were determined by real-time PCR. Values are mean ± Standard error of the mean (SEM) (n=6 for each group). *p<0.05; **p<0.01.*

Pro-inflammatory markers were reduced after 11β-HSD1 inhibition and CMS increased astrogliosis markers

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a protein complex that mainly regulates cytokine production, thus inflammatory signalling and

immune response to infection. Albeit no statistical differences were observed in NF-κB protein levels, there was a tendency to decrease after RL-118 treatment in both treated groups (Fig. 4 A). However, there were differences in several cytokines gene expression controlled by this nuclear factor. Generally, CMS increased interleukin 1β (*Il-1β*), chemokine (C-X-C motif) ligand 2 (*Cxcl-2*) and tumour necrosis factor α (*Tnf-α*) gene expression, although no statistical differences were found. By contrast, 11β-HSD1 inhibition led to decreased cytokine gene expression (Figs. 4 B-D). Moreover, glial fibrillar acidic protein (*Gfap*) gene expression was evaluated and CMS increased its gene expression, which was reversed by 11β-HSD1 inhibition (Fig. 4 E).

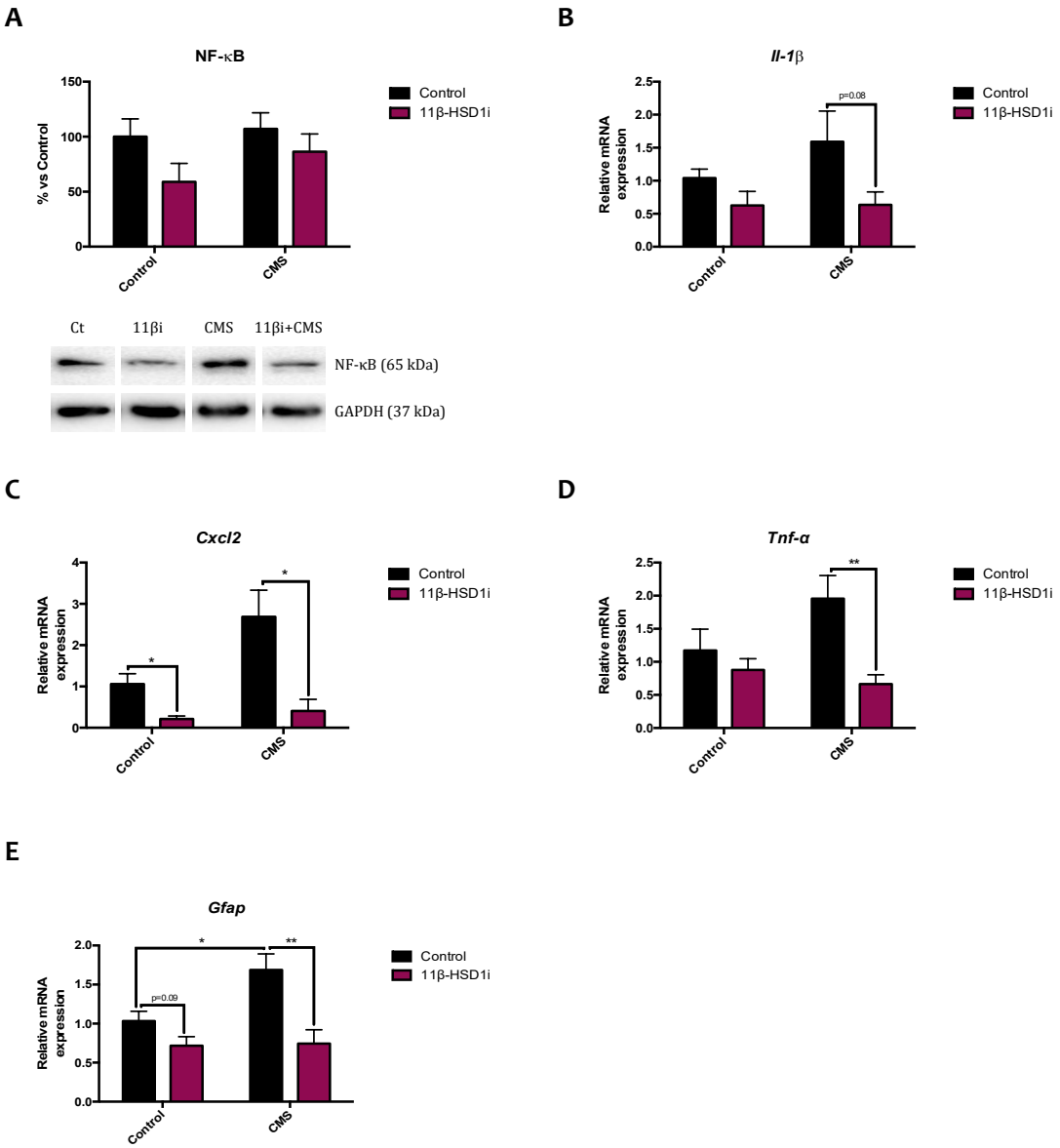


Figure 4. Representative results from inflammatory pathways. *Representative Western Blot for NF-κB protein levels and quantification (A). Relative gene expression of Il-1β (B), Cxcl2 (C),*

*Tnf-α (D) and Gfap (E). Western Blot values in bar graphs are adjusted to 100% for protein levels of SAMP8 Control (Control). Gene expression levels were determined by real-time PCR. Values are mean ± Standard error of the mean (SEM) (n=6 for each group). *p<0.05; **p<0.01.*

11β-HSD1 inhibition and CMS promoted autophagy

Several autophagy markers were evaluated in the present work, such as Beclin1, rapamycin-sensitive TOR complex 1 (TORC1) and microtubule-associated protein 1A/1B light chain 3B (LC3B). Of note, Beclin1 and LC3B are activators of this cellular cleaning process, while TORC1 is associated with autophagy inhibition. After 11β-HSD1 inhibitor treatment, Beclin1 and LC3B protein levels were increased, while serine 151 phosphorylated TORC1 protein levels were decreased. Accordingly, mice under CMS showed higher protein levels of p-TORC1. However and strikingly, mice under CMS also presented higher protein levels of LC3B (Figs. 5 A-C).

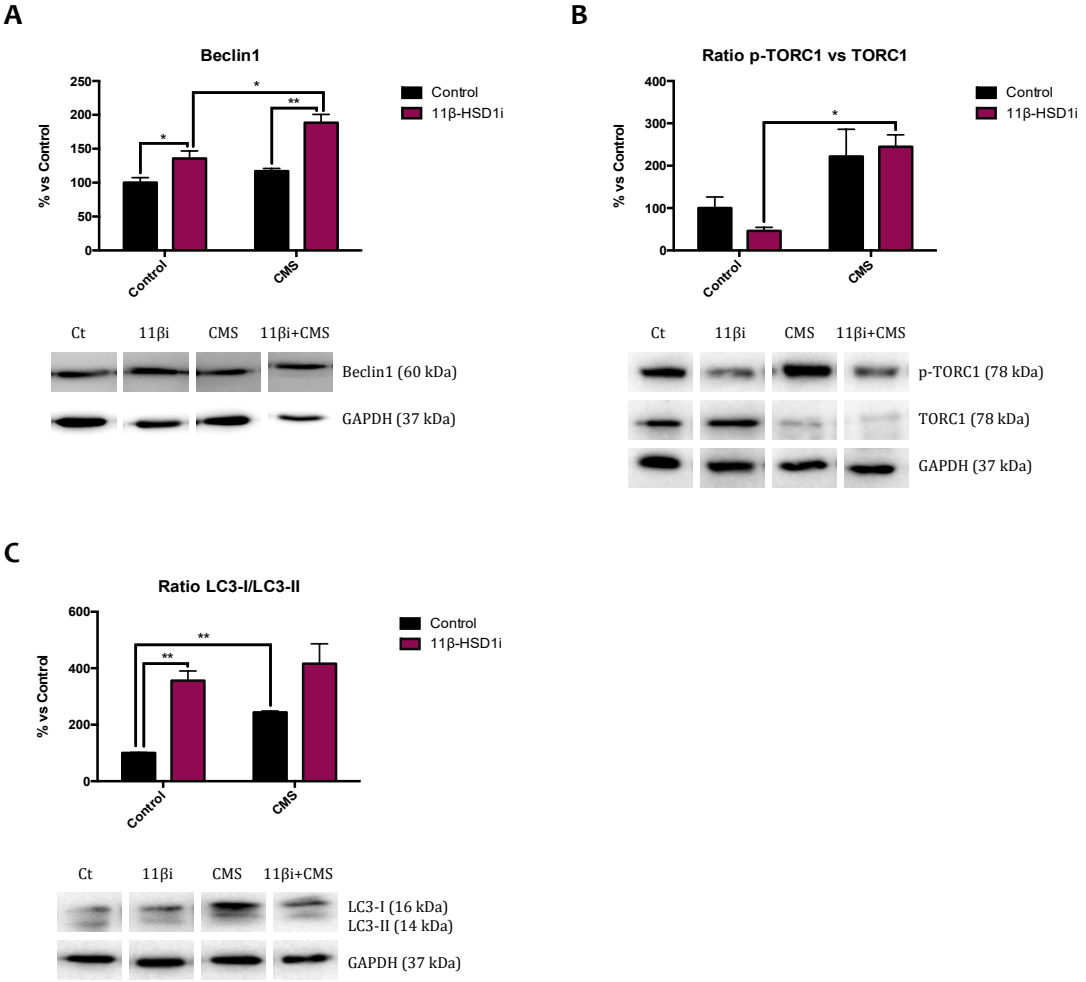


Figure 5. Representative results from autophagy process. *Representative Western Blot for Beclin1 protein levels and quantification (A), the ratio of p-TORC1 protein levels and*

*quantification (B) and the ratio of LC3 protein levels and quantification (C). Values in bar graphs are adjusted to 100% for protein levels of SAMP8 Control (Control). Values are mean ± Standard error of the mean (SEM) (n=4 for each group). *p<0.05; **p<0.01.*

11β-HSD1 inhibition rescued mice from CMS pejorative effects on APP processing

Herein, a disintegrin and metalloproteinase domain protein 10 (*Adam10*) gene expression was increased in mice treated with RL-118 as well as decreased in CMS group (Fig. 6 A). Also, β-secretase 1 (Bace1) gene expression was decreased in both RL-118 treated groups and there was a trend towards increasing Bace1 gene expression after CMS exposure (Fig. 6 B). In line with these results, Aβ-precursor gene expression was increased in CMS group and reversed by 11β-HSD1 inhibition (Fig. 6 C). Finally, β-amyloid degradation was assessed by *Neprilisin12* gene expression. It was found that RL-118 treatment raised its gene expression, while CMS reduced it (Fig. 6 D).

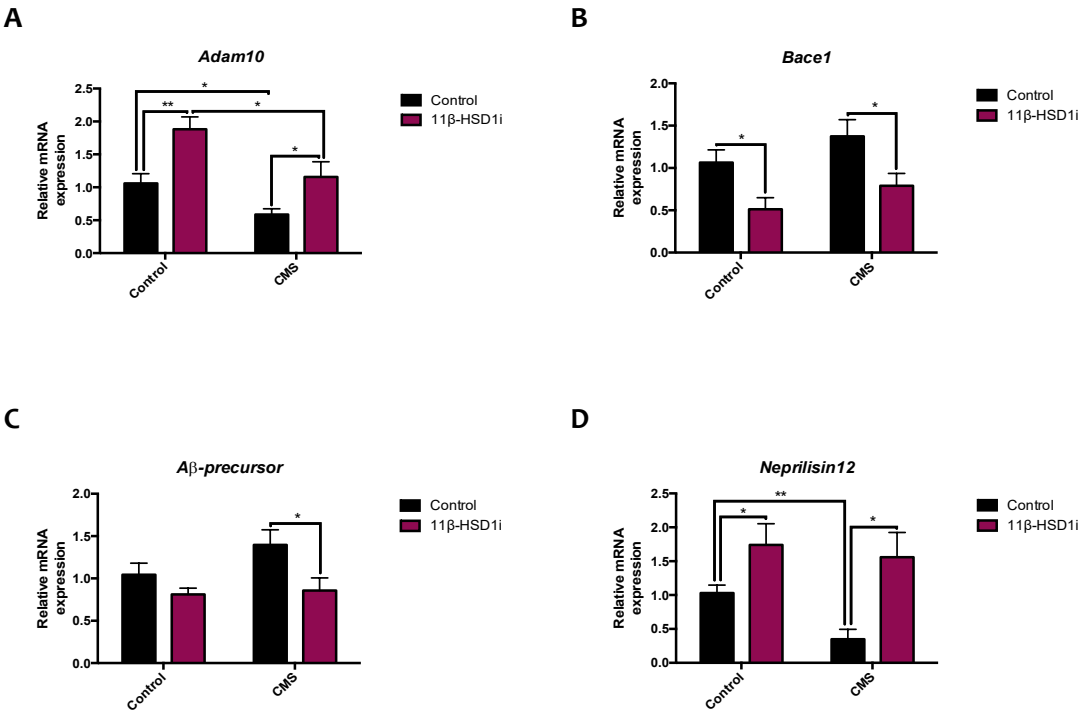
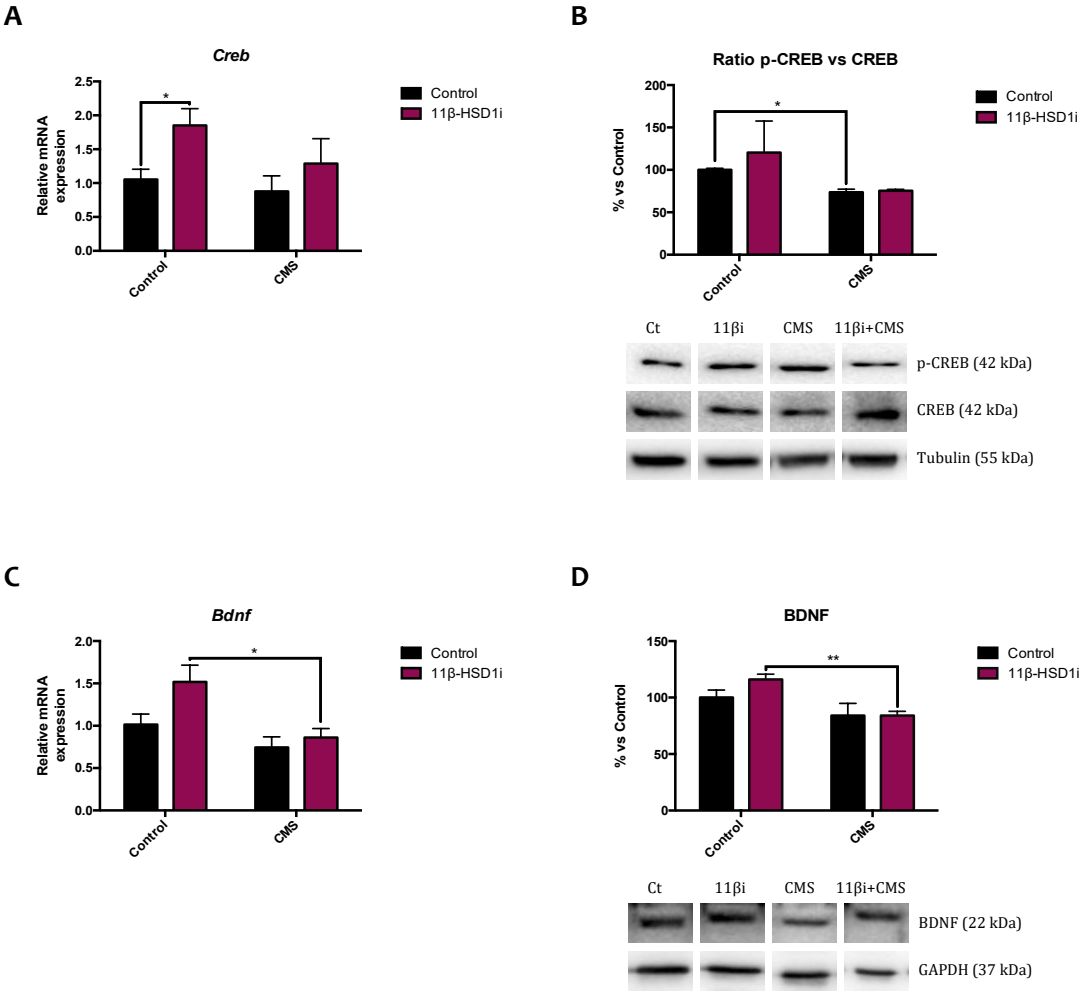


Figure 6. Representative results from APP processing pathways. *Relative gene expression of Adam10 (A), Bace1 (B), Aβ-precursor (C) and Neprilisin12 (D). Gene expression levels were determined by real-time PCR. Values are mean ± Standard error of the mean (SEM) (n=6 for each group). *p<0.05; **p<0.01.*

Changes in synaptic plasticity were produced after GC attenuation

To address whether GC attenuation through 11β-HSD1 inhibition modulated synaptic plasticity, we evaluated different proteins involved in those mechanisms. cAMP response element-binding (*Creb*) gene expression and protein levels were increased in RL-118 treated mice and decreased after CMS exposure. In turn, the same pattern was found in the Creb's downstream mediator, the brain-derived neurotrophic factor (Bdnf) (Figs. 7 A-C). Accordingly, synaptic modulators such as postsynaptic density protein 95 (PSD95), synaptophysin and synaptosomal nerve-associated protein 25 (SNAP25) protein levels were increased after 11β-HSD1 inhibition treatment, although not decreased after CMS (Figs. 7 E-H).



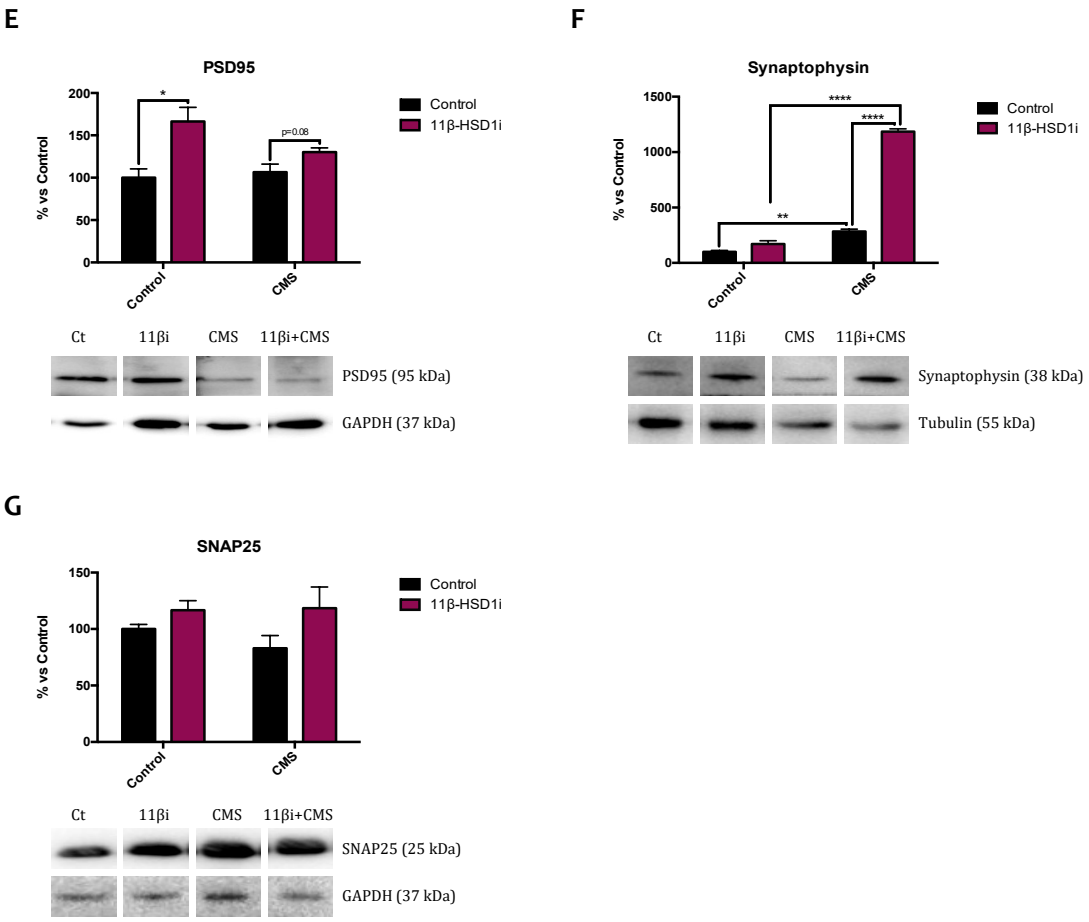
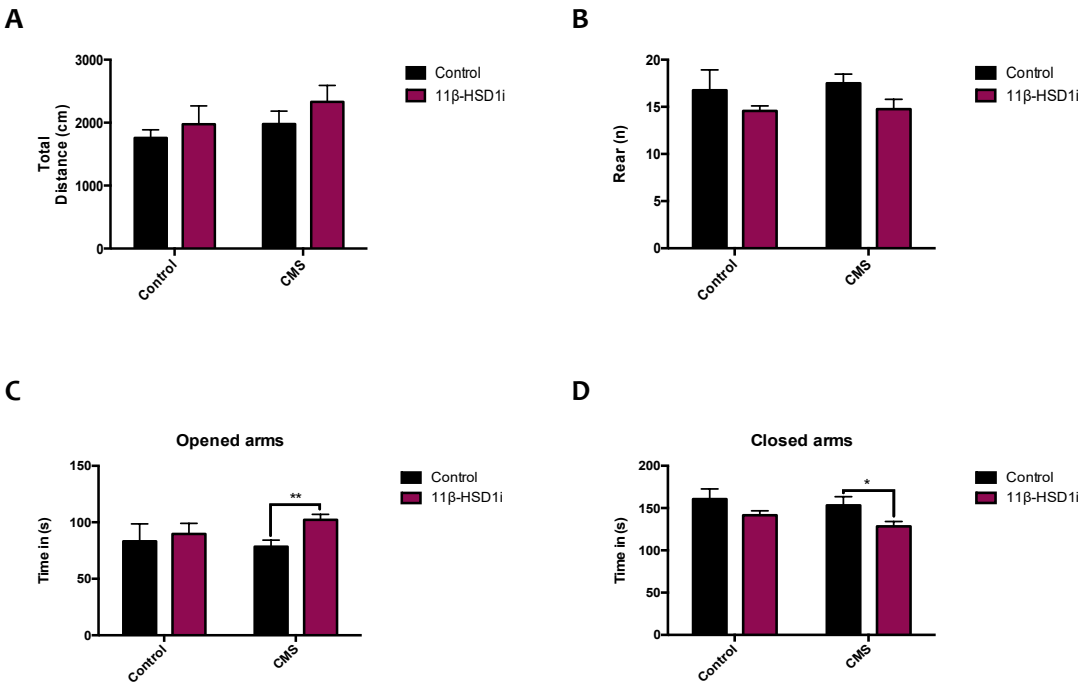


Figure 7. Representative results from neuroplasticity modulators. *Relative gene expression of Creb (A). Representative Western Blot for the ratio of p-CREB protein levels and quantification (B). Relative gene expression of Bdnf (C). Representative Western Blot for BDNF protein levels and quantification (D), PSD95 protein levels and quantification (E), Synaptophysin protein levels and quantification (F) and SNAP25 protein levels and quantification (G). Gene expression levels were determined by real-time PCR. Western Blot values in bar graphs are adjusted to 100% for protein levels of SAMP8 Control (Control). Values are mean ± Standard error of the mean (SEM) (n=6 for each group). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.*

11β-HSD1 inhibition reduced risk-taking behaviour while increased memory and learning abilities and CMS vice verse

As mentioned, stress and GC influence behaviour and accelerate brain aging. CMS paradigm applied did not change any of the parameters studied. However, 11β-HSD1 inhibition by RL-118 produced a tendency to increase horizontal but not vertical locomotor activity, while increased the time spent in the opened arms and decreased the time in the closed arms in the CMS mice, without affecting control groups (Figs. 8 A-D).

Referring to memory, we evaluated the recognition and spatial memory as well as learning abilities, through NORT and MWM. On the one hand, CMS did not induce significant changes in the DI values in reference to control mice in NORT, although recognition memory was slightly reduced by CMS only after 24 h from familiarization (Figs. 8 E-F). Meanwhile, 11β-HSD1 inhibitor treatment increased recognition memory not only at short-term, but also at long-term. On the other hand, MWM learning curve demonstrated that all mice learned where the platform was during the training days, as the latency to the platform was lower than the first day although some groups performed better than others (Fig. 8 G). However, both groups treated with RL-118 showed better learning abilities in reference to CMS group, as the learning curve slope was higher in those groups. Overall, CMS treatment used seemed to have little effect on the tests conducted, as there was a trend towards diminish all the parameters evaluated but not statistical differences were detected. By contrast, RL-118 treatment reduced the distance travelled to reach the platform, albeit there were only statistical significant differences between control groups (Fig. 8 H). In line with these results, 11β-HSD1 inhibition tended to increase target crossings as well as the time spent in the platform zone in both treated groups compared to their control littermates (Figs. 8 I-J).



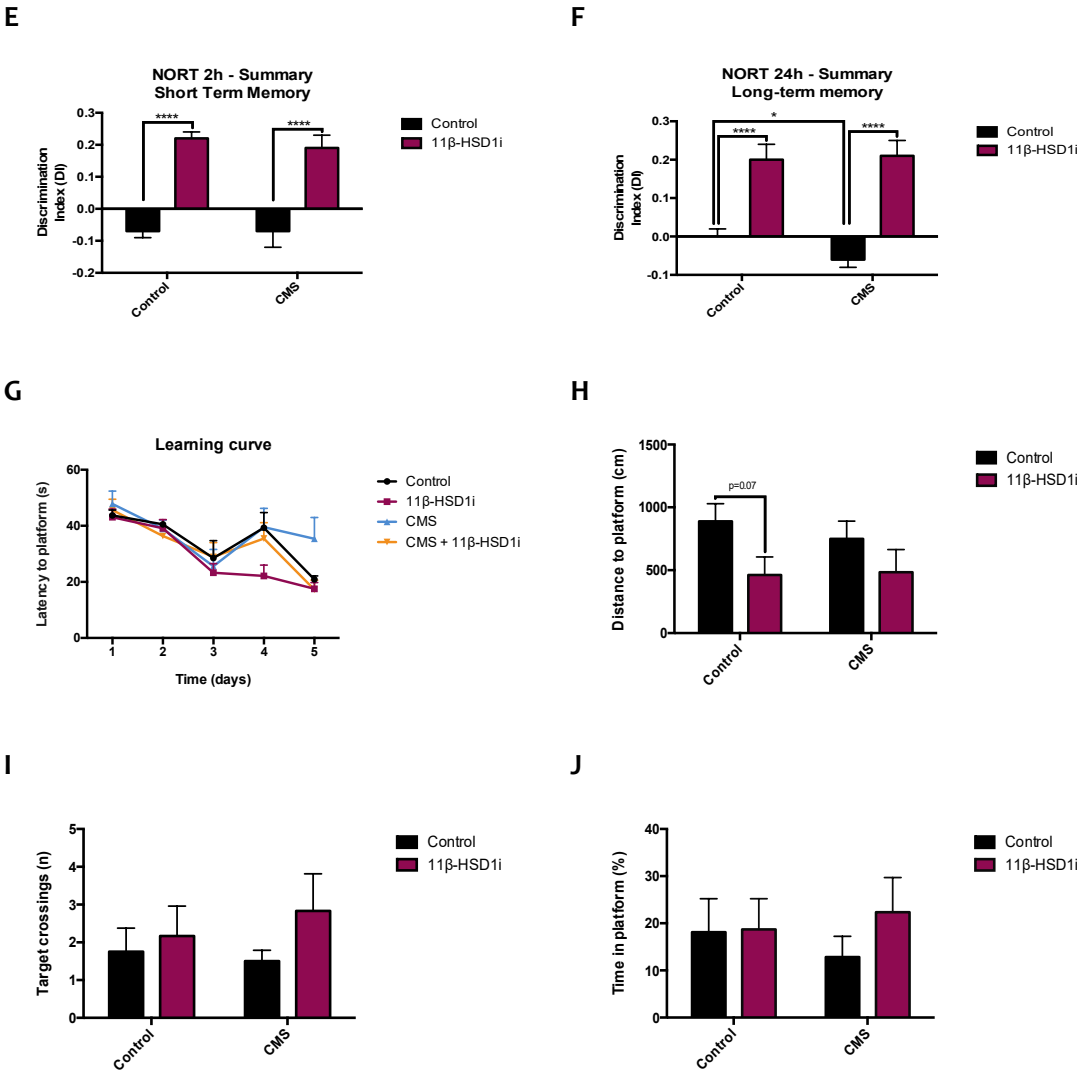


Figure 8. Behavioural tests results from EPM, NORT and MWM respectively. *Total Distance (A), number of Rearing (B), Time in the Opened arms (C), Time in the Closed arms (D), Summary of DI from 2 and 24 hours after familiarization phase (E-F), MWM learning curve (G), Distance to reach the platform (H), number of Entries (I) and Time in the platform (J). Values are mean ± Standard error of the mean (SEM) (n=12 for each group). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.*

3.3.5 Discussion

Taking into consideration the convergence of aging, stress and neurodegenerative diseases, such as AD, there is impaired GC signalling. Therefore, the study of GC response to chronic moderate stressful situations, as account in the daily life, results of huge interest in order to design pharmacological strategies to prevent neurodegeneration.

Several pieces of evidence demonstrate a strong association between prolonged exposure to GC excess and diminished cognitive abilities. It may be due to alterations in hippocampal electrophysiology, structure and function, as well as deleterious effects on neurotransmission, metabolism, cell division and death. GC secretion responds to a feed-forward signalling involving the hypothalamic-pituitary-adrenal (HPA) axis conforming the stress response. As far as stress is concerned, stressful stimuli increase GC secretion and release to confer energy to cope with the stress.

Previously, we affirmed that 11β-HSD1 enzyme regulates the disposure of active GCs (Leiva et al., 2017; Puigoriol-Illamola et al., 2018). Not only that, but also 11β-HSD1 inhibition has been linked to enhance cognitive abilities and AD hallmarks (Mohler et al., 2011; Puigoriol-Illamola et al., 2018, 2020b). In line with these results, it has been described that *11β-HSD1* expression in mouse hippocampus and parietal cortex increases with aging and that its overexpression accelerated age-related cognitive decline (Holmes et al., 2010). By contrast, 11β-HSD1 knockout mice resist age-dependent cognitive loss (Yau et al., 2007, 2015). In line with this, in our hands, we demonstrated that RL-118, a brain penetrant 11β-HSD1 inhibitor drug, treatment promoted autophagy flux, as well as ER stress activation in order to restore the deleterious effects exerted by prolonged exposure to GCs (Leiva et al., 2017; Puigoriol-Illamola et al., 2018, 2020b). In conclusion, it is suggested that it could be a feasible target to fight against cognitive decline in age-related pathologies. In fact, early clinical studies demonstrate that 11β-HSD1 inhibitor (UE2343) is well tolerated in AD patients (Webster et al., 2017) and currently is at phase II. Besides, many selective 11β-HSD1 inhibitors have reached clinical stages for metabolic diseases, for instance AZD8329 and BVT.2733 (Puigoriol-Illamola et al., 2020b).

Up to now, studies demonstrating that RL-118 is able to reach the brain, bind the 11β-HSD1 enzyme and inhibit it leading to therapeutic effects, have not been addressed. Thus, in the present work, the drug-target binding (Target engagement) of RL-118 and 11β-HSD1 was determined through a novel methodology consisting in FACS coupled to MS analysis (Wilson et al., 2017). Effectively, target engagement results showed that RL-118 drug binds to its target and also in a selectively way, since it did not bind to other proteins expressed. In consequence, we have determined that actually the molecular and behavioural effects observed after RL-118 administration were due to its binding to the 11β-HSD1 enzyme.

Stress response may have a genetic and epigenetic origin, reflected in the efficiency of the GC receptor (GR)-mediated negative feedback in the brain and/or the pituitary gland that causes HPA axis hyperactivity (Fink et al., 2017; Harman & Martin, 2019; Sotiropoulous et al., 2008). In an attempt to address whether and which changes produced the chronic presence of stressors we decided to evaluate two main epigenetic marks: DNA methylation and histone post-translational modifications.

Regarding DNA methylation, it is implied in controlling neuronal gene expression and neural development. Dysregulation of this process is linked to a wide range of neuronal disorders, including AD onset and progression. However, the relationship between AD and altered 5-mC levels is not known (Fetahu et al., 2019; Puigoriol-Illamola et al., 2020a). TET family can further oxidize 5-mC to 5-hmC. Generally, 5-mC is associated with gene silencing, while 5-hmC with up-regulation of gene expression (Fetahu et al., 2019; Sherwani & Khan, 2015). Herein CMS reduced those epigenetic marks and accordingly to Puigoriol-Illamola et al. (2020a), attenuating GCs levels led to increase 5-mC as well as 5-hmC percentages in both treated groups. Therefore, it may suggest that RL-118 is able to restore DNA methylation pattern although the influence of detrimental stimuli such as CMS. Although these results seem to contradict what is established for most scientific literature, methylation of specific genes should be evaluated in order to know which translational activity is being repressed. Despite that, DNA hydroxymethylation, which is linked to promote gene expression, was increased in RL-118 treated mice. Overall, those results suggest that further evaluation of gene-promoter specific methylation are required to elucidate the clear epigenetic mechanisms to explain the neuroprotective effects observed in the cognitive tests performed. Likewise, the enzymes responsible for those processes as *Dnmt1* and *Tet2* gene expression were increased in both groups treated with 11β-HSD1 inhibitor, whereas it decreased in the CMS group.

DNA methylation and histone modifications must act coordinately (Zhao et al., 2016). Generally histone acetylation is related to favouring gene transcription through removing histone positive charge and thereby transforming condensed chromatin (heterochromatin) into a relaxed structure (euchromatin) (Turner, 2000; Watson et al., 2014). 11β-HSD1 inhibitor treatment altered *Hdac2* gene expression, promoting a reduction in both groups. In line with this, acetylated H4 studied showed higher protein levels in RL-118 group treated, but not in mice under CMS too. In accordance to Puigoriol-Illamola et al. (2020a), SAMP8 mice under CMS treatment showed higher H3K9 protein levels in respect to control group.

However, it could be reversed after concomitant RL-118 treatment. In turn, the same profile was observed in H3K9me2 protein levels evaluated, suggesting that although the increase in histone acetylation as it is also methylated, it finally results in transcriptional repression or a more compacted chromatin state in CMS groups compared to control mice.

Of relevance, although RL-118 is not a direct epigenetic modulator drug, it modifies the epigenetic landscape. Therefore, our results point out to the paramount importance of studying different drugs at epigenetic level in order to possess a deep knowledge of the pharmacological effects exerted by the drug.

Additional data (Kadmiel & Cidlowski, 2013; Picard et al., 2018; Puigoriol-Illamola et al., 2020a) reports that a stressful environment affects oxidative balance. In normal conditions, pro-oxidant molecules and antioxidant defence mechanisms are balanced; however, under the presence of chronic stressors as well as along with aging and in several disorders, there is a decrease in the capacity of the antioxidant enzymes, allowing ROS accumulation and eventually causing cellular damage and finally dysfunction of the system (Griñán-Ferré et al, 2018). In concordance, in the present work SAMP8 mice under CMS increased ROS accumulation and on the contrary, those animals treated with RL-118 diminished it. OS is frequently invoked as a potential factor in progression of AD, yet whether it is a cause or consequence of the pathology is still debated (Bonet-Costa et al., 2016). Among antioxidant defence mechanisms, NRF2 pathway has been defined as a key indicator and modulator of OS in neurodegeneration (Griñán-Ferré et al., 2018; Johnson et al., 2008; Maeda et al., 2012), given that it regulates the gene expression of different antioxidant enzymes. While NRF2 is a protector against oxidative and electrophilic tissue injury, persistent activation of NRF2 signalling may also contribute to disease pathophysiology (Li et al., 2019). Interestingly, NRF2 protein levels were decreased after RL-118 treatment, suggesting that attenuating GC levels induce lower oxidative damage. However, CMS stimulated the increase in *Aox1* gene expression, which was reduced after concomitant RL-118 treatment, indicating that stressful environment increases pro-oxidant mechanisms while 11β-HSD1 inhibition is able to restore them. Likewise, other OS modulators evaluated as iNOS agreed with the increase in CMS group compared to control group and decreased protein levels of gene expression after RL-118 treatment. Moreover, *iNOS* gene expression has been reported to be regulated by inflammatory signalling, particularly through NF-κB (Arias-Salvatierra et al., 2011). Overall, these results suggest that 11β-HSD1 inhibition promoted antioxidant mechanisms to cope with OS in a mice model of aging, even under CMS conditions.

GCs effects include inflammatory signalling regulation. Although normal GC activity involves immune system suppression, GC excess deploys the contrary effect. Despite differences did not reach statistical significance, 11β-HSD1 inhibition reduced NF-κB protein levels. It appears to be a pivotal mediator of inflammatory responses as it induces the expression of various pro-inflammatory genes, including those encoding cytokines and chemokines, and participates in inflammasome regulation (Liu et al., 2017). It triggers a feed-forward cycle in which increases cytokine production and this in turn result in resistance to the GC immunosuppression, leading to further increases in cytokine release and, therefore, activation of the HPA axis (Irwin & Miller, 2007; Pace et al., 2007). Accordingly, we found decreased pro-inflammatory cytokine gene expression after 11β-HSD1 inhibitor treatment and increased after CMS appliance. Moreover, *Il-10* and *Il-6* gene expression results reinforce the same assertion (Data not shown). Pro-inflammatory cytokines like IFN-α, Il-1, IL-6 and TNF-α have been described to activate the HPA axis and potentiate GC resistance (Sotiropoulous et al., 2008). Additionally, they have been implicated in the genesis of AD, becoming part of the amyloid plaques or triggering amyloid-β (Aβ) production, among others. Dysregulation of inflammatory mediators and astrogliosis are major culprits in the development of chronic inflammation and immunosenescence process, as well as are related to cognitive decline and progression of neurodegenerative diseases (Chung et al., 2019).

Recent studies have indicated that prolonged OS may limit autophagy flux (Bonet-Costa et al., 2016). In addition, altered cellular loss of proteostasis is one of the nine hallmarks of aging postulated by López-Otín et al. (2013). Moreover, our group had previously demonstrated that attenuating GC excess by RL-118 promotes autophagy activation (Puigoriol-Illamola et al., 2018) and, by contrast, CMS treatment induced autophagy deterioration (Puigoriol-Illamola et al., 2020a). In accordance, autophagy flux was decreased in female mice under CMS but those treated with RL-118 showed higher protein levels of autophagy activators, like Beclin1 and LC3B, and lower protein levels of p-TORC1.

11β-HSD1 inhibition has been linked to an extensive number of disorders, among which there is AD. In particular, it has been associated with reduced Aβ neurotoxicity and Tau hyperphosphorylation (Dong & Csernansky, 2009; Ouanes & Popp, 2019), both hallmarks of AD. Analysing deeper this issue, amyloid precursor protein (APP) can be processed through two mechanisms: non-amyloidogenic and amyloidogenic pathways. The

main indicator of the former is Adam10, whereas the later is Bace1 (Shen et al., 2018). In this study, *Adam10* gene expression was diminished and *Bace1* increased after CMS. Importantly, in RL-118 treated groups occurred the opposite, then favouring the slow down formation of Aβ. Moreover, the Aβ degrading enzyme, neprilisin12 gene expression increased after 11β-HSD1 inhibitor treatment but reduced after CMS.

As mentioned above, prolonged exposure to GCs in the brain induces several changes and ample experimental evidence state that repeated exposure to stressful conditions induce structural remodelling of neurons with synaptic loss as well as alterations in glial functions, which are frequently maladaptive (Vyas et al., 2016). In agreement, our results demonstrated a reduction in *Creb* and *Bdnf* gene expression and protein levels after CMS exposure, but conversely RL-118 treatment was not able to prevent it (Steffke et al., 2020). In a similar way, CMS reduced synaptic plasticity markers, like PSD95, synaptophysin and SNAP25, and by contrast 11β–HSD1 inhibition increased them, particularly synaptophysin (Mango et al., 2019).

The effects of GCs on cognition have been widely studied, stating that acute stress enhances cognitive abilities, while chronic stress negatively affects memory and learning processes as well as accelerates brain aging (Ouanes & Popp, 2019; Sandi et al., 2013; Sotiropoulous et al., 2008; Weger & Sandi, 2018). In fact, GC levels have been found to correlate with the severity of the cognitive impairment (Ouanes & Popp, 2019). Assessing EPM results, it is well known that vertical locomotor activity indicates anxiety-like behaviour and, although not statistical differences were observed, together with the total horizontal distance travelled, we could affirm that RL-118 drug treatment helped to reduce the feeling of anxiety and desire to escape from an inhospitable environment, so that risk-taking behaviour becomes reduced. This is supported by the time spent in opened and closed arms, as it can be seen that after 4 weeks of 11β-HSD1 inhibition together to detrimental stimuli such as CMS, animals stood less time in the closed arms and, on the contrary, more time in the opened arms respect to CMS group. In accordance to Puigoriol-Illamola et al. (2020 a & b), mice under RL-118 treatment clearly showed improved recognition memory than control mice. However, the pejorative effect caused by CMS was only detected at long-term recognition memory evaluation. Regarding MWM results, it seemed that CMS treatment might not be strong enough to produce clearly changes in the tests performed. 11β-HSD1 inhibition tended to improve the mice performance, although not statistical differences were detected. However, both groups treated with RL-118 showed better

learning abilities in reference to CMS group, as the learning curve slope is higher in those groups, indicative of a putative role for RL-118 as a neuroprotectant or cognitive enhancer of cognition (nootropic drugs).

3.3.6 Conclusions

In view of these results, target engagement between 11β-HSD1 enzyme and RL-118 drug was demonstrated and therefore, we can surely attribute all the beneficial effects observed in SAMP8 treated with RL-118 to 11β-HSD1 inhibition. Diversely, CMS declined cognitive and behavioural abilities, as well as synaptic plasticity, autophagy and antioxidant mechanisms, while modulated epigenetic markers and increased inflammatory signalling and Aβ formation and accumulation. However and most important, 11β-HSD1 inhibition through RL-118 turned up to restore the majority of these detrimental effects caused by CMS (Fig. 9).

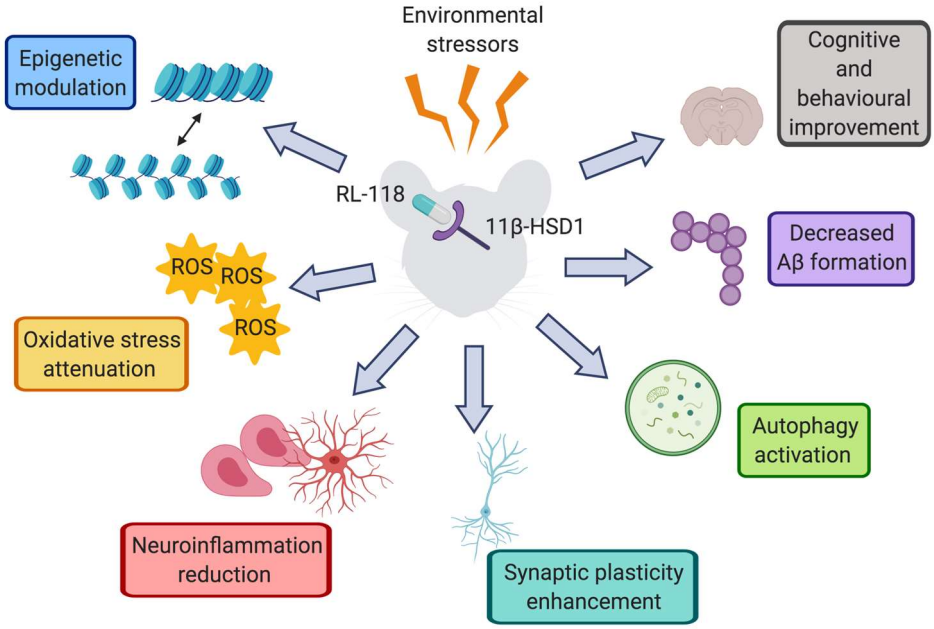


Figure 9. Representative scheme of the molecular pathways altered after RL-118 treatment in SAMP8 mice under chronic mild stress.

3.3.7 Acknowledgements

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3.3.9 Supplementary material

Fig. S1 E2-Crimson-huHSD11B1 fusion protein. Restriction sites for NheI and NotI are marked in pink, Kozak sequence in bold, E2-Crimson gene in red, a linker sequence in blue and HSD11B1 gene in black.

NheI **Kozak**

5'GCTAGCGCCACCATGGCGAGCATGGATAGCACTGAGAACGTCATCAAGCCCTTCATGCGCTTCAA
GGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGTGGGCGAGGGCAAGC
CCTACGAGGGCACCCAGACCGCCAAGCTGCAAGTGACCAAGGGCGGCCCCCTGCCCTTCGCCTGGG
ACATCCTGTCCCCCCAGTTCTTCTACGGCTCCAAGGCGTACATCAAGCACCCCGCCGACATCCCCGAC
TACCTCAAGCAGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGC
GTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCACCCTCATCTACCACGTGAAGTTCATCG
GCGTGAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAGACTCTGGGCTGGGAGCCCTCCACTG
AGCGCAACTACCCCCGCGACGGCGTGCTGAAGGGCGAGAACCACATGGCGCTGAAGCTGAAGGGC
GGCGGCCACTACCTGTGTGAGTTCAAGTCCATCTACATGGCCAAGAAGCCCGTGAAGCTGCCCGGCT
ACCACTACGTGGACTACAAGCTCGACATCACCTCCCACAACGAGGACTACACCGTGGTGGAGCAGT
ACGAGCGCGCCGAGGCCCGCCACCACCTGTTCCAG**GATGACGATGACAAG****ATG**GCTTTTATGAAAA
AATATCTCCTCCCCATTCTGGGGCTCTTCATGGCCTACTACTACTATTCTGCAAACGAGGAATTCAGA
CCAGAGATGCTCCAAGGAAAGAAAGTGATTGTCACAGGGGCCAGCAAAGGGATCGGAAGAGAGAT
GGCTTATCATCTGGCGAAGATGGGAGCCCATGTGGTGGTGACAGCGAGGTCAAAAGAAACTCTACA
GAAGGTGGTATCCCACTGCCTGGAGCTTGGAGCAGCCTCAGCACACTACATTGCTGGCACCATGGA
AGACATGACCTTCGCAGAGCAATTTGTTGCCCAAGCAGGAAAGCTCATGGGAGGACTAGACATGCT
CATTCTCAACCACATCACCAACACTTCTTTGAATCTTTTTCATGATGATATTCACCATGTGCGCAAAA
GCATGGAAGTCAACTTCCTCAGTTACGTGGTCCTGACTGTAGCTGCCTTGCCCATGCTGAAGCAGAG
CAATGGAAGCATTGTTGTCGTCTCCTCTCTGGCTGGGAAAGTGGCTTATCCAATGGTTGCTGCCTATT
CTGCAAGCAAGTTTGCTTTGGATGGGTTCTTCTCCTCCATCAGAAAGGAATATTCAGTGTCCAGGGT
CAATGTATCAATCACTCTCTGTGTTCTTGGCCTCATAGACACAGAAACAGCCATGAAGGCAGTTTCT

GGGATAGTCCATATGCAAGCAGCTCCAAAGGAGGAATGTGCCCTGGAGATCATCAAAGGGGAGC
TCTGCGCCAAGAAGAAGTGTATTATGACAGCTCACTCTGGACCACTCTTCTGATCAGAAATCCATGC
AGGAAGATCCTGGAATTTCTCTACTCAACGAGCTATAATATGGACAGATTCATAAACAAG**TAG**GCG
GCCGC3'
Not1

Table S1. Antibodies used in Western Blot studies.

Antibody	Host	Source/Catalog	WB dilution
Acetyl H4K12	Sheep	R&D Systems/AF5215	1:1000
H4	Rabbit	Cell Signaling/#2592	1:1000
Acetyl H3K9	Rabbit	Millipore/06-599	1:1000
H3	Rabbit	Cell Signaling/#9715	1:1000
H3K9me2	Rabbit	Cell Signaling/#4658	1:1000
NF-κβ	Rabbit	Cell Signaling/DE14E12	1:1000
NRF2	Rabbit	Santa Cruz/sc-722	1:500
Beclin 1	Rabbit	Abcam/ab62557	1:1000
p-TORC1 S151	Rabbit	Cell Signaling/#3359	1:1000
TORC1	Rabbit	Cell Signaling/#2501	1:1000
LC3B	Rabbit	Cell Signaling/#2775	1:1000
p-CREB S133	Rabbit	Cell Signaling/#9198	1:1000
CREB	Rabbit	Cell Signaling/#4820	1:1000
BDNF	Rabbit	Santa Cruz/N-20	1:500
PSD95	Rabbit	Abcam/ab12093	1:2000
Synaptophysin	Mouse	Millipore/MAB5258	1:1000
SNAP25	Mouse	Santa Cruz/ SP-12	1:500
GAPDH	Mouse	Millipore/MAB374	1:2000
β-Tubulin	Mouse	Millipore/Clone AA2	1:2000
TBP	Mouse	Abcam/ab818	1:2000
Goat-anti-mouse HRP conjugated		Biorad/170-5047	1:2000
Goat-anti-rabbit HRP conjugated		Biorad/170-6515	1:2000
Donkey-anti-goat HRP conjugated		Santa Cruz/sc-2020	1:2000

Table S2. Primers and probes used in qPCR studies.

SYBR Green primers

Target	Product size (bp)	Forward primer (5'-3')	Reverse primer (5'-3')
Tet2	113	CCATCATGTTGTGGGACGGA	ATTCTGAGAACAGCGACGGT
Hdac2	280	CTATCCCGCTCTGTGCCCT	GAGGCTTCATGGGATGACCC
G9a	94	TTCCTTGTCTCCCCTCCCAG	CTATGAACTCTCTCGGCGGC
Aox1	286	CATAGGCGGCCAGGAACATT	TCCTCGTTCCAGAATGCAGC

iNOS	101	GGCAGCCTGTGAGACCTTTG	GAAGCGTTTCGGGATCTGAA
Il-1β	179	ACAGAATATCAACCAACAAGT TGATATTCTC	GATTCTTTCCTTTGAGGCCCA
Cxcl2	100	AGCCACACTTCAGCCTAGCG	TGTAGCCTGGTGGTTGGTGG
Tnf-α	157	TCGGGGTGATCGGTCCCCAA	TGGTTTGCTACGACGTGGGCT
Gfap	125	CCTTCTGACACGGATTTGGT	ACATCGAGATCGCCACCTAC
Adam10	125	GGGAAGAAATGCAAGCTGAA	CTGTACAGCAGGGTCCTTGAC
Bace1	67	ACAAGCCTTTCCGCCTCC	TCAGGCCACCATAATCCAGC
Aβ-precursor	99	TCGGGGTGATCGGTCCCCAA	GTCACGTTCACCCTCCCCAG
Neprilisin12	196	TTGGGAGACCTGGCGGAAAC	CATTCCTTGGACCCTCACCCC
Creb	86	GGCTGCTGCTGCCTGT	ACACACCGCGTCAAACTACA
Bdnf	72	TAGCTTGACAAGGCGAAGGG	TCTGGCAAAGATGAGCTCGG
β-actin	190	CAACGAGCGGTTCCGAT	GCCACAGGTTCCATACCCA

Taqman probes

Target	Product size (bp)	Reference
Dnmt1	58	Mm01151063_m1
Gapdh	107	Mm99999915_g1

3.4 11β-HSD1 Inhibition Rescues SAMP8 Cognitive Impairment Induced by Metabolic Stress

Adapted from: **Puigoriol-Illamola, D.**[1,2], Leiva, R.[3,4], Vázquez-Carrera, M.[1,4,5,6], Vázquez, S.[3,4], Griñán-Ferré, C.[1,2], Pallàs, M[1,2]. 11β-HSD1 Inhibition Rescues SAMP8 Cognitive Impairment Induced by Metabolic Stress

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3.4.1 Abstract

In recent years, the interest in the impact of nutrition in health has grown since obesity is one of the features of modern society. Particularly, obesity is a risk factor for many disorders, including diabetes, hypertension, cardiovascular alterations, mild cognitive impairment and AD. In fact, ample evidence provides both clinical and experimental evidence into how obesity and AD may course together. However, the main risk factor for AD is ageing. While ageing, several disturbances lead to impaired GC signalling, resulting in high GC levels. Besides, GCs excess cause metabolic disturbances and have been associated with neurodegeneration. GC activity is regulated by 11β-HSD1 enzyme, which inhibition has been proved to enhance cognitive abilities. The study aimed to determine the potential beneficial effects of RL-118 drug treatment, an 11β-HSD1 inhibitor (11β-HSD1i), after HFD exposure in the SAMP8.

In the present approach, in order to achieve the objective, 4 groups were established: control fed with normal diet (ND), ND-fed treated with the 11β-HSD1i, HFD-fed and HFD-fed treated with the 11β-HSD1i. Since weaning, mice consumed their dietary condition and at the age of 4 months, drug treatment started for 4 weeks. Our results demonstrated improvement in glucose intolerance induced by HFD in mice treated with RL-118, a significant reduction in 11β-HSD1 and GR protein levels. Furthermore, specific modification in the fibroblast growth factor 21 (FGF21) activation after treatment with 11β-HSD1i was found, which induced changes in the SIRT1/ peroxisome proliferator-activated receptor gamma coactivator (PGC) 1α/ AMPKα signalling pathway. OS and ROS, as well as inflammatory markers and microglial activation were significantly diminished in HFD-fed mice treated with 11β-HSD1i. Remarkably, treatment with 11β-HSD1i altered ER stress response in both diet groups, increasing autophagy only in HFD mice group.

RL-118 treatment induced a decrease in GSK3β activation, tau hyperphosphorylation and amyloidogenic APP processing. In agreement, increased non-amyloidogenic APP markers were detected in both treated mice groups, regardless of the dietary condition. Consequently, beneficial effects on social behaviour and cognitive performance were found in treated mice. Thus, our results support the therapeutic strategy of GC excess attenuation by selective 11β-HSD1i for the treatment of age-related cognitive decline and AD through improving metabolic and eventually cognitive disturbances caused by HFD.

3.4.2 Introduction

Aging is the most significant risk factor for a majority of chronic diseases, such as age-related cognitive decline and AD (Guerreiro & Bras, 2015). Obesity has recently been considered to play an essential role in mild cognitive impairment and dementia (Bischof & Park, 2015; Feinkohl et al., 2018). Abundant pieces of evidence suggest that pathological prolonged GCs excess not only is associated with age-related cognitive decline but also with metabolic stress, obesity, and diabetes (Bujalska et al., 2006; De Quervain et al., 2004; Holmes et al., 2010; Kumar & Datusalia, 2018). Thus, it has been described that prolonged exposure to increased levels of GCs exerts deleterious effects on hippocampal electrophysiology, structure and function, both in rodents (Canet et al., 2018) and humans (Tatomir et al., 2014), being part of altered intercellular communication, considered one of the nine hallmarks of aging (López-Otín et al., 2013; Mattson & Arumugam, 2018). The availability of natural GCs in tissues is regulated by corticosteroid-binding globulin in serum and by locally expressed 11β-HSD enzyme, a microsomal enzyme that catalyses the interconversion of active GCs (corticosterone in rodents but cortisol in humans) and inert 11-keto-forms [11-dehydrocorticosterone (11-DHC), cortisone] (Yau & Seckl, 2012). Two isoenzymes have been identified: 11β-HSD type 1 (11β-HSD1) and type 2 (11β-HSD2) (Kadmiel & Cidlowski, 2013). The former acts as a reductase, thus locally potentiating GCs activity, and is the predominant form in the brain, both in rodents and humans. In fact, 11β-HSD1 expression in mouse hippocampus and parietal cortex increases with aging, correlating with impaired spatial memory (Yau et al., 2015) and its overexpression accelerates age-related cognitive decline (Holmes et al., 2010). Conversely, 11β-HSD1 knockout mice resist age-dependent cognitive loss (Yau et al., 2001, 2007). In line with these findings, some preclinical studies demonstrate that 11β-HSD1 inhibition improved cognition and AD hallmarks suggesting a neuroprotective effect (Mohler et al., 2011; Puigoriol-Illamola et al., 2018). On the other hand, preclinical studies have demonstrated that sub-maximal inhibition of central 11β-HSD1 is able to prevent cognitive impairments in AD and ageing animal models (Sooy et al., 2010, 2015) and early preclinical studies demonstrated that administration of brain penetrant 11β-HSD1 inhibitor (UE2343) is well tolerated in humans (Webster et al., 2017). Besides, many selective 11β-HSD1 inhibitors have reached clinical stages for metabolic diseases like type 2 diabetes mellitus (e.g., AZD8329, ABT-384, and BVT-2733).

Indeed, several hypotheses argument a link between altered glucose metabolism and dementia, considering an altered metabolic pathway as a potential contributor to persistent oxidative stress (OS) that culminates into neuronal dysfunction and dementia (Mule & Singh, 2018). Increased OS, neuroinflammation, endoplasmic reticulum (ER) stress, misfolded proteins removal pathways, and autophagy, have been identified as components of neuronal metabolic stress, thus developing and aggravating neurological disorders and cognitive impairment, pointing to their implication in the dysregulated energy metabolism characteristic of ageing (Kumar & Datusalia, 2018). Thus, recent studies have focused on the pathology of cognitive decline and neurodegeneration through HFD feeding that induce metabolic stress (Lee et al., 2018; Wei et al., 2018). For instance, HFD-fed aged animals showed insulin resistance and increased weight gain among others, both signs of pre-diabetes, obesity, cardiovascular disease, depressive-like behaviour and mental health problems (Bowles et al., 2017; Karatsoreos et al., 2010; McEwen et al., 2015). However, the current knowledge about the molecular mechanism responsible for these affections is controversial, even though most of the information is related to reduced insulin sensitivity mediated by HFD. Hence, HFD-induced metabolic stress could be linked with the development of physiopathological conditions, such as those observed in AD and other neurodegenerative diseases (Cai et al., 2012).

Recently, we have developed a brain penetrant 11β-HSD1 inhibitor (RL-118) that was characterized chemically and pharmacologically *in vivo* in Leiva et al. (2017). RL-118 attenuates neuroinflammation, increases the antioxidant defence, promotes autophagy, improves mitochondrial function and reverts memory deficits in senescent mice model (Leiva et al., 2017; Puigoriol-Illamola et al., 2018). Therefore, the targeted inhibition of 11β-HSD1 may become a potential therapeutic strategy for age-related cognitive decline and AD. Here, we assessed the neuroprotective effects of 11β-HSD1 inhibition on metabolic stress through behaviour, cognitive and molecular changes induced by HFD in a mice model of age-related cognitive decline and late-onset AD, the senescence-accelerated mouse prone 8 (SAMP8).

3.4.3 Materials and methods

Animals

Females SAMP8 mice (n=48) were used to carry out behavioural, cognitive and molecular analyses. We divided these animals into 4 groups: normal diet chow (ND Ct,

n=12), ND treated with the RL-118 11β-HSD1 inhibitor (ND+11β-HSD1i, n=12), HFD (HFD Ct, n=12) and HFD treated with RL-118 (HFD+11β-HSD1i, n=12). Animals had free access to food and water and were kept under standard temperature conditions (22±2°C) and 12h: 12h light-dark cycles (300lux/0 lux). Animals were fed with both diets since the weaning (1-month-old) up to sacrifice. RL-118 was administered at 21 (mg/kg/day) by oral gavage from 4 months old to end of behavioural test (Fig. 1 A). ND provided 3,8 Kcal/g meanwhile HFD 4,7 Kcal/g – 45% fatty acids (D12451 Research Diets, Inc.). The weight of the animals and the ingested food were monitored weekly. Before the performance of the cognitive tests, the glucose tolerance test and triglyceride determination were conducted.

Studies and procedures involving mice brain dissection and subcellular fractionation were performed following the institutional guidelines for the care and use of laboratory animals established by the Ethical Committee for Animal Experimentation at the University of Barcelona.

Glucocorticoid, glucose tolerance test and triglyceride determination

Plasma and brain corticosterone concentrations were measured using a commercially available RIA (MP Biomedicals, Irvine, CA). Blood extraction and brain dissection were done among 4 pm and 5 pm in ND+11β-HSD1i and ND Ct mice.

Intraperitoneal (i.p.) glucose tolerance test was performed following 12 weeks of HFD feeding and 4 weeks of 11β-HSD1i/vehicle treatments, as described previously. In brief, mice fasted overnight for 12 hours. The test was performed in a quiet room and 2g/kg i.p. glucose injection was administered (diluted in H_2O) and blood glucose levels were measured at 0, 5, 10, 15, 30, 60 and 120 minutes after the injection with the Accu-Chek® Aviva blood glucose meter (Accu-Chek® Aviva, Roche, Barcelona, Spain). The determination of triglyceride concentration was performed by using a triglyceride meter device (Accutrend® Plus, Cobas, Roche).

Behavioural and cognitive test

Three-Chamber test (TCT)

The Three-Chamber test assesses cognition through sociability and interest in social novelty (Griñán-Ferré, et al., 2016). Testing occurs in a box with three equally dimensioned

rooms. Each test consists of 20 minutes and is recorded with a camera. The animal is placed in the center of the box and allowed to explore the three chambers for 10 minutes (Habituation phase). The time spent in each chamber was evaluated. Afterwards, an intruder (same sex and age) was added to one of the rooms in a metal cage and behaviour is recorded for 10 minutes. In this phase, the time spent in each room is assessed as well as the time interacting with the intruder (e.g., sniffing, grooming).

Morris Water Maze (MWM)

This test evaluates both learning and spatial memory (Vorhees et al., 2006). An open circular pool (100 cm in diameter, 50 cm in height) filled with water was used. Water was painted white with latex in order to make it opaque and its temperature was 22± 1°C. Two main perpendicular axes were established (North-South and East-West), thus configuring 4 equal quadrants (NE, NW, SE, and SW). 4 visual clues (N, S, E, W) were placed on the walls of the tank so that the animal could orientate and could fulfil the objective. The test consists of training a mouse to find a submerged platform (Learning phase) and assess whether the animal has learned and remembered where was the platform the day that it is removed (Test). The training lasts five consecutive days and every day five trials are performed, which have a different starting point (NE, E, SE, S, and SW), with the aim that the animal recognizes the visual clues and learns how to locate the platform, avoiding learning the same path. At each trial, the mouse was placed gently into the water, facing the wall of the pool, allowed to swim for 60 s and there was not a resting time between trials. If the animal was not able to locate the platform, the investigator guided it to the platform and was allowed to rest and orientate for 30 s. The platform was placed approximately in the middle of one of the quadrants, 1.5 cm below the water level. Above the pool there was a camera that recorded the animals' swimming paths and the data was analysed with the statistical program SMART® ver.3.0. During the learning phase, a learning curve was drawn, in which is represented the latency to find the platform every training day. On the day test, more parameters were measured, such as the target crossings and the swum distance in the platform zone.

Novel Object Recognition Test (NORT)

The Novel Object Recognition Test (NORT) protocol employed was a modification of (Ennaceur & Delacour, 1988). In brief, mice were placed in a 90°, two-arms, 25-cm-long, 20-cm-high, 5-cm-wide black maze. Before performing the test, the mice were individually

habituated to the apparatus for 10 min for 3 days. On day 4, the animals were submitted to a 10-min acquisition trial (first trial), during which they were placed in the maze in the presence of two identical, novel objects at the end of each arm. After a delay (2h and 24h), the animal was exposed to two objects one old object and one novel object. The Time that mice explored the Novel object (TN) and Time that mice explored the Old object (TO) were measured. A Discrimination Index (DI) was defined as (TN–TO)/(TN+TO). To avoid object preference biases, objects were counterbalanced. The maze, the surface, and the objects were cleaned with 70% ethanol between the animals' trials to eliminate olfactory cues.

Brain processing

Three days after the behavioural and cognitive tests, 8 animals per group were euthanized for protein extraction, RNA and DNA isolation, and 4 animals per group were euthanized for immunohistochemistry (IHQ).

When the animals were for protein extraction, RNA and DNA isolation, brains were immediately removed and the hippocampus was isolated, frozen on powdered dry ice and maintained at -80°C until procedures. When the animals were for IHQ, mice were intracardially perfused with 4 % paraformaldehyde (PFA) diluted in 0.1M phosphate buffer solution after being anesthetized by intraperitoneal injection of ketamine 100 mg/Kg and xylazine 10 mg/Kg. Afterwards, brains were removed and post-fixed in 4% PFA overnight 4°C then the solution was changed into PFA + 15% sucrose. Finally, brains were frozen burying directly on powdered dry ice (around 5 min) and store at -80°C until sectioned.

Immunodetection experiments

Western blotting

Tissue samples were homogenized in lysis buffer (Tris HCl pH 7.4 50mM, NaCl 150mM, EDTA 5mM and 1X-Triton X-100) containing phosphatase and protease inhibitors (Cocktail II, Sigma-Aldrich) to obtain total protein homogenates.

For Western Blotting (WB), aliquots of 15 µg of hippocampal protein extraction per sample were used. Protein samples were separated by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (8-14%) and transferred onto Polyvinylidene difluoride (PVDF) membranes (Millipore). Afterwards, membranes were blocked in 5% non-

fat milk in Tris-buffered saline (TBS) solution containing 0.1% Tween 20 TBS (TBS-T) for 1 hour at room temperature, followed by overnight incubation at a 4°C with the primary antibodies listed in (Table S1). Then, the membranes were washed and incubated with secondary antibodies listed in (Table S1) for 1 hour at room temperature. Immunoreactive proteins were viewed with the chemiluminescence-based ChemiLucent™ detection kit, following the manufacturer's protocol (ECL Kit, Millipore), and digital images were acquired using ChemiDoc XRS+System (BioRad). Semi-quantitative analyses were done using ImageLab software (BioRad) and results were expressed in Arbitrary Units (AU), considering control protein levels as 100%. Protein loading was routinely monitored by immunodetection of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-tubulin.

Immunofluorescence

Coronal section of 30 µm was obtained by a cryostat (Leica Microsystems CM 3050S, Wetzlar, Germany) and kept in a cryoprotectant solution at -20°C.

First, free-floating slices were selected and placed on a 24-wells plaque. After that, were washed five times with PBS 0.01M + 1% Triton X-100. Then, free-floating sections were blocked with a solution containing 5% fetal bovine serum (FBS), 1% Triton X-100, PBS 0.01M + gelatine 0.2% for 2h at room temperature. Afterwards, slices were washed with PBST (PBS 0.1M, 1% Triton X-100) five times for 5 min each and were incubated with the primary antibodies over-night at 4°C (Table S2). On the following day, coronal slices were washed with PBST 6 times for 5 min and then incubated with the secondary antibodies (Supplementary Table 2) at room temperature for 2h. Later, sections were co-incubated with,1mg/ml DAPI staining solution (Sigma-Aldrich, St. Louis, MI) for 5 min in the dark at room temperature and washed with PBS 0.01M. Finally, the slices were mounted using Fluoromount G (EMS, USA) and image acquisition was performed with a fluorescence laser microscope (Olympus BX41, Germany).

At least 4 images from 4 different individuals by the group were analysed with ImageJ/Fiji software available online from the National Institutes of Health.

RNA extraction and gene expression determination by q-PCR

Total RNA isolation was carried out using TRIsure™ reagent according to the manufacturer's instructions (Bioline Reagent, UK). The yield, purity, and quality of RNA

were determined spectrophotometrically with a NanoDrop™ ND-1000 (Thermo Scientific) apparatus and an Agilent 2100B Bioanalyzer (Agilent Technologies). RNAs with 260/280 ratios and RIN higher than 1.9 and 7.5, respectively, were selected. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed as follows: 2 µg of messenger RNA (mRNA) was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time quantitative PCR (qPCR) was used to quantify mRNA expression of OS and inflammatory genes listed in Table S3. SYBR® Green real-time PCR was performed in a Step One Plus Detection System (Applied-Biosystems) employing SYBR® Green PCR Master Mix (Applied-Biosystems). Each reaction mixture contained 6.75 µL of complementary DNA (cDNA) (which concentration was 2 µg), 0.75 µL of each primer (which concentration was 100 nM), and 6.75 µL of SYBR® Green PCR Master Mix (2X).

Data was analysed utilizing the comparative Cycle threshold (Ct) method (ΔΔCt), where the housekeeping gene level was used to normalize differences in sample loading and preparation. Normalization of expression levels was performed with β-actin for SYBR® Green-based real-time PCR results. Each sample was analysed in duplicate, and the results represent the n-fold difference of the transcript levels among different groups.

Oxidative stress determination

Hydrogen peroxide was measured in hippocampus protein homogenates as an indicator of OS, and it was quantified using the Hydrogen Peroxide Assay Kit (Sigma-Aldrich, St. Louis, MI) according to the manufacturer's instructions.

Data analysis

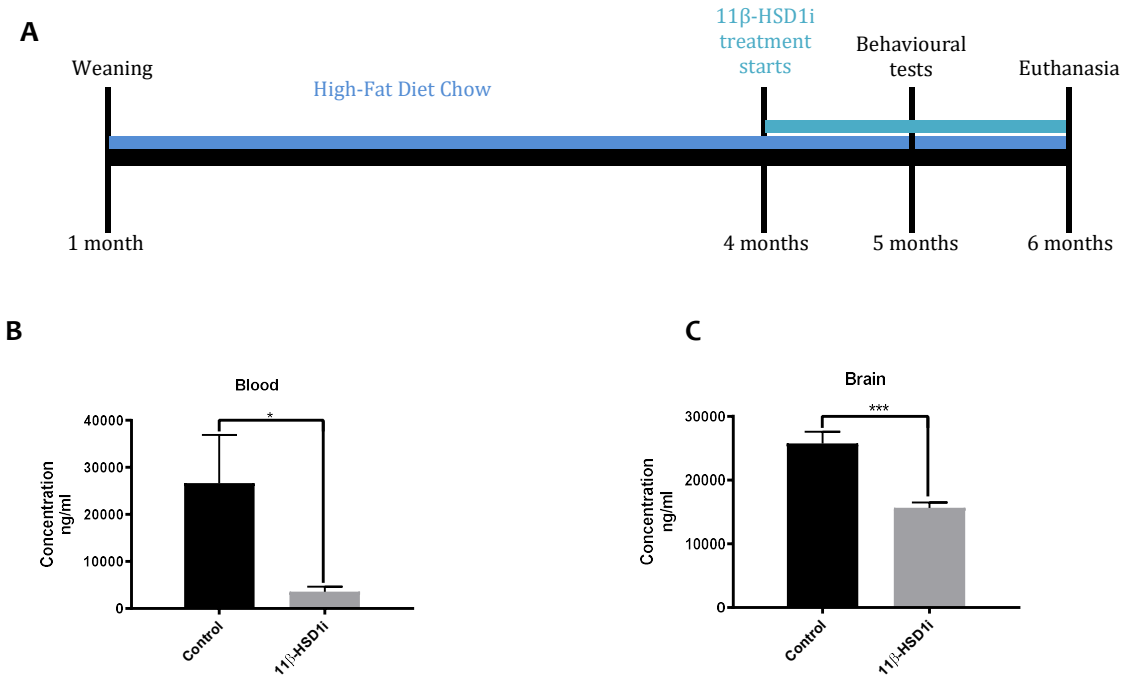
Data analysis was conducted using GraphPad Prism ver. 7 statistical software. Data are expressed as the mean ± standard error of the mean (SEM) of at least 6 samples per group. Diet and treatment effects were assessed by the Two-Way ANOVA analysis of variance, followed by Tukey post-hoc analysis or two-tail Student's t-test when it was necessary. Statistical significance was considered when *p*-values were <0.05. The statistical outliers were determined with Grubbs' test and subsequently removed from the analysis.

3.4.4 Results

Treatment with 11ß-HSD1i decrease GC levels and Improved Glucose Intolerance Induced by HFD

As expected corticosterone levels were significantly reduced in blood and brain tissue after 11β-HSD1i treatment (Figs. 1 B-C). Body weight was measured weekly during the intervention. All animal groups significantly increased body weight over time (Fig. 1 D). Furthermore, both HFD mice groups exhibited increased body weight from 12 weeks to the end time point (Fig. 1 D), correlating with higher caloric intake (Fig. 1 E). However, 11β-HSD1i treatment did not modify those parameters. Likewise, significant higher glucose levels between 15 and 90 min were found in HFD Ct group (Fig. 1 F). Noteworthy, 11β-HSD1i treatment reduced significantly glucose levels in HFD fed mice (Fig. 1 F). On the other hand, triglycerides blood concentration was higher in HFD mice groups but did not differ from 11β-HSD1i treated group (Fig. 1 G). In conjunction, 11β-HSD1i ameliorated glucose metabolism only after metabolic stress induced by HDF feeding.

Finally, WB analysis revealed a significant reduction in 11β-HSD1 protein levels in treated mice, both in ND and HFD fed groups (Figure 1 H). Additionally, the inhibition of 11β-HSD1 diminished GR protein levels significantly regardless of the diet (Fig. 1 I).



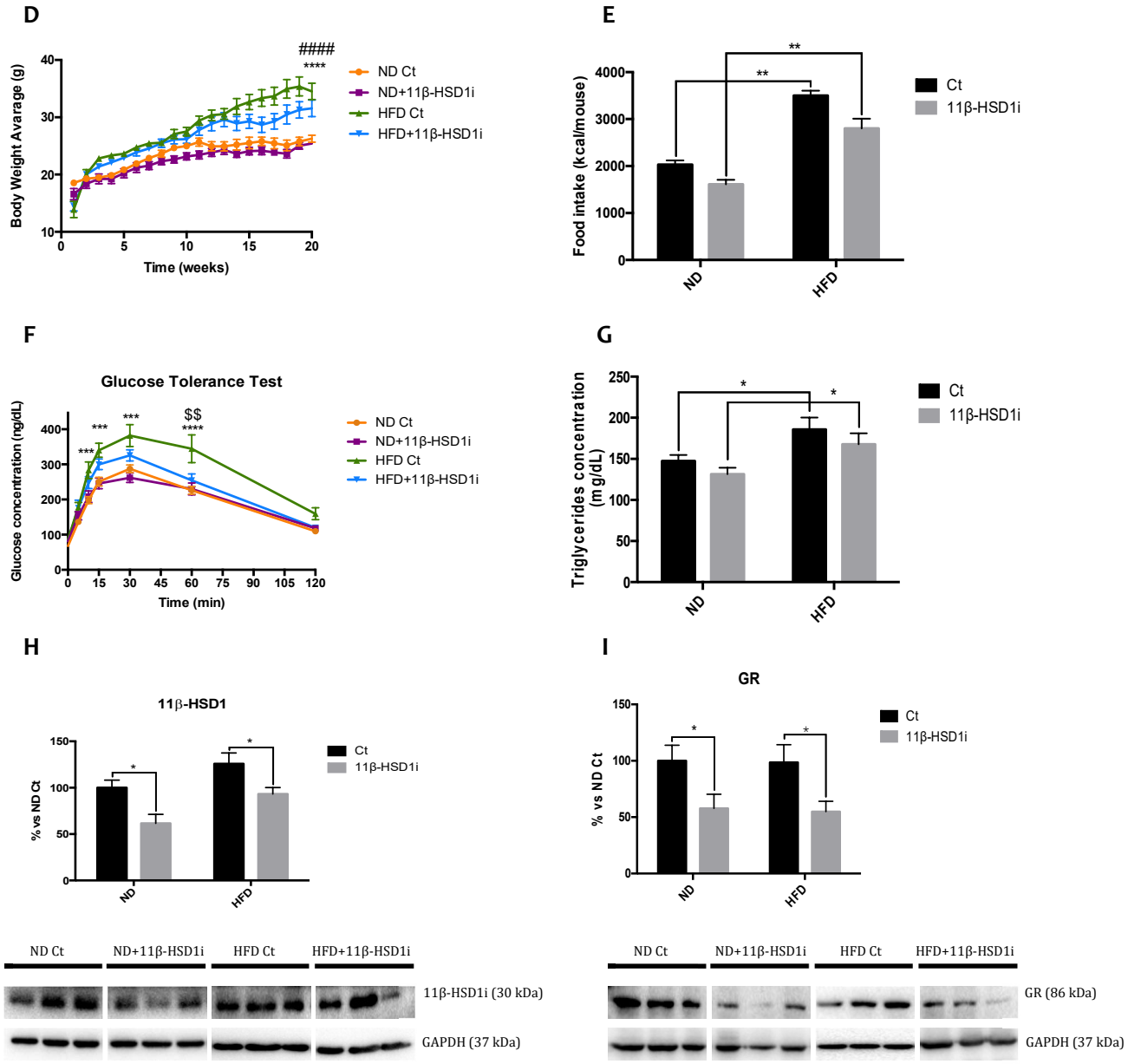


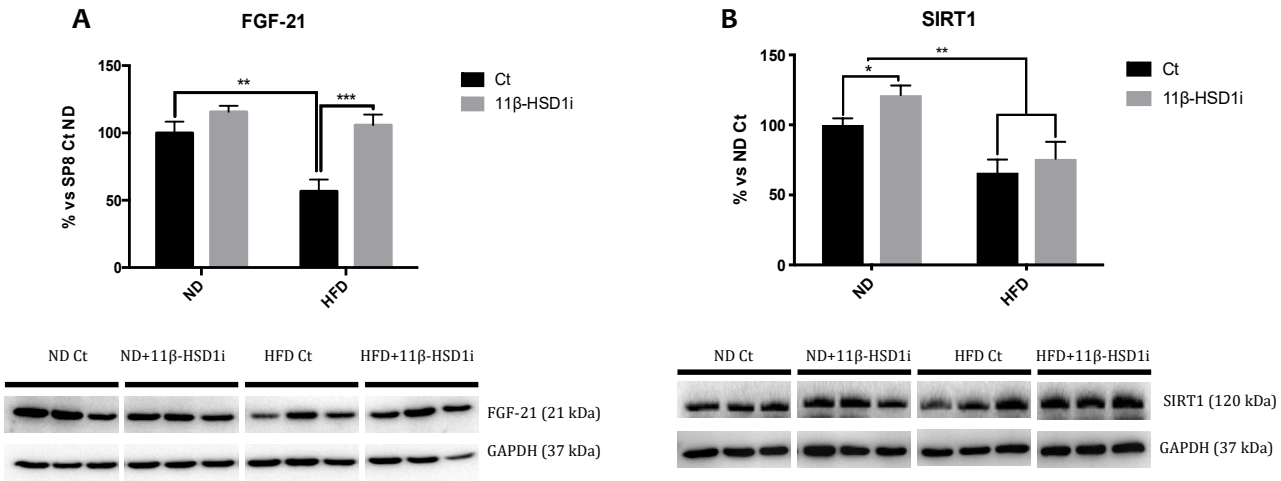
Figure 1. Scheme of experimental design of in vivo experiments (A). Levels of corticosterone in the blood (B) and brain (C) after 11β-HSD1i treatment. Body weight for all mice groups (D). Average caloric intake per mouse for all mice groups (E). Plasma levels of glucose 2g/kg intraperitoneal (i.p.) administration (F). Blood triglyceride concentration (mg/dL) (G). Representative Western Blot and quantification for 11β-HSD1 (H), and GR (I). *Values in bar graphs are adjusted to 100% for protein levels of SAMP8 Normal Diet (ND Ct). Values are mean ± Standard error of the mean (SEM); (n = 12 for each group). *p<0.05; **p<0.01; ***p<0.01; ****p<0.0001. **** Between ND Ct vs HFD Ct; #### between ND+11β-HSD1i vs HFD+11β-HSD1i, and $$ between HFD Ct vs HFD+11β-HSD1i groups.*

Activation of FGF-21 after Treatment with 11β-HSD1i induced changes in Nutrient Sensor

SIRT1/PGC1α/AMPKα axis

Fibroblast growth factor 21 (FGF-21) activator and SIRT1/PGC1α/AMPKα axis were evaluated by WB. A significant reduction in FGF-21 in HFD Ct group compared to the ND Ct group (Fig. 2 A) was observed, demonstrating that HFD reduced FGF-21 protein levels. Of note, a significant increase in HFD+11β-HSD1i group was found in comparison with the HFD Ct group (Fig. 2 A). No significant changes in FGF-21 protein levels were determined in ND+11β-HSD1i compared to ND Ct group, although there was a slight increase. Likewise, analysis of the nutrient sensor axis showed a significant reduction in SIRT1 protein levels in the HFD group, but RL-118 was unable to prevent it. Conversely, ND+11β-HSD1i showed a significant increase in SIRT1 compared to ND Ct group (Fig. 2 B). Importantly, liver kinase B1 (LKB1) was activated in 11β-HSD1i treated groups compared to the control mice regardless of the diet (Fig. 2 C). A significant increase in the phosphorylated activated protein kinase (p-AMPKα) ratio levels in the HFD+11β-HSD1i group was observed compared to the HFD Ct mice (Fig. 2 D). Finally, peroxisome proliferator-activated receptor-gamma coactivator 1-α (PGC1α) protein levels were increased in a significant way only in the ND+11β-HSD1i compared to the ND Ct group, but not to HFD groups (Fig. 2 E).

By contrast, 11β-HSD1 pharmacological inhibition through RL-118 was only able to increase SIRT1, LKB1, and PGC1α in ND-fed animals. Albeit not RL-118 demonstrated apparent changes in SIRT1 and PGC1α in HFD-fed mice, the increase in AMPKα phosphorylation indicated that improvements in nutrient sensing and mitochondrial function after 11β-HSD1 inhibition also occurred after HFD feeding. In whole results, pinpoint the beneficial effect of reducing GC signalling by 11β-HSD1 inhibition in SAMP8 under metabolic stress.



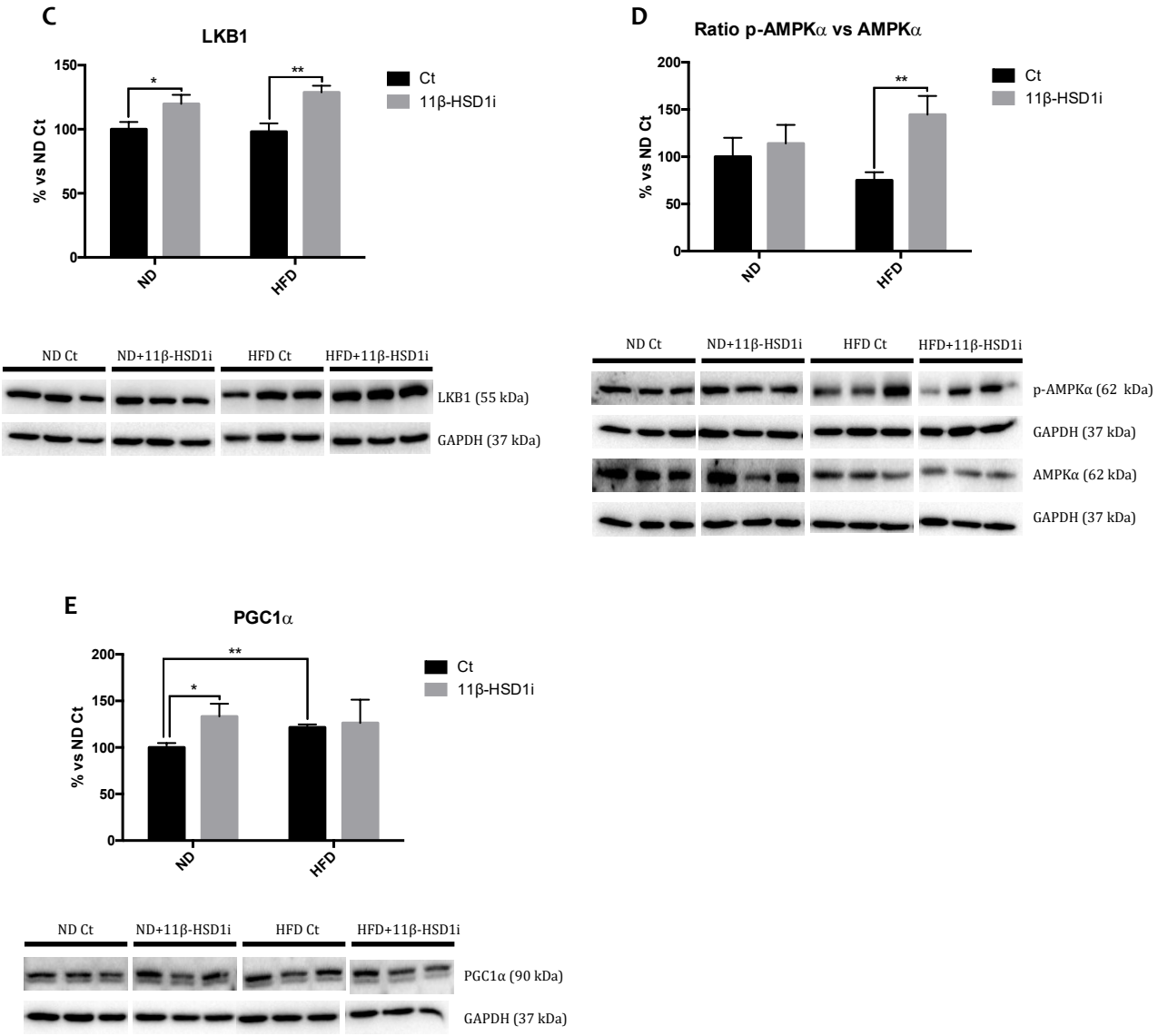


Figure 2. Representative Western Blot and protein levels quantification for FGF21 (A), SIRT1 (B), LKB1 (C), ratio of p-AMPKα (D), and PGC-1α (E). *Values in bar graphs are adjusted to 100% for protein levels of SAMP8 Normal Diet (ND Ct). Values are mean ± Standard error of the mean (SEM); (n = 6 for each group). *p<0.05; **p<0.01.*

Treatment with 11β-HSD1i Reduced OS Markers

HFD induced a significant increase in GPX1 and a moderate increase in SOD1 protein levels that were prevented by RL-118 treatment (Figs. 3 A-B). In addition, *iNOS* gene expression was reduced in 11β-HSD1i treated animals in comparison with the control groups that reached significance in HFD Ct mice (Fig. 3 C). Likewise, analysis of hydrogen peroxide levels showed that 11β-HSD1i can reduce it although in a not significant way (Fig. 3 D).

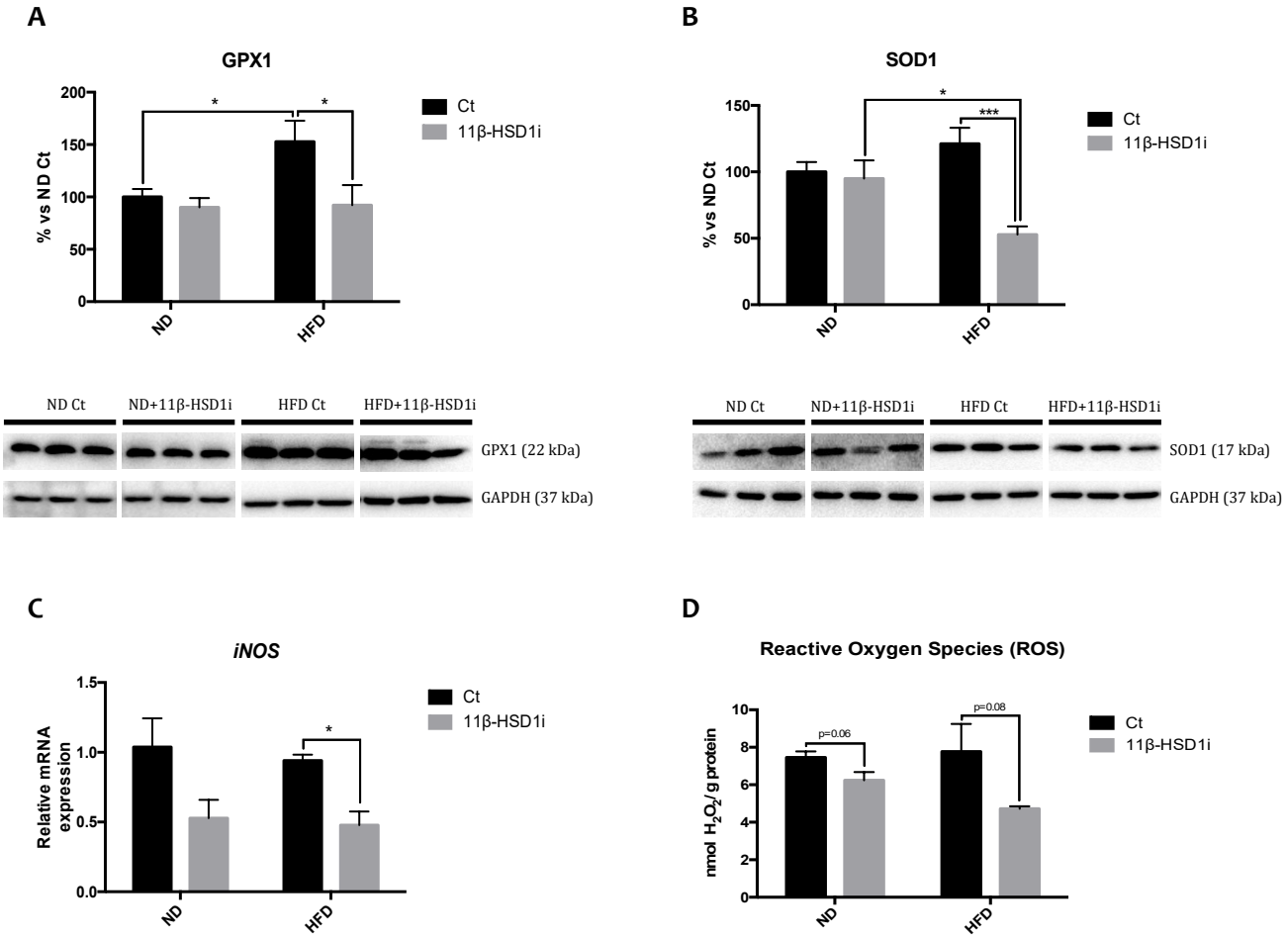


Figure 3. Representative Western Blot and protein level quantification for GPX1 protein (A), SOD1 (B). Representative gene expression of iNOS (C). Representative OS measured as hydrogen peroxide concentration in homogenates of hippocampus tissue (D). *Gene expression levels were determined by real-time PCR. Values in bar graphs are adjusted to 100% for protein levels of SAMP8 Normal Diet (ND Ct). Values are mean ± Standard error of the mean (SEM); (n = 6 for each group). *p<0.05; **p<0.01.*

Reduction of Inflammatory Markers and Microglial Activation after Treatment with 11β-HSD1i

HFD did not modify NF-κB protein levels. However, 11β-HSD1i treatment induced a significant diminution in NF-κB protein levels both in ND and HFD mice (Fig. 4 A). *Il-1β*, *Il-4*, *Il-6*, and *Tnf-α* gene expression was reduced after 11β-HSD1i treatment, being significant in *Il-6*, and *Tnf-α*, regardless of the diet (Fig. 4 B). By last, immunostaining quantification of ionized calcium-binding adapter molecule 1 (Iba1) fluorescence intensity demonstrated that 11β-HSD1i treatment reduced Iba1 staining, especially in the dentate gyrus (DG) and *Cornu Ammonis* 1 (CA1) regions (Figs. 4 C-F).

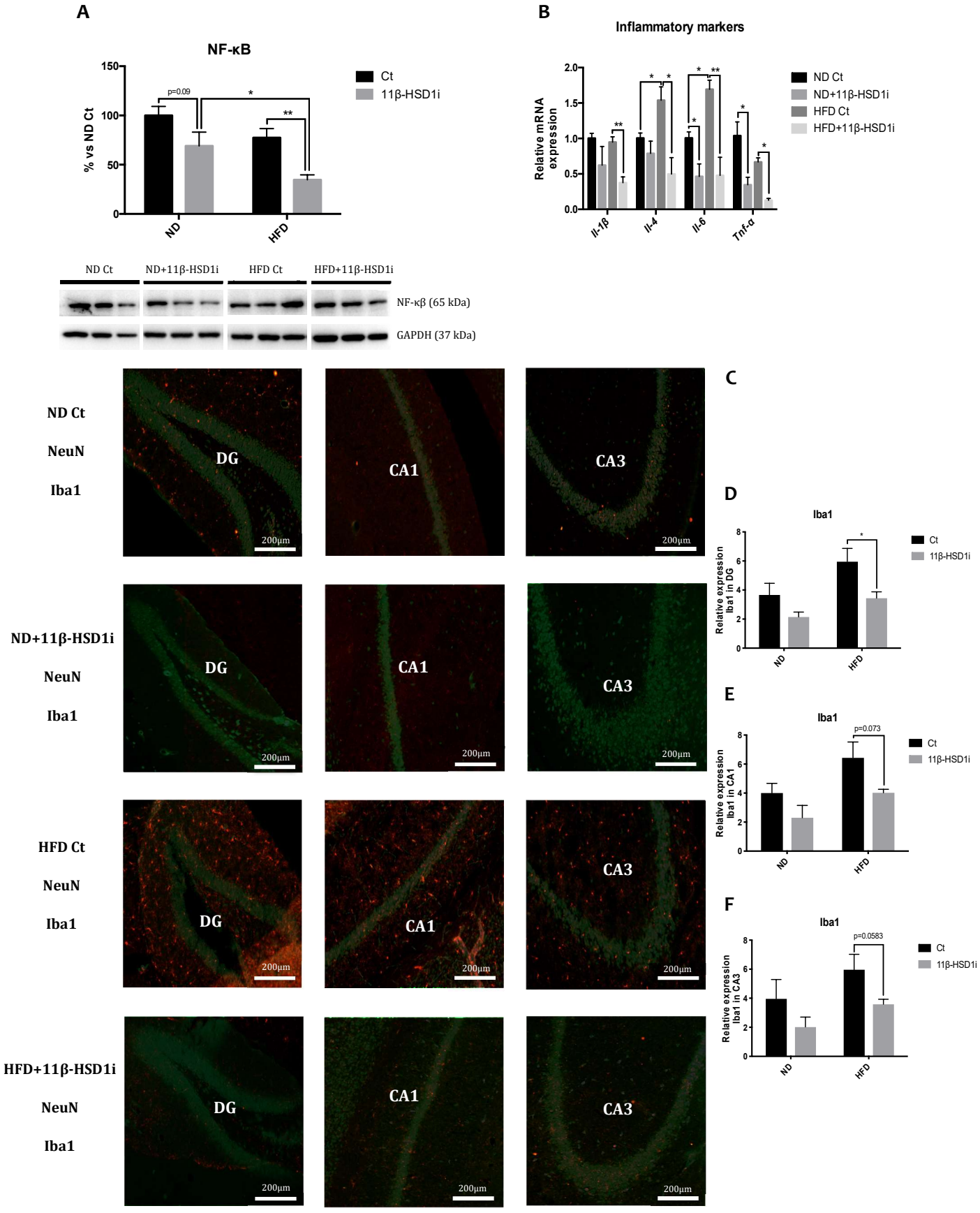
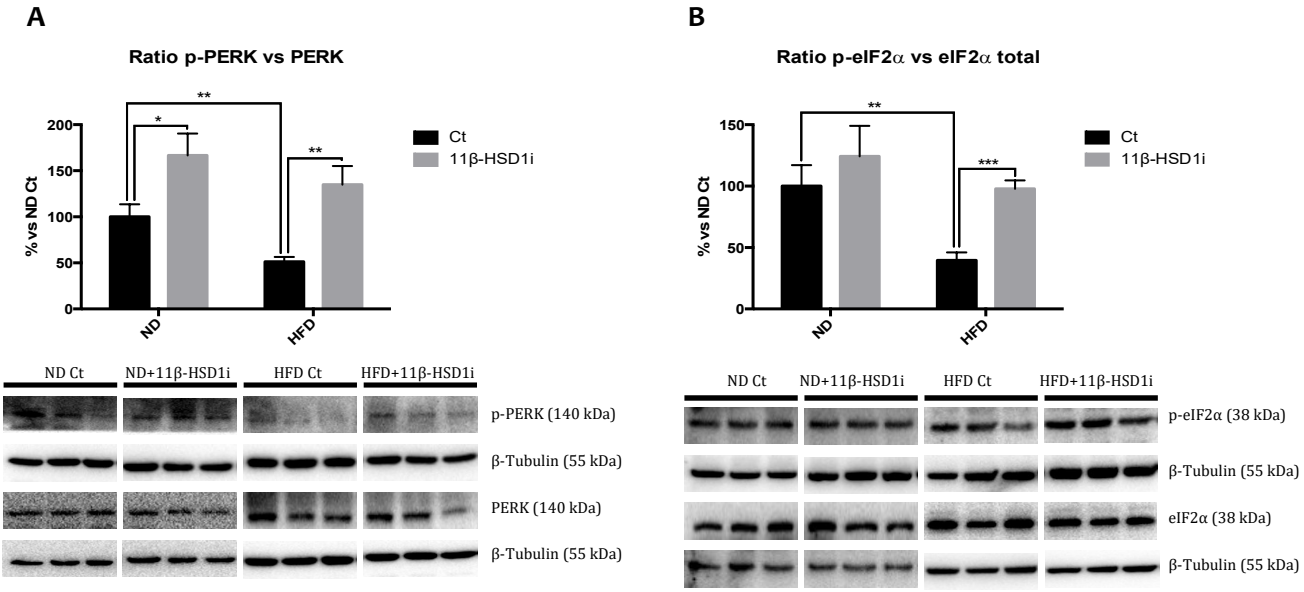


Figure 4. Representative Western blot for NF-kβ protein levels and quantification (A). Representative gene expression of inflammatory markers for Il-1β, Il-4, Il-6 and Tnf-α (B).

Representative images for Iba1 immunostaining (C) and quantification on the bar chart (E-F). *Gene expression levels were determined by real-time PCR. Values in bar graphs are adjusted to 100% for protein levels of SAMP8 Normal Diet (ND Ct). Values are mean ± Standard error of the mean (SEM); (n = 4-6 for each group). *p<0.05; **p<0.01. DG: Dentate Gyrus. Scale bar for immunohistochemical images is 200 µm.*

Treatment with 11β-HSD1i Increased Autophagy through PERK pathway

ER stress response mechanisms were evaluated. PERK pathway revealed changes in phosphorylated PKR-like endoplasmic reticulum kinase (p-PERK), phosphorylated eukaryotic translation-initiation factor 2 (p-eIF2α) activating transcription factor 4 (ATF4), and C/EBP homologous protein (CHOP). All these protein levels were higher in 11β-HSD1i treated groups compared to its Ct group, regardless of the diet (Figs. 5 A-D). Also, the HFD Ct group showed less phosphorylated PERK and eIF2α protein than ND Ct (Figs. 5 A-B). Regarding B cell lymphoma 2 (BCL-2) protein levels no changes were found among groups (Fig. 5 E). However, Beclin 1 protein levels were slightly increased in HFD+11β-HSD1i compared to the HFD Ct group (Fig. 5 F).



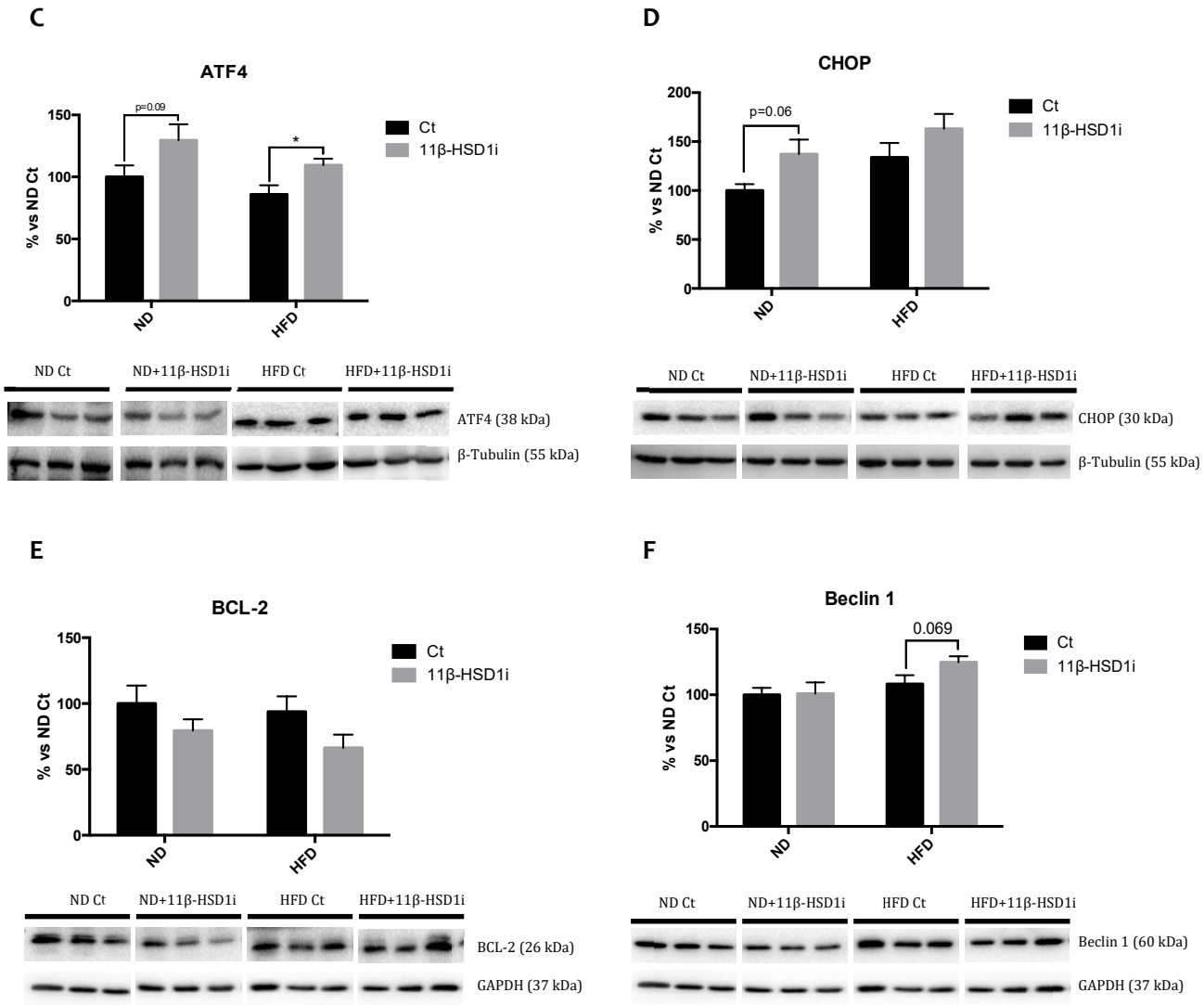
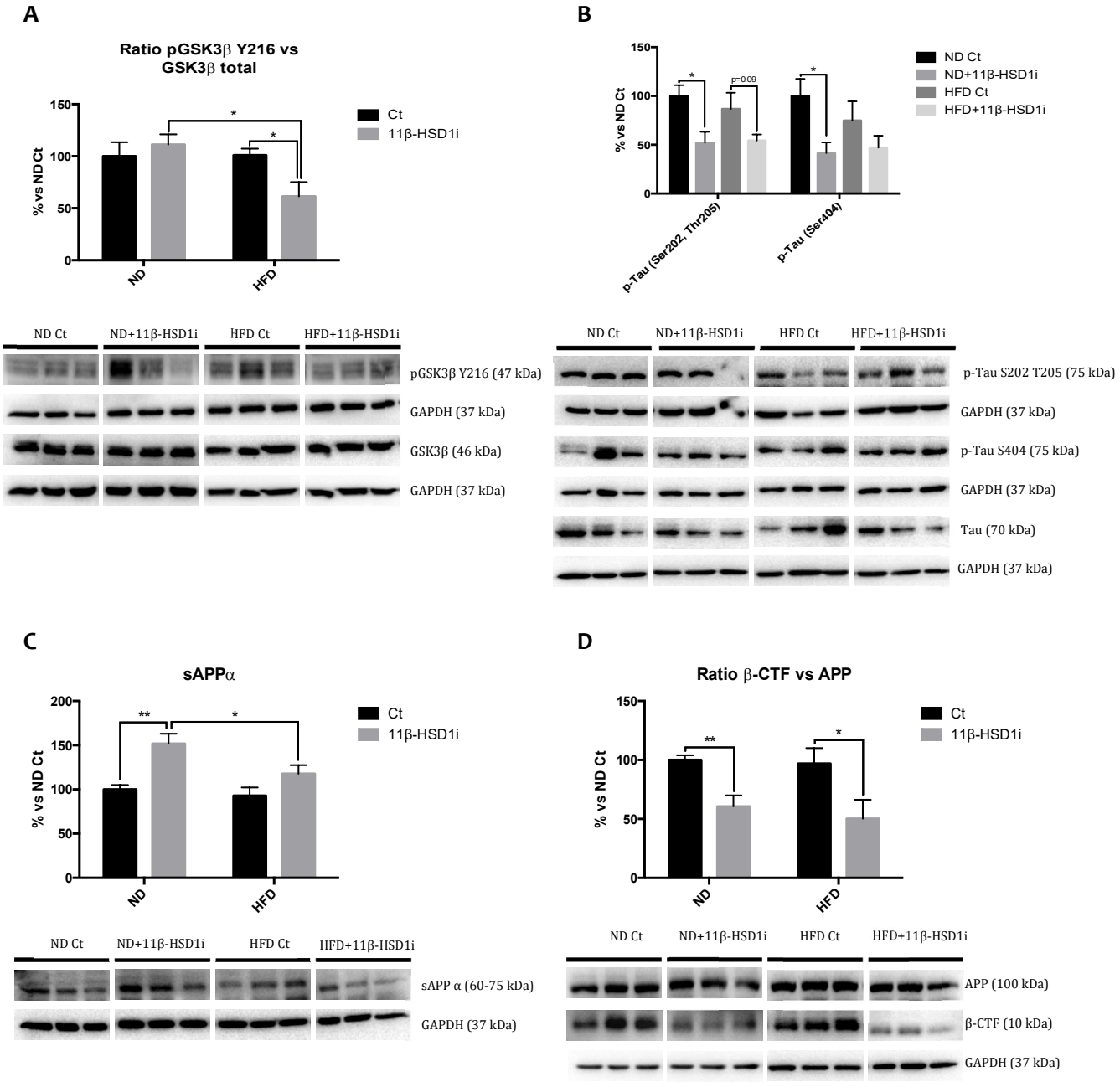


Figure 5. Representative Western Blot and quantification for the ratio of p-PERK (A), the ratio of p-eIF2α (B), ATF4 (C), CHOP (D), BCL-2 (E), and Beclin1 (F). *Values in bar graphs are adjusted to 100% for protein levels of SAMP8 Normal Diet (ND Ct). Values are mean ± Standard error of the mean (SEM); (n = 6 for each group). *p<0.05; **p<0.01; ***p<0.001.*

Reduction of AD Hallmarks after Treatment with 11β-HSD1i are Associated with Glycogen Synthase Kinase 3 Signalling Pathway Induced by HFD

HFD did not alter phosphorylation in tyrosine 217 of glycogen synthase kinase 3 beta (p-GSK3β) and Tau hyperphosphorylation. However, p-GSK3β (Tyr217) was significantly diminished in HFD+11β-HSD1i group compared to HFD Ct (Fig. 6 A). In parallel, a reduction in p-Tau (Ser 202, Thr 205), as well as p-Tau (Ser 404) protein levels after the 11β-HSD1i treatment were found in HFD Ct mice (Fig. 6 B).

HFD was unable to alter APP processing in SAMP8, neither soluble APP fragment alpha (sAPPα) nor the fragment delivered by β-secretase (β-CTF) protein levels were modified. 11β-HSD1i treatment caused a significant increase in sAPPα in ND-fed group but not in HFD-fed mice (Fig. 6 C). β-CTF protein levels were decreased after 11β-HSD1i regardless of the diet (Fig. 6 D). Finally, a significant increase in ADAM10 protein levels was only found in ND+11β-HSD1i group compared to the ND Ct group (Fig. 6 E), whereas a significant reduction in BACE1 protein levels was found in 11β-HSD1i treated animals, reaching significance in ND-fed mice (Fig. 6 F).



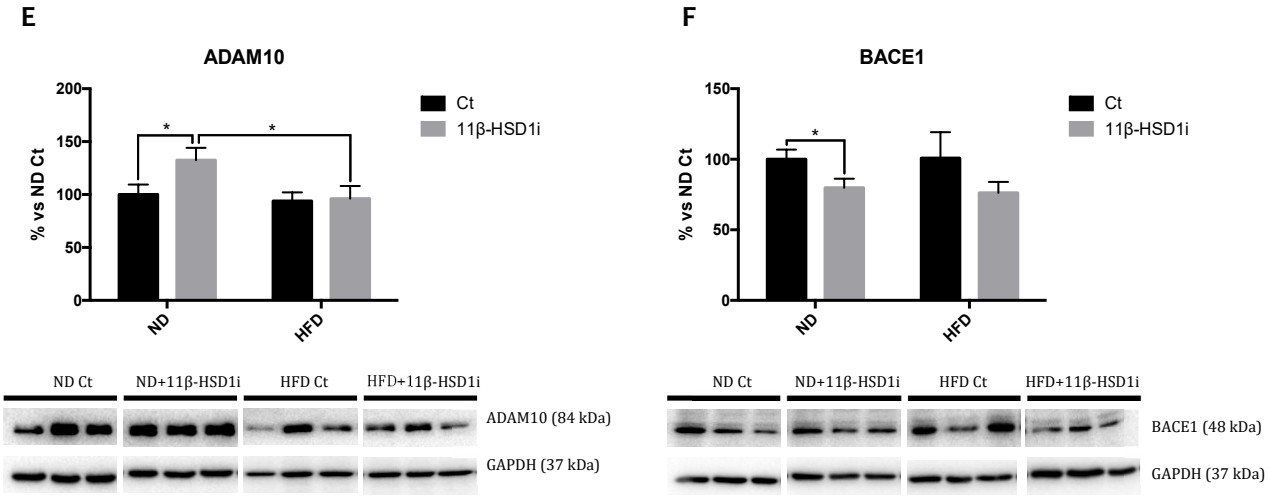


Figure 6. Representative Western Blot and quantification for the ratio of p-GSK-3β (A), the ratio of p-Tau (Ser202, Thr205, and Ser404) (B), sAPPα (C), the ratio of β-CTF (D), ADAM10 (E), and BACE1 (F). *Values in bar graphs are adjusted to 100% for protein levels of SAMP8 Normal Diet (ND Ct). Values are mean ± Standard error of the mean (SEM); (n = 6 for each group). *p<0.05; **p<0.01; ***p<0.001.*

Beneficial Effects on Social Behaviour and Cognitive Performance after Treatment with 11β-HSD1i

Social behaviour was investigated by TCT. HFD did not alter the preference for a specific chamber during the habituation, neither the treatment with 11β-HSD1i (Fig. 7 A). Moreover, in all the experimental groups the presence of an intruder increased significantly the time spent in this chamber, regardless of diet or treatment (Fig. 7 B). Higher interaction in HFD+11β-HSD1i group compared to the HFD Ct group was found, although no changes in the interaction between the resident and the intruder between ND groups occurred (Fig. 7 C).

Furthermore, cognitive performance was measured by the MWM and NORT tests. Regarding MWM, all mice groups were able to learn through the training period as no differences were found among groups (Fig. 7 D). On the test day, 11β-HSD1i treated mice increased the swim length in the platform zone compared to Ct animals indicating higher cognitive abilities (Fig. 7 F). Besides, the number of target crossings was significantly increased in HFD+11β-HSD1i mice in comparison with the ND Ct (Fig. 7 F). In NORT analysis 11β-HSD1i treated mice exhibited a significant improvement in cognitive performance both in short- (2h) and long-term recognition memory (24h) in comparison with Ct groups, obtaining higher DI values (Fig. 7 G).

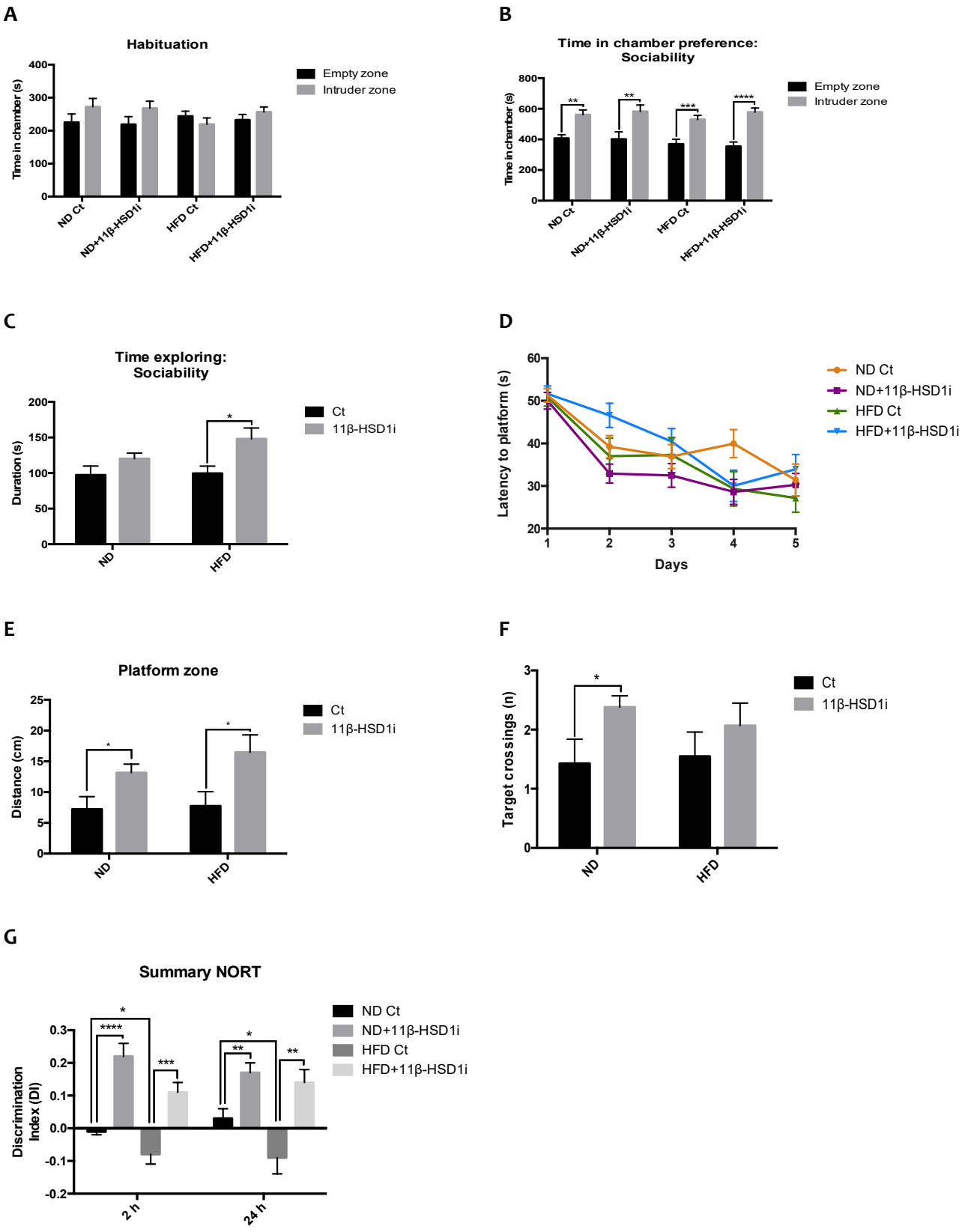


Figure 7. Results of TCT for all mice groups. Habituation phase (A), chamber preference (B), and sociability (C). Results of MWM for all mice groups. Learning curves of MWM during the spatial acquisition phase (D), distance in platform zone during the test (E), and number of

entries in the platform zone during the test (F). Results of NORT for all mice groups. Summary of DI from 2 and 24 hours after the familiarization phase (G). *Values are mean ± Standard error of the mean (SEM) (n=12 for each group). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.*

3.4.5 Discussion

In this study, we provide new evidence that inhibition of 11β-HSD1 improves cognitive impairment after metabolic stress induced by HFD intervention in a mice model of cognitive decline. To this end, various molecular pathways influenced by HFD and GCs, as well as social and cognitive impairment were evaluated to elucidate new mechanisms by which 11β-HSD1 inhibition exerts neuroprotection.

Our group had previously described that HFD induces metabolic stress in a murine model of accelerated senescence, SAMP8 (Palomera-Ávalos et al., 2017a) and in C57BL/6J aged mice (Palomera-Ávalos et al., 2017b), which was prevented by resveratrol (Palomera-Ávalos et al., 2017 a-b). Moreover, in previous reports, the neuroprotective effect of 11β-HSD1 inhibitor (RL-118) was demonstrated in old female SAMP8 (Leiva et al., 2017; Puigoriol-Illamola et al., 2018).

Animals fed with HFD increased body weight in comparison with ND fed mice showing an alteration in glucose metabolism. The hugest increase in animal weight was not only associated with impaired glucose tolerance but also with an alteration in lipid metabolism. Barroso and co-workers described that the excessive consumption of hypercaloric and high saturated fat food causes atherogenic dyslipidaemia (Barroso et al., 2013). Accordingly, our results showed higher blood triglycerides concentration in HFD-fed groups.

As aforementioned, there is extensive evidence that HFD impairs glucose metabolism, altering the insulin signalling (Shoelson et al., 2007; von Frankenberg et al., 2017). Recently, a close relationship between insulin insensitivity and neurodegenerative disorders such as AD has been extensively reported (Griffith et al., 2018; Tumminia et al., 2018) and it is still being discussed whether to consider AD as a type 3 *Diabetes mellitus* (T3DM) (de la Monte and Wands, 2008). Additionally, high GC exposure can mediate insulin resistance response, increasing glucose levels and favouring insulin resistance and obesity

(Geer et al., 2014; Ye, 2013). Thus, selective inhibitors of 11β-HSD1 have been postulated as a neuroprotective strategy in several pathological scenarios, including metabolic disturbances (Joharapurkar et al., 2012). RL-118 treatment reduced the 11β-HSD1 enzyme and GC receptor protein levels both in ND- and HFD-fed SAMP8. In concordance, GC levels were reduced both in blood and in plasma of RL-118 treated animals. Results are in line with reports describing a reduction in gene expression of 11β-HSD1 and GR in diet-induced obese mice after treatment with carbenoxolone, a 11β-HSD1 inhibitor (Liu et al., 2008).

Recently, FGF-21 has been demonstrated to modulate energy homeostasis of glucose and lipid through activation of SIRT1/PGC1α/AMPKα axis, mainly through LKB1 activity (Chau et al., 2010). Consistent with this hypothesis, treatment with 11β-HSD1 inhibitor significantly increased protein levels of FGF-21 and LKB1 under both dietary conditions. Albeit RL-118 did not induce direct changes in SIRT1 and PGC1α protein levels in HFD fed SAMP8, the increase in AMPKα phosphorylation also indicated an improvement in nutrient sensing and mitochondrial function. In sum, these results demonstrate the beneficial effects of reducing GC signalling by 11β-HSD1 inhibition in SAMP8 under HFD-induced metabolic stress.

It has been reported that HFD increases OS and inflammation (Newsholme et al., 2016). Accordingly, in HFD treated groups OS markers, such as GPX1, SOD1 and *iNOS* were increased, and 11β-HSD1i treatment was only able to reduce them when animals were fed with HFD. Whereas a clear tendency to reduce hippocampal ROS levels in all 11β-HSD1i treated animals was found. Regarding neuroinflammation, *Il-6* and *Il-4* gene expression and microglial activation evaluated through Iba-1, increased under HFD confirming the cellular dysfunction induced by impaired energy metabolism. Importantly, 11β-HSD1 inhibition diminished gene expression of *Tnf-α* and *Il-6*, as well as p65 (NF-κB fraction) protein levels, inhibiting microglial reactivity. These findings agree with already published evidence describing that 11β-HSD1 inhibition modulates OS and inflammatory processes (Gathercole et al., 2013; Leiva et al., 2017; Puigoriol-Illamola et al., 2018).

11β-HSD1 inhibition by RL-118 reduced autophagy and apoptosis markers in old SAMP8 (Puigoriol-Illamola et al., 2018), but in the present work, we did not observe significant changes in Beclin 1 or BCL2, probably because of the young age of mice. Because ER stress response activates proteostatic mechanisms, (Cuanalo-Contreras et al., 2013; Hetz et al., 2015) ER stress markers were studied. While HFD did not modify ATF4 protein levels in a significant way, PERK and eIF2α activation were significantly reduced in those animals. Of

interest, inhibition of 11β-HSD1 by RL-118 recovers the ratio of phosphorylation of PERK and eIF2α, and in addition, increases ATF4 protein levels. By contrast, in our hands CHOP showed a narrow but not significant increase after RL-118 treatment (Mahdi et al., 2015). Regarding the accumulation of misfolded proteins, one characteristic of ageing and AD, 11β-HSD1 inhibition reduced GSK3β activation and tau phosphorylation after HFD feeding. Regarding the β-amyloid pathway, RL-118 promoted APP processing by the non-amyloidogenic pathway, decreasing pro-amyloidogenic APP fragments and BACE1 protein levels, as well as increasing ADAM10 protein levels. Results suggested that 11β-HSD1 inhibition reduces the negative impact of HFD on cellular and tissue hallmarks of cognitive decline and AD (Palomera-Ávalos et al., 2017a-b; Petrov et al., 2015).

Of paramount importance, RL-118 promoted changes in sociability behaviour, recognition memory, in both short- and long-term, and spatial memory in both dietary conditions. These results point out that the GC levels have a key role in memory, learning and socialization processes, influencing mood-like behaviours in mice, both physiological and pathological conditions, supported by the molecular results, and in accordance to Raulo & Dantzer (2018).

In conclusion, our results demonstrate that HFD induced systemic metabolic dysfunctions and exacerbated cognitive impairment in adult SAMP8 mediated by alterations in insulin signalling, OS, neuroinflammation, and aberrant protein processing. Decreasing GC levels with RL-118 reduced global stress and led to beneficial effects in most of the markers evaluated in HFD and ND fed mice. Finally, modulation of GC activity by 11β-HSD1 inhibition contribute to enhance cognitive performance in senescent mice regardless of the dietary influence. Because new approaches are needed to fight against cognitive decline and dementia, such as AD, the control of GC levels may open new avenues to prevent these devastating conditions.

3.4.6 Acknowledgements

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3.4.8 Supplementary material

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4. DISCUSSION

Ageing is a physiological and multifactorial process that provokes a gradual cellular deterioration in most living organisms, which is not only associated with compromised lifespan, but it also has a marked effect on healthspan. In fact, it results in increased susceptibility to a variety of neurodegenerative diseases as AD, eating disorders, viral infections and cancers (Jura & Kozak, 2016; Leng & Goldstein, 2010; Niccoli & Partridge, 2012). Additionally, obesity and HFD consumption are two key factors in the development and progression of a wide range of disorders, such as T2DM, metabolic syndrome, and cardiovascular diseases, as well as contribute to neurodegenerative processes like AD (De Felice & Lourenco, 2015; Keshk et al., 2020). Although it is completely assumed that stress is one of the factors that most directly influence the frailty phenotype, there are strikingly few measures to restrain stressful lifestyles at different levels, both metabolically and physically, in order to reduce the progression of pathological towards successful ageing. The study of stress effects on cognition and its relationship with ageing is of the utmost importance in order to unveil what challenges we might have to cope with as a society in a not so far future. Therefore, and taking into account that ageing leads to a gradual decline of cognitive abilities and it is the main risk factor for AD, we firmly believe that it is necessary taking a step forward in the study of the molecular mechanisms underlying neurodegeneration and find a different approach of the disease to achieve an appropriate treatment.

In an attempt to address this issue, several pieces of literature defining stress, stress response and its implications in cognitive decline have been reviewed. It has been established that the environment affects individuals' cognitive performance and brain structure (Crous-Bou et al., 2020; Griñán-Ferré et al., 2016; Oudin, 2020). Indeed, the stress response is actually a very useful and highly adaptive response. Stress exposure activate HPA axis to re-establish homeostasis and release a wide variety of hormones that exert their effects in numerous central and peripheral sites to produce adaptive effects, including the mobilisation of energy from storage, maintenance of the immune system and inhibition of nonessential processes such as reproductive function. Collectively, these functions enable the fight or flight behaviours to remove the organism from immediate danger, while later restoring bodily homeostasis (Goosens & Sapolsky, 2007). However, the experience of too much stress over time can have adverse consequences on health and behaviour, but never experiencing any stress would result in inactivity, boredom and inability to adequately respond to internal and external demands (Stults-Kolehmainen & Sinha, 2014).

As stress response modulates a large constellation of cellular mechanisms implied in ageing and age-related neurodegenerative pathologies, we assessed some proper features of ageing, such as loss of proteostasis, altered nutrient sensing, mitochondrial dysfunction, OS and inflammatory responses, in reference to HPA axis alterations.

Compelling evidence has demonstrated that as ageing, and more pronouncedly when it has been subjected to continuously stressful stimuli exposure, elevated GCs levels due to HPA axis dysregulation have an impact in our cognitive performance and impulse various mechanisms underlying neurodegeneration. In consequence, controlling GC activity by 11β-HSD1 becomes essential to prevent that.

4. 1 11β-HSD1 inhibition

The 11β-HSD1 enzyme has been recognized as an essential modulator in the intracellular control of GC actions, as it catalyses the conversion of inert cortisone to active cortisol in primates or 11-dehydrocorticosterone to corticosterone in rodents. It is anchored in the ER membrane and is present in peripheral tissues, notably in the liver, adipose tissue and vasculature, as well as in multiple regions of the brain; in particular, the anterior pituitary gland, hypothalamic PVN and the hippocampus (Holmes et al., 2010; Peng et al., 2016).

Considering that 11β-HSD1 enzyme presents a high sequence homology between species, particularly within the cofactor-binding region and the catalytic site (Gathercole et al., 2013), we decided to clone the human gene encoding this protein and transfect human embryonic kidney (HEK) 293 cells in order to express that enzyme and in consequence study one of the key factors in drug discovery: the specific binding of the drug to its target, i.e. target engagement. Measuring and quantifying the binding of a drug to a protein target inside living cells and thereby correlating biochemical or biophysical activity with target engagement in cells or tissue represents a key step that should allow for unbiased determination of small molecule-protein interactions with the aim of confirming cellular mechanism-of-action while avoiding major artificial perturbations of cellular homeostasis and integrity (Stefaniak & Huber, 2020).

Up to now, studies demonstrating that RL-118 can reach the brain, bind the 11β-HSD1 enzyme and inhibit it, leading to therapeutic effects have not been addressed. In consequence, in the present work, the target engagement of RL-118 and 11β-HSD1 was

determined, through a novel methodology consisting in FACS coupled to MS analysis called TAPS assay (Wilson et al., 2017). Effectively, target engagement results showed that RL-118 drug binds to its target in a selectively way, since it did not bind to other proteins expressed. Hence, we have determined that actually the molecular and behavioural effects observed after RL-118 administration are due to its binding to the 11β-HSD1 enzyme.

In agreement with that, we have detected lower levels of the active GC in blood and brain of SAMP8 mice treated with the 11β-HSD1 inhibitor in comparison to the control group, and additional data supporting this fact revealed that 11β-HSD1 protein levels were decreased after RL-118 treatment and GR protein levels too, indicative of GC signalling modulation.

4.2 Neurodegeneration

Ageing is closely linked to neuronal changes and cognitive deficits, and therefore to neurodegeneration, being the major risk factor for neurodegenerative disorders, like AD. The ability of misfolded forms of the two key proteins of AD – Aβ and hyperphosphorylated Tau – to disrupt hippocampal synaptic plasticity engenders impending synaptic failure, subsequent structural pathology and eventually contributes to cognitive decline (Balschun & Rowan, 2018). Also, it has been suggested a causal role of stress in the onset and progression of AD, as sustained stress-level GC exposure has been found to increase Aβ formation and hyperphosphorylated Tau accumulation, hallmarks of human AD; and adversely affect behaviour (Bini et al., 2020; Green et al., 2006; MacLullich et al., 2012). Consistently with this, reducing GC exposure in the brain via intracellular inhibition of 11β-HSD1 has emerged as a therapeutic strategy to treat cognitive impairment in early AD (Webster et al., 2017). In consequence, throughout the development of this doctoral dissertation, we decided to study specific markers of AD, as well as other indicators of brain function, such as markers of synaptic plasticity.

Regarding the AD hallmarks, not only APP processing and Tau hyperphosphorylation markers have been evaluated but also other markers controlling them like GSK3-β. It has been reported that GSK3-β regulates Aβ production by down-regulating the activity of the α-secretase and interfering with the γ-secretase activity, thus resulting in Aβ-induced neurotoxicity reduction (Llorens-Martín et al., 2014). Accordingly, GSK3-β participates in Tau phosphorylation and growing evidence indicates that hyperphosphorylated Tau activates GSK3-β through an increase in OS, neuroinflammation and apoptosis (Saeki et al., 2011). On

the one hand, CMS increased Tau hyperphosphorylation and induced changes in APP processing towards an amyloidogenic pathway, especially in SAMP8 mice (Canet et al., 2018; Mravec et al., 2018). Notwithstanding, CMS also decreased Aβ clearance mechanism similar to what is described in Morgese et al. (2017) study. Surprisingly, those results were not observed in SAMP8 under metabolic stress. However, 11β-HSD1 inhibition by RL-118 clearly promoted non-amyloidogenic processing of APP and reduced Tau hyperphosphorilation, allowing the recovery of those marks regardless of the stressful event (Canet et al., 2018).

Diversely, it has been determined that male SAMP8 showed reduced neuronal synaptic plasticity markers in comparison with SAMR1, as well as lower in SAMP8 mice under CMS than the control group (Wang et al., 2016), although in our hands CMS did not impair synaptic plasticity. Previously, our lab demonstrated that RL-118 treatment enhanced synaptic plasticity in SAMP8 mice (Leiva et al., 2017), and in accordance, we observed that although exposure to stressful situations, after 11β-HSD1 inhibition by RL-118 treatment, protein levels of synaptic plasticity markers studied increased. In relation to synaptic function, it has been recently described that CREB-BDNF signalling pathway in the hippocampus mediates learning and memory deficits of rats subjected to CMS, decreasing its activation in CMS groups (Luo et al., 2017). In agreement with that, we found changes in the ratio of p-CREB protein levels but not in *Creb* gene expression in SAMP8 under CMS. However, regarding 11β-HSD1 inhibition effects, similarly to Numakawa et al. (2017), RL-118 treatment increased BDNF protein levels and gene expression.

4.3 Epigenetics

In line with stressful events influence in cognition, the way the environment modifies gene expression is comprised under the term epigenetics. While ageing and in neurodegenerative pathologies, there are several epigenetic modifications, which include DNA methylation, histone modifications and miRNA-mediated gene regulation (Barter & Foster, 2018; Harman & Martín, 2019).

Given that SAMP8 is a spontaneous ageing model, it is suggested that underlying mechanisms orchestrating accelerated senescence must be linked to epigenetics (Griñán-Ferré et al., 2018). In this respect, several studies corroborate the existence of epigenetic modifications related to ageing and neurodegeneration in SAMP8 (Alvarez-López et al., 2014; Cosín-Tomás et al., 2014).

On another note, convergent evidence from both preclinical and clinical studies has demonstrated significant associations between stress and epigenetic alterations, predominantly in genes involved in mediating resilience or vulnerability to stress, including stress-response genes, genes involved in neurotransmission and neurotrophin genes (Jiang et al., 2019; Park et al., 2019). Herein, we evaluated three main epigenetic marks that regulate gene expression, modulating chromatin structure through recruitment, assembly or retention of chromatin-associated factors after CMS exposure.

As far as DNA methylation is concerned, 5-mC DNA contributes to neural development, and its dysregulation is implicated in neuronal disorders, although its relationship is still debated. For instance, heightened 5-mC levels have been related to AD (Mastroeni, 2016), while others described the contrary (Ellison et al., 2017). This difference may be attributed to specific regions of the genome. Typically, 5-mC is associated with gene silencing and deregulated transcription. However, oxidation of 5-mC by TET enzymes to 5-hmC is thought to have the opposite effect (Fetahu et al., 2019). In the present studies, CMS reduced 5-mC and 5-hmC in both mouse strains, although in the second study, there were no significant differences in 5-hmC DNA between CMS groups and their control littermates. TET2 protein levels and gene expression and *Dnmt1* gene expression were agreeable to the above results, although *Dnmt3a* gene expression failed to significance. Referring to RL-118 treatment, it increased 5-mC and 5-hmC in accordance with *Dnmt1* and *Tet2* gene expression, recovering those marks in SAMP8 under CMS treated with the 11β-HSD1 inhibitor studied. In agreement with this, it has been described that GCs can rapidly induce methylation changes in CpG islands of the DNA, most notably global decreases in methylation, although some studies have documented GC-induced DNA hypermethylation in some sites (Zannas & Chrousos, 2017). Of note, global DNA methylation does not represent a precise landscape for specific transcriptional activity as it is misleading which genes are methylated and which are not.

As two major mechanisms for epigenetic regulation, DNA methylation and histone modifications must act coordinately (Zhao et al., 2016). One of the histone PTM is acetylation that is associated with relaxed chromatin structure and thereby greater levels of gene expression (Turner, 2000). CMS and thus heightened GC exposure is supposed to decrease histone acetylation (Bagot et al., 2014; Harman & Martín, 2019). In the same line, we found that CMS reduced acetylated histone protein levels that correlate with increased HDAC2 protein levels. Also, acetylation was decreased in SAMP8 compared to SAMR1

animals. Albeit, higher H3K9 protein levels were found in SAMP8 under CMS group, it might not translate to relaxed chromatin state, as dimethylation of this specific acetylated histone protein levels were increased in this group in both projects conducted. On the contrary, 11β-HSD1 inhibition reduced *Hdac2* gene expression and contributed to increasing acetylated histone protein levels, but not in SAMP8 under CMS too. In addition, other HDACs include SIRT family, which are intimately linked to ageing as they modulate genomic stability, stress resistance and energy metabolism. In particular, activation of SIRT1 enables deacetylation of a variety of proteins resulting in a robust protective cellular response because it regulates processes such as cell death, metabolism or neurodegeneration (Herskovits & Guarente, 2014); while SIRT2 regulates OS, genomic integrity and myelination (Jesko et al., 2017), and *Sirt6* gene expression has been reported to be decreased in the hippocampus and cerebral cortex of aged mice and that is concerned with H3K9 (Khan et al., 2018). Our results agree with those above mentioned, as *Sirt* studied gene expression were decreased in SAMP8 mice compared to SAMR1 and SAMR1 under CMS. Other histone PTM involve methylation and phosphorylation. The former is linked to gene silencing, whereas the later to DNA damage response. The pattern of histone methylation has been shown to change during ageing depending on the tissue and organisms, although a global increase in activation histone methylations concomitant with a reduction of repressive methylated histone marks has been reported (Harman & Martín, 2019). Our results showed increases in these marks in SAMP8 under CMS and trend toward reduction in RL-118 treated groups.

Finally, emerging evidence also supports the role of GC signalling in the regulation of several miRNAs (Zannas & Chrousos, 2017). We evaluated the expression of miRNAs regulating OS and cellular pathways implicated in AD neuronal death, autophagy and neurodegeneration. Altogether, miRNA expression studied correlated with their target genes. In our hands, CMS slightly provided an increase in DNA methylation and amyloidogenic APP processing, while a decrease in Wnt pathway and autophagy. Taking into account that DNMT inhibitors have provided neuroprotection in cell cultures (Chestnut et al., 2011) and β-catenin reduction has been found in the brain of AD patients in accordance to decreased GSK3-β inactive form (Llorens-Martín et al., 2014) that is related to increased Tau hyperphosphorylation, and accounting that CMS favoured amyloidogenic APP processing, while decreased non-amyloidogenic APP processing mediators as well as autophagy flux (Uddin et al., 2018), we concluded that CMS could produce deleterious effects on health.

Noteworthy, stress exposure, which may influence GC signalling, can result in lasting epigenetic modifications that persist throughout life and even across generations (Zannas & Chrousos, 2017). Therefore, it is of paramount importance to study drug actions at the epigenetic level in order to possess a deep knowledge of the pharmacological effects exerted by the drug. To this regard, although RL-118 is not a direct epigenetic modulator drug, it modifies the global epigenetic landscape.

4.4 Proteostasis

In reference to proteostasis mechanisms, it involves autophagy and ER stress response. Under stress conditions, the accumulation of misfolded proteins disrupts ER function and induces its stress response. To respond to ER stress conditions, the UPR signalling pathway activates in order to restore cell homeostasis. However, when it is continued, stress may promote cell damage and death (Karna et al., 2019). The UPR signalling includes ER stress response and autophagy, among others.

ER stress response accounts for several actions to promote correct protein folding and inhibit protein translation. Through different pathways, mediators of this response increase the expression of different genes to cope with OS as well as inflammatory stimuli and promote longevity (Ma et al., 2013; Schmitz et al., 2018). Among others, it has been described that PERK as well as other mechanisms promote NF-κB activation, which up-regulates the expression of pro-inflammatory genes. Similarly, an increase in the ER protein load may lead to an overproduction of ROS and, in turn, may initiate an inflammatory response. Thus, to control OS, the PERK pathway through NRF2 and ATF4 induces transcription of antioxidant and oxidant-detoxifying enzymes (Colla, 2019). Additionally, the eIF2-α factor is activated by AD hallmarks, inflammation and OS pathways, and nutrient imbalance; features of pathological ageing. Also, its reduction in mice has been linked to enhanced synapse plasticity and cognition (Ma et al., 2013). As misfolded proteins accumulate during neurodegeneration, ER capability becomes overrun and eventually proves insufficient leading to proteotoxicity and protein aggregation (Kurtishi et al., 2019). Accordingly, evidence for canonical UPR activation in AD neurons has been determined, and induction of ER stress leads to neuronal metabolic stress, Tau hyperphosphorylation and cognitive impairment in mice (Lourenco et al., 2013; van der Harg et al., 2014). Our results pointed out a reduction in ER stress response mediators in mice under metabolic stress, which was recovered when treated with RL-118, except for C/EBP homologous

protein (CHOP) protein levels that showed a narrow but not significant increase after RL-118 treatment, similarly to Mihailidou et al. (2016).

Diversely, the UPR signalling activates autophagy. ER stress response and autophagy are linked in different ways. Under ER stress conditions, PERK-mediated ATF4 activation is required for up-regulating the expression of genes involved in autophagy, ER chaperone proteins like BIP/GRP78, amino acid biosynthesis, antioxidant response, and the transcription factor CHOP (Kabir et al., 2018). In addition, ATF4 directly binds to cyclic AMP response component binding site of LC3, a vital component of autophagosomal membranes (Luhr et al., 2019). However, in the last few years, research has shown that the ER stress response can not only initiate autophagy cut can also negatively regulate it to maintain cell survival (Kabir et al., 2018). Of interest, autophagy declines with ageing; so that it may participate in the deleterious accumulation of unnecessary or/and harmful components and damaged organelles observed in aged cells, as well as worsens age-related diseases (Gouras, 2019). Accordingly, Li et al. (2020) reported that stress-induced decline of hippocampal neurogenesis and cognitive deficits are mediated by autophagic death. In our hands, SAMP8 mice showed increased mTORC1 and phosphorylated mTOR protein levels compared to SAMR1, suggesting reduced autophagic flux. In concordance, CMS reduced pro-autophagic protein levels, such as Beclin1 and LC3, in SAMR1 but not in SAMP8 in which it triggered them, perhaps as a defence mechanism to try to repair the serious damage of these cells (Kabir et al., 2018). Accordingly, CMS increased anti-autophagic protein levels like mTOR and mTORC1 in SAMP8 mice. In addition, CMS increased B-cell lymphoma 2 (BCL2) protein levels in SAMP8 mice but not in SAMR1. BCL2 not only inhibits apoptosis but also negatively regulates autophagy (Pihán et al., 2017). However, HFD-induced metabolic stress did not modify BCL2 protein expression neither Beclin1. Altogether, this data supports the fact that CMS impairs the autophagic process. By contrast, RL-118 treatment induced the opposite effects, i.e. increased protein levels of pro-autophagic mediators studied recovered after CMS exposure and decreased anti-autophagy markers protein levels, likely Wang et al. (2017) who described that excessive GC levels suppress autophagy.

Noteworthy, we determined significant correlations between some of the autophagy markers evaluated and inflammatory and OS mediators; specifically, between Beclin1, mTOR and ratio of LC3 protein levels and *Il-1β*, *Ccl3*, *Hmox1* and *Aldh2* gene expression in aged mice. Also, correlations between autophagy and cognitive abilities assessed in the

behavioural tests performed were established. Interestingly, Beclin1 and LC3B correlated positively with cognitive improvement, while mTOR negatively. On the same mood, negative correlations were determined between pro-inflammatory and OS markers and pro-autophagic protein levels, but the opposite for mTOR protein levels. Altogether results highlight the importance of proteostasis for cell survival and the evolution of cognitive impairment.

4.5 Nutrient sensing

Likewise, altered nutrient sensing is considered a hallmark of ageing. In recent years, the interest in the impact of nutrition to health has grown, as obesity is a risk factor for many metabolic disorders, such as diabetes, hypertension, cerebrovascular accident, heart diseases, but also for neurodegenerative diseases and cognitive impairment (Edwards III et al., 2019; Palomera-Ávalos et al., 2016). Ample evidence provides both clinical and experimental evidence into how metabolic disorders and AD may course together (Bini et al., 2020; De Felice & Lourenco, 2015). Thus, different pieces of literature have focused on the study of cognitive decline and neurodegeneration through metabolic stress induced by HFD intervention (Wei et al., 2018) and have described that HFD contributes to insulin resistance and obesity; signs related to cardiometabolic disorders and mental health problems (Edwards III et al., 2019). Besides, it is well known that anabolic signalling accelerates ageing and that decreased nutrient signalling achieved with caloric restricted diets or by stimulation of SIRT promotes healthspan and longevity (Farr & Almeida, 2018). Indeed, some of the signalling pathways more intrinsically associated with longevity are those of IIS, mTOR and SIRT1, involved in nutrient sensing and glucose metabolism, autophagy and histone deacetylases, respectively (Salas-Pérez et al., 2019).

Results obtained agree as mice that received HFD increased pronouncedly their body weight gain, consumed more calories and showed higher glucose tolerance curve profile than mice in the control group, thus suggesting that HFD impairs glucose metabolism. Also, blood triglycerides determination revealed higher levels in the groups exposed to HFD. Despite the studies affirming the use of 11β-HSD1 inhibitors for the treatment of metabolic syndrome (Schnackenberg et al., 2013), in our project RL-118 drug did not improve glucose metabolism under normal conditions, although it did in mice receiving concomitant HFD. Interestingly, not only HFD induces changes in glucose metabolism, but also CMS exposure. SAMP8 showed a higher area under the curve (AUC) in the glucose tolerance test compared to SAMR1 mice, although SAMR1 under CMS displayed a similar glucose

metabolism profile to SAMP8 suggesting that CMS also contributes to detrimental glucose metabolism alterations characteristic of aged mice (Chia et al., 2018).

Hormones such as FGF21 and GCs play crucial roles in coordinating the adaptive starvation response. Explicitly, it has been demonstrated that FGF21 modulates energy homeostasis of glucose and lipid through activation of SIRT1/PGC1-α/AMPKα axis, mainly through liver kinase B1 (LKB1) activation, resulting in enhanced mitochondrial activity (Chau et al., 2010; Patel et al., 2014). Consistently, our results determined that HFD feeding decreased their expression, although treatment with the 11β-HSD1 inhibitor significantly increased FGF21 and LKB1 protein levels under both dietary conditions (Lützner et al., 2012; Tezze et al., 2019). Albeit RL-118 did not induce direct changes in SIRT1 and PGC1-α protein levels in HFD-fed SAMP8, the increase in AMPKα phosphorylation suggested an improvement in nutrient sensing and mitochondrial function.

In sum, these results demonstrate the beneficial effects of reducing GC signalling in mice under HFD-induced metabolic stress. Additionally, impaired nutrient sensing has been associated with OS, as it has been reported that high levels of circulating lipids and glucose, present in AD patients, amplify lipid peroxidation that gradually diminishes the antioxidant systems leading to neuronal damage (Rojas-Gutierrez et al., 2017).

4.6 Oxidative Stress

OS reflects an imbalance between the systemic free radical production and the biological system's ability to detoxify the reactive intermediates and repair the resulting damage. As ageing, the cellular antioxidant capacity diminishes and jointly with mitochondrial disruption lead to increased ROS overproduction. Altogether produce oxidative damage to cells causing alterations in their biochemical and physiological functions leading to apoptosis and, therefore to neurodegenerative pathologies (Fivenson et al., 2017; Petersen & Smith, 2016). In line with ageing disturbances, stress exposure produces GCs rise and induces OS (Majer et al., 2019).

To address how OS is affected by HPA axis dysregulation, we assessed various antioxidant and pro-oxidant enzymes and global hippocampal ROS levels. On the one hand, additional data (Farr & Almeida, 2018; Picard et al., 2018) reported that prolonged exposure to stressful events affects cellular oxidative balance, prompting inability of antioxidant enzymes and thus allowing ROS accumulation and eventually causing cellular damage and

finally dysfunction of the system (Schiavone et al., 2013). In concordance, CMS increased ROS levels in both mice strains, although SAMP8 showed heightened levels in comparison to SAMR1 mice. However, HFD-induced metabolic stress tended towards increase ROS levels, but no significant differences were detected. On the contrary, attenuation of GC exposure by 11β-HSD1 inhibition contributed to decreasing ROS levels in control groups, especially in mice fed with HFD, but not in mice under concomitant CMS treatment. Likewise, GPX1, SOD1 and CAT protein levels were decreased in CMS groups of both mouse strains, but to a greater extent in SAMP8 animals. Despite CMS, HFD-fed group showed increased GPX1 and SOD1 protein levels that were decreased after RL-118 treatment.

Besides, NRF2 pathway is defined as an indicator and modulator of OS in neurodegeneration (Griñán-Ferré et al., 2018; Shintani et al., 2015), as it regulates the gene expression of different anti- and pro-oxidant enzymes like *Hmox1* and *Aox1*, respectively. Although ROS levels were increased in mice under CMS, no changes were observed in NRF2 translocation to the nucleus. However, *Aox1* gene expression was up-regulated after CMS exposure in SAMR1 and SAMP8 mice, although only significant differences were detected in SAMP8, and its gene expression was decreased after 11β-HSD1 inhibition. Also, the same profile was observed in another pro-oxidant enzyme assessed: iNOS. Moreover, *iNOS* gene expression was evaluated in HFD study, in which mice that received HFD and RL-118 treatment showed clearly reduced gene expression (Leiva et al., 2017). In line with that, without the presence of stressors and in 12 months-aged SAMP8, RL-118 treatment also reduced antioxidant defence enzymes gene expression like *Hmox1* and *Aldh2* (Schiavone et al., 2013).

Overall, these results suggest that 11β-HSD1 inhibition promoted antioxidant mechanisms to cope with OS in a mice model of ageing, even under CMS or HFD-feeding conditions, corroborating other studies (Kratschmar et al., 2012; Park et al., 2016).

4.7 Neuroinflammation

Following the same line about overall processes linked to senescence, neuroinflammation is correlated with the onset and progression of several neurodegenerative diseases of both an acute and chronic nature. In fact, dysregulation of inflammatory mediators and astrogliosis have been identified as major culprits in the development of chronic inflammation and the immunosenescence process, prompting cognitive deficits and progression of neurodegenerative diseases (Chung et al., 2019;

Cianciulli et al., 2020). Indeed, compelling evidence has observed elevated cytokine and chemokine levels in the brain of individuals under chronic stress or with AD and in animal models of the disease (Fakhoury, 2018; Hou & Yuan, 2014). Moreover, although GCs are the mainstay therapy for several inflammatory diseases, GCs activity alterations are a major problem concerning the treatment of chronic inflammatory disease and their side effects (Ronchetti et al., 2018). Accordingly, in our hands CMS appeared to increase pro-inflammatory cytokine gene expression but not significantly. However, HFD-induced metabolic stress increased them. By contrast, attenuating GC levels decreased pro-inflammatory mediators gene expression, and also RL-118 treatment was able to restore normal expression of those mediators after HFD and CMS (Duque & Munhoz, 2016).

Besides, current evidence strongly suggests that NF-κB, the central core inflammatory mediator, is a key transcriptional factor in the initiation and progression of neurodegeneration (Chung et al., 2019). Particularly, disruption of NF-κB signalling has been linked to AD, as it has been reported that GSK3-β inhibition reduces BACE1-mediated cleavage of APP through NF-κB signalling, so that it reduces Aβ pathology (Ly et al., 2013). Not only that but also it participates in ER stress response. As above mentioned, the UPR and NF-κB pathways converge within the nucleus and coordinate the transcriptional activity or repression of hundreds of genes that collectively determine the balance between metabolic and inflammatory phenotypes and the extent of apoptosis and autophagy (Schmitz et al., 2018). On another note, pro-inflammatory cytokines have been implicated in the cellular and behavioural effects of stress, given that stress activates NF-κB signalling, triggering this nuclear factor to become a critical mediator of antineurogenic and behavioural actions of stress (Koo et al., 2010). Indeed, GCs exhibit anti-inflammatory effects, although some studies have described that under aversive situations, unexpectedly GCs promote survival of altered cells, maybe due to NF-κB over-expression that can cause cellular GC insensitivity. Therefore, the crosstalk between NF-κB and GCs exerts important functions in determining cell survival or apoptosis and it depends on the context (Altonsy et al., 2014; Ling & Kumar, 2012). Surprisingly, the results obtained did not show differences between HFD neither CMS exposed SAMP8 groups and their control littermates, while differences between SAMP8 and SAMR1 animals were found, confirming the intensified inflammatory mediators expression ageing feature. Despite that, NF-κB protein levels were decreased in RL-118 treated groups in both stress experimental approaches in SAMP8 aged 6 months, but not in mice aged 12 months. Of note, RL-118 drug was able to decrease NF-κB signalling after concomitant HFD treatment but not in mice under CMS conditions.

Also, there is abundant evidence showing that activation of glial cells, including microglia and astrocytes, are essential for eliciting the inflammatory signalling pathways involved in neurodegeneration and in particular in AD because it has been described that these cells are found near amyloid burden (Wang et al., 2015). Active microglia have beneficial functions, promoting pathogen clearance and tissue regeneration and contributing to neuronal survival through the release of trophic factors. However, they may change their phenotype and function during neurodegenerative disorders triggering pro-inflammatory responses (Cianciulli et al., 2020). Deleterious effects of HFD-induced metabolic stress on microglia were studied by ionized calcium-binding adapter molecule 1 (Iba1) detection in immunofluorescence experiments. Our results demonstrated increased Iba1 expression in different parts of the hippocampus, although no significant differences were detected, and reduced expression after GC attenuation by 11β-HSD1 inhibition (Liu et al., 2016).

Moreover, brain astrocytes and oligodendrocytes also participate in the inflammatory process by producing or responding to pro-inflammatory mediators (Chung et al., 2019). Indeed, astrocytes serve many basic roles for brain functioning, sensing and integrating environmental signals including GC levels modulation, immune signals and nutritional information (Abbink et al., 2019). In agreement with Naskar & Chattarji (2019), herein mice under CMS exposure showed increased *Gfap* gene expression in both SAMR1 and SAMP8 hippocampal tissue, which was reversed by RL-118 treatment. Moreover, astrocytes have a key role in synaptic regulation, as they can act not only in the formation and maturation of synapses but also in the maintenance, pruning and remodelling of synapses in the development, ageing and diseases (Matias et al., 2019), suggesting a link between reduced neuroinflammation and enhanced synaptic plasticity.

4.8 Cognitive and behavioural changes

Brain ageing involves changes in neuronal and cognitive abilities, like mental speed and executive function, as well as social impairments. One structure of particular interest when considering ageing and cognitive decline is the hippocampus, a brain region that comprises learning and memory consolidation as well as affective behaviours and mood regulation, and where both functional and structural plasticity occur well into adulthood (Bettio et al., 2017). Accordingly when ageing, long-term potentiation results impaired at synapses and immediate early genes for the synaptic plasticity are down-regulated (Liu,

2017). Specifically, the hippocampus is an intricate brain structure part of the limbic system embedded deep into the temporal lobe that plays major roles in learning and memory. In particular, it has been proposed that it participates in the emotional response and in recollecting the experience and imagining future (Anand & Dhikav, 2012). Additionally, mounting evidence states that contribute to the consolidation of information from short-term and long-term declarative and contextual memories, and in spatial memory that enables navigation (Einchenbaum et al., 1996; Vyas et al., 2016). Furthermore, hippocampal hyperexcitability has been observed in individuals with cognitive decline and has been proposed to participate in the development of AD, which interestingly may be attenuated by lifestyle changes (Setti et al., 2017).

Although the cognitive effects of stress are frequently assumed to be detrimental, there are many instances in which cognitive functions are not impaired by stress, or on the contrary, are even improved (Shields et al., 2016). Considering stress intensity, it is believed that low and high stress levels impair memory, whereas intermediate levels facilitate it. Accordingly, several studies, including hippocampal-dependent tasks, have successfully substantiated this hypothesis and confirmed this relationship between hormonal levels and both, learning and synaptic plasticity (Sandi, 2013). Two tests to evaluate hippocampal-dependent memory are the NORT, the MWM and the OLT. Briefly, in the first animals are exposed to familiar and new objects. A discrimination index is calculated referring to the time exploring both objects, and if it is positive indicates that the animal remembers and recognizes the familiar object, so that spends more time exploring the novel object. On the other hand, MWM is based on spatial memory in which animals have to learn a platform location, whereas OLT evaluates the animal's ability to detect a location change.

However, the chronicity of the stressful events is key in determining their effects on memory. In fact, uncontrollable chronic stress has been recognized to influence the hippocampus at various levels of analysis, generally impairing hippocampal-dependent memory tasks, ensuing synaptic plasticity of hippocampal neurons, and changing neuronal morphology, suppressing neuronal proliferation and reducing hippocampal volume (Kim et al., 2015). In accordance, mounting evidence linked GCs to hippocampal atrophy in elderly who exhibited memory impairments (Goosens & Sapolsky, 2007). Not only hippocampal structure but also electrophysiology, metabolism, plasticity and survival have been associated with prolonged exposure to GCs (Vyas et al., 2016). In fact, the idea that GCs might contribute to brain ageing emerged in the early 1970s when studies had revealed that

the hippocampus was especially enriched for GR and particularly vulnerable to both normal and pathological changes observed with ageing (McEwen et al., 1968; Tomlinson & Henderson, 1976). Altogether, scientist proposed the GC hypothesis of ageing, which stated that life-long exposure to normal GC levels would cause deleterious effects of GCs to accumulate in GC-sensitive neurons, such as those of the hippocampus. Moreover, because hippocampal dysfunction could reduce hippocampal-mediated inhibition of the HPA axis, GC secretion was predicted to gradually increase over time, leading to an acceleration of both damage and dysfunction (Goosens & Sapolsky, 2007).

Accordingly to Webster et al. (2017), regulation of GC levels by 11β-HSD1 inhibition appears to be a good strategy for treating age-related cognitive deficits. In the projects of this thesis, aged mice showed worse recognition memory as well as mice under metabolic stress and CMS. In marked contrast, mice treated with the 11β-HSD1 inhibitor under study, RL-118, improved these parameters regardless of the age of the mice and could reverse the aversive effects of both HFD-induced metabolic stress and CMS. Moreover, enhanced spatial memory was found in all mice treated with RL-118. In accordance, Sooy et al. (2015) and Leiva et al. (2017) described that 11β-HSD1 inhibition improved spatial memory abilities and recognition memory in the MWM and NORT tasks.

Indeed, stress and GCs are intimately related to fear and anxiety. The stress response is variable and may hold important clues to individual experiences of mood disorders, as some individuals have lower-than-usual release of GCs in fearful situations, whereas others showed higher release. However, symptoms of anxiety and depression have been linked to heightened levels of GCs (Raglan et al., 2017). Herein, anxious behaviour was assessed using the EPM and the OFT, in which animals are exposed to uncomfortable environments – bright opened arms and an opened arena respectively –and several parameters indicating novelty-induced arousal and anxiety are recorded. In spite of that, in our hands CMS failed to increase recklessness and risk-taking behaviours, albeit 11β-HSD1 inhibition decreased those feelings, similar to Sandi (2011). Regarding the stress effects on SAMP8 animals in comparison to SAMR1, CMS reduced cognitive performance and increased anxiety-like behaviour in both mice strains, as described by Wang et al. (2016).

Besides, fear and recklessness are intrinsically associated with social behaviour. It was evaluated by the TCT, which assesses cognition through sociability and interest in social novelty and consists of adding an intruder to a particular mouse's cage and animals were

allowed to interact. HFD-induced metabolic stress did not modify social abilities and strikingly, RL-118 treatment only increased sociability in HFD-fed mice. However, it has been described that HFD reduces sociability (Hassan et al., 2019) and that GCs are negatively associated with social behaviour (Raulo & Dantzer, 2018). In conclusion, by understanding the mechanism by which stress and GCs exacerbate brain ageing, we also provide insights into ways to promote healthy ageing.

4.9 Final considerations

As observed throughout the studies of this doctoral dissertation, stress modulates a large constellation of cellular mechanisms involved in ageing and neurodegeneration. In stressful situations, the body increases GCs release generating an adaptive response. However, when stress is constant, either HFD or CMS, the synthesis and release of GCs, which in physiological conditions is regulated by strict control of the HPA axis, become altered in such a way that large amounts of GCs are released and produce detrimental effects, in particular, on cognition. Both, the metabolic stress induced by HFD as well as exposure to CMS have contributed to increasing the deregulation of the HPA axis and thus, GC excess and detrimental molecular mechanisms underpinning neurodegeneration. However, this alteration does not occur only under stressful situations, but also as we age. It is widely recognized that as we age, the body's ability to adapt decreases. Taking into consideration all these reasons, in the studies of this doctoral thesis, an animal model of accelerated ageing has been used and the implication of 11β-HSD1 in the senescence demonstrated. To address the objectives planned, mice were treated with a drug that inhibits the 11β-HSD1 enzyme in order to decrease the conversion of inactive to active GCs. RL-118 treatment, by inhibiting 11β-HSD1 and therefore reduce GCs exposure, leads to cognitive improvement, epigenetic changes and decreased OS, neuroinflammation and AD neurodegeneration markers. By contrast, RL-118 increased the UPR response, energy sensing mechanisms and synaptic plasticity markers assessed; therefore, providing a protective cellular and effect (Figure 16). Of note, RL-118 treatment was able to restore most of the deleterious effects produced by either HFD or CMS. Consequently, 11β-HSD1 could be a feasible target to fight against cognitive decline in age-related pathologies.

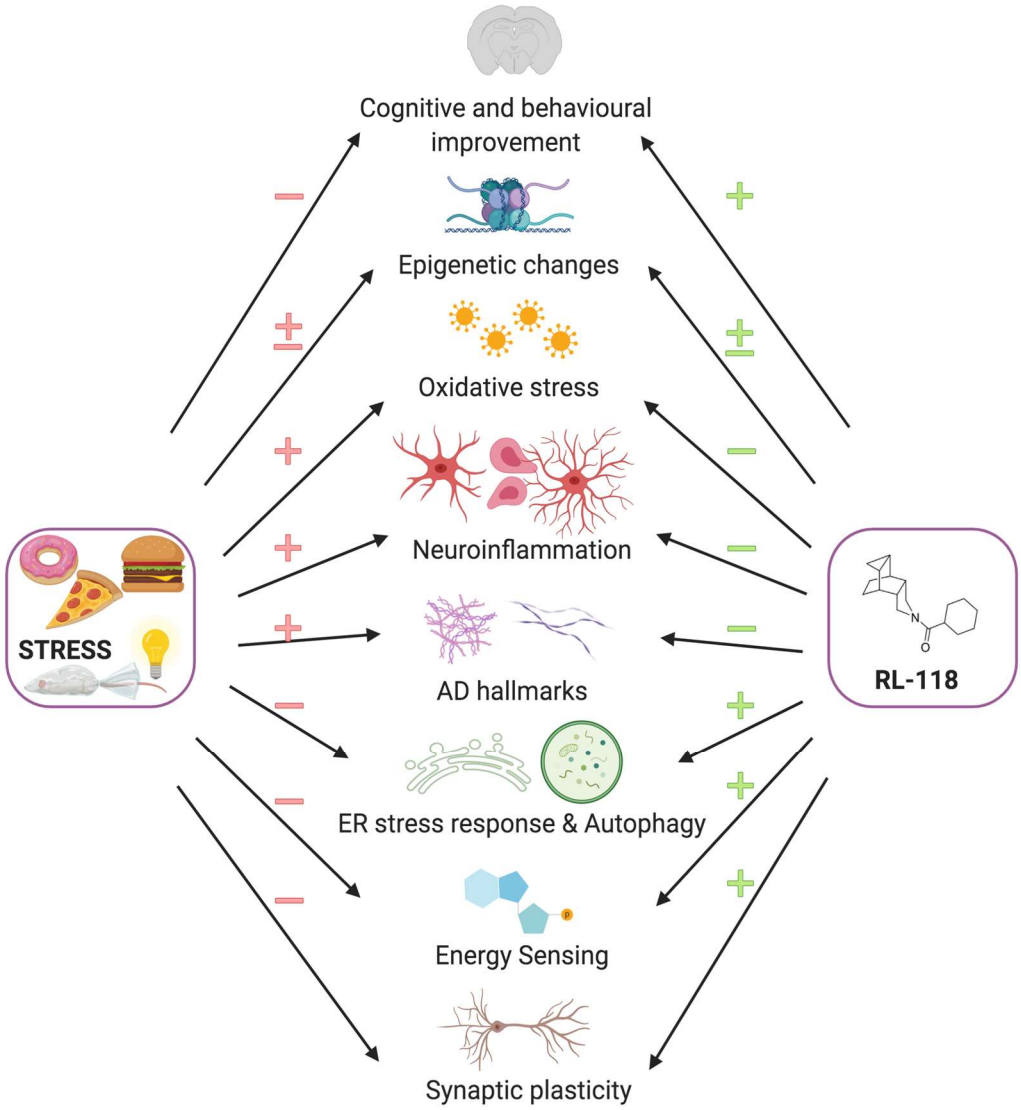

Figure 16. *Schematic representation of the molecular pathways affected after HFD and/or CMS and the protective role of RL-118 drug. Stress has a negative impact on cognitive and behavioural abilities, induces epigenetic changes, increases OS and neuroinflammatory markers, promotes AD hallmarks, attenuates the UPR response and energy sensing as well as has deleterious effects on synaptic plasticity. By contrast, 11β-HSD1 inhibition by RL-118 leads to the opposite actions. (In red the unfavourable effects, whereas in green the beneficial. A positive sign indicates activation or increment, while a negative sign inhibition or decrease, and both signs modifications related to both increases and reductions).*

5. CONCLUSIONS

The general conclusion of the studies conducted in this doctoral dissertation is that RL-118, a brain penetrant drug, treatment promotes healthy ageing by modulating different metabolic pathways underpinning neurodegeneration and affected by metabolic and chronic mild stresses. In particular, RL-118 treatment promotes healthy ageing in 12-month-old SAMP8 mice, but in younger mice, it is able to restore most of the deleterious effects produced by either HFD or CMS. Because new approaches are needed to fight against cognitive decline and dementia, the control of GC levels may open new avenues to prevent these devastating conditions. Consequently, 11β-HSD1 could be a feasible target to fight against cognitive decline in age-related neurodegenerative diseases.

Overall, the results obtained lead to the following conclusions:

1. RL-118 drug is capable of crossing the BBB into the brain of SAMP8 mice, as the effects that result from 11β-HSD1 enzyme inhibition are observed in both blood and cerebral tissues. Besides, modulation of GC activity by 11β-HSD1 inhibition contributes to decreasing 11β-HSD1 and GR protein levels in senescent mice regardless of the dietary influence.

2. RL-118 drug binds specifically to its target, the 11β-HSD1 enzyme. Therefore, all the effects observed in SAMP8 mice treated with the drug described can be attributed to the inhibition of this enzyme.

3. RL-118 treatment decreased anxiety and enhanced the cognitive abilities of aged SAMP8 mice.

4. Inhibition of the 11β-HSD1 enzyme by the RL-118 drug exerts a neuroprotective effect in the brain of aged SAMP8 animals through the promotion of autophagy, favouring the non-amyloidogenic APP processing pathway, the attenuation of tau hyperphosphorylation and reduction of pro-inflammatory as well as OS mediators.

5. The improvement of the autophagic process by RL-118 treatment in aged SAMP8 mice is strongly correlated with enhanced cognitive performance and decreased anxiety-like behaviour. In addition, autophagy activation was demonstrated to have a negative correlation with the neuroinflammation and OS markers evaluated.

6. Significant epigenetic and biochemical changes in SAMR1 animals driving them to an ageing-specific phenotype represented by SAMP8 mice are due to CMS paradigm. CMS adverse effects of SAMP8 are probably limited because its senescence level is already elevated.

7. CMS exposure produces detrimental effects in SAM strains; such as inflammatory signalling activation, loss of antioxidant defence mechanisms, autophagy inhibition, AD hallmarks increases, the decrease of factors that improve neurogenesis, synaptic plasticity and glucose metabolism alterations, as well as, changes in behaviour and cognitive performance. Interestingly, RL-118 treatment is able to restore the majority of the detrimental effects of CMS in adult SAMP8.

8. HFD-induced metabolic stress increases the negative impact of neurodegeneration and energy metabolism present in SAMP8 mice, as on the one hand it causes a decrease in nutrient and energy-sensing through SIRT1/PGC1α/AMPKα axis regulated in part by LKB1 and FGF-21; and on the other hand, increases antioxidant defences to cope with OS and neuroinflammation, while decreasing ER stress response. In contrast, 11β-HSD1 inhibition promotes the recovery to the physiologic profile of these processes as well as causes a slight increase in the analysed autophagy marker.

9. RL-118 treatment in HFD-fed SAMP8 mice reduces GSK3-β active protein levels, reflected by lower tau hyperphosphorylation, and stimulates non-amyloidogenic APP processing in both dietary conditions.

10. Metabolic stress due to HFD feeding exacerbates cognitive impairment in adult SAMP8, which is reversed by RL-118 treatment. Also, 11β-HSD1 inhibition enhances social behavioural abilities.

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