From the Department of Psychiatry and Forensic Medicine,
Autonomous University of Barcelona, Spain

SUSCEPTIBILITY TO EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (MODEL OF MULTIPLE SCLEROSIS) AND ANXIETY IN GENETICALLY HETEROGENEOUS RATS

ESTHER MARTÍNEZ MEMBRIVES

THESIS FOR DOCTORAL DEGREE (PH.D.)

SUPERVISED BY DR. ALBERTO FERNÁNDEZ TERUEL

Universitat Autònoma
de Barcelona

ACKNOWLEDGEMENTS

Gracias Alberto Fernández, mi director, por haberme brindado la oportunidad de entrar en tu grupo. Te admiro como científico y como persona. Eres un investigador y profesor brillante, y además te preocupas por mantener el bienestar de las personas con las que trabajas. No se puede ser más llano y accesible, gracias. Ha sido un honor y un placer trabajar contigo.

Gracias Margarita Díez, mi co-directora, a parte de tener la profesión de científica eres una persona científica, no dejan de asombrarme tu curiosidad y entusiasmo. Más adelante tengo más cosas que decirte.

He tenido la enorme suerte de trabajar codo a codo con personas muy especiales. Elia Vicens, vam passar hores eternes al laboratori i ens vam fer inseparables. Gràcies per la teva amistat vertadera i per totes les aventures tragicòmiques que hem viscut. Tens una sensibilitat extraordinària, gràcies per haver-la compartit amb mi. Yolanda Peña, oficialmente no hemos trabajado juntas, pero todos esos brainstormings científicos puede que sean el principio de algún proyecto... Aunque de lo que realmente estoy agradecida es tu apoyo incondicional y tu amistad sincera. Eres una de las personas más auténticas e interesantes que conozco, además de tener grandes valores. Marc Guitart, és un privilegi haver-te conegut. Ets un professional brillant, però no te n'oblides dels amics. Per això, en especial vull agraïr-te que hagis llegit la meva Tesi i que sempre hagis respós tan ràpid als meus e-mails.

Toni Cañete, tens un gran cor. A part de ser un bon amic, ets un bon company, solidari, actiu, ressolutiu i lluitador. Sempre es pot comptar

amb tu per trobar recolçament moral. Glòria Blázquez, la meva païsana del Llobregat. Ets única. Treballar amb tu ha estat un plaer, el teu somriure (i el teu riure silenciós) ha estat una constant, fins i tot en els moments més durs. Regina López, gràcies per la teva alegria i ímpetu, i també pel teu sentit de l'humor. Carme Mont, ets una persona singular, em quedaria hores escoltant les teves anècdotes, sobretot per com les expliques. També sentint com cantes. Marta Palència, ets com la campaneta, desprens llum. Sóc afortunada de tenir-te com amiga. Sira Díaz, gracias por tu simpatía y por estar siempre dispuesta a ayudar. Andrea, eres fantástica, te deseo mucha suerte en tus inicios como investigadora. Jaume Ferrer, tot i ser tan jove, tens les coses molt clares, i sempre ets respectuós i assertiu. Sandra Sánchez, "pizpireta" i entusiasta, m'encanta la teva capacitat per animar a tothom, no canviis mai. David Torrents, senzill, honest i amable. Miriam Ratia, ets una persona sincera, justa i combatent. Bàrbara Baró, l'exemple de com el seny i la creativitat poden anar de la mà. Joan Basagaña, ple de candor, tolerant i comprensiu. Fina, tienes tanto sentido del humor como sentido común. Lidia Manzo, tu determinación y perseverancia son tan grandes como tu ternura. Heela Saarlus, your conversation seems poetry and you emanate sweetness. Gracias a la gente que ha pasado por el laboratorio y que sin querer he olvidado nombrar. Con algunos de vosotros la interacción en el laboratorio ha sido mínima, pero eso no ha impedido que viviéramos grandes experiencias fuera de él. Viajes, cenas, fiestas, congresos, deportes… que han hecho más llevadero este periodo, que se oscurecía a ratos. Gracias de corazón!!!

Ha estat un plaer treballar aquests anys en el Departament de Psiquiatria i Medicina Legal, gràcies a totes les persones que formen aquest equip. Gràcies a la Montse Albertos, a la Magda Buenaño, a la Sandra Assens, a la Beth Roca i a la Beth Domínguez i a l'Anna. Érau

les primeres cares que veia quan entrava al departament i mai he trobat a faltar un alegre “bon dia!”. Magda gracias por ser tan adorable, y por haber compartido tantos buenos momentos. Joaquín de Histología, a ti te veía incluso antes, y siempre has tenido una frase amable para mí. Tu risa es contagiosa, no la pierdas nunca. Gràcies a tots els professors del departament, en especial a Rafael Torrubia, Beatriz Molinuevo, Yolanda Pardo, Miquel Àngel Fullana, Joan Taberner, Daniel Vega, Maria Victoria Villalta i Lydia Giménez. Gràcies Rosa Maria Escorihuela per les converses sobre la vida i la ciència i també pels consells. Gracias Óscar Andión por ser un ejemplo a seguir como psicólogo clínico y por ser mi mentor. Adolf Tobeña, catedràtic del departament, vull agraïr-te la teva proximitat i amabilitat. Gràcies per aportar el teu consell constructiu, recolçar i donar ànims a tantes reunions de laboratori.

Thanks to collaborators within EURATools, for sharing knowledge and good times in the meetings. Jonathan Flint, you have been so generous with our group, thank you. Tomas Olsson, thank you for the opportunity of researching at your lab. I would like to thank Tim Aitman, to make possible the “Young European Investigators Symposia”, and the EURATOOLS fellowships, providing unique opportunities to the young reseachers of the Consortium. Erik Werner, thank you for taking care of us before-during and after the meetings. Thank you for such a good organization and for being so kind. Thanks to Matthias Heinig for being so friendly and making funnier the meetings. We have had good times. Thanks to Birger Voigt for the interesting conversations about science and life, and for being so kind.

I would like to make an especial mention to those that came to Barcelona to work side by side with us. Pernilla Stridh, Margarita Díez, Amennai Beyeen, Martina Johannesson, Johan Ockinger, Polinka

Hernández, Ulrika Norin, Samreen Falak, Jonatan Tuncel, Alan Gillet, Maja Jagodic, Andre Ortlieb, Nada Abdelmagic, Amelie Baud, Adam Whitley, Imranul Alam, Delith and your group… It has been a pleasure to work so hard with all of you, you guys are admirable. There has been a "before" and an "after" the guiris. You have left a mark on Barcelona and on our hearts, thank you!!! Amennai, *Amelito*, the singer, the dancer, the philosopher and the cartoonist. But above all, you are a good friend, thank you. Martina, you always keep calm in front of difficulties, and luckily it is contagious. Thank you for making easy and funny the long hours of work, and especial thanks for reading my Thesis. Polinka, eres sensata y divertida, gracias por tantos buenos momentos, y por el cactus!! Thanks to Amelie for your enthusiasm and energy, and Adam for your hard work and friendliness. Samreen, warm and caring, I'm glad to know you. Andre, eres encantador, todo un gentleman, and tremendously funny. Maja, you are great, you are as clever as friendly, thanks for the fun times. Nada, thanks for your support, for your kindness, sweetness and for your friendship.

Pernilla and Margarita, if I start listing the things that I'm grateful for, I'll never end, so I'll try to summarize. Thank you for the collaboration of the inbred strains, part of which is my "Study II". Perni, you are a precious person, thanks for your passion, for your braveness, for encouraging me, for listening me, for being so confident, for teaching me in EAE and immunology, for making me laught, for that unforgettable swedish New Year's Eve, etc, etc… Thank you for your frienship! Margarita, I don't have words for express all my grattitude. Eres maravillosa, me apoyas cuando quiero comerme el mundo, me inyectas energía cuando estoy desanimada, me consuelas cuando estoy triste… Gracias por guiarme y enseñarme tantas cosas en el laboratorio y en la vida. Gracias por valorar mis ideas y animarme a perseguir mis metas. Eres una gran amiga, y una gran persona. En tu

casa me he sentido en familia, has cuidado de mí y hemos pasado momentos divertidísimos con Alma, con los gatitos... Alma, tack för de svenska lektionerna!!! Dessutom har jag upptäckt att det är en del av mig som fortfarande är unga och vill inte växa ☺

Thanks to Neuroimmunology Unit (and Rheumatology) people!! Especially, Allan, Melanie, Rickart, Mikael, Petra, Pernilla, Maria, Mikaela, both Marias, Rux and Alex, Cynthia, Roham, Shahin, Patrick and Catherine, Faiez, Cecilia, Sohel, Maja, Venus, Mohsen, Bob Harris and Professor Tomas Olsson. Thank you for teaching me, for answering so many questions and having a lot of patient, you guys are the best guests ever, thanks for making my work and life there so funny. Each single moment that I lived there was marvellous. Sincerelly, thank you.

Gracias a las personas que a lo largo de mi vida han colaborado en mi formación tanto académica como personal. Victoria Díez-Chamizo, gracias por haber sido la primera en abrirme las puertas de la investigación con animales. Nunca olvidaré el año que estuve trabajando contigo, lo mucho que aprendí y lo bien que me trataste. Tere Rodrigo, sempre m'enrecordaré de lo bona i simpàtica que vas ser amb mi. Justi, las ratas eran muy afortunadas de que las cuidaras tan bien. Todo el equipo del laboratorio animal Psicología Básica de la Universidad de Barcelona me trató estupendamente, gracias. Quiero agradecer a también a todos los grupos de investigación-clínica en los que he colaborado y en especial a las personas que me han enseñado personalmente, como Óscar Andión y Natalia Calvo de Vall d'Hebron, Susana Ochoa, de Sant Joan de Déu y Fernando Fernández Aranda, de Bellvitge. Miriam Pérez, eres la directora y profesora que todos querrían para sus hijos. Tienes tantas cualidades que no podría enumerarlas. Eres una persona magnífica y una gran amiga desde

hace tantos años! Hemos pasado momentos de todo, pero tú siempre estás ahí, siempre ayudando, siempre aportándome una sonrisa. Gracias.

El agradecimiento más profundo se lo debo a mi familia. Mi gran y querida familia. Os quiero tanto!!! Mis yayos, sois la piedra angular sobre la que se cimenta esta catedral humana, en que cada pared, cada arco, es imprescindible para los demás. Yaya, eres la matriarca, das tu vida por nosotros. Gracias por tener esa voluntad de hierro, darnos ánimos en todo momento, ser tan cariñosa y cuidarnos a todos y cada uno de nosotros. Yayo, tienes los valores de un héroe épico. Orgulloso y digno, valiente y protector con tu familia. Has sido siempre tan bueno, gracias. Tito Pedro, para mí eres como mi hermano mayor. Gracias por ser tan comprensivo y por preocuparte por todos, eres una de las mejores personas que conozco. Gracias. Tita Tere, tienes un ángel que poca gente tiene, eres capaz de transmitir tu brillo a los demás, gracias por apoyarnos a todos y querernos tanto. Jesusín, eres paciente, y creativo, buena combinación. Derrochas simpatía y puedes conseguir lo que te propongas. Aunque te tomes un descanso este año, yo sé que quedan muchos Montserrats por delante!! Vero, eres preciosa por dentro y por fuera. Gracias por tantos proyectos compartidos, por inyectarme tu ilusión y optimismo cada vez que hablamos. Nuri, eres una persona increíble, cercana y cariñosa. Eres mucho más que una amiga, eres mi CU!!! Eva y Alex, mis otros cuñados, gracias por recibirme con los brazos abiertos en la familia. Sois unas personas magníficas, sois divertidos, inteligentes, dinámicos, cariñosos, y lo más importante de todo, tenéis un corazón que no os cabe en el pecho. Estoy orgullosa de haber extendido mi familia con vosotros. Gracias. Aurora, estoy muy agradecida por lo buena que eres conmigo, y porque siempre has estado ahí, incluso en los malos momentos. Eres una bellísima persona, cariñosa y solidaria, siempre

ayudando a los demás desinteresadamente. No podría tener una mejor suegra, gracias. Dani, mi visino, lo único que nos falta para ser familia es compartir genes. Siempre has estado ahí, para lo bueno y para lo malo. Y cuando digo “siempre” no estoy exagerando. Gracias visino.

Javivi, eres el mejor hermano del mundo. Siempre has estado tocado por la genialidad, eres un artista y estoy orgullosa de ser tu hermana. Admiro tu música, tus dibujos, tus ideas… Pero lo que más me asombra es tu lealtad y bondad, te ganas a todo el que te rodea, porque eres una gran persona. Gracias por tu apoyo incondicional. Papa y Mama, sois los mejores padres del universo. Sólo puedo daros las gracias por todo lo que habéis hecho por mí, que va mucho más allá de las obligaciones parentales. Papa y Mama, gracias por creer en mí, por apoyarme en todo, por respetar mis decisiones, aunque intuyérais que me iba a equivocar. Gracias por ayudarme a levantarme cada vez me caigo. Gracias por enseñarme a superar mis miedos y también por protegerme siempre. Gracias por quererme con todos mis defectos. Gracias.

Si algo he aprendido de la ciencia, es que no puede resolverlo todo. Ahora más que antes tengo la convicción de que siguen existiendo misterios inexplicables. Como la fe, como el amor, como la magia, como lo eterno. Edu, yo tengo fe en ti, en nuestro amor y en que será para siempre. Gracias por haberme demostrado, a lo largo de los años, que tu amor es incondicional, a prueba de bombas (y de tesis). Tú siempre has estado ahí sin fallarme. Gracias por tu amor constante y por la vida que compartimos. Gracias por no dejarme flaquear cuando siento que se desmorona el suelo que piso, y gracias por potenciar mis ilusiones y ayudarme a perseguir mis sueños. Con esta Tesis también me has ayudado mucho a nivel práctico. Gracias por haberme ayudado con el scoring de las ratas los fines de semana, con gráficas y con

pósters, por empujarme a hacer cosas que me daban pereza y por ayudarme a desconectar cuando había entrado en un bucle. Eres la persona que necesito a mi lado, para siempre.

Gracias a todos los animales que contribuyen a que la ciencia avance.

LIST OF ABBREVIATIONS

AIL	Advanced intercross lines
CD74	Cluster of Differentiation 74
Chr	Chromosome
CNS	Central nervous system
CUM	Cummulative score (of EAE)
DA	Dark Agouti
DSM IV	Diagnostic and Statistical Manual IV
DUR	Duration (of EAE)
EAE	Experimental autoimmune encephalomyelitis
EDSS	Extended disability severity scale
EURATools	European Rat Tools for Functional Genomics
EURATRANS	European large-scale functional genomics in the r at for translational research
F2	Intercross Generation 2
GC	Glucocorticoid
HLA	Human Leukocyte Antigen
HPA	Hypothalamic-pituitary-adrenal
HS	Heterogeneous Stock
IFNβ	Interferon-β
IL-6	Interleukin-6
INC	Incidence (of EAE)
IS	Immune system
MAX	Maximum score (of EAE)
MC	Mineralocorticoid
MC2R	Melanocortin 2 Receptor
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
mRNA	Messenger RNA
MS	Multiple Sclerosis

MSSS	Multiple sclerosis severity score
NACTA	Automated novel-cage activity
Nih	National Institutes of Health
N/Nih-HS	National Institutes of Health-Heterogeneous Rat Stock
ONS	Onset (of EAE)
PR	Primary progressive
PTSD	Post traumatic stress disorder
PVG	Piebald Virol Glaxo
PVN	Paraventricular nucleus of the hypothalamus
QTG	Quantitative trait gene
QTL	Quantitative Trait Loci
RA	Rheumatoid arthritits
RHA-I	Roman High Avoidance
RLA-I	Roman Low Avoidance
RT–PCR	Real Time- Polymerase Chain Reaction
RR	Relapsing-remitting
SH	Two-way active, shuttle box avoidance acquisition
SNPs	Single nucleotide polimorphisms
SP	Secondary-progressive phase
TNF	Tumor Necrosis Factor
ZM	Elevated zero-maze test

CONTENTS

1 ABSTRACT

Stress hypothalamic-pituitary-adrenal (HPA) axis responses play a role in both anxiety behaviour and immune system (IS). Enhanced glucocorticoid (GC) levels have shown to play a protective role in experimental autoimmune encephalomyelitis (EAE), a reliable animal model of multiple sclerosis.

In this Thesis, we aimed to investigate if a determined anxious profile could correspond to a specific inflammatory susceptibility. In “Study I”, genetically heterogeneous N/Nih-HS rats of both sexes were immunized with myelin oligodendrocyte glycoprotein (MOG) to evaluate EAE. To assess the effect of anxiety on IS, subgroups of rats scoring extreme values of anxiety were examined on their EAE incidence (INC) and severity. Also, anxious behaviour and relative adrenal weight (RAW) of subgroups selected by resistance or susceptibility was studied was compared. Results indicated a possible relationship between high anxiety and EAE-resistance.

However, the assumed associations between behavioural anxiety and physiological stress needed to be elucidated. Thus, in “Study II” we studied in male and female DA and PVG inbred rats the possible relationships among HPA axis responses and anxiety. DA and PVG strains are respectively susceptible and resistant to a wide range of experimental autoimmune diseases, EAE among others. In the current study, these strains were characterized by their anxiety/inhibition. We further examined their HPA axis function, by means of (basal and post-stress) corticosterone levels, RAW, and via RT–PCR their expression of mRNA adrenocorticotropin receptor (Melanocortin 2 Receptor, MC2R) on adrenal glands. We also studied the mRNA expression of both

CD74 (major histocompatibility complex; MHC-II) and the pro-inflammatory interleukin-6 (IL-6) on paraventricular nucleus of the hypothalamus (PVN), pituitary and adrenal glands.

Together, our data show that in EAE, a high anxious profile accompanied by an enhanced HPA axis may involve the repression of inflammatory responses, providing a certain resistance.

1 RESUMEN

Las respuestas al estrés del eje hipotalámico-pituitario-adrenal (HPA) juegan un papel decisivo tanto en la conducta ansiosa como en el funcionamiento del sistema inmune (IS). Es sabido que los niveles elevados de glucocorticoides (GC) desempeñan un papel protector ante la encefalomielitis experimental autoinmune (EAE), fiable modelo animal de la esclerosis múltiple.

En esta Tesis, nos propusimos investigar si un determinado perfil ansioso podría corresponderse con un perfil específico de sensibilidad a la inflamación. En el "Estudio I", ratas genéticamente heterogéneas N/Nih-HS de ambos sexos fueron inmunizadas con proteína oligodendrocito de la mielina (MOG) para evaluar la EAE. Con el objetivo de valorar los efectos de la ansiedad sobre el IS, examinamos la incidencia (INC) y la severidad de la EAE que presentaban los subgrupos de ratas con puntuaciones extremas en ansiedad. De estos subgrupos (de baja y alta ansiedad) también se comparó la conducta ansiosa y el peso relativo de las glándulas adrenales (RAW). Los resultados indicaron una posible relación entre alta ansiedad y resistencia a la EAE.

Sin embargo, algunas de las asociaciones asumidas en el "Estudio I" entre conducta ansiosa y estrés fisiológico, debían esclarecerse. Para ello, en el "Estudio II" se estudiaron las posibles relaciones entre las respuestas del eje HPA y la ansiedad las ratas inbred DA y PVG de ambos sexos. Las cepas DA y PVG son respectivamente susceptible y resistente a un amplio espectro de enfermedades autoinmunes, entre

otras, la EAE. En el presente estudio, se caracterizaron estas cepas por sus conductas de miedo/ansiedad y actividad ante la novedad. Además se examinó la function del eje HPA, en terminos de niveles de corticosterona (basal y post-stress), peso relativo de las glándulas adrenales, y su expresión mRNA del receptor de la hormona adrenocorticotropa (MC2R). También se estudió la expresión mRNA de CD74 (complejo mayor de histocompatibilidad, clase II); y la interleucina proinflamatoria-6 (IL-6), en el núcleo paraventricular del hipotálamo, la pituitaria y las adrenales.

En conjunto, nuestros resultados muestran que en la EAE, un perfil ansioso se correspondería con un eje HPA incrementado, que podría actuar reprimiendo las respuestas inflamatorias, produciendo un efecto de cierta resistencia a la EAE.

GENERAL INTRODUCTION

2 GENERAL INTRODUCTION

2.1 THE CONTEXT OF THE PRESENT WORK: THE "EUROPEAN RAT TOOLS FOR FUNCTIONAL GENOMICS (EURATOOLS)" CONSORTIUM

The central aim of the "EURATools" European project/consortium (2006-2010), in which our laboratory/group was involved as "Partner 11", has been the development of integrated genome tools that should generate knowledge which could be translated into improvements in healthcare for highly prevalent diseases in the European Union (*http://euratools.rns4u.com/*).

The EURATools aims should be achieved by integrating high-throughput sequencing and genotyping with informatics; by intensive analysis of phenotypes, gene sequence and gene expression to identify genes and regulatory pathways for a wide range of rat disease phenotypes; and by establishment of optimised protocols for rat gene targeting. These new resources would significantly improve our understanding of complex genetic traits, and will enhance prospects for drug development and strategies for preventing and treating some of the commonest diseases in western societies.

Thus, within the framework of "The European Rat Tools for Functional Genomics (EURATools)", our laboratory has played its role as partner of one of the multiple activities carried out by the consortium: to demonstrate the potential of the genetically heterogeneous N/Nih-HS rat stock (see description in next section "1.2") for the fine genetic (QTL; "Quantitative Trait Loci") mapping of multiple quantitative (behavioural,

physiological, disease-related, etc) traits, to such a high resolution level (QTL of ≤ 2 Mb on average) that it would allow candidate gene identification within the QTL peaks.

Within this frame it has been developed a phenotyping protocol capable of capturing many aspects of the rat's behavior and physiology, and it has been shown that the measures we obtain are indeed consistent with those reported for inbred strains (see Table 2.1; and Johannesson *et al.,* 2009). In contrast to standard QTL mapping experiments, where each laboratory maintains, breeds, phenotypes, and genotypes its own animals, in our EURATools Consortium scientists with different phenotyping skills from different laboratories combined together their mastery to a single site where the animals are bred (Medical Psychology Unit, Dept. Psychiatry & Forensic Medicine, Autonomous University of Barcelona). Thus, for this part of the project, the EURATools consortium used only one "operation center" (our laboratory in the Autonomous University of Barcelona), one unequalled tool (the genetically heterogeneous N/Nih-HS stock of rats; see next section) and a "melting pot" of scientists joining forces to study different phenotypes. The phenotypes subjects of study were: hormones, behavior, glucose tolerance, cardiovascular, hematology, immunology, neuroinflammation and tissue harvest (Table 2.1).

Specifically, among its main aims, the work of present Thesis will be devoted to characterize the N/Nih-HS rat stock (see next section for details) as concerns to anxiety/fearfulness-related behaviour (unconditioned anxiety, context-conditioned fear and anxiety, and activity in response to novelty) and regarding susceptibility to autoimmune neuroinflammation (Myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE)).

The analysis of the results and work carried out during the "EURATools" project is currently being continued within the context of the new European project "EURATRANS", in which most of the european laboratories (including ours) participating in EURATools are also "Partners" (*http://www.euratrans.eu/*).

Phenotype group	Test	Week	Responsible laboratory
Hormonal	Serum samples to measure adiponectin and thyroid stimulating hormone (TSH)	1	Medical psychology, UAB, Barcelona
Behavioral	Shuttle box	2	Medical psychology, UAB, Barcelona
	Automated novel cage activity	3	
	Elevated zero maze (an unconditioned test of anxiety)	4	
Glucose tolerance	Glucose values taken before and at 30, 60, and 120 min after intraperitoneal injection of a solution of glucose (a model of diabetes)	5	Genetics of metabolic syndrome and diabetes, WTCHG, Oxford
Cardiovascular	Blood pressure measured with a tail cuff	6	BHF Cardiovascular Research Center, Glasgow University
	Heart size	(post-mortem)	
Hematology	Full blood count	7	Psychiatric genetics, WTCHG, Oxford
Immunology	FACScan analysis of white blood cells	7	Medical Inflammation Research, KI, Stockholm
Neuroinflammation	MOG induced EAE (a model of multiple sclerosis) TNF alpha in serum	7-11	Neuroimmunology, KI, Stockholm
Tissue harvest	Blood, thymus, heart, brain, spinal cord, adrenal glands, liver, spleen, kidney, bone, and ears are collected at the time of sacrifice	12	Neuroimmunology, KI, Stockholm and Psychiatric genetics, WTCHG, Oxford

Table 2.1.- Overview of the high-throughput phenotyping protocol. (Johannesson et al., 2009)

2.2 THE GENETICALLY HETEROGENEOUS –N/NIH-HS-RAT STOCK AS A POWERFUL TOOL FOR FINE-MAPPING LOCI FOR COMPLEX TRAITS: FOCUS ON ANXIETY

Genetic mapping of psychological traits or psychiatric disorders has shown to be harder than initially envisaged. Low heritability, poor characterization of phenotypes, complex "genotype x phenotype" interactions and the probable polygenic heritability are factors that make the molecular dissection of the above mentioned traits a demanding challenge. Conversely, genetic mapping of behavioural variation in laboratory animals has led to robust evidence of genetic linkage, as evidenced by many reports of significant association among a variety of behavioural phenotypes and a wide range of chromosomal regions (for review see Doerge, 2001; Flint and Mott, 2001; Kwitek-Black and Jacob, 2001).

Moreover, and again in contrast to human studies, replication of genetic mapping findings (linked to different phenotypes) in rodents has been consistent. This fact makes it more likely that the additional characterization of the identified chromosomal loci can bring important information about the molecular bases of behavioural traits/phenotypes.

One important limitation in the use of animal models has been, thus far, the need to identify and fine map the genetic loci with enough resolution as to allow the identification of the relevant molecular variants. Actually, the identification of molecular variants that contribute to strain differences (behavioural variations), has shown to be a difficult task. The main problem stems from the fact that the majority of the available mapping techniques (in animals) have a poor resolution. Essentially this is because the proportion of variance explained by a single locus is

rather small - in most cases less than 10% -, even when the total genetic contribution to a trait variation is large.

Thus, using an affordable number of animals in a regular laboratory to perform an inbred cross design (commonly, less than 1000 animals), where two inbred rat strains are crossed to obtain an F2 generation (Flint and Mott, 2001; Mott *et al.,* 2000), it is possible to map genetic effects within the order of 5-15% in intervals that can reach, approximately, half a chromosome. Mapping experiments to resolution levels allowing molecular characterization of quantitative genes would need, nevertheless, more than ten thousand animals (Flint and Mott, 2001; Mott *et al*., 2000). Even so, despite its poor resolution, the identification of QTL in F2 animals (i.e. using the inbred cross design with usually less than 1000 animals) has been useful as a strategy to locate genetic influences in particular chromosomes, and so to allow further studies focused to the fine mapping of these chromosomal intervals or QTL. This was the case of a previous work from our group (Fernández-Teruel *et al*., 2002), in which for the first time we identified several QTL influencing anxiety/fear-related behaviors in rats (F2 generation derived from crossing the RHA-I and RLA-I rat strains), with a very significant pleiotropic QTL in chromosome 5. This study was a first and necessary step to later allow the high resolution fine mapping of QTL in that chromosome.

The first clue to solve the problem of low resolution of QTL mapping studies came with the work from R. Mott and J. Flint group (Mott *et al.,* 2009; Mott and Flint, 2002; Valdar *et al.,* 2006; Yalcin *et al.,* 2004), who demonstrated that genetically heterogeneous (outbred) mouse stocks (derived from crossing eight inbred parental strains) allowed the simultaneous and fine mapping of QTL and even quantitative gene identification (Yalcin *et al.,* 2004). Therefore, there was no reason to

think that the same, or even greater advances, could not be achieved by the use of genetically heterogeneous rats.

At this point, in collaboration with J. Flint's group (Oxford), our group got a colony of 40 matting pairs of genetically heterogeneous N/Nih-HS rats in 2004 (gently provided by Prof. Eva Redei, Center for Comparative Medicine, Nothwestern University, Chicago, USA). The "National Institutes of Health -N/Nih- Genetically Heterogeneous Rat Stock" (hereafter named "N/Nih-HS" rat stock) was created because Hansen and Spuhler (1984) tried to develop a more naturalistic, genetically heterogeneous rat stock, which could yield a broad-range distribution of responses to experimental conditions and could serve as a base population for selection studies. They derived N/Nih-HS rats from eight parental inbred strains: the MR/N, WN/N and WKY/N (these three strains trace their ancestry to the original Wistar stock); the M520/N and F344/N (both established in the 1920s, but of unknown origin); the ACI/N (hybrid between the August and Copenhagen strains), the BN/SsN (derived from a color mutant from a stock of wild rats kept at the Wistar Institute) and the BUF/N strain (Hansen and Spuhler, 1984).

As illustrated in Figure 2.1, genetically heterogeneous stocks of rats (or mice) represent a unique, genetically random mosaic of founding animal chromosomes due to recombinations that have accumulated over many generations. Specifically, the N/Nih-HS has been bred for more than 50 generations using a rotational outbreeding regime to minimize the extent of inbreeding, drift and fixation (Boucher and Cotterman, 1990; Hansen and Spuhler 1984). The N/Nih-HS colony represents a genetically random mosaic of eight founding inbred rat strains (Figure 2.1), with each individual animal being genetically unique. At each generation of breeding there is the potential of new recombinations that could help reduce the size of a QTL.

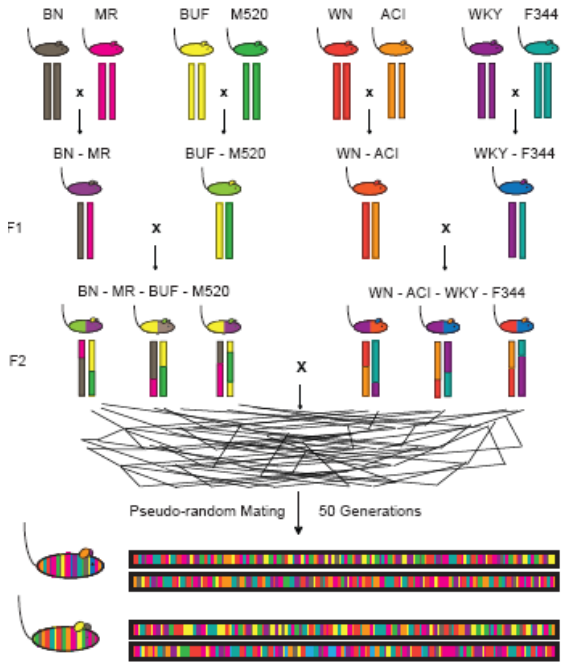


Figure 2.1.- Schematic illustration of N/Nih heterogeneous stock construction (gently provided by Dr.Pernilla Stridh).

After more than 50 generations of breeding, it is the estimated that the average distance between recombination enables the fine-mapping of QTL into subcentimorgan intervals (Mott *et al*., 2000). This estimation is based on the successful methodology followed by Flint and colleagues (Valdar *et al*., 2006), who fine-mapped 843 QTLs for over 100 phenotypes (with an average 95% confidence interval of 2.8 Mb), in which heterogeneous stock of mice were used to elucidate even very small genetic influences on continuous phenotypic characters/traïts (Flint *et al*., 2004; Mott and Flint *et al*., 2002; Mott *et al*., 2000; Valdar *et al*., 2006) to the point that these QTLs can represent chromosomic intervals of ≤ 2-3 Mb, thus even allowing gene identification (Mott *et al*., 2000; Yalcin *et al*., 2004) as well as evaluation of epistatic and gene–environment interactions (Valdar *et al*., 2006a; Valdar *et al*., 2006b). The forementioned works with HS mice are the best recent examples of how, and to what extent, using genetically heterogeneous rodent stocks

has become crucial for genetically-oriented studies that should provide extremely relevant understanding on the genetically influences on complex traits.

Then, taking this work as starting-point, it was carried out the first genetic work (ie. QTL fine mapping) with the N/Nih-HS rats that proved their potential to identify and to fine-map QTLs. It reported the analysis of chromosome 5 in over 800 N/Nih-HS rats for the “avoidance” (anxiety-related) phenotype and the identification of at least one QTL containing nine genes, none of which had been previously shown to influence anxiety-related behaviour (Johannesson *et al.,* 2009; *López*-Aumatell 2008). That work was confined to the analysis of two chromosomes (chr 5 and chr 15). However, they were representative of the genetic structure of other chromosomes because the available genotypes from the progenitors of the N/Nih-HS rat stock indicated that there are no large (>2 Mb) regions without SNPs (“single nucleotide polimorphisms”). After that initial work, we have reasons to believe that the N/Nih-HS can be used for whole genome association studies.

As aforementioned, the N/Nih-HS rats show a unique feature: genetic recombinants (derived from the 8 founder inbred strains) accumulated over many generations of outbreeding. Also, this model is favourable in economical therms, since each single animal provides a considerable amount of information. The breadth of phenotypic and genotypic information can later be combined with expression data to provide the basis for a systems biology approach to complex phenotypes in general. A current major challenge in systems biology is to understand how phenotypes arise from the resulting complex multidirectional net of genes and environment (Barabasi and Oltvai, 2004; Hartwell *et al*., 1999). The combined approach to using the N/Nih-HS rats would provide a significant international resource for systems biology

applications. So, a necessary next step for the widespread use of the N/Nih stock would be the demonstration that phenotypes in the stock are comparable with those obtained using classical laboratory rats.

Besides the above mentioned pioneer study (Johannesson *et al.,* 2009), identifying and fine mapping a QTL for anxiety in the N/Nih-HS rat stock, these rats have been successfully used to fine-map QTLs for diabetes (Solberg *et al*., 2010a), and they have been purposed as a new model to study the genetics of renal phenotypes (Solberg *et al*., 2006b). Furthermore, the N/Nih-HS rat stock constitutes an excellent model for fine mapping and identification of genes underlying bone fragility phenotypes and other complex traits like osteoporosis (Alam *et al*., 2011).

Remarkably, the N/Nih-HS rat behavioural profile has been widely evaluated in our laboratory, in comparison with other inbred rat strains, in terms of unconditioned and conditioned anxiety/fear (López-Aumatell *et al.,* 2011, 2009, 2008a-b), novelty-induced exploration, HPA-axis responses to stress and "depressive"-like behavior (Díaz-Morán *et al.,* 2012; Estanislau *et al.,* 2012 *Poster presented at the 2012 FeSEB, Brazil;* Palència, 2011 master thesis-*unpublished*), aversive instrumental learning and spatial learning (*López*-Aumatell *et al.,* 2008, 2009, 2011; Martinez-Membrives, 2008 master thesis-*unpublished*; Vicens-Costa *et al.,* 2011).

The phenotypic evidence accumulated thus far (by using several hundreds of HS rats in the different studies) allows us to confidently state that the N/Nih-HS rat stock presents a behavioural/endocrine profile which fits well with one of a mildly anxious, passive copper and stress-prone rat which, in turn, present quite good spatial learning/memory ability.

2.3 MULTIPLE SCLEROSIS (MS)

Multiple sclerosis (MS), also known as encephalomyelitis disseminate, is a chronic inflammatory demyelinating disease of the central nervous system (CNS).

There are large individual differences in severity, disease course and clinical symptoms. This variability of symptoms (which are characterized by sensory and motor disturbances, difficulties with coordination/balance, muscular weakness, spasms, optic neuritis, bladder dysfunction as well as pain, fatigue, emotional liability and cognitive impairment (Multiple Sclerosis: Diagnosis, Medical Management, and Rehabilitation. 1ed. New York: Demos Medical Publishing, 2000) between and within a patient probably reflects the location of inflammatory lesions in the central nervous system (CNS).

The prevalence of MS in Spain is 70-80 affected individuals per 100000 individuals (Merck Serono, 2009) and 2.5 million worldwide are known to have MS, with incidence estimates at 1-5 per million worldwide (See the Figure 2.2; National Multiple Sclerosis Society - NMSS, 2006; Noseworthy *et al*., 2000). Like most autoimmune disorders is more prevalent in women than men (ratio of 1:2) (Koch-Henriksen, 1995). Around 80% patients with MS will suffer neurological disability throughout their lives. The aetiology of MS remains elusive, and there is no single test or biomarker that is enough for diagnosis.

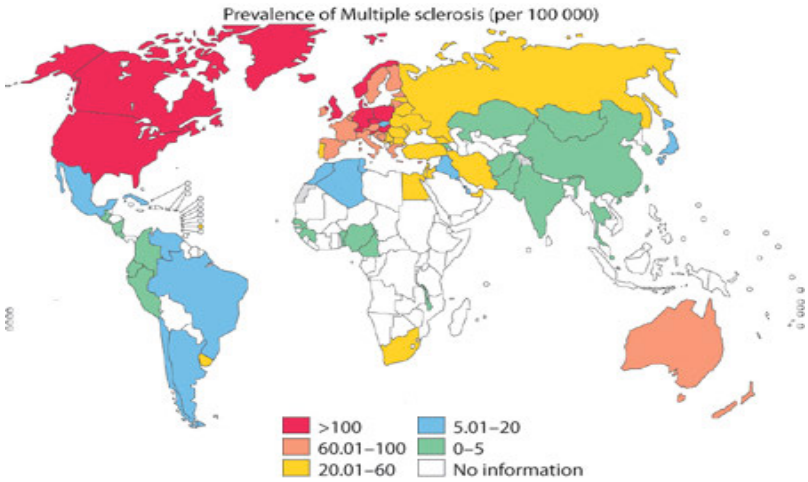


Figure 2.2.- Worldwide prevalence of MS per 100.000 inhabitants. Adapted from the World Multiple Sclerosis Resource Center, *http://www.msrc.co.uk*

2.3.1 Clinical Features

There is no absolute test for the diagnosis of MS, but the current diagnostic criteria state that a patient should have at least two clinical bouts dispersed in time and location, or a single clinical bout but with additional evidence of lesions, using magnetic resonance imaging (MRI) (McDonald *et al.,* 2001). The diagnostics can be further supported by identification of oligoclonal bands in the cerebrospinal fluid (CSF). Disability in MS patients is usually graded using the extended disability severity scale (EDSS) (Kurtzke 1983). The EDSS scale itself does not measure the severity, since it does not take time into consideration, but can be used in combination with duration to calculate the multiple sclerosis severity score (MSSS) based on a cross-sectional disability assessments in a large longitudinal database (Roxburgh *et al* 2005).

MS can exhibit several different forms of progression with symptoms either occurring in discrete attacks or slowly becoming more severe over time. These symptoms sometimes resolve completely between

attacks but permanent neurological problems often persist, especially as the disease advances (Figure 2.3). Initially, the disease course is often characterized by relapses (disease bouts), followed by remissions (periods of recovery). This relapsing-remitting (RR) form of MS is the most common variant affecting 80-95% of the patients, and a majority of these patients eventually progress to the secondary-progressive phase (SP) even though there is a big variation in type and severity of symptoms and the rate of disease of progression, between patients (Compstom *et al* 2002). This course contrasts to the primary progressive (PP) MS, characterized by a steady deterioration and absence of remissions that affects 5-20% MS patients.

Onset of MS occurs between the ages 20-40 years and 50% of MS patients are unable to work 10 years after diagnosis and are thus excluded from the workforce. The magnitude of the socio-economical impact is reflected in that MS, constitutes the shame overall economic burden on society as rheumatoid arthritits (RA), which is five times more prevalent than MS (Beyeen, 2010).

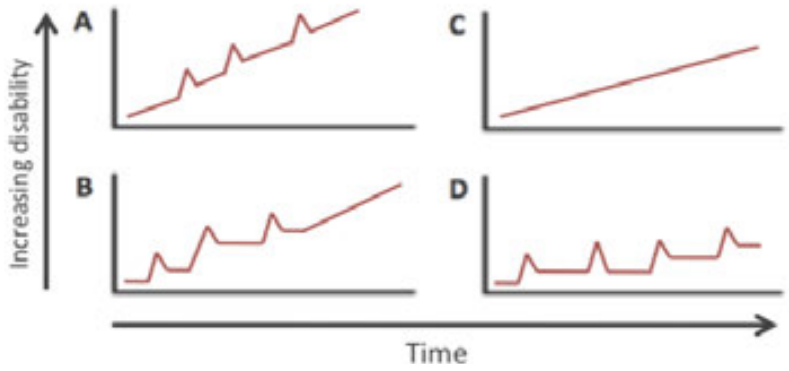


Figure 2.3.- Different disease courses of MS. (A) Progressive-relapsing MS; steady decline with superimposed attacks. (B) Secondary-progressive MS; initially relapse-remitting MS, but then begins to decline without remission. (C) Primary progressive MS; steady decline without remission. (D) Relapse-remitting MS; unpredictable bouts which sometimes cause permanent damage, followed by periods of remission.

2.3.2 Treatment

The available treatments for MS are only disease-modifying and do not cure the disease, and despite over 15 years of usage, the mechanisms for these treatments are still not fully understood. The most commonly used therapies today are the recombinant Interferon-β (IFNβ) and the polypeptide glatiramer acetate, and although they are effective in relapse reduction, they have only modest benefits on progression of disability (Buttman and Rieckmann 2008; Wolinsky 2006). Furthermore, treatment with steroids can temporarily reduce ongoing symptoms (Miller D *et al* 2000). The anti-VLA4 monoclonal antibody (natalizumab) therapy is very efficient in suppressing relapses, but is used restrictively since it has also been associated with more severe adverse effects (Kleinschmidt-deMasters and Tyler 2005; Langer-Gould *et al.,* 2005). New strategies for efficient MS therapies are evaluated continuously and some of these have significantly reduced disease (Cohen JA *et al.,* 2011; Compston *et al.,* 2006 Giovannoni G *et al.,* 2010; Kappos L *et al.,* 2010).

2.4 THE ANIMAL MODEL FOR MULTIPLE SCLEROSIS: EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE)

The experimental autoimmune encephalomyelitis (EAE) is an autoimmune neuroinflammatory disease with clinical and pathological similarities to multiple sclerosis (MS) (Olsson *et al*., 1992). It has been established in several species including rats, mice, guinea pigs, marmosets, rabbits and primates (Baxter, 2007; Freund *et al*., 1947; Lipton and Freund 1952; Morrison 1947; Olitsky and Yager, 1949; Rivers *et al*., 1933). There is also a high degree of genetic similarity between MS and rat EAE, with several MS risk genes being differentially expressed between EAE-susceptible and resistant EAE rat strains (Thessen, 2009).

EAE can be induced by subcutaneous injection of recombinant or purified CNS antigens, synthetic peptides, whole CNS tissue or infection with encephalitogenic viruses (Dal Canto *et al*., 1996; Lorentzen *et al*., 1995). Depending on the antigen and genetic background, these models recapitulate distinct features of human MS, both regarding disease course and pathogenic mechanisms. There are numerous CNS antigens that induce EAE, but the model chosen here is the myelin oligodendrocyte glycoprotein (MOG)-induced EAE model in rats, which appears to accurately reflect the distinct disease courses of MS, although there is no single experimental model that mimics all the aspects of MS. As its name indicates, the induction of the disease is achieved by immunization with MOG, which is a minor glycoprotein exposed on the surface of the myelin sheath. MOG-EAE is characterized by a disease onset at 10-14 days pos-immunization resulting in an ascending paralysis with periods of remission (Storch *et al*., 1998). Indeed, most approved MS therapies used today have first

been characterized in various of those EAE models (Denic *et al*., 2010; Linker and Lee 2009).

In addition to the pathology described above, the MOG-EAE model involves demyelinating processes and components also apparent in MS (Breij *et al*., 2008; Lucchinetti *et al*., 2000; Storch *et al*., 1998; Weissert *et al*., 1998). Immunologically, there are signs of activation of both cellular and humoral anti-MOG specific response, which is also reminiscent of MS, where both T- and B- cell responses to MOG and other myelin antigens are present (Storch, 2002; Steinman *et al*., 1996).

There is a difference depending on genetic regulation, which is demonstrated by inbred rat strains showing different susceptibility to MOG-EAE. This phenomenon has to do with the Major Histocompatibility Complex (MHC). Consistent with MS, the MHC locus (HLA in humans) is the strongest susceptibility locus in EAE (Weissert *et al*., 1998); in other words, the predisposition to MS is conferred by numerous genes, with the HLA complex being the only major risk factor (Chao *et al*., 2009; Lincoln *et al*., 2005; Ramagopalan *et al*., 2008; Sawcer *et al*., 2005). Indeed, when rats face the same MOG challenge, the MHC haplotypes determine the severity of subseqüent disease (Weissert *et al*., 1998). However, additional genes affect disease susceptibility and course (Becanovic *et al*., 2003, De Jaguer *et al*., 2009).

With this model, several non-MHC genome regions have been identified that control either clinical susceptibility and severity, or that more specifically determine defined pathophysiological processes with regard to inflammation, demyelination or axonal loss. Loci that contribute to EAE with smaller effects have been present in several rat

crosses (Becanovic *et al*., 2003; Dahlman *et al*, 1999a-b), showing that the polygenic nature of MS is captured in the MOG-EAE model.

The N/ Nih-HS rat can be used for dissecting EAE, because it contains several MHC types. This could potentially reduce the power for detecting non-MHC QTLs. These QTLs are the primary target study with the N/Nih-HS rat, since the MHC complex, and in particular the class II genes, are well characterized and studied by other means (Lincoln *et al*., 2005). Based on our findings and those reported in literature regarding EAE in the founder strains, we expected the N/Nih-HS to show variation in EAE susceptibility (Becanovic *et al*., 2006; Dahlman *et al*., 1999; Glodmuntz *et al*., 1993; Levine and Wenk, 1965; Stefferl *et al*., 2001; Storch *et al*., 1998; Stridh, 2010; Sundvall *et al*., 1995; Weissert *et al*., 1998;). However, pilot studies were performed with the intended EAE model to establish if there was enough variation in disease outcome depending on non-MHC genes (Johannesson *et al.,* 2009).

Pursuing that aim, rats with MHC AV1 and N types (homozygotes and heterozygotes, respectively) were compared to rats with all other MCH types (B, L, LV1 and D). It was found a lower disease incidence in the N group, and the most probable explanation is that these rats were heterozygous for N and had only part of the susceptibility effect. The MHC influence was present, but did not dictate disease outcome completely, suggesting that part of the influence comes from non-MHC factors (Stridh, 2010). In the N/Nih-HS rats, two homozygous MHC groups were identified, AV1 and L, and another one with a variable MHC. The large phenotype variation within the various MHC haplotype groups strongly suggest that influence from non-MHC genes can be mapped in the N/Nih-HS rats (Stridh, 2010).

A considerable advantage of the N/Nih-HS compared to the advanced intercross lines (AIL; commonly used for these studies) is that the smaller regions are linked to disease in a system that more closely resembles a natural population (8 strains instead of 2). This is the first experimental population used in this thesis that even attempts to mimic features of a human population, because its more diverse and complex genetics than the inbred crosses and it has a wider range of phenotypes.

In the present study we have used a combined approach to take advantage of the singular characteristics of the different populations of rats. As aforementioned, by using the N/Nih-HS rat we expected to be able to capture the complexity involved in multifactorial traits which, likewise, are more susceptible to the parental/family influences.

2.4.1 Genetics of EAE

As in the case of HLA in MS, the MHC locus is the major genetic determinant of the disease –EAE- in rodents (Issazadeh *et al* 1997; Loretzen *et al* 1997; Mustafa *et al* 1994; Weissert *et al* 1998). This effect is fairly profound and some rodent strains carry MHC haplotypes that are only prone to certain CNS antigens (Weissert *et al* 1998). However, there is a substantial non-MHC gene contribution and to date, at least 50 genetic regions are known to regulate EAE in rodents (Baker *et al*., 1995; Becanovic *et al*., 2003; Bergsteinsdottir *et al*., 2000; Butterfield *et al*., 2000; Dahlman *et al*., 1999; Encinas *et al*., 2001; Olsson and Hillert 2008; Roth *et al*., 1999; Sundvall *et al*., 1995). Moreover, a number of other genetic regions have been linked with EAE and are currently being investigated (Becanovic *et al*., 2003a;

Becanovic *et al*., 2006; Jagodic and Olsson, 2006; Ockinger *et al*.,2006).

There is a genetic complexity involved in the inheritance of autoimmune neuroinflammation, and it is known that the phenotypic expressivity of EAE is modulated by multiple genes with a dissociation of effects on different aspects of disease. The polygenic nature of EAE is shown in numerous studies (Marta *et al*., 2010; Storch *et al*., 1998; Stridh *et al*., 2010a-b). QTL studies indicate that these genes interact with other genes and with other factors such as the environment, the induction protocol used and season (Marta *et al*., 2010; Subramanian *et al*., 2005; Stridh 2010; Teuscher *et al*., 2004, 2006).

Furthermore, even genetic influences from one QTL/region can depend on several genes. Such is the case, for instance, with the identification of a QTL for blood pressure in rat that harboured two closely linked genes (also regulating blood pressure in humans; see Glorioso *et al*., 2007).

These QTLs may reflect functionally related genes that are located in the vicinity of each other. Certainly, genetic studies in humans have established the polygenic nature of MS (De Jager *et al*., 2009; Hafler *et al*., 2007; Australia and New Zealand Multiple Sclerosis Genetics Consortium, 2009; International Multiple Sclerosis Genetics Consortium, 2009). The genetic effect in EAE is clearly established as different rat strains display great variation in disease susceptibility under the same environmental conditions (Andersson *et al*, 2004; Olsson and Hillert 2008).

Genome-wide association scan methods have been successful in discovering susceptibility loci for MS and other inflammatory diseases.

For the most part, the susceptibility alleles that have been identified so far fit the profile targeted by genome-wide association studies (de Jager *et al*., 2009). Additionally, it has been demonstrated that the allele combination in the region was a more important determinant for disease outcome that were the effects of each individual QTLs (Stridh *et al*., 2010). To put it another way, the suitable genetic background combinations facilitate a stronger effect than the risk variant by itself alone. Although the HLA has a distinct genetic architecture, the epistatic interactions operating in this region are unlikely to be unique. Indeed, there is also evidence for epistasis involving non-HLA genes, but these interactions are not as well characterized and need further validation (Motsinger *et al*., 2007). Successful examples of identifying the responsible gene for both EAE and other disease models have emerged (Petretto *et al*., 2008; Pravenec *et al*., 2008; Ueda *et al*., 2003). Collectively, these findings indicate great similarities in genetic regulation between EAE and MS, and that gene interactions modify the independent gene effects.

Given the controlled environment and tissue availability in experimental studies, candidate gene investigation in rodent EAE models serves as a powerful complement to analogous human efforts. EAE risk genes can be translated to MS risk genes and can provide valuable insight into the origin of disease mechanisms. Resolution to a small number of candidate genes enables identification of EAE-regulatory genes, which is a way to elucidate the underlying mechanisms responsible for contributing to autoimmune neuroinflammation.

The genetic study of MOG-EAE with advanced intercross lines (AIL; see also next section) and congenic rats has led to the identification of several QTL for EAE. Thus, it has been shown that the 58Mb region on rat chromosome four is composed of four distinct QTLs (Eae24-Eae27)

and the 68Mb region on rat chromosome seventeen is composed of two distinct QTLs (Eae23a and Eae23b) . Furthermore, it was shown that although some QTLs regulate both susceptibility and severity of neuroinflammation (Eae23a and Eae23b), other QTLs show dissociation of genetic influence on different aspects of the disease (e.g. Eae26 regulates severity but does not influence susceptibility, while Eae27 regulates only susceptibility; for review see Marta *et al.,* 2010; Stridth, 2010; Stridh *et al*., 2010; Wallström *et al*., 2007).

Just to finish with the present genetic overview, fine-mapping for Eae23b produced a candidate list of 31 genes to be explored. Of these, the gene most likely to influence neuroinflammation is ZEB1 (Stridh *et al*., 2010) which is an interleukin-2 (IL-2) repressor (Williams *et al*., 1991; Yasui *et al*., 1998). This supports the involvement of the IL-2 pathway, which is already implicated in MS and EAE (Weber *et al*., 2008; for further extensive review see Stridh, 2010).

2.4.2 Two inbred rat strains to study EAE: The susceptible DA vs the resistant PVG strains.

Inbred strains are families which their members are genetically identical or very close to identical. This is achieved by breeding brother and sister pairs for a minimum of 20 generations, which should achieve more than 99% identical genome (Voigt and Serikawa, 2009). The emphasis in studies of inbred strains is to identify a single, often extreme phenotype. The strain can then be exposed to different manipulations to study their effect. It is especially useful when more than one strain are available to be exposed to a compound (i.e. a disease induction), because it allows to identify those who are

susceptible or resistant to that compound, due to the genetic differences.

It has long been documented that various strains of rats differ in susceptibility to induction of experimental allergic encephalomyelitis (EAE) (Hughes and Stedronska, 1973; Kornblum, 1968; Levine and Wenk, 1961, 1965; Perlik and Zidek, 1974). Susceptibility to EAE depends on several factors such as the chosen model of immunization (Staykova *et al.,* 2008) and haplotype (Happ *et al.,* 1988; Weissert *et al.,* 1998). Therefore, we can say that one strain is "resistant" but knowing that it means "relatively resistant".

Dark Agouti (DA) and the Piebald Virol Glaxo (PVG) are the inbred strains used in this thesis (Study II). They are respectively considered as susceptible or resistant to autoimmune neuroinflammation (Weissert *et al* 1998b), since each strain harbours alleles that are disease-promoting and those that are disease-protective. The DA and PVG inbred rats used in this thesis share the MHC haplotype RT1.AV1 (Hedrich, 1990; Figure 2.4). Remember that EAE susceptibility and severity are mostly (but not exclusively) determined by the MHC and MHC-linked effects on the MOG-specific B cell response that mediate severe clinical EAE (Jersild *et al*., 1975; Masterman *et al* 2000; Olerup and Hillert, 1991; Stefferl *et al* 1999). The fact both strains share the MHC haplotype RT1.AV1 (Hedrich, 1990) allows the establishment of intercrosses and congenic strains specifically aimed at identifying non-MHC loci regulating MOG-EAE (Stridh *et al*., 2010).

There are numerous studies with congenic strains, derived from DA and PVG strains, reporting effects of specific gene/region regulating neuroinflammation (Beyeen *et al.,* 2010; Marta, 2007; Marta *et al.,* 2010; Ockinger *et al.,* 2006; Stridh *et al.,* 2010). The DA and PVG rat

strains have also been used to establish backcrosses (Jansson *et al.,* 1999), to allow enough phenotypic variation to identify genomic regions that influence neuroinflammation (Cui *et al.,* 2007). These strains can be used to create intercrosses, better to achieve higher mapping resolution than backcrosses (Darvasi and Soler 1997). Finally, those two strains have also been used to create advanced intercross lines (AIL; Bäckdahl *et al.,* 2009; Jagodic *et al.,* 2004), to enable phenotype mapping at a higher resolution compared to the mentioned backcrosses and F2 intercrosses (Darvasi and Soler, 1995).

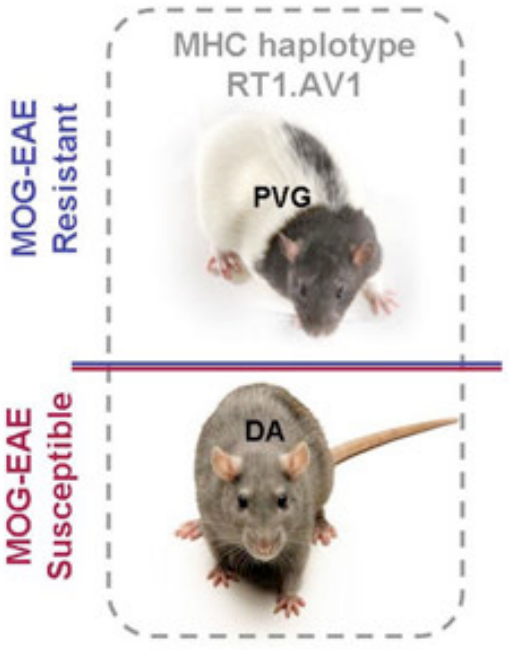


Figure 2.4.- Varying MHC-dependent and non-dependent susceptibilities to EAE the inbred strains relevant for this thesis.

As mentioned earlier, genetic predisposition to EAE and MS also appears to be very similar (in humans and rats), while the major genetic risk factor in both EAE and MS is the major histocompatibility complex (MHC) (Sawcer *et al*., 2005; Weissert *et al*., 1998). Moreover, there is a

significant overlap between non-MHC influences identified in EAE and MS (Serrano-Fernandez *et al*., 2004). Several EAE-regulating genes, identified using the DA and PVG rat strains (identical MHC and different susceptibility), have been suggested to regulate predisposition to human disease (Thessen Hedreul *et al.,* 2009; Harnesk *et al*., 2008; Swanberg *et al*., 2005; Jagodic *et al*., 2004; Vyshkina and Kalman, 2005).

The whole spectrum of MS pathology is closely reflected in MOG-induced EAE in susceptible rat strains. Even clinical and histopathological subforms of MS, such as neuromyelitis optica (Devic's disease), could reproducibly be induced in this model (Storch *et al*., 1998). Hence, DA inbred rats are widely used for the study of experimental autoimmune and/or inflammatory diseases (Carlsen *et al.,* 1998; Gulko *et al.,* 1998; Remmers *et al.,* 1996; Wilder *et al.,* 1999). Specifically, they have been shown to be highly susceptible to experimental autoimmune encephalomyelitis (EAE) induced with whole spinal cord homogenate, with myelin basic protein (MBP-EAE) or with myelin oligodendrocyte glycoprotein (MOG-EAE; see Dahlman, I., *et al* 1998,1999; Lenz, *et al* 1999; Weissert, *et al* 1998). DA rats are susceptible not only to the EAE (Gasser *et al*., 1973) but also to other autoimmune diseases like arthritis (Kleinau *et al*., 1991; Vingsbo *et al*., 1996) and thyroiditis (Rose, 1975). In addition, numerous publications have provided evidence indicating that DA rats are relatively unresponsive to antigen tolerization protocols and are uniquely prone to develop pathogenic autoreactive T cells that produce proinflammatory cytokines, such as tumor necrosis factor alpha (Lenz, *et al* 1999). Conversely, PVG rat is relatively resistant to EAE (Lindh, 1977; Weissert *et al* 1998b) and experimental arthritis (Lorentzen and Klareskog, 1996).

Deciphering functional differences between DA and PVG rats will lead to identification of inherited mechanisms underlying susceptibility to autoimmunity. Studies of EAE in DA and PVG, and in crossings and AIL rats derived from them, can give insight into genetically-driven disease mechanisms of relevance for MS, as it is suggested by the above mentioned genetic (QTL) findings (see previous section) showing several QTLs (Eae24-Eae27) in Chr 4 and Chr 17 (Eae23a and Eae23b) regulating both susceptibility and severity of EAE.

2.5 ANXIETY, STRESS AND NEUROINFLAMMATION

2.5.1 Anxiety

Anxiety is a biologically important mechanism conserved across many species. Normal anxiety serves as an adaptive response to potentially threatening situations (Clement *et al*., 2002; Finn *et al*., 2003; Sandford *et al*., 2000).

Anxiety allows an organism to protect itself against future danger by responding to threatening stimuli through characteristic responses of fight, flight or freezing (Finn *et al*., 2003; Gordon and Hen, 2004; Sandford *et al*., 2000).

In humans anxiety may also be expressed psychologically as worry (Antony and Swinson, 1996; Finn *et al*., 2003). These anxious reactions enable an organism to evaluate a threatening situation and react in an appropriate manner to reduce the risk of harm (Antony and Swinson, 1996).

2.5.2 Anxiety disorders

Although anxiety is an important protective mechanism it can become maladaptive and disruptive (Clément *et al*., 2002; Finn *et al*., 2003; Sandford *et al*., 2000).

Pathological anxiety, as manifested in anxiety disorders, is an anxious response that occurs out of proportion to the threat, becomes disruptive to daily life and causes suffering (Antony and Swinson, 1996; Clement

et al., 2002; Finn *et al*., 2003; Sandford *et al*., 2000). Although many authors see pathological anxiety not as a separate and unique state from normal anxiety but as an extreme expression of it (Finn *et al*., 2003; Lesch, 2001; Sandford *et al*., 2000), anxiety disorders may be defined as a collection of psychological problems that include excessive anxiety, worry, fear and avoidance (Antony and Swinson, 1996). Diagnostically, it can be said that the difference between normal and pathological anxiety lie in the fact that the latter is disruptive and causes suffering for an individual. Anxiety disorders are divided into five major diagnoses according to the Diagnostic and Statistical Manual IV (DSM IV). These five disorders are generalized anxiety disorder, obsessive compulsive disorder, phobias, panic disorder and post traumatic stress disorder (PTSD). While anxiety disorders might be classified into five categories they are not isolated from each other, and many of their behavioural and physiological symptoms overlap (Finn *et al*., 2003; Gross and Hen, 2004). Moreover, many of the disorders respond to the same treatment, highlighting underlying commonalities between disorders. Anxiety disorders affect a large proportion of the population worldwide. An estimated 30 million people in the United States alone will experience an anxiety disorder at some point in their lives (Finn *et al*., 2003; Lepine, 2002). Associated with anxiety disorders are large personal and socio-economic costs ranging from medical treatments to reduced workplace productivity to suicide (Antony and Swinson, 1996; Lepine, 2002). As a result of these (and other) factors there is a great deal of interest in discovering the underlying mechanisms of anxiety disorders in order to improve diagnosis and treatment of these complex disorders.

2.5.3 Animal models of anxiety

Animal behavior models

Anxiety-like behaviours have been observed across many other species. As a result, animal models can be used to obtain information about molecular mechanisms involved in anxiety that would be impossible in humans. Animal models allow investigators to test hypotheses under controlled conditions and using methods that would be difficult to manage in humans (Hitzemann, 2000; Kalueff and Tuohimaa, 2004). The increasing ease of developing rodent and invertebrate models by genetic manipulation or other means has not obviated the difficulties of modeling disorders that often seem uniquely human. Many of the symptoms used to establish psychiatric diagnoses in humans (for example, hallucinations, delusions, sadness and guilt) can not be convincingly ascertained in animals. The truth is that it is difficult to identify analogous behaviours (Kalueff and Tuohimaa, 2004).

It is also difficult to distinguish between fear and anxiety. The behavioural and physiological responses in fear and anxiety are highly similar. The distinction between fear and anxiety lies in the concept that the former is a response to an actual threat while the latter is a response to a potential threat (Belzung and Griebel, 2001; Gordon and Hen, 2004; Gray, 1979, Gray and McNaughton, 2000). This definition is ambiguous in animals so anxiety in animals can only be implied at best.

Another confounding issue for current behavioural tests is the interpretation of anxiety-like phenomena. Many stress-based rodent models exhibit anxiety-like behaviour in a range of assays, such as the elevated plus maze, dark-light test and open field test, all of which were

developed to detect benzodiazepine-like anxiolytic drugs. These tests exploit the balance between the preference of rodents for avoiding open exposure to predators versus exploration for possible rewards. Novelty-suppressed feeding, in which rodents placed in a novel environment show a latency to consume food, responds to chronic, but not acute, doses of antidepressant drugs (the result being decreased latency to feed). It is unclear whether this result demonstrates what is already known in humans, that is, that chronic antidepressant administration treats anxiety disorders as well, or another observation that is well known in humans, the frequent intermixture of symptoms of depression and anxiety. In sum, depression and anxiety-like symptoms both occur in some, but not all, animal models (Gourley, 2008; Krishnan and Nestler, 2008; Wallace et al., 2009).

Conditioned Tests

In the field of anxiety research there are two main categories of animal models: those that involve conditioned responses and those that involve unconditioned responses (Rodgers, 1997; Rodgers and Dalvi, 1997). Conditioned tests combine elements of learning and memory with aversive stimuli and require pre-test training paradigms. They measure a conditioned response, in other words, a specific response that is learned through association with an aversive stimulus (Hitzemann, 2000). In this thesis, it has been used one conditioned anxiety/fear test, the acquisition of two-way active avoidance in a fear conditioning to a context (Escorihuela *et al.,* 1993; Fernandez-Teruel *et al.,* 1991a-c, 2002; Prunell *et al.,* 1994a-b).

Unconditioned tests

On the other hand, unconditioned tests do not require time consuming pre-test training as they measure un-learned, inherent anxiety. They are believed to be more sensitive to stress compared to conditioned tests as the latter tend to use strong and often painful stressors such as foot shock. It is argued that these stressors may suppress activity and cause complex changes in animal behaviors, making the interpretation of the results difficult (Kalueff and Tuohimaa, 2004). The battery used here comprises novelty-induced and habituated exploratory activity, and "elevated zero maze" tests for unconditioned anxiety (Ramos and Mormède, 1998; Shepherd *et al*, 1994; Schwegler *et al* 1997) and the baseline acoustic startle test (e.g. (Aguilar *et al*., 2002, Steimer and Driscoll, 2003).

2.5.4 Anxiety models

Animal models to study the human behaviour form the mainstays for pre-clinically seeking the Neurobiology of Psychiatric Disorders. Nowadays, these models are used to research new therapeutic agents, as well as to study their neurobiological basis (Rodgers *et al*., 1997). An animal model can be defined as an experimental training, which is developed in a specific specie with the aim of study that kind of phenomena typical of this specific specie (and other species). Animal models that are used in Psychobiology have been built on solid evolutionist arguments. Likewise, factors like genetic determinants, neural mechanisms, impact of environment and pharmacological effects, have been assumed to have such an influence on the animal behaviour comparable to those that orchestrate the human (normal and

pathological) behaviour (Hutchinson, 2007; Ramos and Mormède 1998).

Nowadays, there is a wide variety of animal models for mood disorders, like anxiety and depression (Escorihuela and Fernández-Teruel, 1998). Animal models currently used for research into anxiety need to satisfy two criteria: Firstly, predictivity, i.e., manipulations known to influence the pathological state should have the same effects on the model. And in second place, syndrome selectivity, i.e., similarity between the behaviour observed in animals and the human disorder (Clement and Chapouthier, 1998).

Depending on the experimental paradigm the animal models of anxiety have been based, there are different ways to classify these models. There are, for instance, models based on the environmental conditions that can stimulate a specific behaviour or response (Fernández-Teruel and Escorihuela, 1997; Gray, 1981, 1987; Rodgers, 1997). According to Gray (Gray and McNaughton, 2000), animal models of anxiety can be divided into three categories.

First of all, models that are based on a conflict entail the presentation of aversive stimuli (electrical shock) or appetitive stimuli (food, water…) and the contingent response (learned or consummatory). Consequently, there is an approach-avoidance conflict, that will culminate in the inhibition of the current behaviour. For example, the Geller-Seifter and Vogel conflict tests are used to model anxiety state in preclinical studies of anxiety and have selectivity for anxiolytic drugs (Geller *et al.,* 1960).Secondly, models that are based on a novel-context exposure can ease the exploratory behaviours. However, when the novelty-stimuli are aversive (i.e. intense lights, high height, open spaces...), fear inhibits the exploratory behaviour and enhances

anxiety-related behaviours (i.e. defecation, freezing...). The open-field test, the hole-board, the light-dark box or the elevated zero-maze are examples of the exposition to a novel-context (Fernández-Teruel and Escorihuela, 1997). Finally, the animal models of frustration or reward loss are based on the assumption that the lack of the expected reward (or its reduction) constitutes an aversive event with the same reaction than a fear stimulus (Dantzer and Kelley, 2007, Flaherty and Rowan, 1986; Razafimanalina *et al.,* 1996; Papini, 2008; Rabiner *et al.,* 1988). The consummatory successive negative contrast is a model of reward loss that has been widely used in Psicobiology research.

In summary, the overall decrease in interaction shown by captive animals comes to expression in decrease in behavioural variability and an increase in self directed behaviours (Dantzer and Kelley, 2007).

2.5.5 Stress

Strictus *(latin, late 13 century) "compressed, tighted"* → ***estrece*** *(old french) "narrowness, oppression"* → ***destresse*** *(modern french) "distress". The term stress was originlly associated with physical pressure, being different in the beggining from that which is known as psychological "stress" nowadays.*

The nobel Hans Seyle was the first one in writing about the General Adaptation Syndrome (or stress syndrome, the process under which the body confronts "stress") in the British journal Nature in the summer of 1936 (Seyle, 1936). He later coined the term "stress", which has been accepted into the lexicon of various other languages. On the counterpoint, ancient Greek philosophers enunciated terms as

"harmony" or "isonomia" (Warren, 2007) to refer to equilibrium or balance, or, in the modern synonim "homeostasis" (Cannon, 1939; Benison *et al.,* 1987).

Stress is as a state of threatened homeostasis or dysharmony and is counteracted by a complex repertoire of physiologic and behavioral adaptative responses that reestablish homeostasis (Ulrich-Lai and Herman, 2009). Still this homeostasis is constantly challenged by internal or external adverse effects, termed stressors (Chrousos *et al*., 1996; Holsboer and Barden, 1996).

It is common knowledge that factors like the duration, nature, if they are emotional or physical, and controllability vs uncontrollability of the stressor importantly determine how the stress system can change its function from protection to damage and disease (Armario, 2006; Koolhaas *et al.,* 2011; Sorrells and Sapolsky, 2007; Sorrells *et al.,* 2009). Both the magnitude and chronicity are also important (Dhabhar and McEwen, 1999; Keyes *et al.,* 2011). Not to mention the individual differences (Sapolsky, 1998). There is a great variety of stressors that can activate the hypothalamic-pituitary-adrenal (HPA) axis (who really manages the maintainance of homeostasis following stress) through distinct pathways (Hatalski and Baram, 1997; Chen *et al*., 2006; Herman *et al*., 2003; Lupien *et al*., 2005; Rice *et al*., 2008; Shors, 2006).

Over the past decades, it has been established a conceptual framework to explain how the HPA axis and glucocorticoid hormones, in concert with the sympathetic nervous system and various neuropeptides, can coordinate the underlying initial stress response with the management of subsequent stress adaptation (Joëls *et al*., 2008).

The hypothalamic-pituitary-adrenal (HPA) axis begins in the brain with the amygdala, which is involved in recognizing environmental stressors. After a stressor, the amygdala signals the cells in the paraventricular nuclei of the hypothalamus to release corticotropin-releasing factor (CRF) to the pituitary gland (Figure 2.5 shows the HPA axis in response to stress). In response, the pituitary releases adrenocorticotropic hormone (ACTH) into the bloodstream. This hormone travels to the adrenal glands, which sit atop the kidneys. These glands then release cortisol (corticosterone in rodents), which not only helps the body to mobilize energy for the classic fight-or-flight response, but also affects the brain.

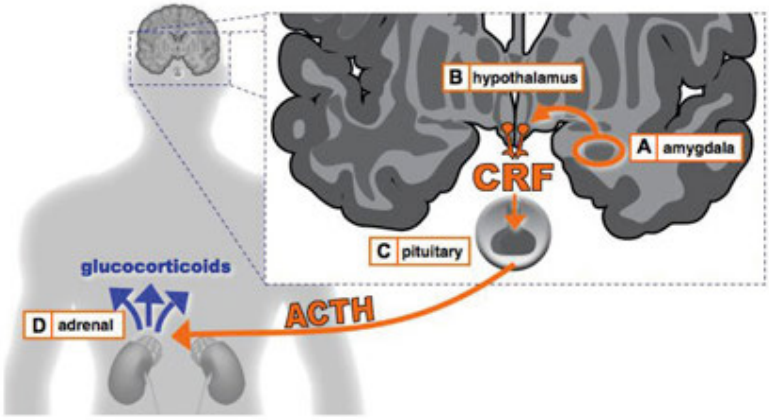


Figure 2.5.- The amygdala (A) is involved in recognizing the stressor and sends out a signal to the paraventricular nuclei of the hypothalamus (B). Cells in these nuclei release CRF to the pituitary gland (C). The pituitary gland releases ACTH to the bloodstream. ACTH then travels to the adrenal glands (D), which release the glucocorticoid cortisol into the bloodstream. Figure taken from Stahl and Wise, 2008

A key brain structure affected by cortisol is the hippocampus. The hippocampus normally can suppress the HPA axis through a pathway to the CRF-containing cells in the hypothalamus (Figure 2.6). However, chronic stress and cortisol can damage the hippocampus (Moretti *et al* 2012). The hippocampus, thus impaired by stress, cannot sufficiently

regulate the HPA axis. Left unchecked by the hippocampus, the HPA stress circuit can ramp up to excessively high levels—an unfortunate feed-forward cycle. In addition, depending on the stressor, various inflammation-related cytokines are secreted and act on hypothalamic, pituitary and/or adrenal components of the HPA axis, mostly to potentiate its activity.

Moreover, there is evidence suggesting that the regulation of cortisol secretion is further influenced by other hormones and/or cytokines, originating from the adrenal medulla or coming from the systemic circulation, and/or by neuronal signals via the autonomic innervation of the adrenal cortex.

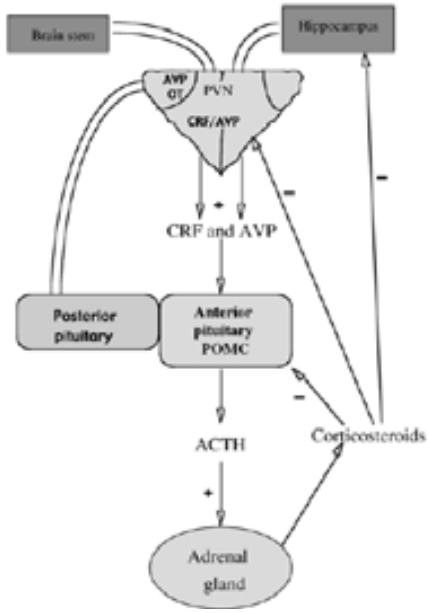


Figure 2.6.- The HPA axis. CRF is synthesised in the hypothalamus PVN. Activation of the axis results in the synthesis/release of corticosteroids from the adrenal cortex (corticosterone in rodents). From Harbuz and Lightman, 1992.

2.5.6 Stress, immune system and effects on autoimmune neuroinflammation.

It is known that complex networks of connections exist among the CNS, neuroendocrine pathways and the immune system (IS). These connections are bidirectional, as CNS and neuroendocrine processes affect IS function in many ways, while the IS influences CNS and CNS-endocrine functions through neuronal and humoral routes as, for instance, via immune mediators and cytokines (e.g. Cohen *et al.,* 2007; Licinio and Wong 1999; Maier and Watkins 1998; see an example of such interactions in Fig. 2.8).

The experimental induction of emotional states (or traits) as anxiety and other stress-related conditions has provided useful tools for the study of the relationships among (chronic or acute) threat and the physiological (CNS, endocrine, IS, disease-related) processes that are activated by the organism in order to cope or to adapt. Along with such processes of adaptation and/or coping the HPA axis acts as a major stress-mediating signalling system and glucocorticoid hormones play a principal role in the control of the stress response (Dhabhar and McEwen, 1999). Thus, for instance, imbalance in GCs/MCs-mediated actions as a result of chronic stress or other triggering factors, is thought to underlie maladaptive behaviour and HPA dysregulation that may lead to impaired immune function (De Kloet *et al.,* 1998; Gesing *et al.,* 2001; Labeur *et al.,* 1995). Through the regulation of GC secretion the HPA axis is considered to be a major modulator of immune function.

Likewise, the HPA axis is crucial in regulating the severity of disease, and the question of susceptibility and/or resistance may be influenced by a variety of factors including behavioural responses, uncontrollability or controllability, individual differences in susceptibility (to stress and/or

to anxiogenic/conflict conditions), exposure to pathogens in early or later life and the behavioural and stress history of the individual (e.g. Sapolsky, 1998; Meaney *et al.,* 1988). Studies in humans have shown that cortisol affects all major homeostatic systems of the body, including innate and acquired immunity (Chrousos and Kino 2005, 2007; Franchimont, 1993). Certainly, in the absence of corticosteroids the immune system is unrestricted and its activation by either acute or chronic immune challenge is likely to be fatal (e.g. Harbuz 1992).

Accumulating evidence suggests, furthermore, that stress might provide a link between anxiety and inflammation. There are numerous studies giving support to this suggestion, by showing that abnormalities in the interactions between the neuroendocrine and immune systems can contribute to the pathogenesis of chronic autoimmune inflammatory diseases (Hall *et al*., 1994; MacPhee and Mason 1988; MacPhee *et al.,* 1989; Schauenstein *et al*., 1987; Sternberg *et al*., 1989). Also, the immunological effects of stressors have been extensively studied (for review see Segerstrom and Miller, 2004), and a wide variety of psychological -anxiety-related- stressors (e.g. restraint, open-field exposure or social isolation) have been shown to increase concentrations of pro-inflammatory cytokines in rodents' brain (O'Connor *et al*., 2003). In the same way, stress-induced alterations of rodents' behaviour can be reversed by treatment with IL-1 inhibitor (Pugh *et al*., 1999). Similarly, acute and chronic stress have both been associated with increased pro-inflammatory cytokines and decreased anti-inflammatory cytokines in humans (Deinzer *et al*., 2004; Goebel *et al*., 2000; Maes *et al*., 1998; Raison and Miller, 2003). Interestingly, stress-induced activation of pro-inflammatory cytokines might provide some insight into the decreases in acquired immune responses found in both stress and depression (Moraska *et al*., 2002). Figure 2.7 illustrates how stress and depression (a stress-related condition), glucocorticoids

(HPA axis) and catecholamines influence aspects of the immune system, and more specifically, the traffic and/or function of leukocytes and immune cells (TNF, IL-1, IL-6).

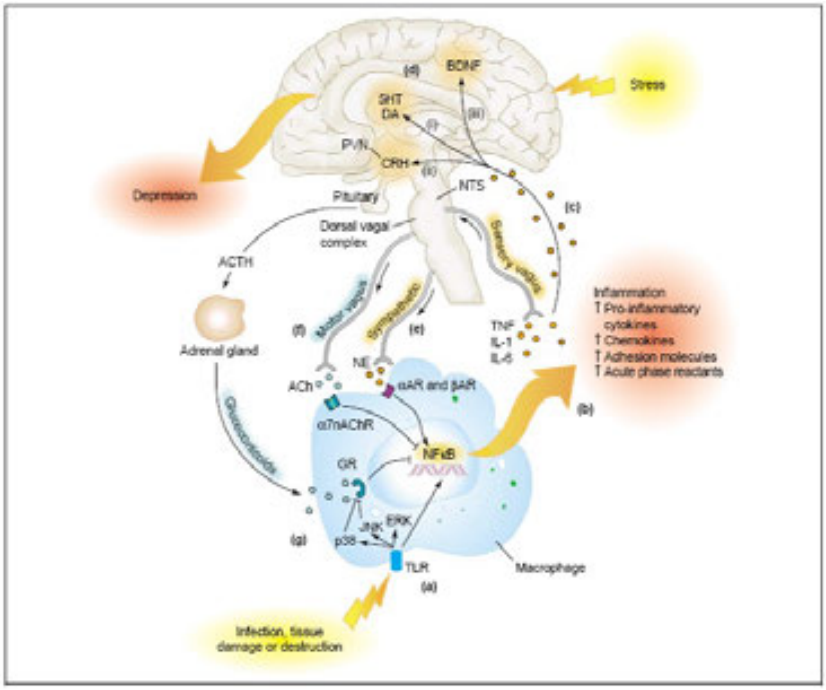


Figure 2.7.- Stress–immune interactions. (a) Activation of nuclear factor-kB (NF-kB) through Toll-like receptors (TLR) during immune challenge leads to an inflammatory response including (b) the release of the proinflammatory cytokines TNF-a, IL-1 and IL-6. (c) These cytokines, in turn, access the brain via leaky regions in the blood–brain barrier, active transport molecules and afferent nerve fibers (e.g. sensory vagus), which relay information through the nucleus tractus solitarius (NTS). (d) Once in the brain, cytokine signals participate in pathways (indicated in orange) known to be involved in the development of depression, including: (i) altered metabolism of relevant neurotransmitters such as serotonin (5HT) and dopamine (DA); (ii) activation of CRH in the paraventricular nucleus (PVN) and the subsequent production and/or release of ACTH and glucocorticoids (cortisol); and (iii) disruption of synaptic plasticity through alterations in relevant growth factors. (e) Exposure to environmental stressors promotes activation of inflammatory signaling (NF-kB) through increased outflow of proinflammatory sympathetic nervous system responses (release of norepinephrine (NE)).(orange). (f) Stressors also induce withdrawal of inhibitory motor vagal input (release of acetylcholine (ACh)). (g) Inhibition of the function of glucocorticoid receptors (GR), thereby releasing NF-kB from negative regulation by glucocorticoids released as a result of the HPA axis in response to stress (blue).

Conversely, several studies support the notion that immune system can affect the HPA axis function. In fact, proinflammatory cytokines stimulate the stress system in several ways and at multiple levels, in both the CNS and peripheral nervous system, including the hypothalamus, central noradrenergic system, pituitary and adrenal glands, which increases glucocorticoid levels and consequently suppresses the inflammatory reaction. In particular, pro-inflammatory cytokines, such as interleukin-6 (IL-6) amongst others, can activate the hypothalamus to produce CRH (Fig. 2.7), which would increase the pituitary ACTH-production, that could indirectly suppress inflammation. Pro-inflammatory cytokine activation also appears to mediate other stress-related biochemical changes in the brain (Frank *et al*., 2006; Musselman *et al*., 2001). These actions form another important negative feedback loop that protects the organism from overshoot of the inflammatory response. Peripheral secretion of CRF (induced by IL6) lead to inflammation and can activate the sickness syndrome - inflammatory reaction, somnolence, fatigue, nausea and depressive mood- (Chrousos, 1996, 2000; Elenkov *et al.,* 1998; Karalis, 1991; Theoharides, 1995). The sickness syndrome includes symptoms such as somnolence, fatigue, nausea and depressive mood and it results from innate processes of the organism that are triggered and sustained by a systemic, inflammatory reaction. Other chronic inflammatory and/or autoimmune and allergic diseases, as well as in in fibromyalgia and chronic fatigue syndrome, have also abnormal neuroendocrine, autonomic and immune functions, an evidence that link these abnormalities to low CRF activity (Clauw and Chrousos,1997; Chrousos and Gold, 1992; Chrousos, 1996, 2000; Elenkov *et al*., 2008; Franchimont, 2003). It also illustrates how some of these immune processes lead to alterations in CNS and neuroendocrine (HPA-axis) functions (Fig. 2.8).

Regarding the autoimmune diseases, there are many examples of studies showing the fundamental role played by the HPA axis in their regulation. It has been demonstrated that glucocorticoids blockers in patients with reumatoid arthritis worsed the disease (Panayi, 1992). In the same way, adrenalectomy in patients with Cushing's syndrome resulted in the development of reumatoid arthritis and autoimmune thyroid disease (Takasu *et al*., 1990; Yakushiji *et al*., 1995). Likewise, adrenalectomy in rodents led to an increase in severity of adjuvant-induced arthritis and experimental allergic encephalomyelitis (EAE) (Harbuz *et al*., 1993; MacPhee and Mason, 1990), which could be prevented by replacement treatment (Sternberg *et al*., 1989). Moreover, several lines of evidence suggest an influence of stress on the manifestation and course of EAE and on the occurrence of relapses in MS (for further review, see Heesen *et al.,* 2007; Gold *et al.,* 2005a-b).

To summarize, it seems obvious that this regulatory system between the IS and CNS plays an important role in susceptibility and resistance to autoimmune, inflammatory, infectious and allergic diseases. In turn, the IS signals the CNS through neuronal and humoral routes, via immune mediators and cytokines (Licinio and Wong, 1999; Maier and Watkins, 1998). On the opposite way, CNS regulates the IS through both neuroendocrine and neuronal pathways. Although the evidence linking stress and inflammation is increasing, the role of HPA axis response dysfunctions in the pathogenesis of this autoimmune disorder is still not totally clear. Nevertheless, and particularly related to the aims of the present work, it is noticeable that stress and/or GCs have been shown to have a modulator role on EAE (and MS in humans). More specifically, higher stress-induced GCs responses have been reported to have protective effects on EAE and MS (e.g. MacPhee *et al.,* 1990; Stefferl *et al.,* 2001; Sternberg *et al.,* 1989).

AIMS

3 AIMS

The initial aim of this thesis was to characterize a large sample of genetically heterogeneous N/Nih-HS rats in multiple phenotypes (i.e. anxiety, fear, exploratory activity, EAE susceptibility/severity, immunology, metabolism, cardiovascular phenotypes, bone fragility, and other complex quantitative traits; see summary in Table 2.1) to subsequently apply high-resolution genetic –QTL- mapping on a whole genome scale to determine the likely molecular causes of quantitative trait variation in all these phenotypes within the context of EURATools and EURATRANS projects. So, the initial aims were:

i) To evaluate the behaviour of a very large sample of genetically heterogeneous N/Nih-HS with regard to their unconditioned and conditioned anxiety/fear.

ii) To characterize N/Nih-HS rats for MOG-EAE susceptibility and severity for the first time.

iii) To study the possible relationships between anxiety and EAE, based on the presumption that increased anxiety levels should be accompanied by an enhanced HPA axis response, which could provide a certain resistance to disease.

However, as the data analyses progressed (along with the experimental batches), it became necessary to assess and complement the suggestive results from the big experiment of the N/Nih-HS rats with a second study. In “Study II”, the presumably coherent relationship between HPA axis function and anxiety (the higher anxiety the higher

glucocorticoids levels) would be investigated in two inbred strains with different profiles of EAE susceptibility. More specifically, the aims were:

iv) To compare the EAE-susceptible DA and EAE-resistant PVG rat strains concerning their anxiety/fear and HPA-axis function profiles.

Results of both studies should allow us to establish associations among anxiety/fearfulness traits, HPA-axis function and EAE.

STUDY I.

N/NIH HETEROGENEOUS RATS: ANXIETY AND EAE

4 STUDY I. N/NIH HETEROGENEOUS RATS: ANXIETY AND EAE

4.1 INTRODUCTION

We have tested N/Nih-HS rats in unconditioned tests for anxiety/fearfulness (i.e. elevated zero-maze and novel cage), as well as for context-conditioned freezing and two-way active avoidance acquisition. This behavioural phenotype is our main target. Our previous work has shown that such an anxiety-driven response (Fernández-Teruel *et al*., 1991, 2002) appears to have a consistent genetic influence, according to recent QTL studies in rat samples (Fernández-Teruel *et al.,* 2002; Johannesson *et al.,* 2008; *López*-Aumatell *et al.,* 2008).

With the aim toward identifying a link between behaviour and biology which renders some individuals with certain anxious profile more prone to EAE, we tested rats in behavioural tests to measure the anxiety levels of the individuals and make predictions based on which about the induced disease. We expected the animals showing higher anxiety profile to be more resistant in front of the immunization.

4.2 MATERIAL AND METHODS

4.2.1 Animals

The subjects were 2006 (1012 female and 994 male) N/Nih-HS rats (*"National Institutes of Health Genetically Heterogeneous Rat Stock",* see (Hansen C, Spuhler K, 1984); progenitors were kindly provided by Dr. Eva Redei in 2004, Center for Comparative Medicine, Northwestern University, Chicago, USA), females weighing 151±19.7g (mean±SD) and males 221±34.2. They were derived from 40 different families which are a breeding colony kept at our laboratory. All litters were culled to 10 pups at birth, trying to keep half of each sex whenever possible. Animals were approximately 8 weeks old at the beginning of behavioural testing. As mentioned above, these rats are part of a high throughput phenotyping protocol in which, besides the behavioural phenotype, a large amount of physiological and disease-related phenotypes are being scored to be submitted to *genome*-wide fine mapping of QTL (see Johannesson *et al.,* 2008). Animals were housed in pairs (males) or groups of three (females), in macrolon cages (50 x 25 x 14 cm), and maintained with food and tap water available ad lib, under conditions of controlled temperature (22±2ºC) and a 12-h light-dark cycle (lights on at 08:00 h).

4.2.2 Procedure and apparatus

Experiments were performed during the light cycle between 09:00 and 19:00h, and in accordance with the Spanish legislation on "Protection of Animals Used for Experimental and Other Scientific Purposes" and the European Communities Council Directive (86/609/EEC) on this subject. Approximately 2-3 weeks elapsed between consecutive behavioural

tests. Three behavioural tests were administered along a 5-6-week period for each of the 8 batches (with n=230-270 rats/batch, approximately half of each sex). Phenotyping of the 8 batches was carried out along 3 years (2007-2009). The sequence and the characteristics of the tests were as follows:

Week	1	2 to 5	6	7	8	9 to 11	12 to 13
Phenotype	1) TSH	2) BEHAVIOUR o ZM o 30-min NACT o SH	3) IPGTT	4) Cardiovascular 5) MOG-Immunization		6) EAE scoring	7) Tissue Harves

Table 3.1.- Schedule of Study I

BEHAVIOURAL TESTS

Elevated zero- maze (ZM)

The maze, similar to that described by Shepherd *et al* (Shepherd *et al*, 1994) comprised an annular platform (105 cm diameter; 10 cm width) made of black plywood and 65 cm above the ground level. It had two open sections (quadrants) and two enclosed ones (with walls 40 cm height). The subject was placed in an enclosed section facing the wall. The apparatus was situated in a black testing room, dimly illuminated with red fluorescent light, and the behaviour was videotaped and measured outside the testing room. Latency to enter into an open section (latency), time spent in the open sections (time), number of entries in the open sections (entries), number of stretched attend postures (SAP), number of head dips (HD), number of line crossings (LC), and number of defecation boluses (def) were measured for 5 minutes (see Shepherd *et al*, 1994; Pähkla *et al* 2000).

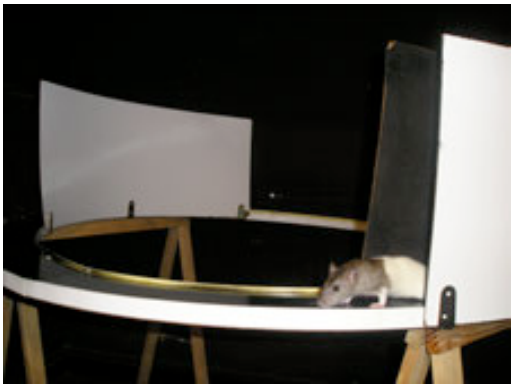


Figure 4.1.- Zero-maze test.

Automated novel-cage activity (NACT)

The apparatus (Panlab, Barcelona, Spain) consisted of a horizontal surface (50 x 50 cm) provided with photobeams that detect and measure movement automatically, loading the data in a computer. The subjects were placed in transparent plexiglas cages (40x40x40 cm). They were situated in a white fluorescent (60 w) illuminated chamber. Spontaneous horizontal activity was measured for 30 minutes (dis 0-30), of which we took for analyses the activity scores of the first 5 minutes (dis 0-5*; as a measure of novelty-induced –open field-like-activity)* and of the last 5 minutes (dis 25-30; as a measure of habituated, or less novelty-affected, activity).

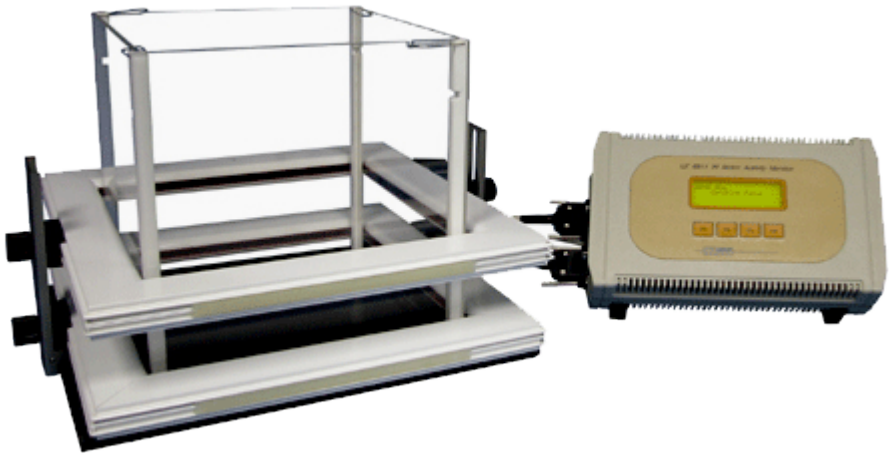


Figure 4.2.- Automated novel-cage activity

Two-way active, shuttle box avoidance acquisition (SH) and context-conditioned freezing (fear)

The experiment was carried out with three identical shuttle boxes (Letica, Panlab, Barcelona, Spain), each placed within independent, sound-attenuating boxes constructed of plywood. A dim and diffuse illumination was provided by a fluorescent bulb placed behind the opaque wall of the shuttle boxes. The experimental room was kept dark. The shuttle boxes consisted of two equally sized compartments (25x25x28 cm), connected by an opening (8x10 cm). A 2400-Hz, 63-dB tone plus a light (from a small, 7-W lamp) functioned as the conditioned stimulus (CS). The unconditioned stimulus (US), which commenced at the end of the CS, was a scrambled electric shock of 0.7 mA delivered through the grid floor. Once the rats were placed into the shuttle box, a 4-min familiarization period elapsed before training commenced. Each training trial consisted of a 10-s CS, followed by a 20-s US. The CS or US was terminated when the animal crossed to the other compartment, with crossing during the CS being considered as an avoidance response, and during the US as an escape response. Once a crossing had been made or the shock (US) discontinued, there was a 60-s inter-trial interval (ITI) during which crossings (ITC) were scored. Training consisted of a single 40-trial session.

The variables recorded were the total number of avoidances (*AV40)*, the number of inter-trial crossings (*ITC), the number of changes in exploration time (CET)* and the average response latency for the whole training session (*LAT40*) (Aguilar R, *et al* 2002; Fernández Teruel A *et al*, 1991). Context-conditioned freezing was measured by two trained observers (between-observer reliability r = 0.98) as the time a rat spent completely motionless except for breathing movements. Freezing was measured during the first five 60-s inter-trial intervals of the 40-trial

acquisition session. No rat made avoidance responses during these first five trials.

Figure 4.3.- Two-way active, shuttle box avoidance acquisition.

Induction and clinical evaluation of experimental autoimmune encephalomyelitis (EAE)

Recombinant rat myelin oligodendrocyte glycoprotein (rMOG), amino acids 1–125 from the N-terminus, was expressed in *Escherichia coli* and purified to homogeneity by metal chelate affinity chromatography (Amor *et al*. 1994) and ion exchange chromatography. Rats were anesthetized with isoflurane (Servicios Genéticos Porcinos) and immunized subcutaneously in the dorsal tail base with 200 µL inoculum containing rMOG (females 50 µg and males 120 µg) in phosphate buffered-saline (PBS; Life Technologies) emulsified 1:1 with Freund's adjuvant (Sigma-Aldrich) containing 200 µg Mycobacterium tuberculosis (H37 RA, Sigma). Signs of EAE and body weight were

monitored daily from day 8 until day 28 post-immunization (p.i.), after which the animals were euthanized by sanguination under anesthesia. The clinical score was graded as follows: 0= no clinical signs or healthy; 1= tail weakness or tail paralysis; 2= hind leg paresis or hemiparesis; 3= hind leg paralysis or hemiparalysis; 4= tetraplegy, urinary, and/or fecal incontinence; and 5= death. If severe disease (score 4) was observed for two consecutive days, the rats were sacrificed due to ethical reasons. The following clinical parameters were assessed: Incidence of EAE as occurance of disease symptoms (INC) and presence of EAE as disease symptoms for a minimum of 2 days (EAE) were defined as 0= absent and 1= present; onset of EAE (ONS) was defined as the first day that clinical signs were observed; duration of disease (DUR) excluding days after death/sacrifice or number of alive days with disease; maximum EAE score (MAX) was the highest score obtained during the experiment; cummulative score or sum of all alive scores (SUM).

Tissue dissection of HS rats

Twenty-eight days after immunization and of clinical scoring for EAE symptoms, rats were euthanized by sanguination under isofluorane anesthesia. The thymus was carefully dissected out and the heart. Thereafter the ears, brain, and spinal cord were dissected in parallel with spleen, liver, adrenal glands, kidneys, and bones.

Tissue was either snap frozen in liquid nitrogen or kept in RNAlater. Blood was incubated for 6 h at room temperature and kept at 4°C overnight and spun at 2000 rpm for 20 min, and sera was aliquoted and

kept at -80°C until use. Tissues were sent to each laboratory responsible for investigating the phenotype of interest.

Adrenal glands

Adrenal glands were immediatelly frozen and sent to Karolinska Institutet for being weighted and used to obtain their gene expression. Each of the glands was accurately weighted on dry ice, where the tweezers used to manipulate them were immersed before in order to ensure that adrenal glands do not warm up. After each pair of adrenal glands of the same animal, tweezers were carefully cleaned with ethanol. The plastic plate was replaced for a new one every single adrenal.

4.2.3 Statistical Analyses

A correlation matrix (Pearson), factor analysis (Direct Oblimin rotation) and "forward stepwise" multiple regression (SPSS Windows, 9.0.1, SPSS Inc; USA) were applied to study the associations among the different and most relevant dependent variables. In order to avoid too much redundancy among variables (and the processes they represent) within the tests, and to select the most relevant variables for factor analysis, we followed the same approach used in our previous works (see Aguilar R *et al* 2000, *López*-Aumatell R *et al* 2008, López-Aumatell *et al* 2009). This led to six variables (2 per test) to which obliqueliy rotated (Direct Oblimin) factor analyses was applied. The next step was to reduce the obtained solution to a two-factor one in order to obtain the minimum meaningful and non test-related factors (Direct Oblimin).

Following our previous works (see López-Aumatell *et al* 2009), subgroups were selected a posteriori by a specific anxiety- or susceptibility- profile were also constituted to evaluate whether the values in that given variable from one test could predict the scores in variables from different tests. Extreme groups of anxiety, consisting of N/Nih-HS animals scoring $\geq$1 standard deviation (SD) or $\leq$1 SD, were made for the following variables, sexes separately (means ± SEM are given in parentheses): SAP (*Males* "+ SAP" n=164, 15.10 ± 0.17; "- SAP" n=212, 3.71 ± 0.09; *females* "+ SAP" n=184, 15.80 ± 0.15; "- SAP" n=207, 4.51 ± 0.10); entries spent into open sections of the zero-maze test (*Males* "+entries" n=227, 10.01 ± 0.13; "-entries" n=186, 0.00 ± 0.00; *females* "+entries" n=388, 10.45 ± 0.13; "-entries" n=270, 0.86 ± 0.05); time spent into open sections of the zero-maze test (*Males* "+ Time" n=191, 125.8 ± 1.58; "- Time" n=212, 0.39 ± 0.08; *females* "+ Time" n=185, 135.22 ± 1.29; "- Time" n=225, 6.36 ± 0.51); head dips, (*Males* "+ HD" n=147, 16.91 ± 0.35; "- HD" n=121, 0.47 ± 0.05; *females* "+ HD" n=185, 18.18 ± 0.28; "- HD" n=203, 1.84 ± 0.08); freezing, (*Males* "+ freezing" n=243, 248.9 ± 0.9; "-freezing" n=306, 139.2 ± 3.2; *females* "+freezing" n=357, 243.8 ± 1.0; "-freezing" n=324, 127.0 ± 2.6); Avoid40, (*Males* "+ avoid40" n=121, 12.5 ± 0.5; "-avoid40" n=443, 0.0 ± 0.0; *females* "+avoid40" n=132, 14.1 ± 0.5; "-avoid40" n=351, 0.0 ± 0.0). Subgroups of different susceptibility to EAE were selected using dichotomic criterium (animals presenting or not presenting EAE symptoms for two consecutive days were considered respectively as relatively-susceptible or relatively-resistant).

Thus, two way ANOVAs for all groups (two sexes and the "superior" and the "inferior" extremes of anxiety, or "EAE-susceptible" and "EAE-resistant"), Duncan's tests for comparison between groups when appropriate (i.e. after significant one-way ANOVA), and Student's t-tests for independent samples for comparisons between extreme

groups and between sexes, were applied in order to test for the a priori hypotheses that, for instance, a relatively higher anxiety would be associated to EAE-resistance, and that females would present lower anxiety levels, as well as higher EAE incidence or severity, than males.

MANOVAs were applied with the aim of analyzing the activity curve in the novel-cage test depending on the susceptibility (repeated measures for intervals of 5 minutes; two factors “EAE-susceptibility” and “sex”). Also MANOVAs were applied to study the disease course in function of anxiety profile (repeated measures for days after immunization; two factors “extreme of anxiety” and “day”). Chi squares were used to compare the percentage of EAE-Incidence in the extreme groups of anxiety.

4.3 RESULTS

4.3.1. Descriptives

Sex-linked differences in behaviour: general HS population

Table 4.2 shows the scores (mean ± S.E.M.) for the main variables in both sexes and Student's t-tests results. It clearly indicates that, compared to males, females generally show significantly lower signs of anxiety, fear and behavioral inhibition in almost all unconditioned (elavated zero-maze -unconditioned anxiety-, novel-cage activity test – behavioral inhibition in response to novelty-) and conditioned (conditioned fear/freezing, two-way avoidance acquisition) anxiety/fear-related variables (all Student's t values fall between -10.75 and 17.44, $p \leq 0.002$), the only exception being "Dis25-30" (i.e. habituated activity in the last 5 minutes of exposure to the "novel-cage activity test"; see Table 4.2).

There also appeared the expected between-sex differences in body weight (lower in females; Table 4.2) as well as in adrenal weight (higher in females; Table 4.2).

There were no differences in MOG-EAE incidence or severity (DUR, CUM, ONS, MAX; see Table 4.2) between the whole male and female samples. However, in the subsamples of animals that got EAE (bottom of Table 4.2) females presented higher severity of the disease (i.e. DUR and CUM variables; see Student's t-tests in bottom of Table 4.2).

	Mean ± S.E.M.		Mean ± S.E.M.		*t*	Sig.
	Males (n=967)	Range	**Females (n=998)**	Range		
ZM						
Latency (s)	104,4 ± 3,6	2,0 - 300,0	70,1 ± 3,1	2,0 - 300,0	7,15	0,000
Time (s)	52,1 ± 1,5	0,0 - 231,0	68,0 ± 1,5	0,0 - 198,0	-7,45	0,000
Entries (n)	4,2 ± 0,1	0,0 - 19,0	6,1 ± 0,1	0,0 - 40,0	-9,92	0,000
SAP (n)	8,8 ± 0,1	0,0 - 27,0	9,8 ± 0,1	0,0 - 27,0	-5,68	0,000
HD (n)	7,1 ± 0,2	0,0 - 30,0	8,8 ± 0,2	0,0 - 32,0	-6,84	0,000
LC (n)	20,0 ± 0,4	1,0 - 56,0	26,2 ± 0,4	0,0 - 67,0	-10,75	0,000
Def (n)	1,4 ± 0,1	0,0 - 7,0	0,7 ± 0,0	0,0 - 9,0	9,50	0,000
NACT						
Dis 0-5 (cm)	1737,7 ± 22,7	63,0 - 5945,0	1941,7 ± 19,3	284,0 - 6288,0	-6,90	0,000
Dis 25-30 (cm)	717,6 ± 22,7	4,0 - 7448,0	780,0 ± 28,4	1,0 - 12962,0	-1,71	*n.s.*
Dis 0-30 (cm)	6912,4 ± 112,7	1347,0 - 35036,0	7394,6 ± 95,5	1565,0 - 35208,0	-3,27	0,001
SH						
Freezing (s)	196,2 ± 1,5	39,0 - 300,0	175,9 ± 1,8	20,0 - 284,0	8,71	0,000
CET (n)	8,5 ± 0,1	0,0 - 21,0	9,2 ± 0,1	0,0 - 23,0	-3,60	0,000
Av40 (n)	2,6 ± 0,1	0,0 - 30,0	3,3 ± 0,2	0,0 - 34,0	-3,03	0,002
Lat40 (s)	15,1 ± 0,2	4,9 - 29,7	11,5 ± 0,1	3,1 - 28,6	17,44	0,000
ITC40 (n)	14,6 ± 0,4	0,0 - 111,0	20,8 ± 0,6	0,0 - 151,0	-8,45	0,000
BW (g)	285,9 ± 1,3	104,0 - 427,0	188,6 ± 0,8	103,0 - 319,0	63,68	0,000
AW (mg)	21,4 ± 0,0	8,4 - 44,9	26,1 ± 0,2	10,0 - 48,0	-14,50	0,000
RAW (mg/100g*BW)	7,6 ± 0,1	3,2 - 20,7	13,8 ± 0,1	5,3 - 38,9	-37,78	0,000
EAE Incidence	*25,30%*		*24,60%*		*Chi square*	*n.s.*
	Mean ± SD		Mean ± SD			
ONS (day)	21,5 ± 0,2	9 - 29	25,7 ± 0,2	9 - 29	-1,22	*n.s.*
MAX (score)	0,7 ± 0,0	0 - 5	0,6 ± 0,3	0 - 5	1,04	*n.s.*
DUR (days)	2,6 ± 0,2	0 - 19	2,62 ± 0,2	0 - 20	-0,28	*n.s.*
CUM (days)	5,3 ± 0,4	0 - 54	5,55 ± 0,4	0 - 50	-0,41	*n.s.*
	Males with EAE (n=245)		**Females with EAE (n=246)**			
	Mean ± S.E.M.		Mean ± S.E.M.			
ONS (day)	15,6 ± 0,3	9 - 29	16,2 ± 0,3	9 - 29	-1,36	*n.s.*
MAX (score)	2,4 ± 0,1	1 - 5	2,4 ± 0,1	1 - 5	0,44	*n.s.*
DUR (days)	9,4 ± 0,3	1 - 19	10,3 ± 0,3	1 - 20	-1,96	0,050
CUM (days)	19,6 ± 0,8	1 - 54	21,9 ± 0,9	1 - 50	-1,89	0,059
BW (g)	265,5 ± 2,9	104,0 - 394,0	178,3 ± 1,6	110,0 - 254,0	25,85	0,000
AW (mg)	23,1 ± 0,5	8,9 - 44,9	27,3 ± 0,1	10,3 - 48,0	-5,33	0,000
RAW (mg/100g*BW)	9,0 ± 0,3	3,2 - 20,7	15,4 ± 0,3	6,3 - 30,9	-15,59	0,000

Table 4.2.- Behavioural scores of male and female of the N/Nih-HS rats across the battery of tests. Means (± S.E.M.), Student's t and p values for the main variables from each test are presented. The d.f.s. were 1 for the number of experimental groups, and 1963 (general sample) or 489 (susceptible rats) for the number of subjects used. Abbreviations of behavioural variables: "ZM", elevated zero-maze test; "latency", latency to enter into an open section (s); "time", time spent in the open sections (s); "entries", number of entries to the open sections (n); "SAP", number of stretch attend postures (n); "HD", number of head dips (n); "LC", number of line crossings (n); "Def", number of defecation boluses (n); "NACT", automated novel-cage activity test; "dis0-5", distance travelled during the first 5 minutes (cm); "dis25-30", distance travelled during the last 5 minutes (cm); "dis0-30", total distance travelled (cm); "SH", two-way shuttle box avoidance conditioning; "freezing", time spent performing freezing (s); "CET", number of changes in exploration time (n); "av40", number of total avoidances (n); "lat40", mean latency of response (s); ITC, number of intertrial crossings (n). Abbreviations of EAE-variables for only the rats that presented the disease: "DUR", duration of the disease (days); "CUM", sum of days with disease (days). Other variables: "BW" body weight at sacrifice (g); "AW", absolute adrenal weight (mg); "RAW", relative adrenal weight (mg/100*BW). n.s. Not significant.

Associations among the behavioural variables in N/Nih-HS.

Correlation coefficients among the most relevant behavioural variables for males (Table 4.3.A) and females (Table 4.3.B), show: 1) predominantly high correlations among measures within the same test, especially among those from the elevated zero-maze test (males: from r=0.37 to r=0.95; females: from r=0.34 to r=0.91), those within the novel-cage activity test (males: from r=0.49 to r=0.82; females: from r=0.27 to r=0.71), as well as among variables reflecting acquisition of the two-way avoidance task (males: from r=-0.49 to r=0.68; females: from r=-0.52 to r=0.74; among AV40, LAT40 and ITC40 variables); 2) low to moderate significant correlations between context-conditioned freezing (during the first 5 intertrial intervals of the two-way avoidance session) and measures of acquisition performance in the avoidance task (males: from r=-0.23 to r=0.35; females: r=0.18 to r=-0.25); 3) some low, although significant correlations among variables from the novel-cage activity test and some of the variables from the SH avoidance task (males: r=-0.07 to r=0.16; females: from r=0.08 to r=0.17); 4) low but significant correlations between variables from the novel-cage activity test and some of the variables of the elevated zero-maze test (males: r=0.15 to r=0.19; females: from r=0.08 to r=0.21); and, 5) very low but significant correlations among elevated zero-maze variables and those from the avoidance task (males: r=0.08 to r=0.09; females: from r=-0.08 to r=0.12) (see Tables 4.3.A and 4.3.B).

The present pattern of correlations (sign and magnitude of "r" coefficients) is also similar to that previously observed in different large samples of N/Nih-HS rats which were behaviourally phenotyped in 2005-2006 (*López*-Aumatell *et al.,* 2008, 2009, 2011).

Target variables to be included in the following factorial analyses were selected either according to theoretical/empirical criteria based on previous works (*López*-Aumatell *et al.,* 2008, 2009, 2011), or after applying independent orthogonally-rotated (Varimax) factor analyses to ZM, NACT and SH tests/variables (according to the procedure used by *López*-Aumatell *et al.,* 2009, 2009, 2011). After such a selection process 6 variables were chosen as the most relevant ones representing the underlying structure of the different behavioural procedures.

Obliquely-rotated factor analyses (Direct Oblimin) were then applied to those 6 target variables. By doing so we obtained a three-fold factor structure in both the male and the female samples of HS rats. In any case, these factors represented better the pattern seen in the correlation matrix than any theoretically meaningful construct of anxiety, i.e. in both sexes, each factor corresponded exclusively to measures of one single test: Factor 1 reflected NACT measures of activity, Factor 2 reflected conditioned SH measures and Factor 3 grouped measures of unconditioned anxiety in the ZM test (data not shown here). When the analysis was forced to only two factors (Table 4.4), the emerging structures were slightly different in males and females. Thus, in males, Factor 1 grouped unconditioned anxiety in the elevated zero-maze and conditioned freezing/fear and anxiety (i.e. response latency) in the two-way avoidance task, suggesting that males' behaviour is preferentially influenced by "conflict", while Factor 2 only consisted of activity measures from the novel-cage activity test (see Table 4.4). Conversely, in females, Factor 1 grouped unconditioned anxiety in the elevated zero-maze and activity in the novel cage, thus suggesting that females' responses are predominantly modulated by activity-related processes, while Factor 2 included conditioned freezing/fear and conditioned anxiety (response latency in the two-way avoidance task) with a lower

contribution (loading = -.30) of “entries” into open sections (unconditioned anxiety) of the elevated zero-maze (see Table 4.4).

We also performed, only in “EAE-resistant” rats (separated by sex), the same correlation and factor analyses with “relative adrenal weight” (RAW) included as a variable, and no significant correlations appeared between RAW and any of the behavioural variables (data not shown), while the 2-factor structure for the 6 behavioural variables remained exactly the same as in Table 4.4 (data not shown).

All HS males. Correlation matrix among the behavioural variables

	ZM						NACT			SH			
	Latency	Time	Entries	SAP	HD	LC	Dis 0-5	Dis 25-30	Dis 0-30	Freezing	CET	Av40	Lat40	ITC40
ZM														
Latency	1													
Time	**-.649****	1												
Entries	**-.657****	**.872****	1											
SAP	**-.387****	**.395****	**.440****	1										
HD	**-.469****	**.629****	**.575****	**.676****	1									
LC	**-.588****	**.823****	**.946****	**.373****	**.489****	1								
NACT														
Dis 0-5	-.046	**.174****	**.170****	**.148****	**.146****	**.189****	1							
Dis 25-30	.024	.029	.004	.009	.056	.003	**.488****	1						
Dis 0-30	.035	.047	.029	.025	.049	.038	**.765****	**.816****	1					
SH														
Freezing	.031	-.069	-.074	-.035	-.059	**-.089***	**-.084***	.025	-.008	1				
CET	**-.109****	**.161****	**.199****	**.213****	**.164****	**.206****	**.161****	.024	.06	-.041	1			
Av40	.029	.022	.013	**.082***	**.078***	.015	**.082***	.018	.032	**-.226****	.06	1		
Lat40	-.022	-.049	-.05	.002	-.006	**-.067***	**-.065***	-.009	-.019	**.353****	-.06	**-.488****	1	
ITC40	-.022	**.088***	**.075***	.058	**.087***	**.075***	**.125****	**.073***	**.093****	**-.296****	**.109****	**.685****	**-.544****	1

Table 4.3.- Correlations ≥ .07 among the behavioural variables are shown in bold letters. All HS males (n=967)
* $p \leq .05$, Pearson's correlation coefficient.
** $p \leq .01$, Pearson's correlation coefficient.

All HS females. Correlation matrix among the behavioural variables B)

	ZM						NACT			SH			
	Latency	Time	Entries	SAP	HD	LC	Dis 0-5	Dis 25-30	Dis 0-30	Freezing CET	Av40	Lat40	ITC40
ZM													
Latency	1												
Time	**-.644****	1											
Entries	**-.632****	**.825****	1										
SAP	**-.385****	**.384****	**.408****	1									
HD	**-.468****	**.664****	**.585****	**.647****	1								
LC	**-.574****	**.775****	**.906****	**.338****	**.500****	1							
NACT													
Dis 0-5	**-.108****	**.165****	**.095****	**.206****	**.179****	**.109****	1						
Dis 25-30	-.048	.028	.006	**.087****	.062	.021	**.267****	1					
Dis 0-30	-.05	**.081***	.038	**.121****	**.109****	.041	**.710****	**.496****	1				
SH													
Freezing	.046	-.019	-.037	.075	.007	-.031	-.003	.009	.006	1			
CET	**-.160****	**.163****	**.184****	**.244****	**.236****	**.145****	**.174****	.05	**.084****	.031	1		
Av40	-.048	.019	.032	.034	**.087****	.003	.021	.019	.013	**-.201****	**.064***	1	
Lat40	.053	-.051	-.064	-.032	**-.079***	-.036	-.024	-.034	-.04	**.181****	-.048	**-.591****	1
ITC40	-.053	.048	.041	.053	**.117****	.008	**.081***	.002	.037	**-.249****	**.144****	**.743****	**-.523****	1

Table 4.3.- Correlations ≥ .06 among the behavioural variables are shown in bold letters. All HS females (n=998)
* $p \leq .05$, Pearson's correlation coefficient.
** $p \leq .01$, Pearson's correlation coefficient.

All HS males		*n=967*
ZM	Factor 1	Factor 2
Entries	.65	-
SAP	.62	-
NACT		
Dis 0-5	-	.84
Dis 25-30	-	.84
SH		
Freezing	-.61	-
Lat40	-.56	-
Eigenvalues	1.68	1.36
% of accumulated explained variance:	31.6	50.5
Correlation between factors= .060		

All HS females		*n=998*
ZM	Factor 1	Factor 2
Entries	.66	-.30
SAP	.76	-
NACT		
Dis 0-5	.60	-
Dis 25-30	.43	-
SH		
Freezing	-	.70
Lat40	-	.72
Eigenvalues	1.61	1.18
% of accumulated explained variance:	26.8	46.6
Correlation between factors= .034		

Table 4.4.- Correlation between factors = 0.001. Values ≥ 0.30 are shown. Oblique two factor solution (direct oblimin) with the selected variables (factors with eigenvalues greater than 1).

Tables 4.5 A-B, 4.6 A-B, 4.7 A-B and 4.8 A-B show the scores (mean + sem) and Student's t-tests ("A" tables) as well as the ANOVAs ("B" tables) applied to behavioural data from subgroups of rats selected for their extreme values in unconditioned anxiety measures (i.e. extremes in SAP –Table 4.5-, extremes in "Entries" –Table 4.6-, extremes in "Time" –Table 4.7-, extremes in "HD" –Table 4.8-). Selection by extreme values of a given variable consisted of HS animals scoring ±1 standard deviation in the selected variable (see "Methods"). These four tables (Table 4.5–4.8.) generally and consistently show that, regardless the selection anxiety variable (i.e. SAP, Entries, Time or HD), rats displaying increased anxiety (in the elevated zero-maze) according to any of those 4 selection variables will also show enhanced anxiety in the remaining dependent measures of the same test as well as in "Dis0-5" (novel-cage activity test: activity in the first 5 minutes of exposure) and "CET" (free exploration of the shuttle box before the conditioning session), as it is confirmed by the significant Student's t-tests (part "A" of tables 4.5–4.8) between the extreme groups and by the "2 x 2" ANOVAs (including also "sex" as a factor; part "B" of tables 4.5–4.8). Moreover, it is remarkable that selection for extreme values in "SAP" or "HD" in the elevated zero-maze leads to selection of two-way avoidance acquisition ability, i.e. the subgroups of "Superior SAP" or "Superior HD" show significantly better acquisition of the two-way avoidance task (see "AVOID40" variable in Table 4.5A and 4.8A, and ANOVAs in Table 4.5B and Table 4.8B) than the respective "Inferior SAP" or "Inferior HD" subgroups. It is worth noting that the selection for extreme values in "Entries" and "Time" (elevated zero-maze) led to differential context-conditioned "Freezing", i.e. the higher the "Entries" or "Time" the lower the "Freezing" levels (see tables 4.6A and 4.7A, and 4.6B and 4.7B for ANOVAs results).

On the other hand, and mostly in congruency with the abovementioned results, selection for extremes in conditioned “Freezing” led to differential anxious/fearful behaviour in the three tests/tasks, as shown by the fact that the “Superior Freezing” subsample displays significantly higher signs of anxiety (and inhibited activity) in almost all variables of the elevated zero-maze, novel-cage activity test and two-way avoidance session (see Table 4.9A, and ANOVAs in Table 4.9B). Still in line with the these results, selection by extremes in “Avoid40” led mainly to selection of “SAP” and “HD” behaviors (see Table 4.10A, and especially the ANOVAs in Table 4.10B), as well as to differential levels of conditioned “Freezing” in the shuttle box (Table 4.10A; ANOVAs in Table 4.10B).

We have to say that separation by extremes in anxious behaviour (i.e. all the variables seen in Tables 4.5-4.10), using “EAE-resistant” rats, did not lead to significant effects/differences in “RAW” (relative adrenal weight; data not shown). Likewise, subgroups separated by extreme RAW values did not show significant differences in any of the anxiety- (or activity-) related behavioural variables (data not shown).

A)

	Mean ± S.E.M.	Mean ± S.E.M.	*t*	Sig.
MALES				
	Superior SAP (n=164)	Inferior SAP (n=212)		
ZM				
Latency	51,8 ± 5,7	184,2 ± 8,7	11,83	0,000
Time	74,2 ± 2,9	20,5 ± 2,7	-13,45	0,000
Entries	6,4 ± 0,3	1,5 ± 0,2	-15,31	0,000
HD	13,0 ± 0,4	2,5 ± 0,2	-25,31	0,000
LC	25,6 ± 0,8	12,5 ± 0,6	-13,29	0,000
NACT				
Dis 0-5	1918,8 ± 61,8	1575,0 ± 44,0	-4,65	0,000
Dis 25-30	681,7 ± 53,7	662,9 ± 43,2	-0,28	n.s.
Dis 0-30	6965,8 ± 276,3	6564,7 ± 200,3	-1,20	n.s.
SH				
Freezing	189,6 ± 4,4	198,4 ± 2,9	1,73	n.s.
CET	9,6 ± 0,3	7,5 ± 0,2	-5,06	0,000
Av40	3,6 ± 0,4	2,0 ± 0,3	-3,53	0,000
Lat40	14,9 ± 0,5	15,2 ± 0,4	0,65	n.s.
ITC40	16,2 ± 1,1	13,5 ± 0,8	-2,07	0,039
FEMALES				
	Superior SAP (n=184)	Inferior SAP (n=207)		
ZM				
Latency	33,9 ± 4,0	149,5 ± 9,2	11,01	0,000
Time	87,2 ± 2,9	32,9 ± 3,3	-12,20	0,000
Entries	8,1 ± 0,3	2,9 ± 0,3	-12,04	0,000
HD	15,1 ± 0,4	3,8 ± 0,2	-24,00	0,000
LC	30,2 ± 0,8	17,3 ± 0,9	-10,29	0,000
NACT				
Dis 0-5	2140,8 ± 52,1	1731,5 ± 40,7	-6,25	0,000
Dis 25-30	935,7 ± 84,3	662,6 ± 36,4	-3,09	0,002
Dis 0-30	8023,5 ± 280,1	6922,5 ± 202,0	-3,24	0,001
SH				
Freezing	177,0 ± 4,7	170,8 ± 4,6	-0,94	n.s.
CET	10,8 ± 0,3	8,0 ± 0,3	-7,41	0,000
Av40	3,9 ± 0,4	3,0 ± 0,3	-1,75	n.s.
Lat40	11,2 ± 0,2	11,5 ± 0,2	1,20	n.s.
ITC40	23,6 ± 1,3	20,6 ± 1,2	-1,66	n.s.

Table 4.5.- Behavioural scores of superior and inferior extreme (in stretch-attend postures) males and females across the battery of tests. Means (± S.E.M.), Student's t and p values for the main variables from each test are presented. The d.f.s. were 1 for the number of experimental groups, and 374 for males and 389 for females used. Abbreviations of behavioural variables: “ZM”, elevated zero-maze test; “latency”, latency to enter into an open section (s); “time”, time spent in the open sections (s); “entries”, number of entries to the open sections (n); “HD”, number of head dips (n); “LC”, number of line crossings (n); “NACT”, automated novel-cage activity test; “dis0-5”, distance travelled during the first 5 minutes (cm); “dis25-30”, distance travelled during the last 5 minutes (cm); “dis0-30”, total distance travelled (cm); “SH”, two-way shuttle box avoidance conditioning; “freezing”, time spent performing freezing (s); “CET”, number of changes in exploration time (n); “av40”, number of total avoidances (n); “lat40”, mean latency of response (s); ITC, number of intertrial crossings (n). n.s. Not significant. See ANOVAs in table 4.5.B.

B)

		Factor "Sex"	Factor "SAP"	"Sex"x"SAP"
ZM				
Latency	F=	11,8	261,6	1,2
	p≤	0,001	0,001	n.s.
Time	F=	17,9	324,2	0,0
	p≤	0,001	0,001	n.s.
Entries	F=	33,7	347,8	0,3
	p≤	0,001	0,001	n.s.
HD	F=	28,8	1200,2	1,1
	p≤	0,001	0,001	n.s.
LC	F=	33,9	262,5	0,0
	p≤	0,001	0,001	n.s.
NACT				
Dis 0-5	F=	14,7	58,3	0,4
	p≤	0,001	0,001	n.s.
Dis 25-30	F=	5,1	6,8	5,1
	p≤	0,02	0,001	0,02
Dis 0-30	F=	8,8	9,9	2,2
	p≤	0,001	0,001	n.s.
SH				
Freezing	F=	23,1	0,1	3,2
	p≤	0,001	n.s.	n.s.
CET	F=	9,1	76,5	1,7
	p≤	0,001	0,001	n.s.
Av40	F=	3,7	13,4	1,1
	p≤	0,05	0,001	n.s.
Lat40	F=	126,8	1,1	0,0
	p≤	0,001	n.s.	n.s.
ITC40	F=	42,9	6,6	0,0
	p≤	0,001	0,01	n.s.

Table 4.5.- Factorial ANOVA analyses, “2 sex x 2 sap extremes” , for ZM test, novel-cage activity and shuttlebox variables. See table 4.5.A for other details and for variable symbols.

A)

	Mean ± S.E.M.	Mean ± S.E.M.	*t*	Sig.
MALES				
	Superior Entries (n=227)	Inferior Entries (n=186)		
ZM				
Latency	22,0 ± 1,3	300,0 ± 0,0	189,59	0,000
Time	110,7 ± 2,0	0,0 ± 0,0	-50,09	0,000
SAP	10,7 ± 0,3	5,7 ± 0,2	-13,96	0,000
HD	11,3 ± 0,4	2,5 ± 0,2	-19,02	0,000
LC	37,8 ± 0,5	8,5 ± 0,2	-52,09	0,000
NACT				
Dis 0-5	1964,6 ± 46,8	1606,7 ± 54,8	-5,00	0,000
Dis 25-30	729,4 ± 41,7	706,7 ± 59,4	-0,32	n.s.
Dis 0-30	7199,8 ± 219,8	6843,6 ± 284,6	-1,01	n.s.
SH				
Freezing	190,2 ± 3,2	199,6 ± 3,1	2,05	0,041
CET	9,5 ± 0,3	7,6 ± 0,3	-5,07	0,000
Av40	2,8 ± 0,3	2,7 ± 0,4	-0,18	n.s.
Lat40	14,6 ± 0,4	14,7 ± 0,4	0,24	n.s.
ITC40	16,3 ± 1,2	14,3 ± 1,0	-1,31	n.s.
FEMALES				
	Superior Entries (n=386)	Inferior Entries (n=269)		
ZM				
Latency	18,5 ± 0,9	183,7 ± 7,4	26,36	0,000
Time	109,1 ± 1,4	11,4 ± 0,8	-53,14	0,000
SAP	11,2 ± 0,2	6,8 ± 0,2	-15,18	0,000
HD	12,4 ± 0,3	3,7 ± 0,2	-23,47	0,000
LC	39,3 ± 0,4	11,1 ± 0,2	-52,23	0,000
NACT				
Dis 0-5	1987,4 ± 28,4	1787,2 ± 35,2	-4,45	0,000
Dis 25-30	768,5 ± 37,7	704,1 ± 42,8	-1,12	n.s.
Dis 0-30	7480,0 ± 137,2	7057,5 ± 165,3	-1,97	0,049
SH				
Freezing	174,2 ± 2,7	181,8 ± 3,7	1,70	n.s.
CET	9,7 ± 0,2	8,1 ± 0,2	-5,45	0,000
Av40	3,4 ± 0,3	3,2 ± 0,3	-0,67	n.s.
Lat40	11,4 ± 0,1	11,7 ± 0,2	1,73	n.s.
ITC40	21,8 ± 1,0	20,6 ± 1,2	-0,80	n.s.

Table 4.6.- Behavioural scores of superior and inferior extreme (in entries) males and females across the battery of tests. Means (± S.E.M.), Student's t and p values for the main variables from each test are presented. The d.f.s. were 1 for the number of experimental groups, and 412 for males and 653 for females used. Abbreviations of behavioural variables: “ZM”, elevated zero-maze test; “latency”, latency to enter into an open section (s); “time”, time spent in the open sections (s); “SAP”, number of stretch attend postures (n); “HD”, number of head dips (n); “LC”, number of line crossings (n); “NACT”, automated novel-cage activity test; “dis0-5”, distance travelled during the first 5 minutes (cm); “dis25-30”, distance travelled during the last 5 minutes (cm); “dis0-30”, total distance travelled (cm); “SH”, two-way shuttle box avoidance conditioning; “freezing”, time spent performing freezing (s); “CET”, number of changes in exploration time (n); “av40”, number of total avoidances (n); “lat40”, mean latency of response (s); ITC, number of intertrial crossings (n). n.s. Not significant. See ANOVAs in table 4.6.B.

B)

		Factor "Sex"	Factor "Entries"	"Sex"x"Entries"
ZM				
Latency	F=	228,5	3127,0	202,8
	p≤	0,001	0,001	0,001
Time	F=	11,5	5173,6	20,4
	p≤	0,001	0,001	0,001
SAP	F=	13,5	414,0	1,6
	p≤	0,001	0,001	n.s.
HD	F=	14,2	870,3	0,0
	p≤	0,001	0,001	n.s.
LC	F=	26,3	5028,3	2,2
	p≤	0,001	0,001	n.s.
NACT				
Dis 0-5	F=	6,4	48,4	3,9
	p≤	0,01	0,001	0,05
Dis 25-30	F=	0,2	0,9	0,2
	p≤	n.s.	n.s.	n.s.
Dis 0-30	F=	1,6	4,0	0,0
	p≤	n.s.	0,05	n.s.
SH				
Freezing	F=	25,5	6,4	0,1
	p≤	0,001	0,01	n.s.
CET	F=	2,0	54,8	0,4
	p≤	n.s.	0,001	n.s.
Av40	F=	2,7	0,3	0,1
	p≤	n.s.	n.s.	n.s.
Lat40	F=	141,0	0,9	0,2
	p≤	0,001	n.s.	n.s.
ITC40	F=	27,1	2,1	0,1
	p≤	0,001	n.s.	n.s.

Table 4.6.- Factorial ANOVA analyses, “2 sex x 2 entries extremes” , for ZM test, novel-cage activity and shuttlebox variables. See table 4.6.A for other details and for variable symbols.

A)

	Mean ± S.E.M.	Mean ± S.E.M.	*t*	Sig.
MALES				
	Superior Time (n=191)	Inferior Time (n=212)		
ZM				
Latency	25,4 ± 1,9	270,6 ± 5,6	39,57	0,000
SAP	9,2 ± 0,2	0,2 ± 0,0	-47,33	0,000
Entries	10,3 ± 0,3	5,8 ± 0,2	-13,17	0,000
HD	12,2 ± 0,4	2,7 ± 0,2	-20,64	0,000
LC	34,8 ± 0,7	8,9 ± 0,2	-36,67	0,000
NACT				
Dis 0-5	1927,0 ± 49,0	1606,4 ± 49,9	-4,57	0,000
Dis 25-30	825,1 ± 63,2	681,5 ± 53,2	-1,75	n.s.
Dis 0-30	7404,8 ± 274,5	6734,1 ± 255,5	-1,79	n.s.
SH				
Freezing	185,5 ± 3,4	198,0 ± 3,1	2,75	0,006
CET	9,4 ± 0,3	7,7 ± 0,3	-4,42	0,000
Av40	2,8 ± 0,3	2,8 ± 0,3	-0,13	n.s.
Lat40	14,2 ± 0,4	14,8 ± 0,4	0,98	n.s.
ITC40	16,8 ± 1,4	14,1 ± 0,9	-1,67	n.s.
FEMALES				
	Superior Time (n=185)	Inferior Time (n=224)		
ZM				
Latency	19,0 ± 1,2	201,4 ± 8,0	20,63	0,000
SAP	10,4 ± 0,2	0,8 ± 0,1	-47,38	0,000
Entries	10,7 ± 0,3	6,5 ± 0,2	-11,27	0,000
HD	14,1 ± 0,4	3,4 ± 0,2	-24,46	0,000
LC	38,2 ± 0,8	11,0 ± 0,3	-34,10	0,000
NACT				
Dis 0-5	2031,1 ± 46,9	1745,2 ± 39,9	-4,67	0,000
Dis 25-30	783,3 ± 66,2	676,2 ± 35,1	-1,50	n.s.
Dis 0-30	7697,0 ± 260,8	6949,5 ± 180,8	-2,42	0,016
SH				
Freezing	174,3 ± 4,0	180,0 ± 4,3	0,96	n.s.
CET	10,0 ± 0,3	8,2 ± 0,3	-4,82	0,000
Av40	3,5 ± 0,4	3,2 ± 0,4	-0,49	n.s.
Lat40	11,4 ± 0,2	11,6 ± 0,2	0,60	n.s.
ITC40	24,1 ± 1,8	20,8 ± 1,4	-1,47	n.s.

Table 4.7.- Behavioural scores of superior and inferior extreme (in entries) males and females across the battery of tests. Means (± S.E.M.), Student's t and p values for the main variables from each test are presented. The d.f.s. were 1 for the number of experimental groups, and 401 for males and 407 for females used. Abbreviations of behavioural variables: “ZM”, elevated zero-maze test; “latency”, latency to enter into an open section (s); “SAP”, number of stretch attend postures (n); “HD”, number of head dips (n); “LC”, number of line crossings (n); “NACT”, automated novel-cage activity test; “dis0-5”, distance travelled during the first 5 minutes (cm); “dis25-30”, distance travelled during the last 5 minutes (cm); “dis0-30”, total distance travelled (cm); “SH”, two-way shuttle box avoidance conditioning; “freezing”, time spent performing freezing (s); “CET”, number of changes in exploration time (n); “av40”, number of total avoidances (n); “lat40”, mean latency of response (s); ITC, number of intertrial crossings (n). n.s. Not significant. See ANOVAs in table 4.7.B.

B)

		Factor "Sex"	Factor "Time"	"Sex"x"Entries"
ZM				
Latency	F=	48,8	1562,2	33,7
	p≤	0,00	0,00	0,00
SAP	F=	5,2	295,6	0,3
	p≤	0,02	0,00	n.s.
Entries	F=	42,6	4475,5	4,7
	p≤	0,00	0,00	0,03
HD	F=	17,7	1013,0	3,9
	p≤	0,00	0,00	0,05
LC	F=	26,4	2478,7	1,6
	p≤	0,00	0,00	n.s.
NACT				
Dis 0-5	F=	6,8	42,6	0,1
	p≤	0,01	0,00	n.s.
Dis 25-30	F=	0,2	5,3	0,1
	p≤	n.s.	0,02	n.s.
Dis 0-30	F=	1,1	8,6	0,0
	p≤	n.s.	0,00	n.s.
SH				
Freezing	F=	15,3	5,9	0,8
	p≤	0,00	0,02	n.s.
CET	F=	4,0	42,7	0,1
	p≤	0,05	0,00	n.s.
Av40	F=	2,2	0,2	0,1
	p≤	n.s.	n.s.	n.s.
Lat40	F=	93,1	1,3	0,4
	p≤	0,00	n.s.	n.s.
ITC40	F=	27,0	4,8	0,0
	p≤	0,00	0,03	n.s.

Table 4.7.- Factorial ANOVA analyses, “2 sex x 2 time extremes” , for ZM test, novel-cage activity and shuttlebox variables. See table 4.7.A for other details and for variable symbols.

A)

	Mean ± S.E.M.	Mean ± S.E.M.	*t*	Sig.
MALES				
	Superior HD (n=146)	Inferior HD (n=121)		
ZM				
Latency	32,6 ± 3,1	216,9 ± 11,0	17,50	0,000
Time	100,4 ± 3,0	9,2 ± 2,3	-23,36	0,000
Entries	7,6 ± 0,3	0,7 ± 0,1	-22,68	0,000
SAP	12,9 ± 0,3	4,2 ± 0,2	-22,51	0,000
LC	28,8 ± 0,9	9,9 ± 0,4	-18,04	0,000
NACT				
Dis 0-5	1929,6 ± 66,8	1685,5 ± 62,7	-2,62	0,009
Dis 25-30	806,3 ± 78,7	747,6 ± 66,0	-0,56	n.s.
Dis 0-30	7314,8 ± 347,3	7200,0 ± 348,4	-0,23	n.s.
SH				
Freezing	186,0 ± 4,8	196,9 ± 4,3	1,66	n.s.
CET	9,4 ± 0,3	7,8 ± 0,3	-3,30	0,001
Av40	3,3 ± 0,4	1,9 ± 0,3	-2,58	0,010
Lat40	14,8 ± 0,5	15,4 ± 0,5	0,92	n.s.
ITC40	16,6 ± 1,4	12,5 ± 0,9	-2,17	0,031
FEMALES				
	Superior HD (n=183)	Inferior HD (n=203)		
ZM				
Latency	20,8 ± 1,8	170,0 ± 8,8	15,80	0,000
Time	110,5 ± 2,4	18,5 ± 1,8	-31,01	0,000
Entries	9,6 ± 0,3	1,8 ± 0,2	-23,99	0,000
SAP	13,5 ± 0,3	5,9 ± 0,2	-23,78	0,000
LC	34,7 ± 0,8	14,1 ± 0,6	-21,08	0,000
NACT				
Dis 0-5	2078,8 ± 46,6	1771,4 ± 44,2	-4,79	0,000
Dis 25-30	838,3 ± 66,6	641,4 ± 33,8	-2,69	0,007
Dis 0-30	7843,7 ± 266,0	6869,4 ± 220,0	-2,84	0,005
SH				
Freezing	176,0 ± 4,6	173,8 ± 4,4	-0,34	n.s.
CET	10,8 ± 0,3	8,2 ± 0,3	-6,80	0,000
Av40	4,2 ± 0,5	3,2 ± 0,4	-1,75	n.s.
Lat40	11,2 ± 0,2	11,7 ± 0,2	1,94	0,053
ITC40	24,1 ± 1,5	19,7 ± 1,2	-2,24	0,026

Table 4.8.- Behavioural scores of superior and inferior extreme (in entries) males and females across the battery of tests. Means (± S.E.M.), Student's t and p values for the main variables from each test are presented. The d.f.s. were 1 for the number of experimental groups, and 265 for males and 384 for females used. Abbreviations of behavioural variables: “ZM”, elevated zero-maze test; “latency”, latency to enter into an open section (s); “time”, time spent in the open sections (s); “entries”, number of entries to the open sections (n); “SAP”, number of stretch attend postures (n); “LC”, number of line crossings (n); “NACT”, automated novel-cage activity test; “dis0-5”, distance travelled during the first 5 minutes (cm); “dis25-30”, distance travelled during the last 5 minutes (cm); “dis0-30”, total distance travelled (cm); “SH”, two-way shuttle box avoidance conditioning; “freezing”, time spent performing freezing (s); “CET”, number of changes in exploration time (n); “av40”, number of total avoidances (n); “lat40”, mean latency of response (s); ITC, number of intertrial crossings (n). n.s. Not significant. See ANOVAs in table 4.8.B.

B)

		Factor "Sex"	Factor "HD"	"Sex"x"HD"
ZM				
Latency	F=	16,8	540,3	6,0
	p≤	0,001	0,001	0,02
Time	F=	16,0	1440,9	0,0
	p≤	0,001	0,001	n.s.
Entries	F=	43,3	997,4	3,1
	p≤	0,001	0,001	n.s.
SAP	F=	22,2	1058,4	5,3
	p≤	0,001	0,001	0,02
LC	F=	47,6	727,7	1,3
	p≤	0,001	0,001	n.s.
NACT				
Dis 0-5	F=	4,6	25,4	0,3
	p≤	0,03	0,001	n.s.
Dis 25-30	F=	0,4	4,2	1,2
	p≤	n.s.	0,04	n.s.
Dis 0-30	F=	0,1	3,5	2,2
	p≤	n.s.	n.s.	n.s.
SH				
Freezing	F=	12,4	0,9	2,0
	p≤	0,001	n.s.	n.s.
CET	F=	8,3	47,4	2,8
	p≤	0,004	0,001	n.s.
Av40	F=	6,8	8,5	0,2
	p≤	0,01	0,004	n.s.
Lat40	F=	109,8	3,0	0,0
	p≤	0,001	n.s.	n.s.
ITC40	F=	27,8	9,1	0,0
	p≤	0,001	0,003	n.s.

Table 4.8.- Factorial ANOVA analyses, “2 sex x 2 HD extremes” , for ZM test, novel-cage activity and shuttlebox variables. See table 4.8.A for other details and for variable symbols.

A)

	Mean ± S.E.M.	Mean ± S.E.M.	*t*	Sig.
MALES				
	Superior Freezing (n=243)	Inferior Freezing (n=306)		
ZM				
Latency	55,6 ± 5,3	196,1 ± 7,3	14,96	0,000
Time	93,6 ± 3,1	24,6 ± 2,5	-17,39	0,000
Entries	7,1 ± 0,3	2,0 ± 0,2	-15,83	0,000
SAP	9,9 ± 0,2	6,7 ± 0,2	-9,93	0,000
LC	10,0 ± 0,4	4,2 ± 0,3	-12,16	0,000
HD	28,3 ± 0,8	14,1 ± 0,6	-14,15	0,000
NACT				
Dis 0-5	1808,0 ± 42,4	1691,5 ± 40,8	-1,97	0,050
Dis 25-30	772,9 ± 49,1	675,1 ± 41,4	-1,53	n.s.
Dis 0-30	7071,6 ± 216,3	6807,9 ± 200,3	-0,89	n.s.
SH				
CET	8,9 ± 0,2	8,1 ± 0,2	-2,55	0,011
Av40	2,2 ± 0,3	3,4 ± 0,3	2,92	0,004
Lat40	16,7 ± 0,4	13,9 ± 0,3	-5,59	0,000
ITC40	12,2 ± 0,9	16,9 ± 0,9	3,58	0,000
FEMALES				
	Superior Freezing (n=357)	Inferior Freezing (n=324)		
ZM				
Latency	35,2 ± 3,1	141,8 ± 7,0	14,33	0,000
Time	101,8 ± 2,1	31,4 ± 2,2	-22,73	0,000
Entries	8,6 ± 0,2	2,9 ± 0,2	-19,52	0,000
SAP	11,6 ± 0,2	7,5 ± 0,2	-14,16	0,000
LC	13,5 ± 0,3	4,6 ± 0,3	-22,58	0,000
HD	33,1 ± 0,7	17,1 ± 0,6	-17,90	0,000
NACT				
Dis 0-5	2004,7 ± 32,2	1828,7 ± 34,0	-3,75	0,000
Dis 25-30	799,0 ± 40,3	673,7 ± 29,7	-2,45	0,014
Dis 0-30	7663,7 ± 170,9	7015,0 ± 167,0	-2,70	0,007
SH				
CET	9,9 ± 0,2	8,4 ± 0,2	-5,26	0,000
Av40	3,1 ± 0,3	4,0 ± 0,3	2,19	n.s.
Lat40	11,6 ± 0,1	11,3 ± 0,2	-1,19	n.s.
ITC40	20,3 ± 0,8	23,6 ± 1,4	2,13	0,034

Table 4.9.- Behavioural scores of superior and inferior extreme (in entries) males and females across the battery of tests. Means (± S.E.M.), Student's t and p values for the main variables from each test are presented. The d.f.s. were 1 for the number of experimental groups, and 547 for males and 679 for females used. Abbreviations of behavioural variables: “ZM”, elevated zero-maze test; “latency”, “time”, time spent in the open sections (s); “entries”, number of entries to the open sections (n); “SAP”, number of stretch attend postures (n); “LC”, number of line crossings (n); “NACT”, automated novel-cage activity test; “dis0-5”, distance travelled during the first 5 minutes (cm); “dis25-30”, distance travelled during the last 5 minutes (cm); “dis0-30”, total distance travelled (cm); “SH”, two-way shuttle box avoidance conditioning; “CET”, number of changes in exploration time (n); “av40”, number of total avoidances (n); “lat40”, mean latency of response (s); ITC, number of intertrial crossings (n). n.s. Not significant. See ANOVAs in table 4.9.B.

B)

		Factor "Sex"	Factor "Freezing"	"Sex"x"Freezing"
ZM				
Latency	F=	39,8	436,5	8,2
	p≤	0,001	0,001	0,004
Time	F=	9,3	790,1	0,1
	p≤	0,002	0,001	n.s.
Entries	F=	29,9	613,9	2,3
	p≤	0,001	0,001	n.s.
SAP	F=	31,4	283,4	4,9
	p≤	0,001	0,001	0,03
HD	F=	41,1	574,2	26,1
	p≤	0,001	0,001	0,001
LC	F=	32,7	504,9	1,7
	p≤	0,001	0,001	n.s.
NACT				
Dis 0-5	F=	20,0	15,4	0,6
	p≤	0,001	0,001	n.s.
Dis 25-30	F=	0,1	7,6	0,1
	p≤	n.s.	0,01	n.s.
Dis 0-30	F=	4,5	5,9	1,0
	p≤	0,03	0,02	n.s.
SH				
CET	F=	8,6	29,5	2,9
	p≤	0,003	0,000	n.s.
Av40	F=	6,2	12,8	0,3
	p≤	0,01	0,001	n.s.
Lat40	F=	222,2	35,8	25,0
	p≤	0,001	0,001	0,001
ITC40	F=	50,1	14,8	0,4
	p≤	0,001	0,001	n.s.

Table 4.9.- Factorial ANOVA analyses, “2 sex x 2 freezing extremes” , for ZM test, novel-cage activity and shuttlebox variables. See table 4.9.A for other details and for variable symbols.

A)

	Mean ± S.E.M.	Mean ± S.E.M.	*t*	Sig.
MALES				
	Superior Avoid40 (n=121)	Inferior Avoid40 (n=443)		
ZM				
Latency	115,3 ± 10,5	103,5 ± 5,4	-1,00	n.s.
Time	55,8 ± 4,6	50,8 ± 2,2	-1,04	n.s.
Entries	4,4 ± 0,4	4,1 ± 0,2	-0,73	n.s.
SAP	9,7 ± 0,4	8,5 ± 0,2	-2,82	0,005
LC	8,0 ± 0,5	7,0 ± 0,3	-1,80	n.s.
HD	20,8 ± 1,1	19,9 ± 0,6	-0,73	n.s.
NACT				
Dis 0-5	1866,0 ± 67,7	1694,3 ± 33,4	-2,38	0,018
Dis 25-30	664,7 ± 78,2	706,6 ± 31,6	0,58	n.s.
Dis 0-30	6785,3 ± 337,5	6801,4 ± 153,0	0,05	n.s.
SH				
Freezing	177,9 ± 4,3	204,4 ± 2,1	6,14	0,000
CET	9,4 ± 0,4	8,4 ± 0,2	-2,55	0,011
Lat40	9,3 ± 0,1	18,7 ± 0,3	17,35	0,000
ITC40	32,1 ± 2,1	8,8 ± 0,3	-18,94	0,000
FEMALES				
	Superior Avoid40 (n=132)	Inferior Avoid40 (n=351)		
ZM				
Latency	62,9 ± 8,1	73,9 ± 5,4	1,10	n.s.
Time	69,0 ± 3,9	68,6 ± 2,5	-0,08	n.s.
Entries	6,7 ± 0,5	5,9 ± 0,2	-1,60	n.s.
SAP	10,2 ± 0,4	9,7 ± 0,2	-1,17	n.s.
LC	9,7 ± 0,6	8,5 ± 0,3	-2,15	0,032
HD	27,0 ± 1,2	26,1 ± 0,7	-0,67	0,503
NACT				
Dis 0-5	1948,2 ± 61,0	1956,4 ± 30,6	0,13	n.s.
Dis 25-30	747,6 ± 72,6	746,3 ± 43,7	-0,02	n.s.
Dis 0-30	7299,4 ± 296,5	7400,9 ± 140,1	0,35	n.s.
SH				
Freezing	157,5 ± 4,9	187,5 ± 2,9	5,53	0,000
CET	9,3 ± 0,4	8,9 ± 0,2	-1,28	n.s.
Lat40	8,6 ± 0,1	12,9 ± 0,2	16,16	0,000
ITC40	46,4 ± 2,6	12,9 ± 0,4	-19,52	0,000

Table 4.10.- Behavioural scores of superior and inferior extreme (in entries) males and females across the battery of tests. Means (± S.E.M.), Student's t and p values for the main variables from each test are presented. The d.f.s. were 1 for the number of experimental groups, and 547 for males and 679 for females used. Abbreviations of behavioural variables: “ZM”, elevated zero-maze test; “latency”, “time”, time spent in the open sections (s); “entries”, number of entries to the open sections (n); “SAP”, number of stretch attend postures (n); “LC”, number of line crossings (n); “NACT”, automated novel-cage activity test; “dis0-5”, distance travelled during the first 5 minutes (cm); “dis25-30”, distance travelled during the last 5 minutes (cm); “dis0-30”, total distance travelled (cm); “SH”, two-way shuttle box avoidance conditioning; “CET”, number of changes in exploration time (n); “freezing”, time spent performing freezing (s); “lat40”, mean latency of response (s); ITC, number of intertrial crossings (n). n.s. Not significant. See ANOVAs in table 4.10.B.

B)

		Factor "Sex"	Factor "Avoid40"	"Sex"x"Avoid40"
ZM				
Latency	F=	27,8	0,0	2,2
	p≤	0,001	n.s.	n.s.
Time	F=	21,0	0,6	0,5
	p≤	0,001	n.s.	n.s.
Entries	F=	48,1	2,9	0,6
	p≤	0,001	n.s.	n.s.
SAP	F=	9,3	7,9	1,3
	p≤	0,002	0,01	n.s.
HD	F=	15,9	7,8	0,1
	p≤	0,001	0,01	n.s.
LC	F=	46,2	1,0	0,0
	p≤	0,001	n.s.	n.s.
NACT				
Dis 0-5	F=	12,8	2,9	3,5
	p≤	0,001	n.s.	n.s.
Dis 25-30	F=	1,2	0,1	0,2
	p≤	n.s.	n.s.	n.s.
Dis 0-30	F=	6,1	0,1	0,0
	p≤	0,014	n.s.	n.s.
SH				
Freezing	F=	29,6	68,0	0,2
	p≤	0,001	0,001	n.s.
CET	F=	0,7	7,3	0,7
	p≤	n.s.	0,01	n.s.
Lat40	F=	110,3	490,2	64,7
	p≤	0,001	0,001	0,001
ITC40	F=	79,2	753,6	24,7
	p≤	0,001	0,001	0,001

Table 4.10.- Factorial ANOVA analyses, “2 sex x 2 avoid40 extremes” , for ZM test, novel-cage activity and shuttlebox variables. See table 4.10.A for other details and for variable symbols.

Figure 4.4 shows percentages of EAE incidence and comparisons between “extreme anxiety” subgroups of N/Nih-HS males and females (i.e. extremes in SAP –Figure 4.4.A-, extremes in “Entries” –Figure 4.4.B-, extremes in “Time” – Figure 4.4.C-, extremes in “HD” – Figure 4.4.D-). Chi-square tests show that EAE incidence is significantly higher in low anxious rats (i.e. the “Superior” groups, in Fig 4.4.A-D) than in high anxious animals (i.e. the “Inferior” groups in Fig. 4.4.A-D), such an effect being the clearest between extreme “SAP” and “HD” groups.

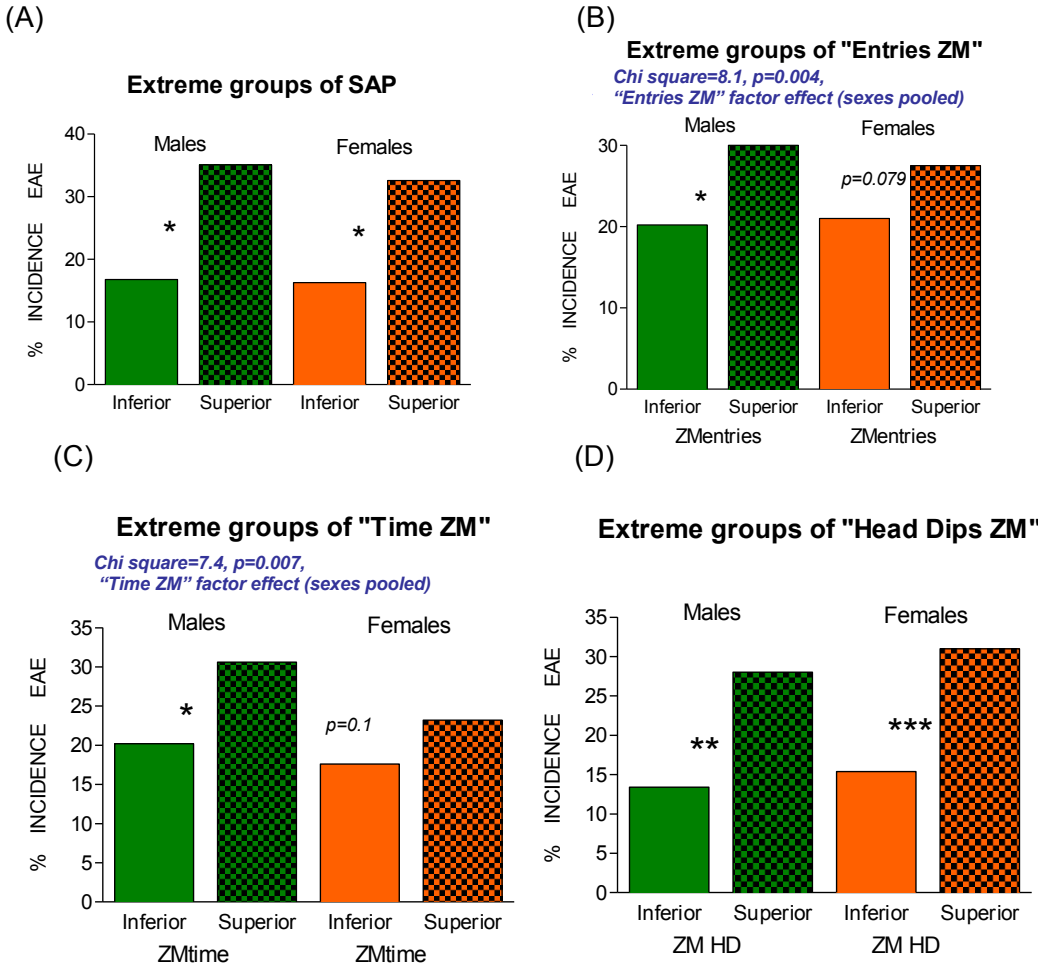


Figure 4.4.- Comparison of the extreme groups of anxiety-related behaviours in the elevated "zero-maze" with respect to EAE incidence. (A) Extreme groups of "SAP". Superior group, males, n= 164 and females, n= 184; Inferior group, males, n= 212 and females, n= 207. (B) Extreme groups of "entries. Superior group, males, n= 227 and females, n= 386; Inferior group, males, n= 186 and females, n= 269 (C) Extreme groups of "Time". Superior group, males, n= 191 and females, n= 185; Inferior group, males, n= 212 and females, n= 224. (D) Extreme groups of "HD". Superior group, males, n= 146 and females, n= 183; Inferior group, males, n= 121 and females, n= 203. * $p \leq 0,05$; ** $p \leq 0,01$; *** $p \leq 0,001$ "extreme group" effect ("*", "**", "***", following Chi-square tests $\geq$ 4.9).

Figures 4.5 - 4.8 show the EAE course, along the 20 days of scoring, for subgroups of N/Nih-HS rats (sexes pooled) selected for extreme anxiety levels in the elevated zero-maze test (Figure 4.5, SAP; Figure 4.6, “Entries”; Figure 4.7, “Time”; Figure 4.8, HD). In the whole sample of rats, and regardless of the anxiety target used to built the extreme subgroups, MANOVAs show significant effects of the “anxiety-extreme” factor (i.e. Factors: SAP, “Entries”, “Time” or “HD”), as well as of “day” factor and ““anxiety-extreme” x “day”” interactions (see MANOVAs in Fig. 4.5A, 4.6A, 4.7A and 4.8A). The common pattern observed is that the relatively less anxious (i.e. “Superior SAP”, “Superior Entries”, “Superior Time”, “Superior HD”, in the elevated zero-maze) rat subgroups display significantly higher “disease severity”. When only “susceptible” rats -divided by the same anxiety extremes- are analysed, the “day” effect is still significant in all cases (see Fig. 4.5B, 4.6B, 4.7B and 4.8B), while the “anxiety-extreme” effect loses significance and there appear ““Day” x “SAP”” (see Fig. 4.5B) and ““Day” x “HD”” (see Fig. 4.8B) significant interactions. These interactions mean that the low anxious rats reach higher levels of disease severity earlier than the relatively high anxious rats (see the comparison of disease severity in days 12 to 16 in Fig. 4.5B and 4.8B; significant Student’s t-tests following MANOVAs).

(A) **(B)**

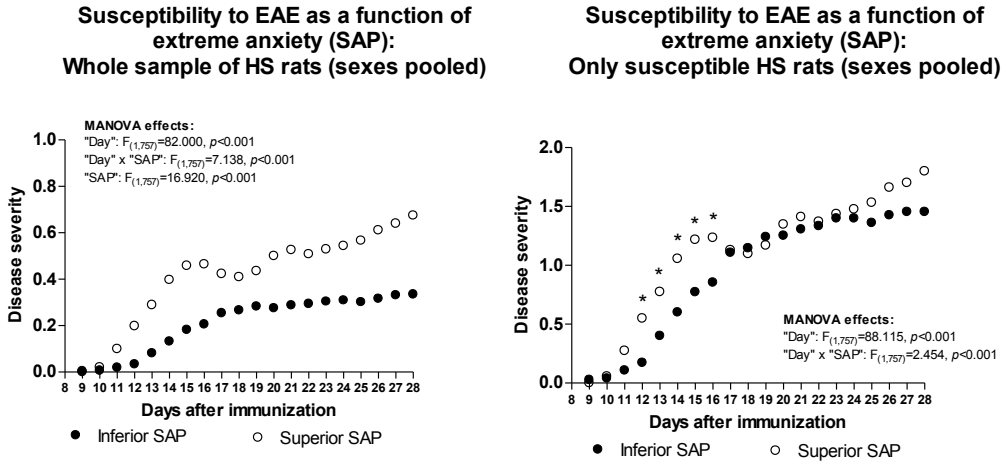


Figure 2B.

Figure 4.5.- Comparison of "SAP" extreme subgroups regarding the course of EAE through days of scoring (scale from 0 to 5, see material and methods). A) Whole sample of HS rats. B) Susceptible HS rats.

(A) **(B)**

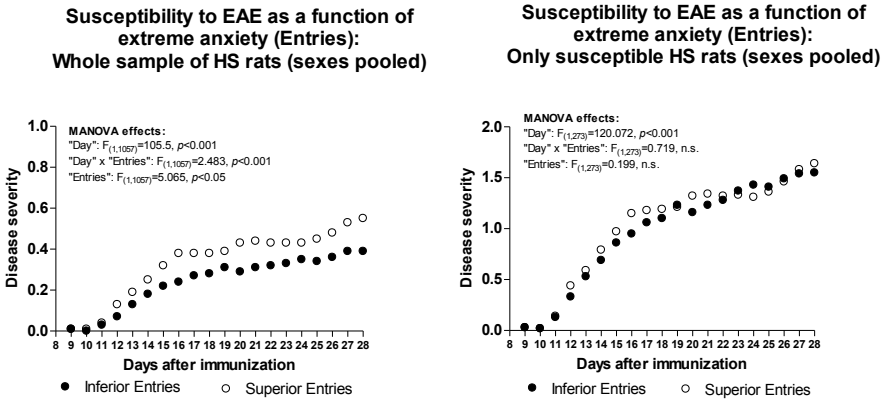


Figure 4.6.- Comparison of "Entries" extreme subgroups regarding the course of EAE through days of scoring (scale from 0 to 5, see material and methods). A) Whole sample of HS rats. B) Susceptible HS rats.

(A) **(B)**

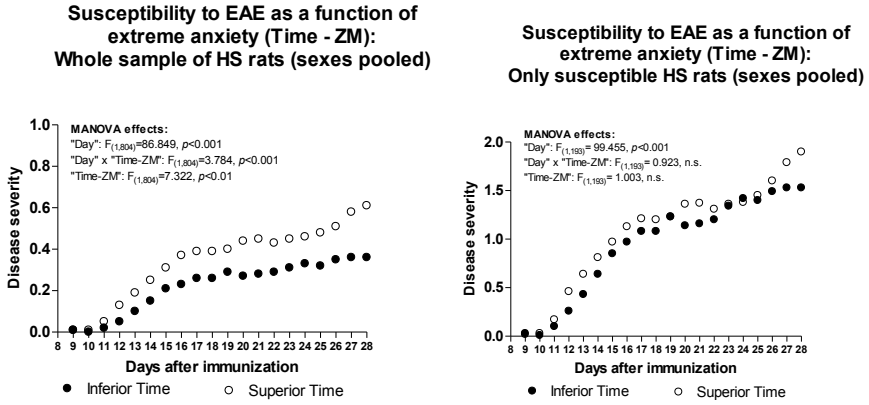


Figure 4.7.- Comparison of "Time" extreme subgroups regarding the course of EAE through days of scoring (scale from 0 to 5, see material and methods). A) Whole sample of HS rats. B) Susceptible HS rats.

(A) **(B)**

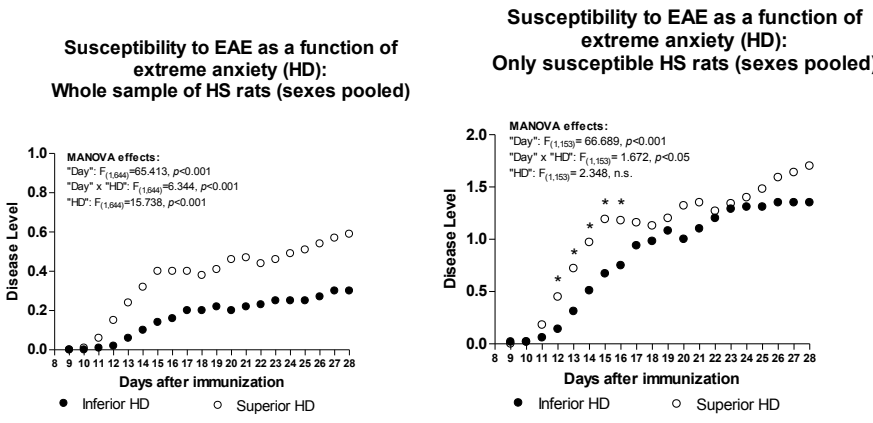


Figure 4.8.- Comparison of "HD" extreme subgroups regarding the course of EAE through days of scoring (scale from 0 to 5, see material and methods). A) Whole sample of HS rats. B) Susceptible HS rats.

The “forward stepwise” logistic regression analyses (Table 4.11) show that EAE is positively predicted by the unconditioned anxiety variables SAP, “Time” and CET, with a lower but negative influence of AV40. Remarkably, the fact that “SAP” is a significant predictor in all these models, in both sexes, and that “Time” is also an important predictor variable in males, confirms with the present regression analyses what we have observed in the previous analyses (Fig. 4.4 – 4.8) through the comparisons of “anxiety-extreme” subgroups.

Dependent variable	Step	Predictor variable	β	Wald's coefficient	gl	*p*
Males (n=967)						
EAE	1	SAP	.072	13.11	1	.001
	4	Time	.004	3.89	1	.049
		SAP	.047	4.40	1	.036
		CET	.060	7.50	1	.006
		Av40	-.048	5.97	1	.015
Females (n=998)						
EAE	1	SAP	.049	6.08	1	.014

Table 4.11.- Forward stepwise logistic regression models relating EAE (dependent variable) to measures of unconditioned anxiety (ZM) and conditioned anxiety (SH) in the whole sample of N/Nih-HS rats. This model is represented as a summary of the first step (i.e. the first significant model with the maximum number of predictor variables included) and the last step (i.e. the last significant model with the minimum number of predictor variables included).

Behavioural differences of “resistant” and “susceptible” HS rats

Figures 4.9-4.11 show the scores of the N/Nih-HS males and females with different EAE susceptibility in the different variables of the behavioural battery of tests. Thus, two-way ANOVAs (2 x “sex” and 2 x “EAE”) and Duncan’s test for comparisons between groups when appropriate (i.e. after significant one-way ANOVA) were applied to observe the differences in behavioural inhibition/activity and

unconditioned anxiety (i.e. novel-cage test and elevated zero-maze test) as well as conditioned anxiety/fear (i.e. fear/freezing, two-way avoidance acquisition).

Figure 4.9 represents the most important variables of the zero-maze test. Regarding the latency to enter into open sections (Fig. 4.9A), there was a significant "sex" effect [$F_{(1,2004)}$=37.109, p≤0.001], with females showing less latency. There was also a significant "EAE" effect [$F_{(1,389)}$=13.352, p≤0.001] as susceptible rats presented less latency. Further, there was a significant "sex" effect [$F_{(1,2004)}$=32.617, p≤0.001] in time to enter into open sections(Fig. 4.9B), with females spending more time into open sections, as well as an "EAE" effect [$F_{(1,389)}$=7.226, p =0.007] with susceptible rats travelling longer. In addition, regarding the number of entries (Fig. 4.9C), there was a "sex" effect [$F_{(1,2004)}$=72.581, p≤0.001], as females performed more entries into the open sections, as well as an "EAE" effect [$F_{(1,389)}$=10.190, p≤0.001] with susceptible rats displaying more number of entries than resistant rats. Moreover, concerning the number of SAP (Fig. 4.9D), there was a "sex" effect [$F_{(1,2004)}$=28.439, p≤0.001], with females doing a higher number of SAPs and an "EAE" effect [$F_{(1,389)}$=45.909, p≤0.001] with susceptible rats performing more SAPs. Similarly, in the number of head dips (Fig. 4.9E), there were also significant effects of "sex" [$F_{(1,2004)}$=40.074, p≤0.001] and "EAE" [$F_{(1,389)}$=18.577, p≤0.001], with females performing more head dips, as well as susceptible rats. A similar pattern and direction of effects was found with respect to the number of line crossings, which females performed more number ["EAE" effect; $F_{(1,389)}$=6.613, p≤0.001, and "sex" effect; $F_{(1,2004)}$=480.653, p≤0.001; Fig. 4.9F] and also the susceptible rats.

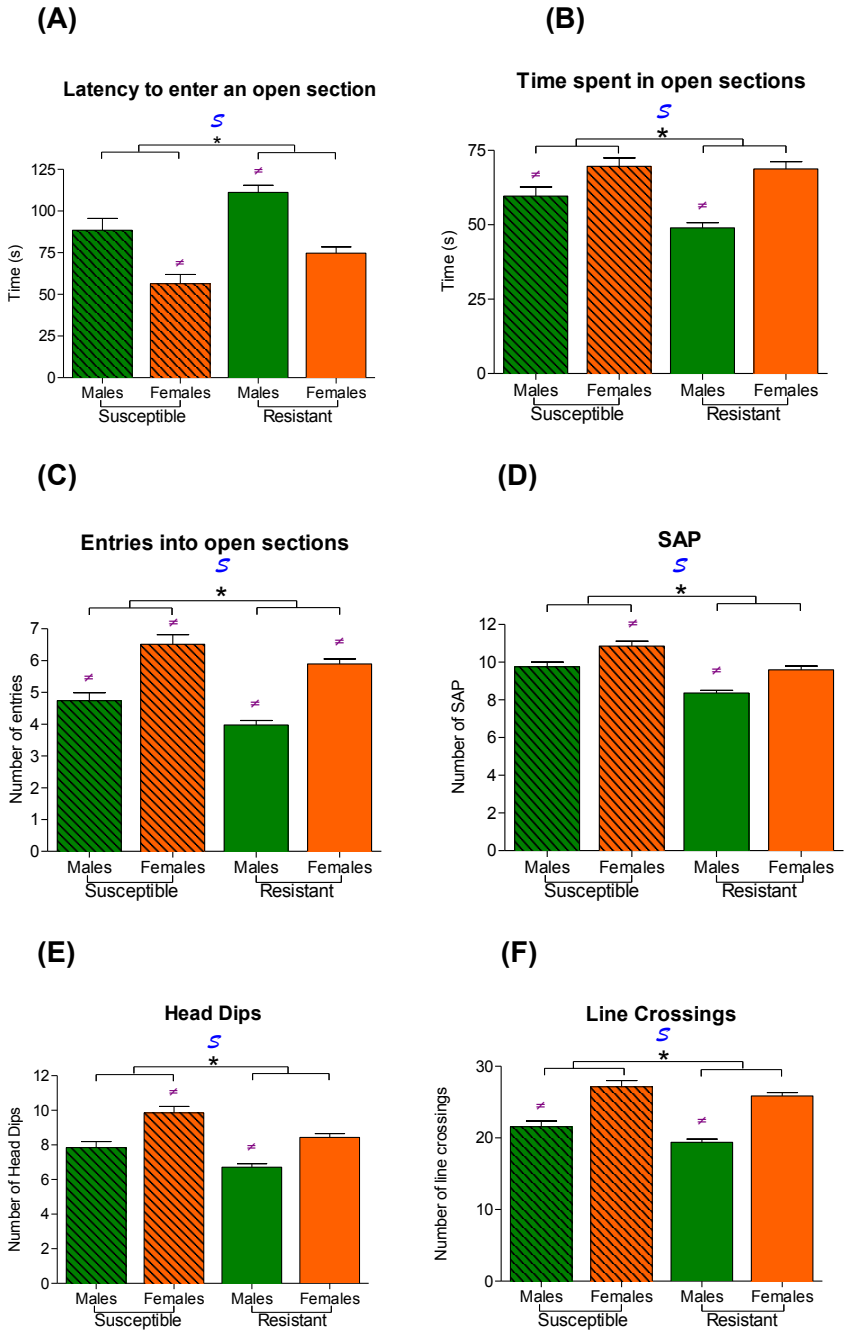


Figure 4.9.- Scores (means ± S.E.M.) in the ZM. (A) latency to enter to open sections of the ZM, (B) time spent in open sections of the ZM, (C) number of entries into open sections of the ZM, (D) number of stretch-attend postures (SAP), (E) number of head dips (HD), (F) number of line crossings. (Group symbols: "Resistant males", n= 722 ; "Susceptible males", n= 245; "resistant females", n= 752; "susceptible females", n= 246). * p <0.05, "EAE" effect; **S** p <0.05, "sex" effect;

≠ p <0.05, Duncan's post-hoc after significant ANOVA, the marked group is different from all the others.

Figure 4.10 shows the acquisition of the novel cage activity test , by distance travelled in all the five-minute intervals, for all susceptibility groups (2 x "sex" and 2 x "EAE"). MANOVAs analyses show significant "EAE" and "Sex" effects, indicating that EAE "susceptible" rats are overall more disinhibited (i.e. display higher levels of activity/distance in the novel-cage test) than "resistant" rats, while it also means that females are generally more active than males (see MANOVAs in Fig. 4.10).

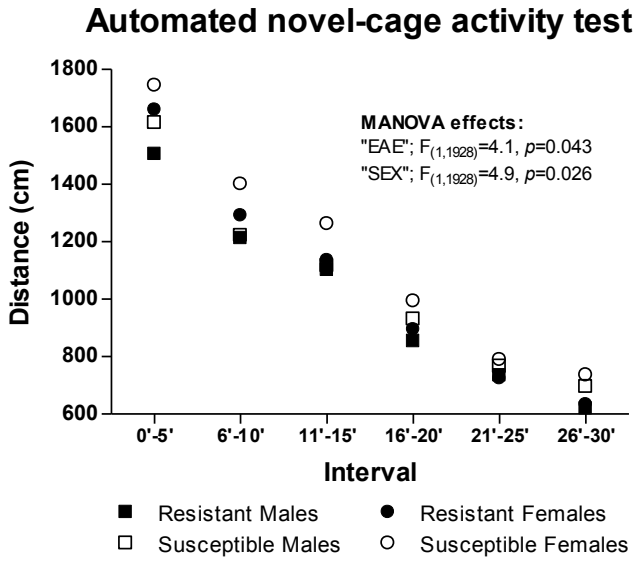


Figure 4.10.- Scores (means ± S.E.M.) in the automated novel-cage test. (A) distance travelled within the first 5 minutes, (B) distance travelled during the last 5 minutes, (C) total distance travelled. (Manovas for: "Resistant males", n= 722 ; "Susceptible males", n= 245; "resistant females", n= 752; "susceptible females", n= 246).

Figure 4.11 shows the differences differences between groups on fear/freezing and conditioned-anxiety. Regarding “freezing” at the beginning of the test (Fig. 4.11A), there was a significant “sex” effect [$F_{(1,2004)}$=62.396, p<0.001] with males displaying a higher freezing behaviour. Concerning the number of crossings in exploration time (Fig. 4.11B), there was a “sex” effect [$F_{(1,2004)}$=5.865, p=0.016], as females performed more crossings, as well as an “EAE” effect [$F_{(1,389)}$=20.119, p<0.001], with susceptible rats displaying higher number of crossings. Furthermore, there was a marked “sex” effect in several conditioned-anxiety variables, such as number of avoidances in the whole session [$F_{(1,2004)}$=9.874, p =0.002; Fig. 3.11C] and mean latency to escape [$F_{(1,2004)}$=242.375, p=0.002; Fig. 3.11D], with females showing the best conditioning acquisition of the task (and less anxiety).

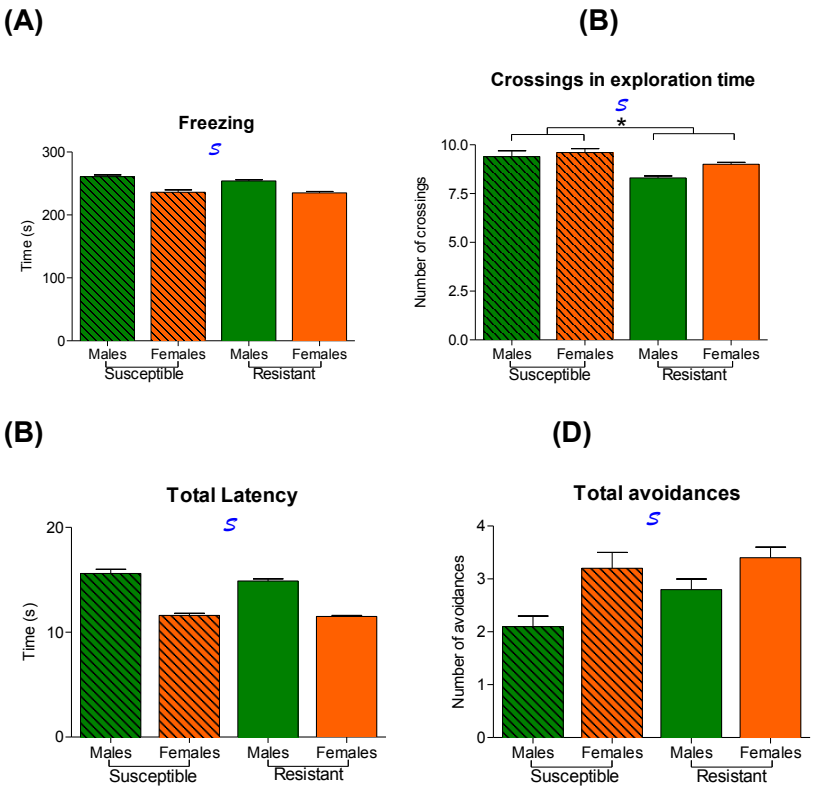


Figure 4.10.- Scores (means ± S.E.M.) in two-way shuttlebox avoidance conditioning. (A) time spent doing freezing, (B) total number of crossings in exploration time, (C) total latency, (D)number of total avoidances. (Groups: "Resistant males", n= 722 ; "Susceptible males", n= 245; "resistant females", n= 752; "susceptible females", n= 246). * p <0.05, "EAE" effect; **S** p <0.05, "sex" effect; ≠ p <0.05, Duncan's post-hoc after significant ANOVA, the marked group is different from all the others.

Differences in adrenal weight of "resistant" and "susceptible" HS rats

Once ruled out any possible difference of laterality between each pair of adrenal glands (data not shown), it was done a mean of both adrenal glands weight (AW= absolute adrenal weight) as well as the relative adrenal weight (RAW= (AW/Body weigh)*100). Figure 4.12 represents the differences between groups regarding the relative adrenal weight, and there was a significant "sex" effect [$F_{(1,2004)}$=1088.502, p<0.001], as females showed heavier adrenals, and also a significant "EAE" effect [$F_{(1,389)}$=124.611, p<0.001], with susceptible rats showing higher weight.

In order to observe the associations between the relative adrenal weight and the most relevant measures of EAE a Pearson's correlation coefficient was applied to EAE-susceptible males and females (Table 4.12). Correlations show: 1) moderate to high correlations among EAE measures (males: from r=0.32 to r=0.94; females: from r=0.51 to r=0.92), and 2) low to moderate correlations between relative adrenal weight and EAE measures (males: from r=0.28 to r=0.50; females: from r=0.34 to r=0.45).

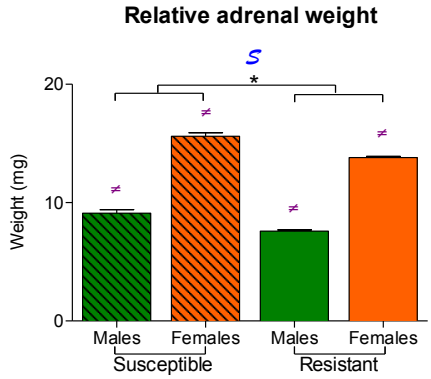


Figure 4.12.- Relative adrenal weight is represented by means ± S.E.M. (Groups: "Resistant males", n= 722 ; "Susceptible males", n= 245; "resistant females", n= 752; "susceptible females", n= 246) * p <0.05, "EAE" effect; **S** p <0.05, "sex" effect; ≠ p <0.05, Duncan's post-hoc after significant ANOVA, the marked group is different from all the others.

Correlation matrix between RAW and EAE

	RAW	MAX	DUR	CUM
Susceptible males				
RAW	1			
MAX	.501**	1		
DUR	.281**	.324**	1	
CUM	.348**	.481**	.941**	1
Susceptible females				
RAW	1			
MAX	.451**	1		
DUR	.338**	.518**	1	
CUM	.391**	.701**	.917**	1

Table 4.12.- Correlations ≥.28 among the behavioural variables are shown. ** p <0.001, Pearson's correlation coefficient.

4.5 DISCUSSION

BEHAVIOURAL CHARACTERIZATION OF N/Nih-HS RATS

As shown in our previous work (*López*-Aumatell *et al.,* 2008), and consistent with the literature (see Aguilar *et al.,* 2003; *López*-Aumatell *et al.,* 2011; Vicens-Costa *et al.,* 2011), females show significantly less signs of unconditioned anxiety/fearfulness and higher exploratory drive than males. Likewise, in variables related to learned anxiety or fear, females also show less signs of behavioural inhibition. Thus these sex differences appear, respectively, in variables or responses supposed to reflect unconditioned anxiety or fearfulness, as these measured in the elevated zero-maze test and in the novel-cage test during the initial five minutes (i.e. the automated novel-cage activity test; see (*López*-Aumatell *et al.,* 2008, 2009), and in conditioned responses in the shuttle box task, i.e. conditioned fear (i.e. context-conditioned freezing during the initial stages of the task) and conditioned two-way avoidance acquisition (as indicated by total avoidances and mean response latency in the whole session). These tests, particularly the elevated zero-maze and the acquisition of two-way active avoidance, are well-validated measures of unconditioned anxiety and conditioned anxiety/fear, respectively (see Fernández-Teruel *et al.,* 1991; *López*-Aumatell *et al.,* 2008, 2009; Pähkla *et al.,* 2000; Shepherd *et al.,* 1994). The measure of context-conditioned freezing/fear is also relevant, because similar procedures are used in humans to study “pavlovian”/classical aversive conditioning (even if in human studies the usual dependent variable is not freezing, but for example skin conductance, heart rate changes or startle responses), and because classical aversive conditioning shares common neuroanatomical bases in different species (e.g. Gray and McNaughton, 2000; Davis and Whalen, 2001; LeDoux, 2000).

Exploration of a novel, open field-like environment (i.e. the "novel-cage" activity test), has been traditionally considered as related to fearfulness (i.e. the lower exploration, the higher the level of fearfulness; see, for instance, Aguilar *et al.,* 2002; Escorihuela *et al.,* 1999; Fernandez-Teruel *et al.,* 1992; Gray, 1981), a contention which is also supported from our previous work showing associations between activity during 5 minutes in the novel cage and typical anxiety responses in the light-dark test and the elevated zero-maze test (see *López*-Aumatell *et al.,* 2008, 2009).

The results of the present study are also consistent with previous multitest studies of fearfulness in rodents (for review see, for instance, Aguilar *et al.,* 2002, 2003; Prunell *et al.,* 1994; Ramos and *Mormède* 1998), as refers to the fact that significant across-tests correlations exist but they are generally of not very high magnitude, and also because factor analysis shows a multidimensional structure of anxiety/fear-related behaviors in our rat sample (see below). Still, despite that pattern of low correlations, it is nevertheless outstanding that they actually appear consistent: with the sign that could be expected from the hypothesis that some of these different measures of fearfulness should share some common components (see Table 4.3A-B). Thus, for instance, there are low but significant correlations (ranging 0.08 to 0.12) between some variables of the elevated zero-maze test and those reflecting acquisition of the two-way active avoidance task, indicating that relatively lower unconditioned anxiety in the zero-maze test is associated to a better acquisition of the two-way avoidance task (e.g. see correlations between SAP and/or HD variables of the zero-maze test and AVOID40 or LAT40 in Table 4.3A-B). On the other hand, still higher correlations (ranging from 0.1 to 0.2) appear among variables from the initial 5 minutes of exposure to the novel-cage (activity test) and anxiety measures from the elevated

zero-maze test, consistently indicating that the higher the activity levels during the first 5 minutes in the novel cage the lower the anxiety level observed in the zero-maze test (i.e. higher levels of "SAP", "Entries", "Time", etc.; see Table 4.3A-B). These results are in close agreement with those already reported from our group using other rat samples (*López*-Aumatell *et al.,* 2008, 2009, 2011; Vicens-Costa *et al.,* 2011).

Furthermore, the present factorial results (Table 4.4) lend general support to the abovementioned associations, while at the same time simplifying the structure of the correlation matrix and giving further conceptual meaning to it. Thus, anxiety in the elevated zero-maze, conditioned fear/freezing and shuttle box avoidance acquisition are those loading on the first factor in males (it could appear to be a "Conflict/anxiety" factor), while the second factor retains only (novel-cage) activity measures. Conversely, activity measures from the novel-cage test load jointly with zero-maze test variables on the first factor in females (it could be described as a "flight/disinhibition" factor), while the second factor is dominated by shuttle box measures and a lower loading of "Entries in the open sections" of the zero-maze. Therefore, these factor analyses bring evidence on some between-sex differences in the factor structure underlying the anxiety/fearfulness traits of male and female N/Nih-HS rats.

Previous results from factor-analytical studies have suggested that females' responses in unconditioned anxiety-related tests (e.g. the elevated plus-maze, the hole-board; see (Fernandes *et al.,* 1999; Johnston and File, 1991; but see also Aguilar *et al.,* 2003) might be predominantly influenced by locomotor activity (i.e. tendency to "flight" responses), whereas males' behaviour would appear to be more

dependent on anxiety (i.e. tendency to "freezing" responses when facing a conflict). The present factorial results appear to lend support to that, as "conflict" appears to dominate the first factor in males while "activity-related responses" seem to be relatively more important in the first factor in females. It remains possible that our present sex differences are importantly modulated through these divergences in activity-driven behaviour between females and males, an issue that should be evaluated by using tests or tasks not dependent upon locomotor activity. It is worth noting, in this context, the finding that females from the N/Nih-HS rat stock and from other strains have been even found to be more anxious/fearful than males in tests which do not depend on locomotor activity, such as the baseline acoustic startle response and the context-conditioned acoustic startle response (*López*-Aumatell *et al.,* 2008; Aguilar *et al.,* 2003).

ASSOCIATIONS AMONG DIFFERENT ANXIETY MEASURES/RESPONSES

To further explore associations (or even relationships) among anxiety/fear variables across the different tests we performed comparisons between subgroups showing extreme values in relevant variables (Tables 4.5 – 4.10). The first four tables (Table 4.5 – 4.8) generally and consistently show that, regardless the selection anxiety variable (i.e. SAP, Entries, Time or HD), rats displaying increased anxiety (in the elevated zero-maze) according to any of those 4 selection variables will also show enhanced anxiety in the remaining dependent measures of the same test as well as in "Dis0-5" (novel-cage activity test: activity in the first 5 minutes of exposure) and "CET" (free exploration of the shuttle box before the conditioning session).

Remarkably, the selection for extreme values in “SAP” or “HD” in the elevated zero-maze leads to selection of two-way avoidance acquisition ability, i.e. the subgroups of “Superior SAP” or “Superior HD” show significantly better acquisition of the two-way avoidance task (see Table 4.5 A-B and 4.8 A-B) than the respective “Inferior SAP” or “Inferior HD” subgroups. It is worth noting that the selection for extreme values in “Entries” and “Time” (elevated zero-maze) led to differential context-conditioned “Freezing”, i.e. the higher the “Entries” or “Time” the lower the “Freezing” levels (see Table 4.6 A-B and 4.7 A-B).

On the other hand, and mostly in congruency with the abovementioned results, selection for extremes in context-conditioned “Freezing” led to differential unconditioned anxious behaviour in the elevated zero-maze and conditioned anxiety (AV40 and LAT40 variables) in the shuttle box task. This is illustrated by the fact that the “Superior Freezing” subsample displays relatively higher signs of unconditioned anxiety, as reflected by reduced values of “Entries”, “Time” and “Line crossings” in the elevated zero-maze test, and impaired acquisition of the two-way avoidance task (see AV40, LAT40 and ITC40 variables; Table 3.9 A-B). Still in line with these results, selection by extreme values in “AV40” (total number of avoidances in the two-way avoidance session) led mainly to selection of “SAP” and “HD” behaviours from the elevated zero-maze (see Table 4.10 A-B), as well as to differential levels of context-conditioned “Freezing” in the shuttle box (Table 4.10 A-B).

To sum up, among the main general findings of the present study it is noteworthy that unconditioned anxiety responses (as measured in the zero-maze test) show a significant degree of predictive capacity over conditioned fear- (i.e. context-conditioned freezing) or anxiety-related (two-way avoidance acquisition) responses, while these conditioned

fear/anxiety measures are able, in turn, to predict unconditioned anxious responses in a hypothetically congruent direction.

The results of selection by extreme values in conditioned fear deserve further mention, as it is outstanding that context-conditioned freezing (i.e. classically conditioned fear to a context) is an important negative predictor of the ability that a rat will show to solve the double "passive avoidance/active avoidance" conflict, which is prominent during the initial phases of the two-way active avoidance task. It has been a long-standing contention that such a (passive avoidance/active avoidance) conflict during the initial stages of acquisition involves high levels of anxiety and a dominant tendency for freezing responses which run against the appearance of active escape/avoidance behavior (Fernandez-Teruel *et al.,* 1991a-b; Gray *et al.,* 1981; Gray and McNaughton 2000; Weiss *et al.,* 1968; Wilcock, J. and Fulker, D.W. 1973). In support of that, anxiolytic drugs (which decrease conditioned freezing) improve, and anxiogenic drugs (which increase conditioned freezing) impair, two-way avoidance acquisition, septohippocampal lesions improve two-way avoidance acquisition and several anxiety-reducing environmental treatments clearly and positively affect the acquisition of the two-way avoidance task (Criswell *et al* 1993; Escorihuela *et al.,* 1993, 1994, 1995; Fernandez-Teruel et el 1988; 1991a-c; Gray, 1982; Gray and McNaughton 2000; Prunell *et al.,* 1994a-b; Savic *et al.,* 2005).

However, testing the contention that the initial conditioned freezing/fear was predictive of avoidance acquisition means that freezing had to be measured in the very beginning of the two-way avoidance task and in the same shuttle box apparatus were the training session is being ran (Fernandez-Teruel *et al*, 1991a-b; Gray, 1981), a type of study that have not been performed thus far (except for a preliminary study from

our laboratory which used a small rat sample; Vicens-Costa *et al.,* 2011). In summary, what is remarkable from the present study is the finding of a significantly impaired acquisition of two-way active avoidance in a very large sample of N/Nih-HS rats displaying increased levels of context conditioned freezing during the first five inter-trial intervals of the two-way avoidance session, as compared to rats displaying lower freezing levels (which show better acquisition of the task). This indicates that context-conditioned fear is a relevant process at the beginning of such a conflict-driven task which (at least partly) influences acquisition, thus suggesting that neurobiological mechanisms underlying both processes (i.e. context-conditioned fear and actual two-way avoidance responses/acquisition) should share at least some common aspects, an idea that has already been proposed by J.A. Gray (Gray 1982; Gray and McNaughton 2000). In this regard, it is well-known, for instance, that septo-hippocampal lesions attenuate context-conditioned freezing and improve two-way active avoidance acquisition (e.g. Gray and McNaughton 2000). There is however, evidence on divergences between the neural mechanisms governing contextual fear-conditioning and conditioned anxiety-related (i.e. avoidances) responses: basolateral and central amygdala lesions or inactivation (by injection of NMDA -N-methyl-D-aspartate- antagonists) impair acquisition of two-way shuttle box avoidance (Savonenko *et al.,* 1999; Werka 1997; Werka and Zielinski 1998). It has been reported that such disruptions of amygdala function impair shuttle box avoidance acquisition by deteriorating the directionality of escape responses and the attentional reactions to the conditioned stimulus (i.e. to the fear cue) (Savonenko *et al.,* 1999). But the treatment does not affect contextual fear as measured by freezing responses to the context during the intertrial intervals of shuttle box training (Savonenko *et al.,* 1999).

To summarize, the anxiety- (conflict) and fear-driven acquisition of two-way active avoidance is negatively influenced by unconditioned anxiety levels and, still more clearly, it is negatively influenced (i.e. it is impaired) by context conditioned fear/freezing occurring during the very early stages of the task.

Anxiety or fearfulness (as measured in laboratory rodents) are not unitary nor simple processes, but complex traits involving different subtypes of behavioral/psychological dimensions which in turn are likely to involve (at least partly) different neurobiological and genetic mechanisms (e.g. Fernández-Teruel *et al.,* 2002; Gray and McNaughton, 2000; Ramos and Mormède, 1998; Aguilar *et al.,* 2002).

The utility of the N/Nih-HS -HS- rats for dissecting EAE and associations with anxiety trait profiles

The HS population is genetically much more diverse and complex than classical inbred crosses (usually derived from only two strains) and has a wider range of phenotypes. This experimental population even attempts to mimic features of a human population: a considerable advantage of the HS, compared to the AIL –congenic- rats, is that smaller chromosomal regions are linked to disease/phenotype in a system that more closely resembles a natural population –as HS rats derive from 8 inbred rat strains, rather than 2 strains, as is the case of AIL rats-.

One particular concern was whether the HS could be used for dissecting EAE, because it contains several MHC types. This could potentially reduce the power for detecting non-MHC QTLs. These QTLs

are the primary target for the HS studies since the MHC complex, and in particular the class II genes, are well characterized and studied by other means. Based on our findings and those reported in literature regarding EAE in the founder strains, we expected the HS to show variation in EAE susceptibility (Becanovic *et al.,* 2006; Dahlman *et al.,* 1999; Goldmuntz *et al.,* 1993; Levine *et al.,* 1965; Stefferl *et al.,* 1999; Sun *et al.,* 1999; Weissert *et al.,* 1998). However, it was important to perform a pilot study with the intended EAE model to establish if there was enough variation in disease outcome depending on non-MHC genes (Johannesson *et al.,* 2009). Thus, to ensure that influence from non-MHC genes could be mapped in this population, we performed a study on 25 rats to determine the variance of phenotypes between and within MHC types. Two homozygous MHC groups could be identified (AV1 and L), while the other group contains MHC heterozygotes. The large phenotype variation within the various MHC haplotype groups strongly suggested influence of non-MHC genes that can be mapped in the NIH-HS, which is why we went ahead and phenotyped EAE also in the bigger NIH-HS experiment, using the present 2000 HS rats (Johanesson *et al.,* 2009). As already mentioned in this Thesis (see "General Introduction"), provided that a enough "n" is used the gene recombination level of HS rats makes it possible to identify QTLs of less than 1 cM, which may in turn allow gene identification for some regions and phenotypes (Yalcin *et al.,* 2004). Another advantage of this procedure is the capacity to elucidate small-effect QTLs, explaining even less than 2% of the variance (Flint, 2004; Mott *et al.,* 2000; Mott and Flint, 2002; Valdar *et al.,* 2006a). A third advantage is that epistatic interactions and gene-environment interactions can be evaluated (Valdar *et al.,* 2006a-b).

Based on our previous pilot study (Johannesson *et al.,* 2009) we expected around 40% of incidence in the general HS rat sample.

However, we have found a 25% of incidence. Moreover, even though there were slight between-sex differences in severity (duration), as females presented a tendency to show longer disease course than males (p=0.05; see Table 4.2), there were not gender differences in incidence nor in other disease parameters. This result is striking, as the literature regarding EAE incidence and severity very robustly shows that females are more sensitive to neuroinflammation, including MOG-EAE (e.g. Heesen *et al.,* 2007; Kokras *et al.,* 2011; Massella *et al.,* 2012).

A really outstanding result from the present study has been the consistent associations found between anxiety levels and EAE incidence. Thus, when comparing subgroups of rats showing extreme anxiety levels (and regardless of sex) in the elevated zero-maze, all the "superior" groups (selected by extremely high values in SAP, "Entries ZM", "Time ZM" and "Head dips ZM"; see Fig. 4.2), i.e. the relatively low-anxious groups, showed an EAE incidence close to 30%, while the "inferior" (i.e. high-anxious) groups showed approximately a 15-20% of EAE incidence (see chi-square significant differences in the four cases in Fig. 4.2). Confirming these results, the four "Superior" (less anxious) groups present a "Disease level" progression across days which indicates significantly increased EAE severity as compared to the respective "Inferior" (relatively high anxious) subgroups (see Fig. 4.3A, 4.4A, 4.5A, 4.6A). That's to say, the less anxious animals showed a significantly increased MOG-EAE incidence and severity progression (across days) in comparison to the high anxious animals, i.e. a relatively higher level of unconditioned anxiety appears to have a protective effect on MOG-EAE susceptibility.

Still lending further support to that contention, when taking into account only the EAE-affected rats our results indicated that the relatively less

anxious rats, particularly the “Superior SAP” and “Superior HD” (elevated zero-maze variables; see Fig. 4.3B and 4.6B) rat subgroups, displayed significantly higher “disease level” across days, as indicated by the displacement of the “disease level” curves to the left in the “Superior SAP” and “Superior HD” groups as compared to the respective “Inferior” groups (Fig. 4.3B and 4.6B), i.e. the low anxious rats reached higher levels of disease severity earlier than the relatively high anxious rats (Fig. 4.3B and 4.6B).

Additionally, and also providing support to the above mentioned results, logistic regression analyses indicated that EAE incidence in males was positively predicted by the anxiety variables SAP, “Time” and CET, while SAP was also the best predictor of EAE incidence in females (see Table 4.9).

As said above, the systematic “relatively low anxiety –> relatively high EAE incidence” association is striking, but it appears to be very consistent, provided the different statistical tests applied and the large “n” used. Moreover, such an “anxiety-EAE” association is further strengthened by the findings showing that selection of rats as a function of being EAE-susceptible or EAE-resistant leads to completely congruent selection of anxiety levels, that is to say, the “Susceptible” rats present significantly lower anxiety levels according to all the zero-maze test variables (Fig. 4.8A-F) and are overall more disinhibited (i.e. activity in the novel-cage test –Fig. 4.9- and in the “exploration time in the shuttle box” –Fig. 4.10B-) than the “Resistant” rats.

Anxiety is associated with EAE: Which could be the neurobiological mechanisms underlying such an association?

As we are facing an emotional process (as measured in an unconditioned animal model of anxiety) on one side, and an autoimmunological process on the other side, and provided that we have not measured truly "intermediate" neurobiological processes which could establish some link between the two studied processes, it becomes obvious that our discussion on some possible links has to rely on hypothetical intermediate mechanisms (which should, in any case, be tested in further specific studies). One of such hypothetical neurobiological mechanisms is "stress", as far as it is a state/response which is known to be associated to anxiety-related states and traits (for reviews see, for instance, Herrero *et al.,* 2006; Sandi and Richter-Levin 2009).

In fact, there is a considerable body of evidence indicating that anxiety responses in rats are positively associated to (or paralleled by) stress hormone responses, especially of the HPA-axis. Evidence in support of this indicates that: i) psychogenetically-selected rat lines/strains presenting divergent trait-anxiety profiles also present congruent HPA-axis responses to stress, i.e. as compared to low anxious animals, the relatively anxious rat lines/strains present enhanced HPA-axis responses to stress (e.g. Carrasco *et al.,* 2008; Driscoll *et al.,* 2009; Landgraf and Wigger 2002; Neumann *et al.,* 1998; see review by Sandi and Richter-Levin 2009); ii) anxiolytic-like environmental treatments, like neonatal handling or environmental enrichment, reduce HPA-axis responses to stress in parallel to a reduction of anxiety responses (e.g. Fernandez-Teruel *et al.,* 2002; Levine 1968 ; Meaney *et al.,* 1988; Núñez *et al.,* 1996; Steimer *et al.,* 1998; Peña *et al.,* 2009); iii)

subsamples of rats -from a given strin- showing elevated anxiety also show increased stress-induced HPA-axis responses (Herrero *et al.,* 2006; Salehi *et al.,* 2010; Sandi and Richter-Levin 2009 for review); iv) in other samples of N/Nih-HS rats we have previously found associations (through factor analyses) between post-stress corticosterone levels and unconditioned anxiety measured in several tests, indicating that relatively higher anxiety levels correspond to more elevated post-stress corticosterone responses (Díaz-Morán *et al.,* 2012).

All the above evidence enables us to make the logical assumption that, in the present study, "high anxious" HS rats should be expected to display higher HPA-axis responses to stress than "low anxious" HS rats (Diaz-Morán *et al.,* 2012; Sandi and Richter-Levin 2009), that is to say, our "high anxious" HS rats would be relatively "more stressed" rats as compared to the "low anxious" HS animals (Diaz-Morán *et al.,* 2012; Herrero *et al.,* 2006; Sandi and Richter-Levin 2009).

The previous reasoning brings us to the next question, i.e. what underlying mechanisms could be involved in differential EAE susceptibility as a function of differential anxiety or stress sensitivity?

In a pioneering work, Levine *et al.* (1962) were the first to address such a question. Assuming that stress might increase the resistance to immune-mediated diseases, as stress should increase glucocorticoid levels (and so their immunosuppressive capacity), those authors demonstrated that chronic stress prior to EAE induction reduced the incidence and severity of the disease (Levine and Wenk*,* 1961; Levine and Saltzman, 1987). Further studies involving HPA-axis function/manipulation and its effects on EAE in rats tend to give support to that contention, as moderate stress or increases in HPA-axis function

have been generally shown to suppress or to attenuate EAE severity when administered before induction (e.g. for review see Heesen *et al*. 2007; Levine and Wenk, 1961; MacPhee *et al*. 1989; Stefferl *et al*. 1999). Thus, these results are consistent with the immunosupressive effects of glucocorticoids (and their therapeutic effects on neuroinflammatory diseases) and with the current concept that an enhanced HPA-axis function can be protective against EAE in animals and MS in humans (e.g. for review see Heesen *et al*. 2007; Levine *et al.,* 1961; MacPhee *et al*. 1989; Stefferl *et al*. 1999).

Therefore, in light of the above evidence, it now appears congruent that the present “high anxious” (as said, presumably “more stressed”) HS rats are relatively protected against MOG-EAE as compared with the “low anxious” (“less stressed”) HS rats. Even if this conclusion appears quite (or the most) logical, we have to remind that further work is deserved to better and more exhaustively elucidate the nature and mechanisms of the present “Anxiety – EAE” relationship.

STUDY II.

INBRED STRAINS: ANXIETY AND STRESS CHARACTERIZATION

5 STUDY II. INBRED STRAINS: ANXIETY AND STRESS CHARACTERIZATION

5.1 INTRODUCTION

Multiple sclerosis (MS) is a heterogeneous inflammatory and neurodegenerative disease with a proposed autoimmune aetiology (Hemmer *et al.,* 2002; McQualter and Bernard, 2007; Sospedra and Martin, 2005). The pathological hallmarks are perivascular inflammation, demyelination and axonal loss in the CNS. This chronic, disabling disease of the central nervous system (CNS), affects more than two million people worldwide. Experimental autoimmune encephalomyelitis (EAE) is an autoimmune neuroinflammatory disease with clinical and pathological similarities to MS (Wallström, 2007). However, while several models of EAE exist, they only mimic certain aspects of MS and the discussion of its value in understanding MS is still ongoing (Steinman and Zamvil, 2006). Factors such as the mode of induction, the genetic constitution, and the myelin autoantigen used, age, weight, and sex influence the outcome shape the clinical course and the histopathological and immunological features of MS captured. There is a great need for more efficient and safe MS treatments, which requires a better understanding of disease mechanisms. Therefore, disease appropriate animal models are indispensable for further progress. Indeed, the most recent treatments approved for MS have been developed in EAE, demonstrating its predictive value when appropriately applied (Becanovic *et al* 2006).

Due to the extreme similarities in pathogenesis, the study of the genetic regulation of MOG-induced EAE in rats is aimed to achieve advances in the study of MS in humans (Wallström, 2007). Rats are immunized with

MOG, which is a minor glycoprotein exposed on the surface of the myelin sheath, to induce the EAE disease. In addition to the pathology described above, this model involves demyelinating plaques and glial scar formation, also apparent in MS (Breij *et al.,* 2008; Lucchinetti *et al.,* 2000; Storch *et al.,* 1998; Weissert *et al.,* 1998). Immunologically, there are signs of activation of both cellular and humoral anti-MOG specific response, which is also reminiscent of MS, where both T- and B-cell responses to myelin antigens are present (Olsson, 1992; Steinman, 1996).

Inbred rats show varying susceptibility to MOG-EAE, demonstrating a difference dependent on genetic regulation. Consistent with MS, the MHC locus (HLA in human) is the strongest susceptibility locus in EAE (Weissert *et al.,* 1998). Indeed, when rats face the same MOG challenge, the MHC haplotypes determine the severity of subsequent disease (Weissert *et al.,* 1998). With this model, several non-MHC genome regions have been identified that control either clinical susceptibility or severity. Thus, loci that contribute to EAE with smaller effects have been found in several crosses from inbred rats (Dahlman *et al.,* 1999a-b; Jagodic *et al.,* 2001; Becanovic *et al.,* 2003), showing that the polygenic nature of MS is captured in the MOG-EAE model.

In this second study, we have used EAE-susceptible DA and EAE-resistant PVG strains. As these strains are well characterized by the MOG-EAE model (Stridh, 2010; Stridh *et al.,* 2010), we have not immunized them. Typically, the DA debuts with clinical symptoms around two weeks after immunization and presents a relapsing-remitting EAE (see “General Introduction”; Wallström, 2007). Conversely, PVG rats are resistant to MOG-EAE.

The search for physiological/neural processes which could modulate EAE (in animals) or MS (in humans) led to very early –and pioneer- results when Levine *et al.* (1962), assuming that stress might increase the resistance to immune-mediated diseases (provided that stress should increase glucocorticoid levels), demonstrated that chronic stress prior to EAE induction reduced the incidence and severity of the disease (Levine and Wenk, 1961; Levine and Saltzman, 1987).

Further studies involving HPA-axis function/manipulation and its effects on EAE in rats tend to give support to that contention, as moderate stress or increases in HPA-axis function have been generally shown to suppress or to attenuate EAE severity when administered before induction (e.g. for review see Heesen *et al.,* 2007; Levine and Wenk, 1961; McPhee *et al.,* 1989; Stefferl *et al.,* 1999). Thus, these results are consistent with the immunosupressive effects of glucocorticoids (and their therapeutic effects on neuroinflammatory diseases) and with the current concept that an enhanced HPA-axis function can be protective against EAE in animals and MS in humans (e.g. for review see Heesen *et al.,* 2007; Levine and Wenk, 1961; McPhee *et al.,* 1989; Stefferl *et al.,* 1999).

The results obtained in “Study I” suggest that anxiety has a relationship with the susceptibility/resistance to MOG-EAE, i.e. N/Nih-HS rats that never got EAE (i.e. EAE-resistant rats) are more anxious in several unconditioned anxiety-related variables than EAE-susceptible rats. No hormonal measures were taken in “Study I” but, provided that relatively elevated anxiety levels in that type of behavioural tests are commonly associated with an enhanced HPA-axis response to stress (e.g. Díaz-Morán *et al.,* 2011; López-Aumatell *et al.,* 2009; Núñez *et al.,* 1996; Steimer *et al.,* 1998), these results can be interpreted as indicating that relatively high anxious -and EAE-resistant- N/Nih-HS rats might

present increased HPA-axis responses. Results from our laboratory tend to give support to that, by showing relatively higher corticosterone levels in the "low-exploring" or "high-anxious" N/Nih-HS rats (Díaz-Morán *et al.,* 2011, and unpublished data).

Taking all the previous into account we could propose the following hypothesis: if an increased HPA-axis response is related with enhanced anxiety, and if both were associated with higher resistance to EAE, the EAE-resistant PVG rat strain should present enhanced HPA-axis and anxious responses as compared with the EAE-susceptible DA rat strain.

Despite they are two strains of reference for the study of EAE (and other inflammatory diseases) at the genetic level, DA and PVG rats have not been compared thus far with regard to their respective stress hormone responses nor with respect to their anxiety/fear behavioural profiles, in spite that such a hormonal and behavioural evaluation could provide relevant insight as to what other neurobiological processes are different between both strains.

Therefore, in the present "Study II" we aim to achieve a characterization of DA and PVG rats with regards to their levels of (unconditioned and conditioned) anxiety and learned fear. Further, we aim to evaluate their basal and post-stress corticosterone levels. As said above, the assumption is that the susceptible DA rats should show lower anxiety and stress hormone responses than the resistant PVG rats.

Moreover, provided their divergent "pro-inflammatory" profiles/traits, i.e. their differential susceptibility to MOG-EAE, we aimed at studying the relative expression of the inflammation-related markers CD74 and IL-6 in both rat strains. In this regard, it is known that the "MHC class II-

associated invariant chain", also known as CD74, is involved in the communication between dendritic cells (the so-called "sentinels of the immune system") and microglia to mediate inflammation of the CNS, and participates in several key processes of the immune system, including B-cell differentiation, T-cell selection and inflammatory signalling (Beswick *et al.,* 2005; Faure-Andre *et al.,* 2008; Leng *et al.,* 2003; Matza *et al.,* 2003; Segura *et al.,* 2005, Stumptner-Cuvelette and Benaroch, 2002; Ye *et al.,* 2008; for review see Borghese and Clanchy, 2011). On the other hand, inflammatory cytokines, especially IL-6 – interleukin 6- (but also IL-1 and TNF-α), are secreted by several cells (including monocytes, macrophages, astrocytes, and others) in response to infectious stimuli and are involved in inflammation, in a way that they activate HPA-axis function (e.g. Chrousos, 2000). In line with that, CD74 has been found to be over-expressed in several forms of inflammation and autoimmune diseases (or disease models), including atherosclerosis and possibly MS (for review see Borghese and Clanchy 2011). Similarly, increased expression of CD74 and IL-6, in spinal cord and lymph nodes (respectively), was found in EAE-susceptible DA rats with respect to resistant PVG rats (Thesen *et al.,* 2009).

Thus, in order to evaluate baseline pro-inflammatory parameters of our DA vs PVG rats, in the present "Study II" we measured the expression of CD74 and IL-6 (see more about it in "Introduction") in hypothalamus, pituitary gland and adrenals. Our hypothesis was that the EAE-susceptible DA rats would show increased expression levels of those two inflammation-related factors.

MATERIAL AND METHODS

5.2 MATERIAL AND METHODS

5.2.1 Animals

Subjects used in this study were 22 susceptible DA male and female rats and 27 resistant PVG.1AV1 male and female rats obtained from the Neuroimmunology Unit (Karolinska Institutet, Sweden), where they were bred and maintained until their arrival to our laboratory (Department of Psychiatry and Forensic Medicine, Autonomous University of Barcelona).

Since then, animals were paired in macrolon® cages (50 cm x 25 cm x 14 cm) and maintained with food and tap water available ad lib, under conditions of controlled temperature (22 + 2ºC) and a 12h light-dark cycle (lights on at 08:00h). Weights were monitored during the first 15 days, as well as the body weight gain over the experimental period. Rats were 2 months old at the beginning of the experiments (weight: 150-300 g).

5.2.2 Procedure and apparatus

Experiments were performed during the light cycle between 09:00 and 19:00h, and in accordance with the Spanish legislation on “Protection of Animals Used for Experimental and Other Scientific Purposes” and the European Communities Council Directive (86/609/EEC) on this subject. Approximately 2-3 weeks elapsed between consecutive behavioural

tests. Three behavioural tests were administered along a 5-6-week period for each of the 2 batches (with n≤ 50 rats/batch, approximately half of each sex).

Experiments were performed during the light cycle, between 09:00h and 19:00h in accordance with the Spanish legislation on "Protection of Animals Used for Experimental and Other Scientific Purposes" and the European Communities Council Directive (86/609/EEC) on this subject. The sequence and the characteristics of the tests were as follows:

DAY	**0**	**7**	**14**	**24**
TEST	Basal Cort	ZM	SH	• 20-min NACT • Post-stress Cort • Harvesting

Table 5.1.- Schedule of study II.

CORTICOSTERONE

Blood collection procedure

The blood collection procedures were always done in the morning between 9:30 a.m. and 1:00 p.m., when resting and stress levels of HPA hormones are very stable. Samples were taken by a tail nick that consisted of gently wrapping the animals with a cloth, making a 2 mm incision at the end of the tail veins and then massaging the tail while collecting, within 2 min, 300 ml of blood into ice-cold EDTA capillary tubes (Starsted, Granollers, Spain). Blood samples were centrifuged at 3000rpm for 10 min at 4ºC. The plasma was stored at -20ºC until it was

sent to the Veterinary Hematology Service to determine the corticosterone levels by RIA.

This procedure has been extensively used (Garcia *et al*., 2000; Gagliano *et al*., 2008) due to the obtained levels of hormones are similar to those obtained after decapitation without anesthesia (Vahl *et al*., 2005). During the next week, wounds were carefully inspected to rule out infections and to assure that rats were in good health.

Day 0. Basal corticosterone levels

Starting 15 days after their arrival to our lab, naive rats were bleeded following the aforementioned procedure.

Day 24. Poststress levels of corticosterone

Blood samples were taken immediately after the 20 min in the automated novel-cage activity to evaluate HPA responsiveness to a mild stressor. Blood collection was performed following the same procedure.

Biochemical analysis

Plasma corticosterone levels were determined by double-antibody radioimmunoassay (RIA) procedures currently used at the Veterinary Hematology Service (Servei d'Hematologia Clínica Veterinària, Universitat Autònoma de Barcelona) using *ELISA (enzyme-linked immunosorbent assay)* in sera and supernatants. The labeled antigen was Corticosterone EIA –Immunodiagnostic Systems ITD, IDS Ltd; Boldon, UK.

BEHAVIOURAL TESTS

Elevated zero- maze (ZM)

It was applied an identical procedure than Study I (see “3.2.2, Study I”).

Automated novel-cage activity (NACT)

The apparatus (Panlab, Barcelona, Spain) consisted of a horizontal surface (50 x 50 cm) provided with photobeams that detect and measure movement automatically, loading the data in a computer. The subjects were placed in transparent plexiglas cages (40x40x40 cm). They were situated in a white fluorescent (60 w) illuminated chamber. Spontaneous horizontal activity was measured for 30 minutes (Dis 0-20), of which we took for analyses the activity scores of the first 5 minutes (Dis 0-5*; as a measure of novelty-induced –open field-like-activity)* and of the last 5 minutes (Dis 15-20; as a measure of habituated, or less novelty-affected, activity).

Two-way active, shuttle box avoidance acquisition (SH) and context-conditioned freezing (fear)

It was applied an identical procedure than Study I (see Section 4.2.2).

Tissue dissection of inbred rats

Twenty-four days after the basal blood collection and immediatelly after the poststress blood collection, rats were euthanized by decapitation under isofluorane anesthesia (Servicios Genéticos Porcinos). Pituitary, adrenals and brain were carefully dissected out. Glands were snap frozen in liquid nitrogen. After replaced, brains were immediately snap frozen (on dry ice) and stored in aluminium foil to prevent freeze-drying. Tissues were stored (−80°C) until the shipment to KI laboratory for further mRNA and cDNA analyses.

Cryostad sectioning: Paraventricular nucleus of the hypothalamus

Prior to dissection, brains were placed for 15 to 20 minutes to increase their temperature from -80ºC to -20ºC and were mounted onto a holder in the cryostat. After the tissue reached a stable temperature, brain was manually sectioned with a razor blade by using a Zivic Rat Brain Slicer. Paraventricular nuclei of the hypothalamus were obtained according to the Patxinos and Watson (1998) rat brain atlas. Each section was collected in an eppendorf tube, and stored at -80°C until further use (Real time quantitative-PCR).

Relative quantification of mRNA by real-time quantitative PCR

Cells for each PVN sample were lysed, and total RNA was extracted was isolated from homogenized tissues using a RNeasy total RNA extraction kit (Qiagen). RNA samples underwent 15 min on-column DNase digestion (27 Kunitz units; Qiagen) before cDNA synthesis to

avoid amplification of genomic DNA. Reverse transcription was performed with 10 µl of total RNA, random hexamer primers (0.1 µg; Invitrogen Life Technologies), and superscript reverse transcriptase (200 U; Invitrogen Life Technologies). Amplification was performed on an iQ5 real-time PCR detection system (Bio-Rad). All primers were designed using Beacon Designer software (Bio-Rad). Primer specificity was assessed by analyzing amplicon dissociation curves in each sample. Relative amounts of mRNA levels were calculated using the standard curve method, constructed by using serial dilutions (1/1, 1/10, 1/100, and 1/1000) of cDNA. All samples were analyzed in duplicates. The transcript level in each sample was calculated as the ratio between the relative amount of the specific marker investigated to the endogenous housekeeping gene, *Hprt,* which was our control.

The following primers were used for RT-PCR are represented in table 5.2:

Gene	**Forward primer 5′ → 3′**	**Reverse primer 5′ → 3′**
IL6	CTTCCAGCCAGTTGCCTCT3	GAGAGCATTGGAAGTTGGGG
CD74	GTGATGCACCTGCTTACGAAGT	CTCCGGGAAGCTCCCCT
CRF	TGATCCGCATGGGTGAAGAATACTTCCTC	CCCGATAATCTCCATCAGTTTCCTGTTGCT
MC2R	GTTCGTCCTCTCTTTGCTGG	GAGGTGAAGGTGAGCACTGT
HPRT	CTCATGGACTGATTATGGACAGGAC	GCAGGTCAGCAAAGAACTTATAGCC

Table 5.2.- Sequences of primers used for quantitative real-time PCR. Primers designed using Primer Express software v1 (Applied Biosystems)

5.2 Statistical analysis

Two way ANOVAs for all experimental groups (two sexes and two strains), Duncan's tests for comparison between groups when appropriate (i.e. after significant one-way ANOVA) were applied in order to test for the a priori hypotheses that, for instance, the DA assumed EAE-susceptibility would be consistent with increased inflammatory mRNA expression and associated to a relatively decreased HPA axis activity (corticosterone levels, HPA mRNA expression and anxiety). Females were expected to present an enhanced HPA axis as well as high levels of inflammatory markers (as they are more susceptible to inflammation) than males.

Overall MANOVAs were applied to each pro-inflammatory marker (two sexes, two strains and three measures), with the aim of analyzing the possible trend associated to "sex" or "strain" regarding mRNA expression across the three structures.

5.3 RESULTS

Behavioural differences of DA and PVG rats

Figures 5.1 - 5.3 show the scores of DA and PVG strains (each sex separately) in different variables of the behavioural battery of tests. Thus, two-way ANOVA (2 x "strain" and 2 x "sex") followed by Duncan's test when appropriate (i.e. after significant ANOVA) were applied to observe the differences in behavioural inhibition/activity and unconditioned anxiety (i.e. novel-cage test and elevated zero-maze test) as well as conditioned anxiety/fear (i.e. fear/freezing, two-way avoidance acquisition).

There were no differences between groups in the unconditioned anxiety variables of the elevated zero-maze test (Fig. 5.1).

Figure 5.2 represents the most important variables of the novel-cage activity test. Concerning the initial distance travelled (first 5 minutes; Figure 5.2A), there was a significant "strain" effect [$F_{(1,45)}$=32.6, p≤0.001], with DA strain travelling higher distance, and a significant "sex" effect [$F_{(1,45)}$=5.0, p≤0.05], with females showing higher ambulation in the first five minutes (Fig. 5.2A). Even though there were no differences between groups in distance travelled during the last 5-min interval (Figure 5.2B), the "total distance" travelled during the whole 20-min test (Figure 2C) was also higher for DA rats ["strain" effect, $F_{(1,45)}$=16.1, p≤0.001].

There appeared no differences between the strains in conditioned "freezing" (Fig. 5.3A) nor in conditioned anxiety as measured by "Latency 40" (Fig. 5.3C) and "Total avoidances" (Fig. 5.3D) in the two-way active –shuttle box- avoidance task. There were no between-strain differences in "crossings in exploration time" (Fig. 5.3C), i.e.

unconditioned activity during 4 minutes of free exploration of the shuttle box, although there was a significant "sex" effect [$F_{(1,45)}$=6.2, p≤0.02], as females performed more crossings than males (Fig. 5.3B).

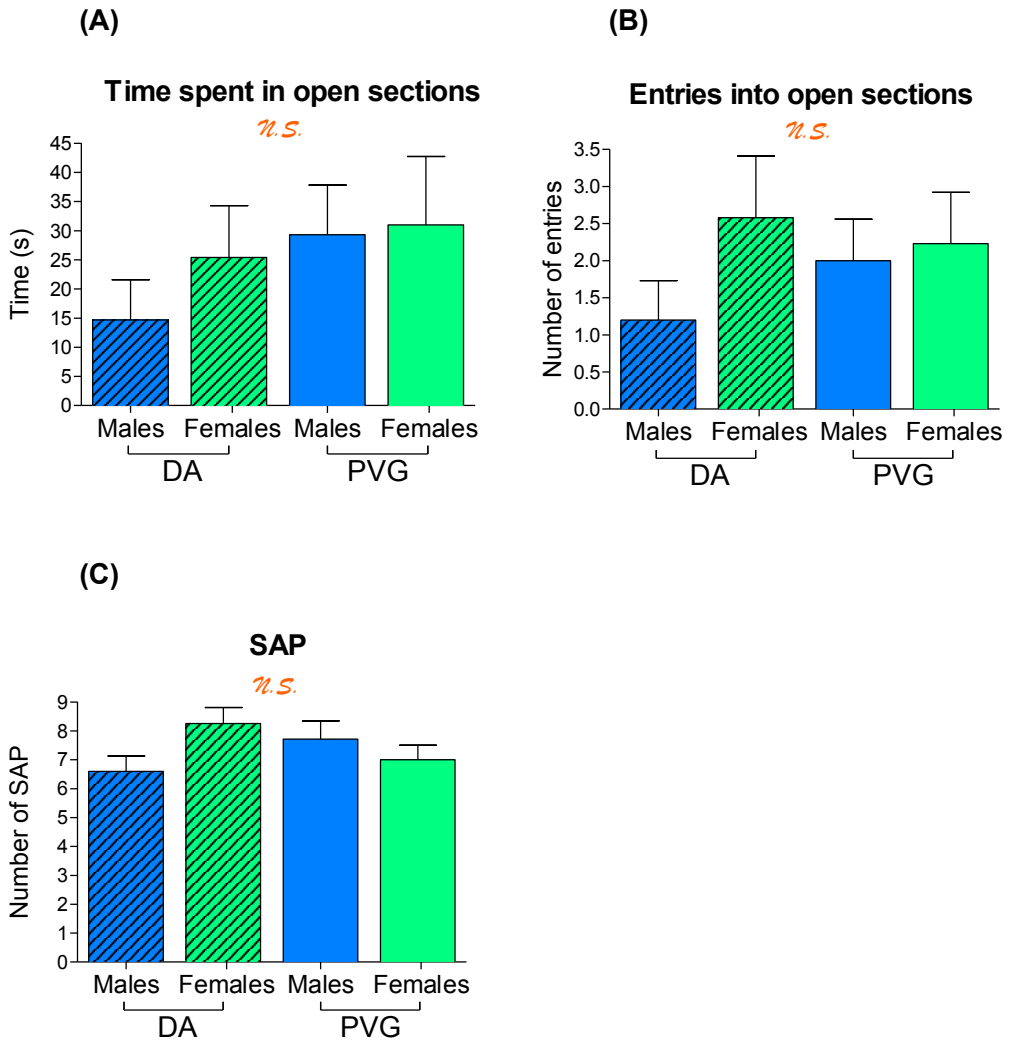


Figure 5.1.- Scores (means ± S.E.M.) in the ZM. (A) time spent in open sections of the ZM, (B) number of entries into open sections of the ZM, (C) number of stretch-attend postures (SAP) (Group colour: DA males, striped blue, n= 14; PVG males, plain blue, n= 13; DA females, striped green, n= 10; PVG females, plain green, n= 12). n.s. Not significant differences.

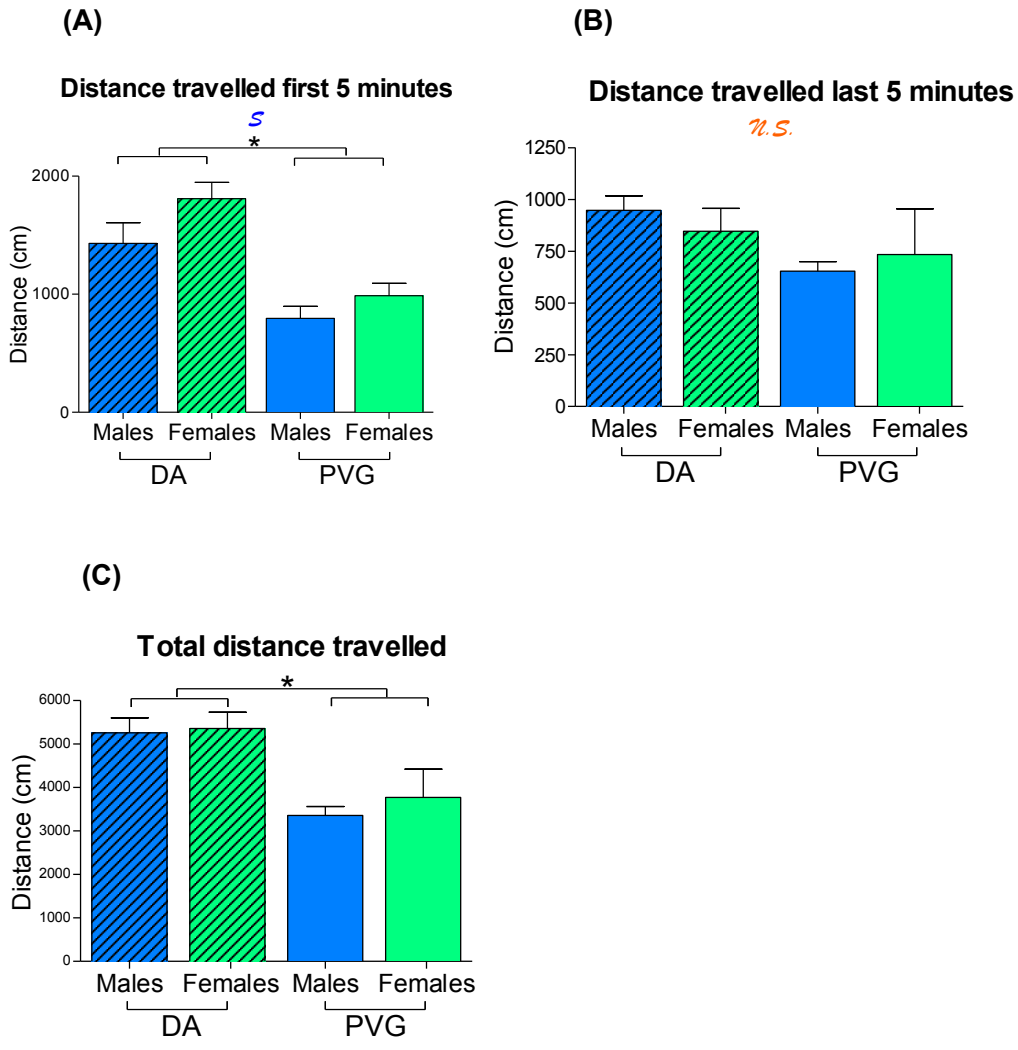


Figure 5.2.- Scores (means ± S.E.M.) in the in the automated novel-cage test. (A) distance travelled within the first 5 minutes, (B) distance travelled within the last 5 minutes, (C) total distance travelled. (Group colour: DA males, striped blue, n= 14; PVG males, plain blue,n= 13; DA females, striped green, n= 10; PVG females, plain green, n= 12) * $p \leq 0.05$, "Strain" effect; **S** $p \leq 0.05$, "sex" effect; n.s., not significant differences.

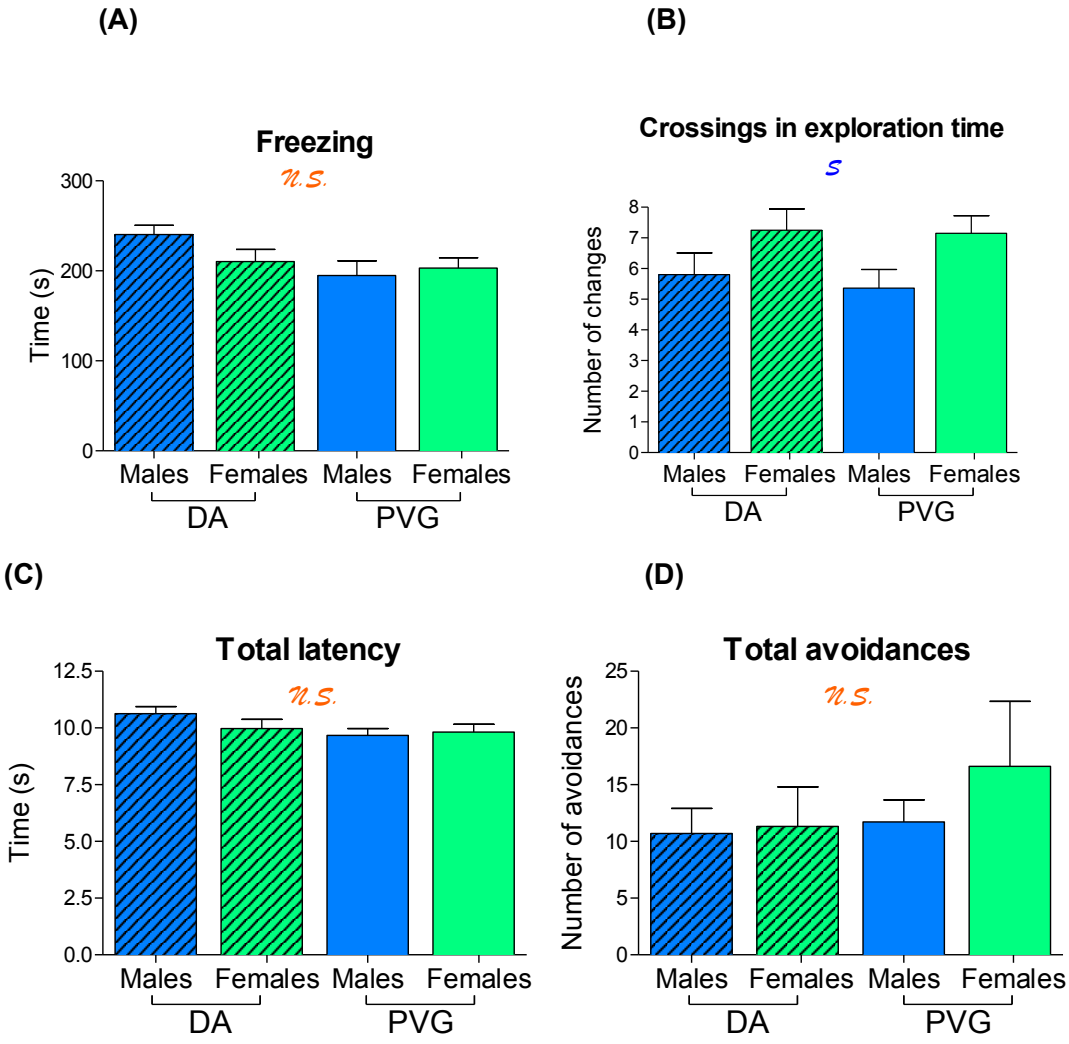


Figure 5.3.- Scores (means ± S.E.M.) in two-way shuttlebox avoidance conditioning. (A) time spent doing freezing, (B) total number of changes in exploration time, (C) mean of latency response, (D) number of total avoidances. (Group colour: DA males, striped blue, n= 14; PVG males, plain blue,n= 13; DA females, striped green, n= 10; PVG females, plain green, n= 12). **S** $p \leq 0.05$, "sex" effect; n.s., not significant differences.

Differences in adrenal weight of DA and PVG rats

There was a significant "sex" effect [$F_{(1,45)}$= 107.386, p≤0.001] on the relative adrenal weight, with females showing larger adrenal glands, and also a significant "strain" effect [$F_{(1,45)}$=90.922, p≤0.001], with the resistant PVG rats showing heavier adrenals (Fig. 5.4). "Strain" x "sex" interaction was also significant [$F_{(1,45)}$= 19.026, p≤0.001], as female PVG rats showed especially enhanced adrenal relative weight.

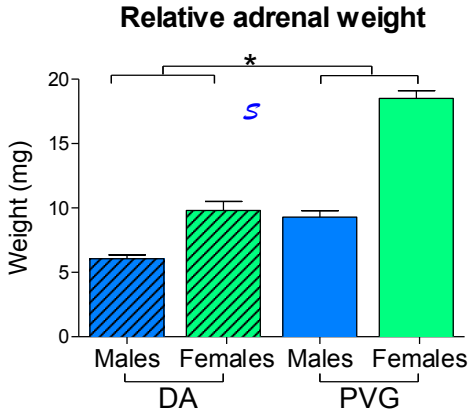


Figure 5.4.- Scores (means ± S.E.M.) in relative adrenal weight. (Group colour: DA males, striped blue, n= 14; PVG males, plain blue,n= 13; DA females, striped green, n= 10; PVG females, plain green, n= 12). **S** *p* ≤0.05, "sex" effect.

Differential HPA response of DA and PVG rats

Figure 5.5 shows the differences between groups in basal corticosterone (Fig. 5.5A), post-stress corticosterone (Fig. 5.5B) and differential corticosterone (Fig. 5.5C). Regarding basal corticosterone levels (Figure 5.5A), there was a significant "sex" effect [$F_{(1,45)}$=12.5, p≤0.01], as female corticosterone levels were higher than in males, but there was no "strain" effect. Concerning post-stress corticosterone (Fig. 5.5B), there was a significant "strain" effect [$F_{(1,45)}$=5.9, p=0.019], with PVG rats showing higher levels, as well as a "sex" effect [$F_{(1,45)}$=10.4, p=0.002] indicating that corticosterone levels were higher in females than in males. Likewise, there were significant "strain" [$F_{(1,45)}$= 6.3, p=0.015] and "sex" effects [$F_{(1,45)}$=4.7, p=0.035] on differential corticosterone levels (Fig. 5.5C), in the same direction as the effects observed for post-stress corticosterone.

Figure 5.6 shows the HPA markers expressed in both central and peripheral structures, i.e. gene expression of CRF in the hypothalamus (Fig. 5.6A) and gene expression of ACTHr –MC2 receptors- in the adrenal glands (Fig. 5.6B). There were no "strain" effects neither in CRF expression in the hypothalamus (Fig. 5.6A) nor in ACTHr in the adrenal glands (Fig. 5.6B), while there was a "sex" effect on ACTHr [$F_{(1,43)}$=24.163, p≤0.001], with females showing higher expression levels than males (Fig. 5.6B).

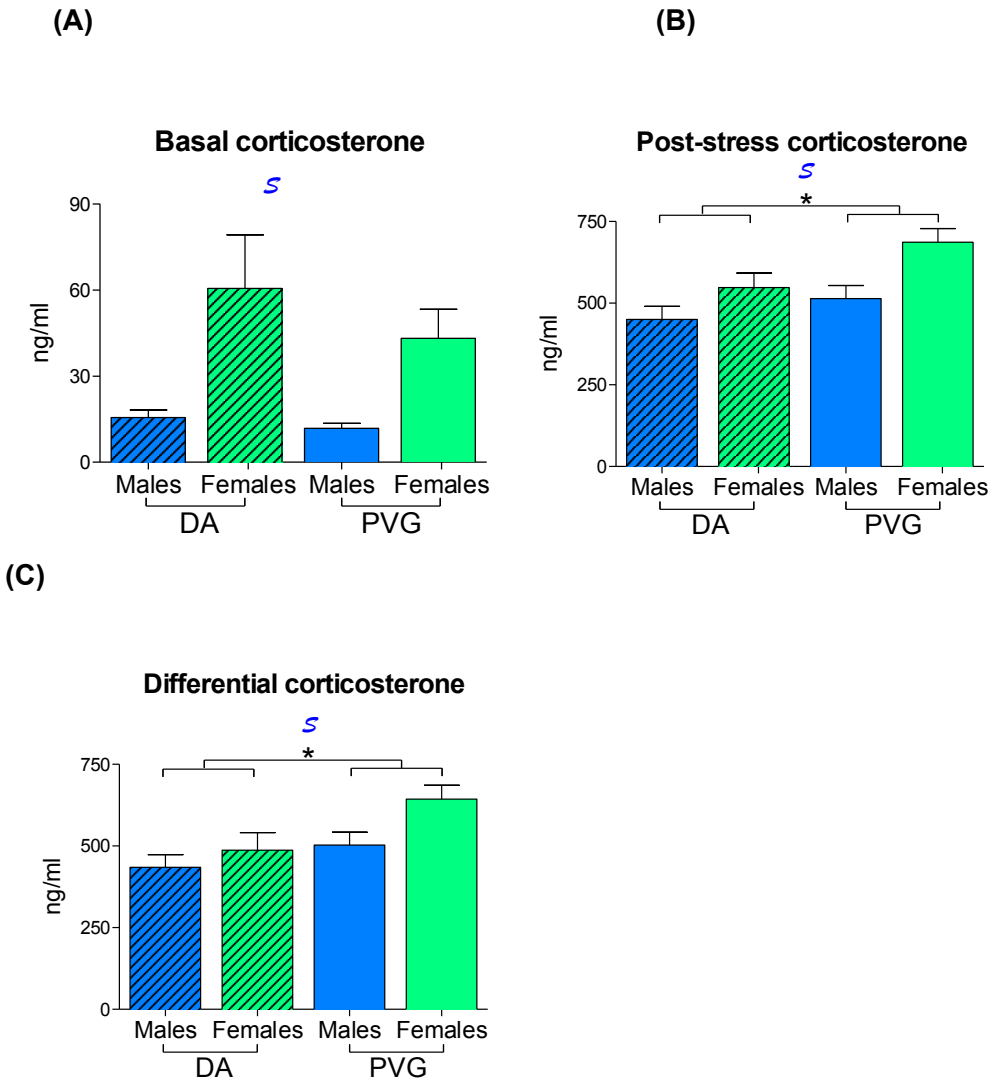


Figure 5.5.- Scores (means ± S.E.M.) in corticosterone levels. (A) basal levels, (B) post-stress levels, (C) differential levels. (Group colour: DA males, striped blue; PVG males, plain blue; DA females, striped green; PVG females, plain green; n= 5-8.). * $p \leq 0.05$, "Strain" effect; **S** $p \leq 0.05$, "sex" effect.

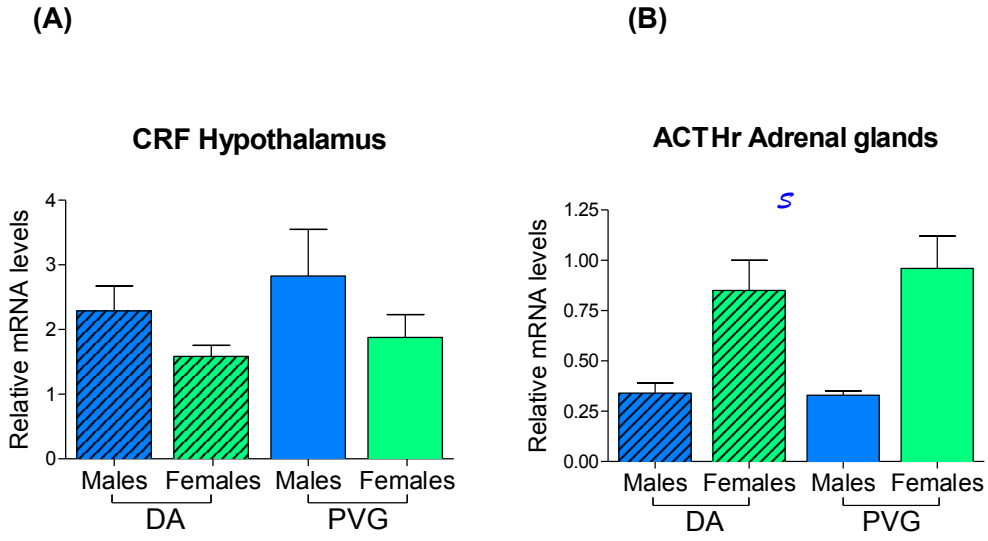


Figure 5.6.- Scores (means ± S.E.M.) in relative HPA Mrna expression of: (A) CRF in hypothalamus (n=5-8), and (B) ACTHr in adrenal glands (n= 7-13). Group colour: DA males, striped blue; PVG males, plain blue; DA females, striped green; PVG females, plain green). **S** $p \leq 0.05$, "sex" effect; n.s., not significant differences.

Differences on inflammatory response of DA and PVG rats

Figure 5.7 represents the between-strain differences in gene expression of the pro-inflammatory factors Interleukin-6 (IL-6) and the MHC–related Cluster of Differentiation 74 (CD74), measured in three structures (hypothalamus, adrenal glands and pituitary gland).

When analyzing separately mRNA expression in each structure, ANOVAs showed no statistical effects on hypothalamic expression levels (Figure 5.7A-B). Conversely, there were "strain" effects on IL6 in the pituitary [$F_{(1,24)}$=47.9, p≤0.001], reflecting that DA rats had higher expression, and also "sex" effects [$F_{(1,24)}$=21.4, p≤0.001] indicating that males showed overall higher expression than females (Fig. 5.7C). A similar pattern and direction of effects was found with respect to CD74 expression in the pituitary ["Strain" effect: $F_{(1,24)}$=79.8, p≤ 0.001; "sex" effect: $F_{(1,24)}$=30.0, p≤ 0.001; Fig. 7D].

There were no main factor (nor interaction) effects on IL6 expression from adrenals (Fig. 5.7E), while there appeared "strain" [$F_{(1,24)}$=16.8, p≤0.001] and "strain X sex" [$F_{(1,24)}$=4.3, p=0.043] effects on adrenal CD74 expression, indicating that such a strain effect in CD74 is predominantly due to the marked expression difference between DA and PVG females (Fig. 5.7F).

Finally, when applying overall MANOVAs to each pro-inflammatory marker (i.e. IL6 or CD74 measured in each of the 3 structures; factorial MANOVAs "2 strains" x "2 sexes" x "3 structures/structures"), in order to observe the possible "strain" or "sex" tendency to show a characteristic pattern of mRNA expression across the 3 structures, there were "strain" effects on both inflammatory markers (see both

MANOVAs results at the bottom of Fig. 5.7), indicating that the susceptible DA rats presented overall higher mRNA levels.

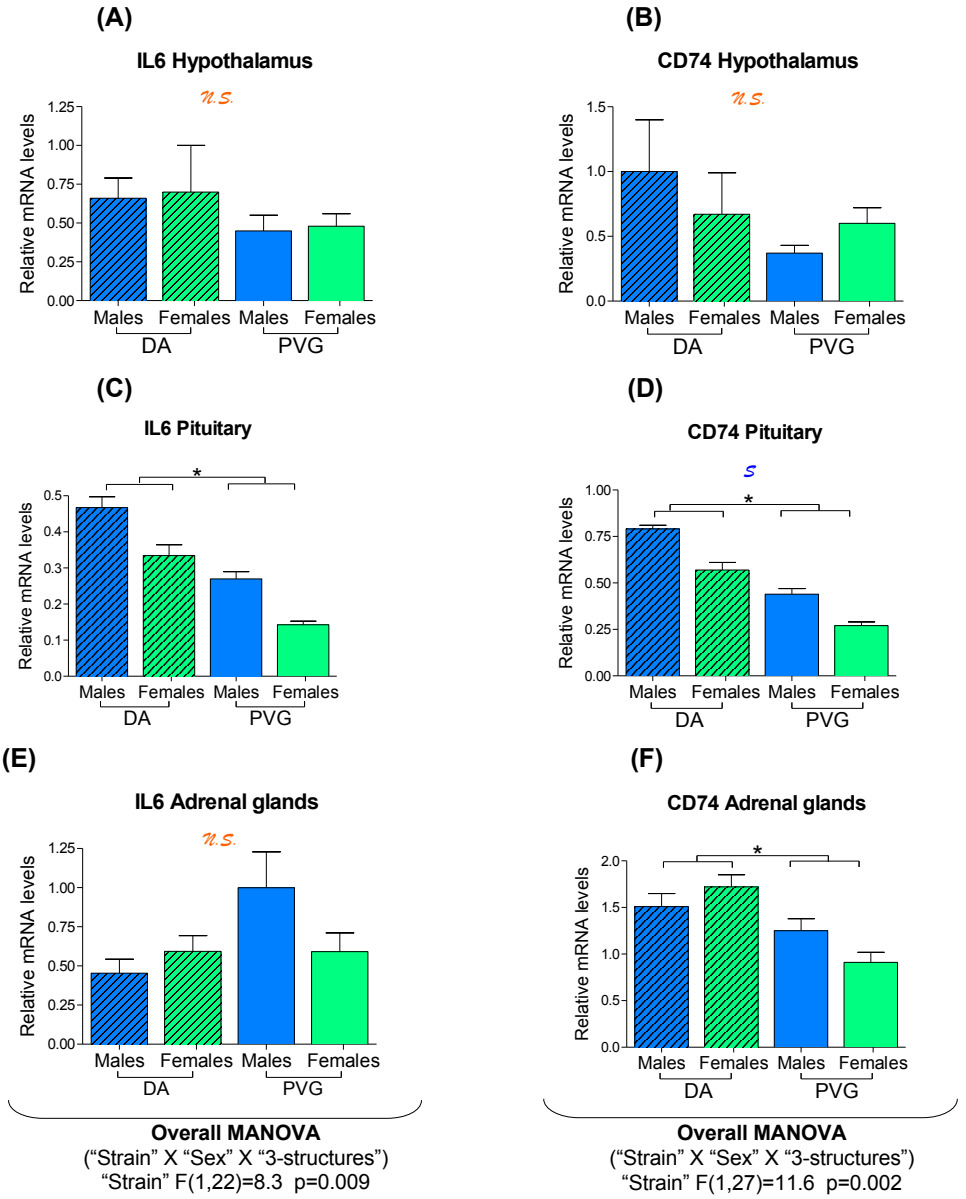


Figure 5.7.- Scores (means ± S.E.M.) in relative mRNA expression of inflammatory markers. (A) IL6 in hypothalamus (n= 4-9), (B) CD74 in hypothalamus (n=4-10), (C) IL6 in pituitary (n= 10-14), (D) CD74 in pituitary (n=10-14), (E) IL6 in adrenal glands (n=10-14), (F) CD74 in adrenal glands (n=10-14). ***** p ≤0.05, "Strain" effect; **S** p ≤0.05, "sex" effect; n.s., not significant differences.

5.4 DISCUSSION

As compared to the EAE-resistant PVG rat strain, the EAE-susceptible DA strain shows: i) more disinhibited behavior (i.e. increased activity) in the novel-cage test, a result which is partially in line with the results found in "Study I", i.e. more disinhibited behavior (i.e. activity) in the EAE-susceptible N/Nih-HS rats in the novel-cage test (and in the elevated zero-maze); ii) lower stress-induced corticosterone responses, and lower adrenal weight; iii) no differences regarding adrenal MC2Rs (ACTH receptors) nor CRF expression in hypothalamus; and iv) overall increased IL-6 and CD74 expression levels in hypothalamus, pituitary and adrenal glands.

Thus, as far as disinhibited exploratory behaviour in the "novel-cage activity test" can be assumed to be an index of lowered unconditioned anxiety, it would appear that EAE-susceptible DA rats are somehow less anxious and less sensitive to stress-induced HPA-axis responses than EAE-resistant PVG.1AV1 rats. The lower adrenal weight of DA rats is also in line with that. These results would appear to be congruent with the findings that prior corticosterone (McPhee *et al.,* 1989) or stress (for review see Heesen *et al.,* 2007; Levine and Wenk, 1961) reduce the incidence and severity of EAE in rats. What is new from the present study is that for the first time HPA-axis stress (and anxious behavioral) responses have been compared between DA and PVG.1AV1 rats, which are reference strains in the study of EAE, while most previous studies on the association of HPA-axis function and EAE have been carried out with Lewis or Wistar rats, or otherwise by using only a single rat strain (see review by Heesen *et al.,* 2007).

The absence of between-strain differences in hypothalamic CRF expression and adrenal MC2Rs, tends to suggest that the present

HPA-axis divergences are peripheral (or not mainly due to central differences), though further studies should also measure ACTH levels, among other central and peripheral parameters, in order to definitively establish the origin of the observed differences. The results of DA-PVG differences in neuroinflammatory IL-6 and CD74 (MHC-II) markers are in line with our expectancies, i.e. DAs should show enhanced expression levels of these two markers, according to their higher MOG-EAE susceptibility, as compared to (EAE-resistant) PVG rats.

In summary, as said above there is compelling evidence indicating that enhanced HPA axis responses might be protective against inflammatory diseases as EAE in laboratory animals or MS in humans (e.g. MacPhee and Mason, 1990; Stefferl *et al.,* 1999; Sternberg *et al.,* 1989; see further references above). The present results are in line with that contention as concerns to the observed between-strain (DA vs PVG) differences in stress-induced HPA-axis responses and adrenal weight. However, the observed sex effects deserve especial mention, as females of both strains presented 1) higher baseline and stress-induced corticosterone response, 2) heavier adrenal glands, and 3) higher adrenal MC2R mRNA expression than males (chronic adrenal hyperactivity is associated to increases in both adrenal volume and expression of genes involved in steroidogenesis, like MC2R ; e.g. see Dalla *et al.,* 2005, 2008; Drossopoulou *et al.,* 2004; Galea *et al.,* 1997; Kitay, 1961; Kokras *et al.,* 2009; Lehoux *et al*., 1998 Pitychoutis *et al.,* 2009; Raone *et al*. 2007; Ulrich-Lai *et al*. 2006).

While these sex-related differences are mostly in line with the literature (see Askari, 1970; Da Silva *et al.,* 1999; Handa *et al* 2002; Karandrea *et al*., 2000; Kudielka and Kirschbaum, 2005; Pfaff *et al*., 2004; Solem, 1966; Turner, 1990, 1997; Bowers JM *et al*, 2010; Wigger and Neumann, 1999), these results could seem contradictory with the well-

known fact that females are more susceptible to MOG-EAE than males (Heesen *et al.,* 2007; Kokras *et al.,* 2011; Massella *et al.,* 2012), as their apparently more activated HPA axis would be expected to provide more protection against MOG-EAE in females than in males. Hence, these apparent sex-related inconsistencies indicate that factors other than HPA-axis function should be involved in conferring disease susceptibility or protection depending upon the gender.

It is assumed that sex hormones and/or sex chromosomes may be partly responsible for the enhanced susceptibility to EAE (or human MS or other autoimmune diseases) in females. In general, it has been well documented that women have more robust immune responses than men, as well as that the XX sex chromosome is disease-promoting as compared to the XY (Duma *et al.,* 2010; Libert *et al.,* 2010). In males, MS onset tends to be relatively later in life, in parallel with the beginning of the decline in bioavailable testosterone (Weinshenker, 1994), while MS onset in females mostly coincides with the beginning of the reproductive age (Duquette *et al.,* 1992).

Compared to males, females have a stronger humoral response and a greater antibody response to various antigens after immunization (Butterworth *et al.,* 1967). Generally speaking, women with MS present more robust immune responses than men (Honjo, 2010; Moldovan *et al.,* 2008; Kadioglu *et al.,* 2011; Kantarci *et al.,* 2008; Kataranovski *et al.,* 2009). Furthermore, neuroimaging studies have shown that women have more inflammatory markers in the CNS (Pozilli *et al.,* 2003). The current view is that these sex differences in MS (or EAE, in animals) may at least partly result from the protective effects of testosterone in males, as shown by the EAE murine model (Bebo *et al.,* 1998; Foster *et al.,* 2003; Gold and Voskuhl 2009; Smith *et al.,*1999). Studies with other autoimmune diseases have shown a similar gender dimorphism

(Ahmed and Penhale, 1982; Harbuz *et al.,* 1995; Fitzpatrick *et al.,* 1991; Fox, 1992). Altogether, these data support the hypothesis that endogenous androgens may be protective at physiological levels, as well as exogenous androgen treatment involves effects on cytokine production which lead to EAE protection (Dalal *et al.,* 1997; Liva and Voskuhl, 2001;). On the other hand, several studies have shown that the clinical severity of EAE in mice is reduced by estrogens (estriol or 17!-estradiol), through mechanisms which involve anti-inflammatory processes (for review see Gold and Voskuhl, 2009; Grossman, 1993; Olsen and Kovacs, 1997; Pozzilli *et al.,* 1993). Finally, another general finding that is considered to be of great relevance to explain the autoimmunity-protective effects of testosterone and estrogens is the fact that both types of sex hormones display neuroprotective effects in animal models (see review by Gold and Volskuhl, 2009).

The present is, to the best of our knowledge, the first study comparing HPA-axis responses to stress, as well as anxiety-related behavioural responses, between DA and PVG rats. This is of particular relevance, as both rat strains have the same MHC haplotype and very different response to MOG-induced EAE, and they have served as a tool for genetic (QTL) studies of EAE as well as for developing congenic rat strains and advanced intercross rat lines for that purpose (e.g. Beyeen *et al.,* 2010; Huberle *et al.,* 2009; Ockinger *et al.,* 2010; Stridh *et al.,* 2010). Thus, the present results also suggest that studying HPA-axis responses and their relationship with EAE in these congenic inbred strains from a genetic standpoint could be worthwhile. On the other hand, a complementary relevant study would be to evaluate MOG-EAE sensitivity in rat strains showing the same MHC haplotype and showing extreme differences in (unconditioned and conditioned) anxiety and HPA responses to stress, as the RHA-I/RLA-I rat strains.

The contribution of sex hormones (androgens and estrogens) to EAE in rats should also be taken into account in future studies, as the involvement of those hormones and of sex chromosomes is thought to be of great relevance in complex autoimmune diseases such as MS (for review see Gold and Voskuhl 2009).

GENERAL DISCUSSION

6 GENERAL DISCUSSION

6.1 OVERVIEW OF RESULTS

This Thesis presents the behavioural characterization of anxiety/fear-related phenotypes in a very large (n=2000) sample of rats from the N/Nih-HS stock, which constitutes a replication and important extension of our previous work carried out in smaller rat samples (Díaz-Morán *et al.,* 2012; López-Aumatell *et al.,* 2008, 2009, 2011; Vicens-Costa *et al.,* 2011). For the first time, these genetically heterogeneous rats have been characterized for their susceptibility and severity of MOG-EAE, an animal model of multiple sclerosis, with the final aims of both performing (within the context of the EURATools and EURATRANS projects) high-resolution genetic (QTL) mapping of EAE and searching for quantitative trait genes (QTGs) influencing EAE (and MS in humans). The characterization of such a large sample of N/Nih-HS rats in anxiety/fear as well as in EAE, has allowed us to study the associations between both phenotypes, while the abovementioned genetic analyses are currently being finished by the EURATRANS project Consortium.

As detailed in previous sections (see 4.3 and 4.4) the main results from Study I could be summarized as follows:

i) Taken together, and taking also into account the results from our previous works (e.g. Díaz-Moran *et al.,* 2012; López-Aumatell *et al.,* 2008, 2009, 2011), the behavioural –anxious/fearful- profiles of the genetically heterogeneous N/Nih-HS rat stock are clearly reminiscent of a rather anxious/fearful and passive copper rat type (Table 6.1). Moreover, and also agreeing with the above reports, females

are consistently less anxious/fearful (and more behaviourally disinhibited) than males.

	N/Nih-HS males (n=967)	DA males (n=10)	PVG males (n=14)	RLA-I males (n=13)	RHA-I males (n=8)
CET (n)	8,5 ± 0,1	8,2 ± 0,5	7,1 ± 0,6	5,3 ± 0,8	10,9 ± 1,1
Freezing (s)	**196,2** ± 1,5	240,3 ± 10,2	194,8 ± 16,4	**170,0** ± 12,9	**28,2** ± 10,2
Av40 (n)	**2,6** ± 0,1	10,7 ± 2,2	11,7 ± 1,9	**3,2** ± 1,2	**32,9** ± 2,8
ITC40 (n)	14,6 ± 0,4	18,6 ± 1,9	33,6 ± 4,9	20,9 ± 4,4	**70,8** ± 11,8

Table 6.1.- Comparison of behavioural measures (means ± S.E.M) among the N/Nih-HS males (Study I), DA and PVG males (Study II) and the Roman High- and Low-avoidance rats (RHA-I –low anxious- and RLA-I –high anxious-, respectively). All these groups of rats were tested during the period of testing of the last 500 N/Nih-HS group. Means ± S.E.M are represented. "CET", number of (unconditioned) crossings in exploration time (n); "Freezing", time (s) spent performing freezing, conditioned to the context (measured during the first 5 intertrial intervals of the shuttle box training session); "Av40", number (n) of total avoidances in the 40-trial shuttle box training sessions (n); ITC40, number of intertrial crossings in the 40-trial shuttle box training sessions. The results (compare especially the numbers in bold and those underlined) clearly indicate that N/Nih-HS rats present values of context-conditioned fear ("Freezing"), total avoidances ("Av40") and total intertrial crossings ("ITC40") which are much more similar to the "high anxious" RLA-I rats –and to DA and PVG rats- than to the "low anxious" RHA-I rat strain, thus supporting the conclusion that N/Nih-HS rats are, as a population/stock, relatively anxious, fearful and passive coppers (for further evidence see Díaz-Morán *et al.,* 2012; López-Aumatell *et al.,* 2009).

ii) The significant associations among the different unconditioned and conditioned anxiety and fear responses, established through different statistical analyses, lead to the suggestion that some common factors (or traits) appear to be shared by unconditioned anxiety and conditioned fear/anxiety-related responses, although such associations are different between sexes and the within-test associations (i.e. variables from the same test) are stronger than those found across tests/tasks.

iii) N/Nih-HS rats presented a 25% of EAE incidence, which was somewhat lower than expected based on our previous

pilot study (Johanesson *et al*., 2009). There were no consistent sex differences in EAE incidence or severity, although females presented just a slight trend (p=0.05) for a longer disease course. The absence of consistent sex-related differences in the various EAE disease parameters is at odds with the literature showing that females generally present higher EAE (and MS) incidence and severity as compared to males (Heesen *et al.,* 2007; Kokras *et al.,* 2011; Massella *et al.,* 2012).

iv) One of the most outstanding results has been the finding, through different types of analyses, that rats selected by their high anxiety profile showed an incidence close to 30%, while low anxious rats showed 15-20% of incidence. The result is very consistent and was confirmed through regression analyses. That is to say, the relatively less anxious animals showed an increased incidence in comparison to the high anxious animals. In addition, EAE severity was also higher in low anxious rats.

These findings, especially because of their consistency in such a large rat sample, strongly support the hypothesis that a resistant EAE profile would be associated to relatively elevated anxiety levels. If a relatively high level of trait anxiety is paralleled by predisposition to relatively increased HPA-axis responses and increased GCs release, as seen in previous sections (see references in support of that in sections 4.4 and 5.1), such a neuroendocrine profile could be a (or at least one) mechanism providing protection against neuroinflammation, specifically against EAE in the relatively high anxious N/Nih-HS subsample.

Therefore, starting from that assumption, we formulated the hypothesis that rats with different EAE susceptibility/resistance profiles would, in parallel, show differential anxiety/fearfulness traits and divergent stress-induced HPA-axis response profiles. “Study II” was devoted to behaviourally (i.e. anxiety/fear) evaluate two inbred strains, the EAE-susceptible DA and the EAE-resistant PVG rats, and to characterize them regarding both HPA-axis function and pro-inflammatory markers. To sum up, the results of Study II indicate that the DA rat strain shows an increased exploratory activity level during the 20-min exposure to the “novel-cage activity” test. Thus, as far as disinhibited exploratory behaviour under a novelty situation (i.e. the 20-min “novel-cage activity” test) can be assumed to be an index of relatively lowered unconditioned anxiety, it would appear that EAE-susceptible DA rats are somehow less anxious at least in some specific situations (as the “novel-cage” test) and, in parallel, they show less sensitivity to stress-induced corticosterone (HPA-axis) responses than EAE-resistant PVG rats.

Moreover, compared to PVGs, DA rats presented overall lighter adrenals and (as expected) overall enhanced pro-inflammatory factor (CD74 and IL-6) levels in hypothalamus, pituitary and adrenals, while no between-strain differences appeared in CRF mRNA expression (hypothalamus) nor in MC2R mRNA expression (adrenal glands).

Both rat strains have been previously compared with other strains (e.g. Sprague-Dawley, Lister hooded, etc) with regard to their anxiety profiles (e.g. plus-maze testing, open field testing), but they have never been compared to each other (i.e. DA vs PVG) as concerns to HPA-axis function or to unconditioned and conditioned anxiety/fear (King, 1999; Mechan *et al.,*2001; Schmitt and Hiemke, 1998), which makes very difficult to compare the results from those laboratories with the present ones.

Taken together, and comparing with PVG rats, that pattern of results appears to cohere with the EAE-susceptible DA rats presenting a profile of (i) certain level of behavioral disinhibition, or lower "timidity" (when facing some specific novelty/aversive situations), along with (ii) relatively lowered HPA-axis responses to stress (i.e. lowered corticosterone responses, lighter adrenals) and (iii) higher pro-inflammatory marker levels, which is actually consistent with the fact that they are (autoimmune-) neuroinflammation-prone rats. These results, in particular the fact that PVG rats are resistant to EAE, would appear to be congruent with the findings that glucocorticoid or stress administration prior to EAE induction, as well as corticosterone replacement (see review by Heesen *et al.,* 2011; Levine and Wenk, 1961; McPhee *et al.,* 1989; McPhee and Mason, 1990) reduce the incidence and severity of EAE in rats (for more detailed references see sections 5.1 and 5.4).

What can we learn from behavioural and genetic studies concerning the anxiety/fearfulness profiles of N/Nih-HS ?

Along the last decade, and collaborating with the EURATools and EURATRANS projects since 2004, our group has devoted a large part of its efforts and capacity to the identification of QTLs and quantitative trait genes (QTGs) implicated on the regulation of anxiety and fear in rats. As summarized in Figure 6.1, the pathway followed by our laboratory along the last decade in the search for (and identification) of QTLs –and, let's hope, QTGs- for anxiety in rats has led to the following main findings:

i) We identified a QTL (a section of almost half a chromosome) in Chr 5, for two-way avoidance acquisition (conditioned anxiety) and cue- and context-conditioned freezing (conditioned fear) in a F2 cross of RHA-I (low anxious) and RLA-I (high anxious) rats (Fernandez-Teruel *et al.,* 2002).

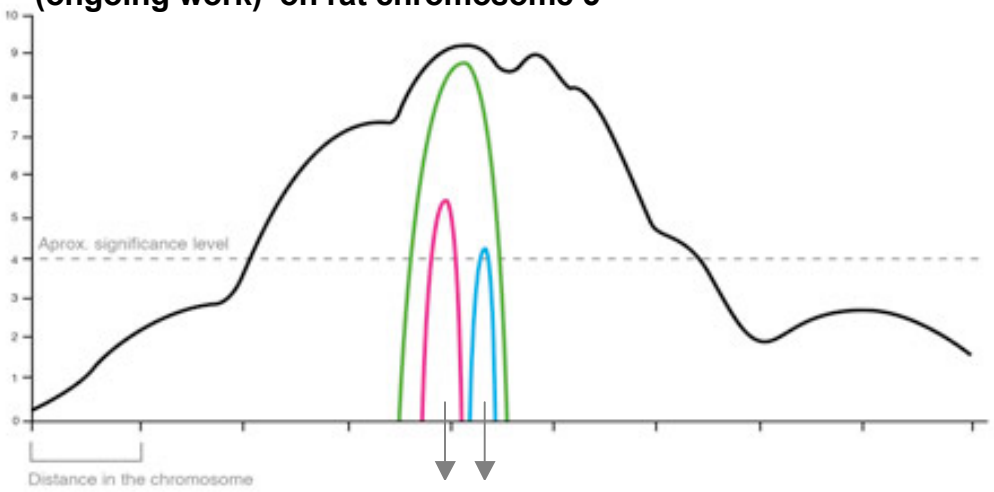
ii) We demonstrated, for the first time, that the genetically heterogeneous N/Nih-HS rat stock is a valuable and unique resource for the genome-wide simultaneous fine mapping of quantitative trait loci to gene-level resolution –QTL intervals $\leq$ 2 Mb- . As said, such a fine-mapping allows quantitative trait gene (QTG) identification, as very few genes are usually contained in such small chromosomal regions (Alam *et al.,* 2011; Johanneson *et al.,* 2009; see as an example of QTG identification the case of Rsg2 gene, Yalcin *et al.,* 2004b, and its confirmation in humans by Smoller *et al.,* 2008).

iii) Next, we fine-mapped the abovementioned QTL for anxiety (the one identified in the work of Fernandez-Teruel *et al.,* 2002) and identified a QTL, just in the middle section of that previous one (containing 9 genes; illustrated in Figure 6.1), influencing two-way avoidance acquisition in N/Nih-HS rats (Johannesson *et al.,* 2009).

iv) The current ongoing work (non-definitive results, as final analyses are still uderway), in the context of EURATools and EURATRANS european projects, indicates that two (provisional) QTLs, for "Head dips" in the elevated zero-maze test (unconditioned anxiety/conflict) and for context-conditioned freezing (conditioned fear), fall within the Chr 5 section defined by the QTL identified by Johannesson *et al.,* (2009; see illustration in Figure 6.1).

v) Several other "suggestive" QTLs for anxiety have been provisionally identified in various chromosomes, but these QTLs await confirmation from the final analyses which are currently underway (the final results and final version of the paper are going to be discussed at the "2nd EURATRANS project meeting", 4th-6th June, Tutzing, Munich, Germany).

Figure 6.1.- Anxiety QTLs and "suggestive/provisional" QTLs (ongoing work) on rat chromosome 5



Possible non-definitive QTLs for unconditioned anxiety and conditioned fear being currently submitted to the final analyses to confirm them (EURATRANS project Consortium)

PUBLISHED QTLs FOR CONDITIONED AND UNCONDITIONED ANXIETY/FEAR IN RAT CHROMOSOME 5.-

Shuttlebox avoidances and conditioned fear; QTL on Chr 5 in a F2 cross from RHA-I and RLA-I rats. (Fernández-Teruel *et al.,* "Genome Research" 2002)

Shuttlebox avoidances (conditioned anxiety); QTL on Chr 5 in N/Nih-HS rats (Johannesson *et al.,* "Genome Research" 2009)

SUGGESTIVE/PROVISIONAL QTLs FROM ONGOING WORK (to be confirmed).-

Head dips in the elevated zero-maze (unconditioned anxiety/conflict). Suggestive QTL on Chr 5 in N/Nih-HS rats. (EURATRANS project, currently under analysis)

Context-conditioned freezing in the shuttlebox (conditioned fear/anxiety). Suggestive QTL on Chr 5 in N/Nih-HS rats (EURATRANS project, currently under analysis)

As necessary steps to achieve the previous targets we have been working in characterizing N/Nih-HS rats for multiple phenotypes along several years. Concerning their anxious/fearful profile, coping style, and stress hormone responses, from our previous work (e.g. Díaz-Morán *et al.,* 2012; Johannesson *et al.,* 2009; López-Aumatell *et al.,* 2008, 2009, 2011; Vicens-Costa *et al.,* 2011) and from the results of the present "Study I" we are confident to conclude that N/Nih-HS rats could be positioned as relatively high anxious/fearful, passive copper and stress-prone rats. In spite of this, N/Nih-HS rats show, as a population, a wide range of score distribution in all the behavioural variables obtained in Study I (e.g. see Table 4.2), which makes them an optimal base population for selection studies (i.e. studies aimed at generating sub-populations of heterogeneous –or even inbred- rats with extreme values in given/particular complex traits/phenotypes; Hansen and Spuhler, 1984).

The study of the genetic basis/mechanisms of complex traits, even in rodents, has demonstrated to be more difficult than initially envisaged. Few complex trait genes per se have been identified, relative to the number of QTLs. A classical obstacle to progress has been the difficulty to refine QTL intervals, initially identified in inbred strain crosses, to gene-level resolution (e.g. Flint and Mott 2008). Within the framework of the EURATools and EURATRANS projects, we apply a strategy developed in the mouse that combines the identification and fine-mapping of QTLs in one population (Johannesson *et al.,* 2009; Mott, 2000; Mott *et al.,* 2000; Solberg et al., 2006; Valdar *et al*. 2006a). An HS makes genome-wide genetic association studies possible, as has been shown in the mouse, where 843 fine-mapped QTLs (chromosomal intervals averaging 2.8 Mb) were identified for 97 phenotypes and the first quantitative trait gene for anxiety was

identified also using that approach (Valdar *et al*. 2006b ; Yalcin *et al.,* 2004).

It is to be expected that the work from our laboratory (within the frame of EURATools and EURATRANS consortiums), which this Thesis is part of, will contribute with a step forward in the field of (quantitative) genetic mechanisms involved in anxiety/fear and related conditions in rats, and predictably, in the analogue human traits (e.g. anxiety disorders, depression, etc; see Flint 2004; Willis-Owen and Flint, 2007). Following the successful approach used in HS mice (Valdar *et al.,* 2006), and after confirming that N/Nih-HS rats allow genome-wide QTL fine-mapping (Johannesson *et al.,* 2009), we –the EURATRANS consortium- have applied next generation sequencing jointly with high-resolution genetic –QTL- mapping to determine the likely molecular causes of quantitative trait variation (i.e. traits like anxiety, fear, exploratory activity, EAE susceptibility/severity, immunology, metabolism, cardiovascular phenotypes, bone fragility, and other complex quantitative traits; see summary in Table 2.1; EURATRANS "Rat sequencing consortium" , paper in preparation, 2012).

We have reasons to believe that such an approach will provide the tools to start to tease out, on a whole genome scale, the complex relationships of factors contributing to anxiety/fearfulness, by showing not only the main genetic effects, but also the gene-by-environment interaction effects (that could even be more frequent and larger than the pure genetic effects; see Valdar *et al.,* 2006; Valdar *et al.,* 2003; see also López-Aumatell *et al.,* 2011). In fact, besides gene-by-environment effects, this approach also allows the discovery of gene-by-gene (epistatic) interaction effects (e.g. Valdar *et al.,* 2006), and thus it

makes possible not only to identify a given single genetic mechanism but to characterize genetic-physiological networks/processes underlying complex phenotypes/traits. Such a network-based approach is nowadays possible because new important resources enable the study of all genes, gene networks, messenger RNAs and proteins involved in (or influencing) complex traits. This strategy is currently being applied to mouse complex-trait genetics, as well as to the ongoing analyses of results from our N/Nih-HS rat sample (e.g. Flint and Mott, 2008; Valdar *et al.,* 2003, 2006; EURATRANS, paper in preparation).

What can we learn from MOG-EAE phenotypic and genetic studies with N/Nih-HS rats?

As mentioned earlier, the rat N/Nih-HS is a population that holds recombinants derived from eight inbred strains that have accumulated over many generations of out-breeding to create a genetic mosaic (Valdar *et al.,* 2006). In agreement with the data from previous mouse experiments (Huang GJ, *et al.,* 2009; Valdar *et al.,* 2006), the N/Nih-HS rat can provide high mapping resolution, allowing fine-mapping of QTLs to intervals smaller than a cM (Johannesson *et al.,* 2009; Valdar *et al.,* 2006). So, with the main aim of further investigating EAE QTLs and quantitative genes, the evaluation of the MOG-EAE model in the N/Nih-HS rat colony was necessary to confirm that the N/Nih-HS rat stock (as well as the traditionally used inbred strains and crosses) can deliver stable neuroinflammatory phenotypes (MOG-EAE). According to previous findings, EAE phenotype have a wider distribution in N/Nih-HS rats than in traditional inbred strains or crosses (Becanovic *et al.,* 2006; Dahlman *et al.,* 1999; Goldmuntz *et al.,* 1993; Levine and Wenk, 1965; Stefferl *et al.,* 1999; Storch *et al.,* 1998; Stevens *et al.,* 2002; Sun

et al., 1999; Weissert *et al.,* 1998). Hence, the EAE evaluation of N/Nih-HS rats would demonstrate its usefulness to map EAE, which could bring us a better (compared to inbred strains or crosses) comprehension of the complexity involved in autoimmune disease.

In addition, another great advantage of the complex genetic background of the N/Nih-HS rat is that contains several MHC types (heterozygotes and the homozygous AV1 and L). This could presumably allow to map non-MHC genes influencing on EAE (Johannesson 2009). In fact, the MHC class II genes have been well characterized, so, the primary target for the N/Nih-HS studies would be the NON-MHC QTLs containing genes that regulate autoimmune neuroinflammation (with the expectation of achieving QTL intervals of at best 1-3 genes).

Future proposals on anxiety-stress and EAE (MS).

There are two noteworthy limitations of this Thesis: First, the lack of an evaluation of HPA axis function in N/Nih-HS rats ("Study I"). It could be acknowledged and addressed by a future study in which N/Nih-HS rats would be characterized by their basal and post-stress HPA-axis activity, by other peripheral and central HPA-axis-related parameters, as well as by their anxiety/fear profile. Moreover, they would be MOG-EAE induced and evaluated (here we would like to remark that all adrenal samples from the present N/Nih-HS rats are stored, waiting for analyses, in the Neuroimmunology Unit, Karolinska Institutet, Sweden).

The second limitation is related to the "Study II", in which EAE was not induced because of the well-known differential inflammatory profiles of the inbred DA and PVG rats. Thus, it would constitute a likely significant advance, following a similar approach to that described above, to evaluate an advanced intercross line (for a description of AIL see Darvasi and Soller, 1995; Stridh, 2010) derived from DA and PVG rats (established at the Neuroimmunology Unit, Karolinska Institutet) that has been widely used to fine-map and identify genomic regions regulating MOG-EAE (Marta *et al.,* 2010; Stridh *et al.,* 2010). To be complete, such a characterization should take into account anxiety profile, HPA axis responses, EAE and mRNA levels regarding HPA axis and inflammation.

Additionally, since "Study II" addressed the issue of whether an elevated anxiety could provide a certain resistance to EAE, future studies could also consider the use of inbred rats selected by different anxious profiles which also present coherent HPA-axis response

phenotypes. For example, the previously mentioned RLA-I and RHA-I rats (e.g. Broadhurst and Bignami, 1965; Carrasco *et al.,* 2008; Díaz-Morán *et al.,* 2012; Steimer *et al.,* 1998), as well as the HAB and LAB rats from Landgraf's group (e.g. Landgraf and Wigger, 2003), present parallel profiles regarding anxiety and HPA axis responsiveness, i.e. the more anxious RLA-I and HAB present higher ACTH and corticosterone responses to stress than their RHA-I and LAB counterparts. A comparative study of EAE induction in these four strains, with divergent anxious/fearful and HPA-axis profiles, would likely add relevant information to one of the main issues addressed in this Thesis, i.e. the association between relatively low anxiety and stress hormone levels with an increased EAE susceptibility.

The conjunction of those different approaches, studying EAE susceptibility/severity (jointly with anxiety and HPA-axis function) with N/Nih-HS and AIL rats, as well as with pairs of selectively-bred inbred strains displaying differential anxiety and stress hormone responses, could provide valuable additional information regarding the relationships between anxiety/stress-sensitivity and EAE susceptibility.

CONCLUSIONS

7 CONCLUSIONS

The results presented here provide new information about anxiety/fearfulness, stress and neuroinflammation. While also generating new questions that remain to be addressed in future studies, the main conclusions we can draw are the following:

v) A very large sample of genetically heterogeneous N/Nih-HS rats has been characterized with regard to their unconditioned and conditioned anxiety/fear profiles as well as to MOG-EAE (a model of multiple sclerosis). Concerning their behavioural profiles, it is shown that N/Nih-HS rats are, as a population, relatively high anxious and passive coppers, while females show a consistent pattern of decreased anxiety and behavioural inhibition with respect to males.

vi) The different unconditioned anxiety/exploration variables and conditioned anxiety/fear measures show consistent association patterns in N/Nih-HS rats. Thus, unconditioned anxiety responses show a significant capacity to predict conditioned fear and anxiety-related responses (e.g. the higher the unconditioned anxiety the worse the acquisition of the two-way active avoidance task). Conditioned fear/anxiety measures also predict some unconditioned anxiety/exploration responses.

vii) N/Nih-HS rats have been characterized for MOG-EAE susceptibility and severity for the first time. They show approximately 25% EAE incidence, with a range of disease scores that allows genetic QTL studies (underway). There were not consistent sex-differences in incidence or severity of EAE, in spite of a slightly significant difference in EAE duration.

viii) An especially outstanding and consistent result has been the finding that relatively low anxious N/Nih-HS rats (regardless of sex) show significantly increased EAE incidence and severity progression, in comparison to the relatively high anxious animals. Congruently, EAE-susceptible N/Nih-HS rats are those showing the lowest levels of unconditioned anxiety.

ix) Such an association, between anxiety and EAE susceptibility, suggests that the presumably enhanced (stress-related) HPA-axis function of high anxious N/Nih-HS rats, could be a (or one of the) mechanism mediating anti-inflammatory or autoimmune-protective activity in that rat subsample.

x) In that connection, Study II was devoted to compare the well-known EAE-susceptible DA and EAE-resistant PVG rat strains (both sexes) with regard to their anxiety/fear and HPA-axis function profiles. Compared to the EAE-resistant PVG rats, susceptible DA rats show less behavioural

inhibition in specific novelty situations, no differences in typical anxiety/fear measures, reduced HPA-axis response to stress and increased expression levels of pro-inflammatory (CD74 and IL-6) markers.

xi) The connection or association among anxiety/fearfulness traits, HPA-axis function and EAE, and the underlying mechanisms subserving interactions among them, would likely benefit from future studies in which all three mentioned aspects are taken into account in the same population. In this regard, it is proposed that such an approach, either by using N/Nih-HS or AIL rats (both outbred), or utilizing pairs of selectively-bred strains differing in anxiety and in HPA-axis responses, would bring valuable information to better understand the "hidden" aspects of the discussed "Anxiety → HPA → EAE" associations.

REFERENCES

8 REFERENCES

Aguilar R, Gil L, Tobeña A, Escorihuela RM, Fernández-Teruel A (2002) Differential effects of cohort removal stress on the acoustic startle response of the Roman/Verh rat strains. Behav Genet. 30:71-5.

Aguilar R, Escorihuela RM, Gil L, Tobeña A, Fernández-Teruel A (2002) Differences between two psychogenetically selected lines of rats in a swimming pool matching-to-place task: long-term effects of infantile stimulation. Behav Genet.32:127-34.

Aguilar R, Gil L, Gray JA, Driscoll P, Flint J, Dawson GR, Giménez-Llort L, Escorihuela RM, Fernández-Teruel A, Tobeña A (2003) Fearfulness and sex in F2 Roman rats: males display more fear though both sexes share the same fearfulness traits. Physiol Behav.78:723-32.

Ahmed SA, Penhale WJ (1982) The influence of testosterone on the development of autoimmune thyroiditis in thymectomized and irradiated rats. Clin Exp Immunol. 48:367-74.

Alam I, Koller DL, Sun Q, Roeder RK, Cañete T, Blázquez G, López-Aumatell R, Martínez-Membrives E, Vicens-Costa E, Mont C, Díaz S, Tobeña A, Fernández-Teruel A, Whitley A, Strid P, Diez M, Johannesson M, Flint J, Econs MJ, Turner CH, Foroud T(2011) Heterogeneous stock rat: a unique animal model for mapping genes influencing bone fragility. Bone. 48:1169-77.

Amor S, Groome N, Linington C, Morris MM, Dornmair K, Gardinier MV, Matthieu JM, Baker D (1995) Identification of epitopes of myelin oligodendrocyte glycoprotein for the induction of experimental allergic encephalomyelitis in SJL and Biozzi AB/H mice.J Immunol.153:4349-56.

Antony, M.M. and R.P Swinson (1996) Anxiety Disorders and their Treatment: A Critical Review of the Evidence-Based Literature, Health Canada.

Armario A (2006) The hypothalamic-pituitary-adrenal axis: what can it tell us about stressors? CNS Neurol Disord Drug Targets. 5:485-501. Review.

Askari HA (1970) Sexual differences in the biogenesis of the androgens by the adrenal cortex in rat. Endocrinology. 87:1377-80.

Bäckdahl L, Guo JP, Jagodic M, Becanovic K, Ding B, Olsson T, Lorentzen JC (2009) Definition of arthritis candidate risk genes by combining rat linkage-mapping results with human case-control association data. Ann Rheum Dis. 68:1925-32.

Baker D, Rosenwasser OA, O'Neill JK, Turk JL (1995) Genetic analysis of experimental allergic encephalomyelitis in mice.J Immunol.155:4046-51.

Barabási AL, Oltvai ZN (2004) Network biology: understanding the cell's functional organization. Nat Rev Genet. 5:101-13. Review.

Baxter AG (2007) The origin and application of experimental autoimmune encephalomyelitis. Nat Rev Immunol. 7:904-12.

Bebo BF Jr, Zelinka-Vincent E, Adamus G, Amundson D, Vandenbark AA, Offner H (1998) Gonadal hormones influence the immune response to PLP 139-151 and the clinical course of relapsing experimental autoimmune encephalomyelitis. J Neuroimmunol.84:122-30.

Becanovic K, Wallstrom E, Kornek B, Glaser A, Broman KW, Dahlman I, Olofsson P, Holmdahl R, Luthman H, Lassmann H, Olsson T (2003) New loci regulating rat myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis. J Immunol. 170:1062-9.

Becanovic K, Jagodic M, Sheng JR, Dahlman I, Aboul-Enein F, Wallstrom E, Olofsson P, Holmdahl R, Lassmann H, Olsson T (2006) Advanced intercross line mapping of Eae5 reveals Ncf-1 and CLDN4 as candidate genes for experimental autoimmune encephalomyelitis. J Immunol. 176:6055-64.

Belzung C, Griebel G (2001) Measuring normal and pathological anxiety-like behaviour in mice: a review. Behav Brain Res. 125:141-9. Review.

Benison SA, Barger C, Wolfe EL (1987) *Walter B. Cannon: the Life and Times of a Young Scientist*. pp.16-32, Belknap Press.

Bergsteinsdottir K, Yang HT, Pettersson U, Holmdahl R (2000) Evidence for common autoimmune disease genes controlling onset, severity, and chronicity based on experimental models for multiple sclerosis and rheumatoid arthritis. J Immunol. 164:1564-8.

Beswick EJ, Bland DA, Suarez G, Barrera CA, Fan X, Reyes VE (2005) Helicobacter pylori binds to CD74 on gastric epithelial cells and stimulates interleukin-8 production. Infect Immun. 73:2736-43.

Beyeen AD, Adzemovic MZ, Ockinger J, Stridh P, Becanovic K, Laaksonen H, Lassmann H, Harris RA, Hillert J, Alfredsson L, Celius EG, Harbo HF, Kockum I, Jagodic M, Olsson T (2010) IL-22RA2 associates with multiple sclerosis and macrophage effector mechanisms in experimental neuroinflammation. J Immunol. 185:6883-90.

Borghese F, Clanchy FI (2011) CD74: an emerging opportunity as a therapeutic target in cancer and autoimmune disease. Expert Opin Ther Targets.15:237-51.

Boucher W, Cotterman CW (1990) On the classification of regular systems of inbreeding. J Math Biol. 28:293-305.

Bowers JM, Waddell J, McCarthy MM (2010) A developmental sex difference in hippocampal neurogenesis is mediated by endogenous oestradiol. Biol Sex Differ. 2010. 1:8.

Breij EC, Brink BP, Veerhuis R, van den Berg C, Vloet R, Yan R, Dijkstra CD, van der Valk P, Bö L (2008) Homogeneity of active demyelinating lesions in established multiple sclerosis. Ann Neurol. 63:16-25.

Broadhurst PL, Bignami G (1965) Correlative effects of psychogenetic selection: a study of the Roman high and low avoidance strains of rats. Behav Res Ther. 3:273-80.

Butterfield RJ, Blankenhorn EP, Roper RJ, Zachary JF, Doerge RW, Teuscher C (2000) Identification of genetic loci controlling the characteristics and severity of brain and spinal cord lesions in experimental allergic encephalomyelitis. Am J Pathol. 157:637-45.

Butterworth M, McClellan B, Allansmith M (1967) Influence of sex in immunoglobulin levels. Nature. 214:1224-5.

Buttmann M, Rieckmann P (2008) Treating multiple sclerosis with monoclonal antibodies. Expert Rev Neurother. 8:433-55. Review.

Cannon WB (1939) The argument for chemical mediation of nerve impulses. Science. 90:521-7.

Carrasco J, Márquez C, Nadal R, Tobeña A, Fernández-Teruel A, Armario A (2008) Characterization of central and peripheral components of the hypothalamus-pituitary-adrenal axis in the inbred Roman rat strains. Psychoneuroendocrinology. 33:437-45.

Clément Y, Calatayud F, Belzung C (2002) Genetic basis of anxiety-like behaviour: a critical review. Brain Res Bull. 57:57-71. Review.

Chao MJ, Ramagopalan SV, Herrera BM, Lincoln MR, Dyment DA, Sadovnick AD, Ebers GC (2009) Epigenetics in multiple sclerosis susceptibility: difference in transgenerational risk localizes to the major histocompatibility complex. Hum Mol Genet.18:261-6.

Chen CP, Boyadjieva NI, Advis JP, Sarkar DK (2006) Ethanol suppression of the hypothalamic proopiomelanocortin level and the splenic NK cell cytolytic activity is associated with a reduction in the expression of proinflammatory cytokines but not anti-inflammatory cytokines in neuroendocrine and immune cells. Alcohol Clin Exp Res. 30:1925-32.

Chrousos GP, Gold PW (1992) The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. JAMA. 267:1244-52. Review.

Chrousos GP, Castro M, Leung DY, Webster E, Kino T, Bamberger C, Elliot S, Stratakis C, Karl M (1996) Molecular mechanisms of glucocorticoid

resistance/hypersensitivity. Potential clinical implications. Am J Respir Crit Care Med. 154:S39-43. Review.

Chrousos GP (2000) The HPA axis and the stress response. Endocr Res. 26:513-4.

Chrousos GP, Kino T (2005) Interactive functional specificity of the stress and immune responses: the ying, the yang, and the defense against 2 major classes of bacteria. J Infect Dis. 192:551-5.

Chrousos GP, Kino T (2007) Glucocorticoid action networks and complex psychiatric and/or somatic disorders. Stress. 10:213-9. Review.

Clauw DJ, Chrousos GP (1997) Chronic pain and fatigue syndromes: overlapping clinical and neuroendocrine features and potential pathogenic mechanisms. Neuroimmunomodulation. 4:134-53.

Clément Y, Calatayud F, Belzung C (2002) Genetic basis of anxiety-like behaviour: a critical review. Brain Res Bull. 57:57-71. Review.

Clément Y, Chapouthier G (1998) Biological bases of anxiety. Neurosci Biobehav Rev. 22:623-33. Review.

Cohen JA, Chun J (2011) Mechanisms of fingolimod's efficacy and adverse effects in multiple sclerosis.Ann Neurol. 69:759-77. Review.

Cohen AS, Pfister BJ, Schwarzbach E, Grady MS, Goforth PB, Satin LS (2007) Injury-induced alterations in CNS electrophysiology. Prog Brain Res. 161:143–169.

Compston A, Coles A (2002) Multiple sclerosis. Lancet. 25;372:1502-17.

Compston A (2006) The basis for treatment in multiple sclerosis. Acta Neurol Scand Suppl.183:41-7. Review.

Criswell LA, Such CL, Neuhaus JM, Yelin EH (1997) Variation among rheumatologists in clinical outcomes and frequency of office visits for rheumatoid arthritis. J Rheumatol. 24:1266-71.

Dahlman I, Lorentzen JC, de Graaf KL, Stefferl A, Linington C, Luthman H, Olsson T (1998) Quantitative trait loci disposing for both experimental arthritis andencephalomyelitis in the DA rat; impact on severity of myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis and antibody isotype pattern. Eur J Immunol. 28:2188-96.

Dahlman I, Wallström E, Weissert R, Storch M, Kornek B, Jacobsson L, Linington C, Luthman H, Lassmann H, Olsson T (1998) Linkage analysis of myelin oligodendrocyte glycoprotein-induced experimental

autoimmune encephalomyelitis in the rat identifies a locus controlling demyelination on chromosome 18. Hum Mol Genet. 8:2183-90.

Dal Canto MC, Melvold RW, Kim BS, Miller SD (1995) Two models of multiple sclerosis: experimental allergic encephalomyelitis (EAE) and Theiler's murine encephalomyelitis virus (TMEV) infection. A pathological and immunological comparison. Microsc Res Tech. 32:215-29. Review.

Dalal M, Kim S, Voskuhl RR (1997) Testosterone therapy ameliorates experimental autoimmune encephalomyelitis and induces a T helper 2 bias in the autoantigen-specific T lymphocyte response. J Immunol. 159:3-6.

Dalla C, Antoniou K, Drossopoulou G, Xagoraris M, Kokras N, Sfikakis A, Papadopoulou-Daifoti Z (2005) Chronic mild stress impact: are females more vulnerable? Neuroscience. 135:703-14.

Dalla C, Antoniou K, Kokras N, Drossopoulou G, Papathanasiou G, Bekris S, Daskas S, Papadopoulou-Daifoti Z (2008) Sex differences in the effects of two stress paradigms on dopaminergic neurotransmission. Physiol Behav. 93:595-605.

Dantzer R, Kelley KW (2007) Twenty years of research on cytokine-induced sickness behavior. Brain Behav Immun. 21:153-60.

Darvasi A, Soller M (1995) Advanced intercross lines, an experimental population for fine genetic mapping. Genetics. 141:1199-207.

Da Silva JA (1999) Sex hormones and glucocorticoids: interactions with the immune system. Ann N Y Acad Sci. 876:102-17; discussion 117-8. Review.

David L. Rabiner, J. W. Kling and Patricia A. Spraguer (1988) Modulation of taste-induced drinking: The effects of concentration shifts and drinking interruptions. Anim Learn Behav16: 365-376, DOI: 10.3758/BF03209374

Davis M, Whalen PJ (2001) The amygdala: vigilance and emotion. Mol Psychiatry. 6:13-34. Review.

De Jager PL, Chibnik LB, Cui J, Reischl J, Lehr S, Simon KC, Aubin C, Bauer D, Heubach JF, Sandbrink R, Tyblova M, Lelkova P; Steering committee of the BENEFIT study; Steering committee of the BEYOND study; Steering committee of the LTF study; Steering committee of the CCR1

study, Havrdova E, Pohl C, Horakova D, Ascherio A, Hafler DA, Karlson EW (2009) Integration of genetic risk factors into a clinical algorithm for multiple sclerosissusceptibility: a weighted genetic risk score. Lancet Neurol. 8:1111-9. Epub 2009 Oct 29.

Deinzer R, Granrath N, Stuhl H, Twork L, Idel H, Waschul B, Herforth A (2004) Acute stress effects on local Il-1beta responses to pathogens in a human in vivo model. Brain Behav Immun. 18:458-67.

De Kloet ER, Vreugdenhil E, Oitzl MS, Joëls M (1998) Brain corticosteroid receptor balance in health and disease. Endocr Rev. 19:269-301. Review.

Denic A, Johnson AJ, Bieber AJ, Warrington AE, Rodriguez M, Pirko I (2011) The relevance of animal models in multiple sclerosis research. Pathophysiology. 18:21-9.

Dhabhar FS, McEwen BS (1999) Enhancing versus suppressive effects of stress hormones on skin immune function. Proc Natl Acad Sci U S A. 96:1059-64.

Díaz-Morán S, Palència M, Mont-Cardona C, Cañete T, Blázquez G, Martínez-Membrives E, López-Aumatell R, Tobeña A, Fernández-Teruel A (2012) Coping style and stress hormone responses in genetically heterogeneous rats: comparison with the Roman rat strains. Behav Brain Res. 228:203-10.

Doerge DR (2001) Placental transfer of the soy isoflavone genistein following dietary and gavage administration to Sprague Dawley rats. Reprod Toxicol. 15:105-10.

Drossopoulou G, Antoniou K, Kitraki E, Papathanasiou G, Papalexi E, Dalla C, Papadopoulou-Daifoti Z (2004) Sex differences in behavioral, neurochemical and neuroendocrine effects induced by the forced swim test in rats. Neuroscience. 126:849-57.

Duma D, Collins JB, Chou JW, Cidlowski JA (2010) Sexually dimorphic actions of glucocorticoids provide a link to inflammatory diseases with gender differences in prevalence. Sci Signal. 3:ra74.

Duquette P, Pleines J, Girard M, Charest L, Senecal-Quevillon M, Masse C (1992) The increased susceptibility of women to multiple sclerosis. Can J Neurol Sci. 19:466-71. Review.

Elenkov IJ, Webster E, Papanicolaou DA, Fleisher TA, Chrousos GP, Wilder RL (1998) Histamine potently suppresses human IL-12 and stimulates IL-10 production via H2 receptors. J Immunol. 161:2586-93.

Escorihuela RM, Fernández-Teruel A, Zapata A, Núñez JF, Tobeña A (1993) Flumazenil prevents the anxiolytic effects of diazepam, alprazolam and adinazolam on the early acquisition of two-way active avoidance. Pharmacol Res. 28:53-8.

Fernández-Teruel A, Escorihuela RM (1997) Modeling emotional reactivity and sensation/novelty seeking with the Roman/Verh rat lines/strains: an introduction. Behav Genet. 27:499-501.

Encinas JA, Lees MB, Sobel RA, Symonowicz C, Weiner HL, Seidman CE, Seidman JG, Kuchroo VK (2001) Identification of genetic loci associated with paralysis, inflammation and weight loss in mouse experimental autoimmune encephalomyelitis. Int Immunol. 13:257-64.

Faure-André G, Vargas P, Yuseff MI, Heuzé M, Diaz J, Lankar D, Steri V, Manry J, Hugues S, Vascotto F, Boulanger J, Raposo G, Bono MR,Rosemblatt M, Piel M, Lennon-Duménil AM (2008) Regulation of dendritic cell migration by CD74, the MHC class II-associated invariant chain. Science. 12;322:1705-10.

Flaherty CF, Rowan GA (1986) Successive, simultaneous, and anticipatory contrast in the consumption of saccharin solutions. J Exp Psychol Anim Behav Process. 12:381-93.

Fernandes C, González MI, Wilson CA, File SE (1999) Factor analysis shows that female rat behaviour is characterized primarily by activity, male rats are driven by sex and anxiety. Pharmacol Biochem Behav. 64:731-8.

Fernández-Teruel A, Escorihuela RM, Driscoll P, Tobeña A, Bättig K (1991) Infantile (handling) stimulation and behavior in young Roman high- and low-avoidance rats. Physiol Behav. 50:563-5.

Fernández-Teruel A, Escorihuela RM, Núñez JF, Zapata A, Boix F, Salazar W, Tobeña A (1991) The early acquisition of two-way (shuttle-box) avoidance as an anxiety-mediated behavior: psychopharmacological validation.
Brain Res Bull. 26:173-6.

Fernández-Teruel A, Escorihuela RM, Boix F, Tobena A (1991) Effects of different handling-stimulation procedures and benzodiazepines on two-way active avoidance acquisition in rats. Pharmacol Res. 24:273-82.
Fernández-Teruel A, Escorihuela RM, Núñez JF, Gomà M, Driscoll P, Tobeña A (1992) Early stimulation effects on novelty-induced behavior in two psychogenetically-selected rat lines with divergent emotionality profiles. Neurosci Lett. 137(2):185-8.

Fernández-Teruel A, Driscoll P, Gil L, Aguilar R, Tobeña A, Escorihuela RM (2002) Enduring effects of environmental enrichment on novelty seeking, saccharin and ethanol intake in two rat lines (RHA/Verh and RLA/Verh) differing in incentive-seeking behavior. Pharmacol Biochem Behav. 73:225-31.
Finn DP, Martí O, Harbuz MS, Vallès A, Belda X, Márquez C, Jessop DS, Lalies MD, Armario A, Nutt DJ, Hudson AL (2003) Behavioral, neuroendocrine and neurochemical effects of the imidazoline I2 receptor

selective ligand BU224 in naive rats and rats exposed to the stress of the forced swim test. Psychopharmacology (Berl). 167:195-202.

Fitzpatrick F, Lepault F, Homo-Delarche F, Bach JF, Dardenne M (1991) Influence of castration, alone or combined with thymectomy, on the development of diabetes in the nonobese diabetic mouse. Endocrinology. 129:1382-90.

Flint J, Mott R (2001) Finding the molecular basis of quantitative traits: successes and pitfalls. Nat Rev Genet. 2:437-45.

Flint J (2004) The genetic basis of neuroticism. Neurosci Biobehav Rev. 28:307-16. Review.

Foster SC, Daniels C, Bourdette DN, Bebo BF Jr (2003) Dysregulation of the hypothalamic-pituitary-gonadal axis in experimental autoimmune encephalomyelitis and multiple sclerosis. J Neuroimmunol. 140:78-87.

Fox HS (1992) Androgen treatment prevents diabetes in nonobese diabetic mice. J Exp Med. 175:1409-12.

Franchimont D, Kino T, Galon J, Meduri GU, Chrousos G (2003) Glucocorticoids and inflammation revisited: the state of the art. NIH clinical staff conference. Neuroimmunomodulation. 10:247-60. Review.

Freund J, Stern ER, Pisani TM (1947) Isoallergic encephalomyelitis and radiculitis in guinea pigs after one injection of brain and Mycobacteria in water-in-oil emulsion. J Immunol. 57:179-94.

Galea LA, McEwen BS, Tanapat P, Deak T, Spencer RL, Dhabhar FS (1997) Sex differences in dendritic atrophy of CA3 pyramidal neurons in response to chronic restraint stress. Neuroscience. 81:689-97.

Gagliano H, Fuentes S, Nadal R, Armario A (2007) Previous exposure to immobilisation and repeated exposure to a novel environment demonstrate a marked dissociation between behavioral and pituitary-adrenal responses. Behav Brain Res. 187:239-45.

García A, Martí O, Vallès A, Dal-Zotto S, Armario A (2000) Neuroendocrinology. 72:114-25.

Geller I, Demarco AO, Seifter J (1960) Delayed effects of nicotine on timing behavior in the rat. Science. 131:735-7.

Gesing A, Bilang-Bleuel A, Droste SK, Linthorst AC, Holsboer F, Reul JM (2001) Psychological stress increases hippocampal mineralocorticoid receptor levels: involvement of corticotropin-releasing hormone. J Neurosci. 21:4822-9.

Giovannoni G, Comi G, Cook S, Rammohan K, Rieckmann P, Soelberg Sørensen P, Vermersch P, Chang P, Hamlett A, Musch B, Greenberg SJ; CLARITY Study Group (2010) N Engl J Med. 362:416-26.

Glorioso N, Argiolas G, Filigheddu F, Troffa C, Cocco F, Bulla E, Bulla P, Zaninello R, Degortes S, Pitzoi S, Frau F, Fadda S, Pinna Parpaglia P, Bernini G, Bardini M, Fallo F, Malatino L, Regolisti G, Ferri C, Cusi D, Sciacqua A, Perticone F, Degli Esposti E, Baraccani C, Parati G, Veglio F, Mulatero P, Williams TA, Macciardi F, Stancanelli B; Study Group on Cardiovascular Pharmacogenomics of Italian Society of Hypertension (2007) Conceptual basis and methodology of the SOPHIA study. Pharmacogenomics. 8:1497-509.

Frank MG, Barrientos RM, Biedenkapp JC, Rudy JW, Watkins LR, Maier SF (2006) mRNA up-regulation of MHC II and pivotal pro-inflammatory genes in normal brain aging. Neurobiol Aging. 27:717-22.

Goebel MU, Mills PJ, Irwin MR, Ziegler MG (2000) Interleukin-6 and tumor necrosis factor-alpha production after acute psychological stress, exercise, and infused isoproterenol: differential effects and pathways. Psychosom Med. 62:591-8.

Gold SM, Voskuhl RR (2009) Estrogen and testosterone therapies in multiple sclerosis. Prog Brain Res. 175:239-51. Review.

Gold SM, Mohr DC, Huitinga I, Flachenecker P, Sternberg EM, Heesen C (2005) The role of stress-response systems for the pathogenesis and progression of MS. Trends Immunol. 26:644-52. Review.

Gold SM, Raji A, Huitinga I, Wiedemann K, Schulz KH, Heesen C (2005) Hypothalamo-pituitary-adrenal axis activity predicts disease progression in multiple sclerosis. J Neuroimmunol. 165:186-91.

Goldmuntz EA, Remmers EF, Zha H, Mathern P, Du Y, Crofford LJ, Wilder RL (1993) Genetic map of seven polymorphic markers comprising a single linkage group on rat chromosome 5. Mamm Genome. 4:670-5.

Gordon JA, Hen R (2004) Genetic approaches to the study of anxiety. Annu Rev Neurosci. 27:193-222. Review.

Gourley SL, Wu FJ, Kiraly DD, Ploski JE, Kedves AT, Duman RS, Taylor JR (2008) Regionally specific regulation of ERK MAP kinase in a model of antidepressant-sensitive chronic depression. Biol Psychiatry.63:353-9.

Gray JA (1979) Emotionality in male and female rodents: a reply to Archer. Br J Psychol. 70:425-40.

Gray JA (1981) A critique of Eysenck's theory of personality. In H. J. Eysenck (Ed.), A model for personality. New York: Springer. 246–276.

Gray JA (1987). The psychology of fear and stress. Cambridge: Cambridge University Press.

Gray JA and McNaughton N (2000) The Neuropsychology of Anxiety: An Enquiry into the Functions of the Septo-Hippocampal System. Oxford Psychology Series. 33. Oxford: Oxford University Press.

Gross C and Hen R (2004) Genetic and environmental factors interact to influence anxiety. Neurotox Res 6: 493-501.

Grossman CJ, Nienaber M, Mendenhall CL, Hurtubise P, Roselle GA, Rouster S, Weber N, Schmitt G, Gartside PS (1993) Sex differences and the effects of alcohol on immune response in male and female rats. Alcohol Clin Exp Res. 17:832-40.

Gulko PS, Kawahito Y, Remmers EF, Reese VR, Wang J, Dracheva SV, Ge L, Longman RE, Shepard JS, Cannon GW, Sawitzke AD, Wilder RL, Griffiths MM (1998) Identification of a new non-major histocompatibility complex genetic locus on chromosome 2 that controls disease severity in collagen-induced arthritis in rats. Arthritis Rheum. 41:2122-31.

Huberle A, Beyeen AD, Ockinger J, Ayturan M, Jagodic M, de Graaf KL, Fissolo N, Marta M, Olofsson P, Hultqvist M, Holmdahl R, Olsson T, Weissert R (2009) Advanced intercross line mapping suggests that ncf1 (ean6) regulates severity in an animal model of guillain-barre syndrome. J Immunol. 182:4432-8.

Hall J, Morand EF, Medbak S, Zaman M, Perry L, Goulding NJ, Maddison PJ, O'Hare JP (1994) Abnormal hypothalamic-pituitary-adrenal axis function in rheumatoid arthritis. Effects of nonsteroidal antiinflammatory drugs and water immersion. Arthritis Rheum. 37:1132-7.

Handa RJ, Ogawa S, Wang JM, Herbison AE (2012) Roles for oestrogen receptor β in adult brain function. J Neuroendocrinol. 24:160-73.

Hansen C, Spuhler K (1984) Development of the National Institutes of Health genetically heterogeneous rat stock. Alcohol Clin Exp Res. 8:477-9.

Happ MP, Wettstein P, Dietzschold B, Heber-Katz E (1988) Genetic control of the development of experimental allergic encephalomyelitis in rats. Separation of MHC and non-MHC gene effects. J Immunol. 141:1489-94.

Harbuz MS, Lightman SL (1992) Stress and the hypothalamo-pituitary-adrenal axis: acute, chronic and immunological activation. J Endocrinol. 134:327-39. Review.

Harbuz MS, Leonard JP, Lightman SL, Cuzner ML (1993) Changes in hypothalamic corticotrophin-releasing factor and anterior pituitary pro-opiomelanocortin mRNA during the course of experimental allergic encephalomyelitis. J Neuroimmunol. 45:127-32.

Harbuz MS, Perveen-Gill Z, Lightman SL, Jessop DS (1995) A protective role for testosterone in adjuvant-induced arthritis. Br J Rheumatol. 34:1117-22.

Harnesk K, Swanberg M, Ockinger J, Diez M, Lidman O, Wallström E, Lobell A, Olsson T, Piehl F (2008) Vra4 congenic rats with allelic differences in the class II transactivator gene display altered susceptibility to experimental autoimmune encephalomyelitis. J Immunol.180:3289-96.

Hartwell LH, Hopfield JJ, Leibler S, Murray AW (1999) From molecular to modular cell biology. Nature. 402:C47-52.

Hatalski CG, Baram TZ (1997) Stress-induced transcriptional regulation in the developing rat brain involves increased cyclic adenosine 3',5'-monophosphate-regulatory element binding activity. Mol Endocrinol. 11:2016-24.

Hedrich HJ, Fangmann J, Reetz IC, Klöting I, Kornerup Hansen A, Wonigeit K (1990) Genetic characterization of different diabetes-prone and -resistant BB-rat strains with special reference to RT6. Transplant Proc. 22:2570-1.

Heesen C, Gold SM, Huitinga I, JMHM Reul (2007) Stress and hypothalamic-pituitary-adrenal axis function in experimental autoimmune encephalomyelitis and multiple sclerosis-A review. Psychoneuroendocr. 32:604-618

Hemmer B, Cepok S, Nessler S, Sommer N (2002) Pathogenesis of multiple sclerosis: an update on immunology. Curr Opin Neurol. 15:227-31. Review.

Herman JP, Figueiredo H, Mueller NK, Ulrich-Lai Y, Ostrander MM, Choi DC, Cullinan WE (2003) Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. Front Neuroendocrinol. 24:151-80. Review.

Herrero AI, Sandi C, Venero C (2006) Individual differences in anxiety trait are related to spatial learning abilities and hippocampal expression of mineralocorticoid receptors. Neurobiology of learning and memory. 86:150-159.

Hitzemann R (2000) Animal models of psychiatric disorders and their relevance to alcoholism. Alcohol Res Health. 24:149-58.

Holsboer F, Barden N (1996) Antidepressants and hypothalamic-pituitary-adrenocortical regulation. Endocr Rev. 17:187-205. Review.

Honjo T, Kuribayashi T, Seita T, Mokonuma Y, Yamaga A, Yamazaki S, Yamamoto S (2012) The effects of interleukin-6 and cytokine-induced neutrophil chemoattractant-1 on α-macroglobulin production in rats. Exp Anim. 59:589-94.

Huang GJ, Shifman S, Valdar W, Johannesson M, Yalcin B, Taylor MS, Taylor JM, Mott R, Flint J (2009) High resolution mapping of

expression QTLs in heterogeneous stock mice in multiple tissues. Genome Res. 19:1133-40.

Hutchinson, E. (2007) *Dimensions of human behavior: The changing life course*, (3rd Edition). Sage: USA.

Hughes RA, Stedronska J (1973) The susceptibility of rat strains to experimental allergic encephalomyelitis. Immunology. 24:879-84.

International Multiple Sclerosis Genetics Consortium, Hafler DA, Compston A, Sawcer S, Lander ES, Daly MJ, De Jager PL, de Bakker PI, Gabriel SB, Mirel DB, Ivinson AJ, Pericak-Vance MA, Gregory SG, Rioux JD, McCauley JL, Haines JL, Barcellos LF, Cree B, Oksenberg JR, Hauser SL (2007) Risk alleles for multiple sclerosis identified by a genomewide study. N Engl J Med.357:851-62.

Jagodic M, Kornek B, Weissert R, Lassmann H, Olsson T, Dahlman I (2001) Congenic mapping confirms a locus on rat chromosome 10 conferring strong protection against myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis.Immunogenetics.53:410-5.

Jagodic M, Becanovic K, Sheng JR, Wu X, Bäckdahl L, Lorentzen JC, Wallström E, Olsson T (2004) An advanced intercross line resolves Eae18 into two narrow quantitative trait loci syntenic to multiple sclerosis candidate loci. J Immunol. 173:1366-73.

Jagodic M, Olsson T (2006) Combined-cross analysis of genome-wide linkage scans for experimental autoimmune encephalomyelitis in rat. Genomics. 88:737-44.

Jansson AM, Jacobsson L, Luthman H, Lorentzen JC (1999) Susceptibility to oil-induced arthritis is linked to Oia2 on chromosome 4 in a DA(DA x PVG.1AV1) backcross. Transplant Proc. 31:1597-9.

Jersild C, Dupont B, Fog T, Platz PJ, Svejgaard A (1975) Histocompatibility determinants in multiple sclerosis. Transplant Rev. 22:148-63.

Joëls M, Karst H, DeRijk R, de Kloet ER (2008) The coming out of the brain mineralocorticoid receptor. Trends Neurosci. 31:1-7.

Johannesson M, Lopez-Aumatell R, Stridh P, Diez M, Tuncel J, Blázquez G, Martinez-Membrives E, Cañete T, Vicens-Costa E, Graham D, Copley RR, Hernandez-Pliego P, Beyeen AD, Ockinger J, Fernández-Santamaría C, Gulko PS, Brenner M, Tobeña A, Guitart-Masip M, Giménez-Llort L, Dominiczak A, Holmdahl R, Gauguier D, Olsson T, Mott R, Valdar W, Redei EE, Fernández-Teruel A, Flint J (2009) A resource for the simultaneous high-resolution mapping of multiple quantitative trait loci in rats: the NIH heterogeneous stock. Genome Res. 19:150-8.

Johnston AL, File SE (1991) Sex differences in animal tests of anxiety. Physiol Behav. 49:245-50.

Kadioglu A, Cuppone AM, Trappetti C, List T, Spreafico A, Pozzi G, Andrew PW, Oggioni MR (2011) Sex-based differences in susceptibility to respiratory and systemic pneumococcal disease in mice. J Infect Dis. 204:1971-9.

Kalueff AV, Tuohimaa P (2004) Experimental modeling of anxiety and depression. Acta Neurobiol Exp (Wars). 64:39-48. Review.

Kantarci OH, Hebrink DD, Schaefer-Klein J, Sun Y, Achenbach S, Atkinson EJ, Heggarty S, Cotleur AC, de Andrade M, Vandenbroeck K, Pelfrey CM, Weinshenker BG (2008) Interferon gamma allelic variants: sex-biased multiple sclerosis susceptibility and gene expression. Arch Neurol. 65:349-57.

Kappos L, Radue EW, O'Connor P, Polman C, Hohlfeld R, Calabresi P, Selmaj K, Agoropoulou C, Leyk M, Zhang-Auberson L, Burtin P; FREEDOMS Study Group (2010) A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis. N Engl J Med. 362:387-401.

Karalis K, Sano H, Redwine J, Listwak S, Wilder RL, Chrousos GP (1991) Autocrine or paracrine inflammatory actions of corticotropin-releasing hormone in vivo. Science. 254:421-3.

Karandrea D, Kittas C, Kitraki E (2000) Contribution of sex and cellular context in the regulation of brain corticosteroid receptors following restraint stress. Neuroendocrinology. 71:343-53.

Kataranovski M, Janković S, Kataranovski D, Stosić J, Bogojević D (2009) Gender differences in acute cadmium-induced systemic inflammation in rats. Biomed Environ Sci. 22:1-7.

Keyes KM, Hatzenbuehler ML, Hasin DS (2011) Stressful life experiences, alcohol consumption, and alcohol use disorders: the epidemiologic evidence for four main types of stressors. Psychopharmacology (Berl). 218:1-17. Review.

King SM (1999) Escape-related behaviours in an unstable elevated and exposed environment. I. A new behavioural model of extreme anxiety. Behav Brain Res. 98:113-26.

Kitay J (1961) Sex differences in adrenal cortical secretion in the rat. Endocrinology. 68:818-24.

Kleinau S, Erlandsson H, Holmdahl R, Klareskog L (1991) Adjuvant oils induce arthritis in the DA rat. I. Characterization of the disease and evidence for an immunological involvement. J Autoimmun. 4:871-80.

Kleinschmidt-DeMasters BK, Tyler KL (2005) Progressive multifocal leukoencephalopathy complicating treatment with natalizumab and interferon beta-1a for multiple sclerosis. N Engl J Med. ;353:369-74.

Kwitek-Black AE, Jacob HJ (2001) The use of designer rats in the genetic dissection of hypertension. Curr Hypertens Rep. 3:12-8.

Kokras N, Antoniou K, Dalla C, Bekris S, Xagoraris M, Ovestreet DH, Papadopoulou-Daifoti Z (2009) Sex-related differential response to clomipramine treatment in a rat model of depression. J Psychopharmacol. 23:945-56.

Koolhaas JM, Bartolomucci A, Buwalda B, de Boer SF, Flügge G, Korte SM, Meerlo P, Murison R, Olivier B, Palanza P, Richter-Levin G, Sgoifo A, Steimer T, Stiedl O, van Dijk G, Wöhr M, Fuchs E (2011) Stress revisited: a critical evaluation of the stress concept. Neurosci Biobehav Rev. 35:1291-301. Review.

Kornblum J (1968) The application of the irradiated hamster test to the study of experimental allergic encephalomyelitis. J Immunol. 101:702-10.

Krishnan V and Nestler J (2008) The molecular neurobiology of depression. Nature. 455: 894–902.

Kudielka BM, Kirschbaum C (2005) Sex differences in HPA axis responses to stress: a review. Biol Psychol. 69:113-32.

Kurtzke JF (1983) Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). Neurology. 33:1444-52.

Landgraf R, Wigger A (2003) Born to be anxious: neuroendocrine and genetic correlates of trait anxiety in HAB rats. Stress. 6:111-9. Review.

Langer-Gould A, Moses HH, Murray TJ (2004) Strategies for managing the side effects of treatments for multiple sclerosis. Neurology. 63:S35-41. Review.

LeDoux JE (2000) Emotion circuits in the brain. Annu Rev Neurosci. 23:155-84. Review.

Lehoux JG, Fleury A, Ducharme L (1998) The acute and chronic effects of adrenocorticotropin on the levels of messenger ribonucleic acid and protein of steroidogenic enzymes in rat adrenal in vivo. Endocrinology. 139:3913-22.

Leng L, Metz CN, Fang Y, Xu J, Donnelly S, Baugh J, Delohery T, Chen Y, Mitchell RA, Bucala R (2003) MIF signal transduction initiated by binding to CD74. J Exp Med. 197:1467-76.

Lenz DC, Wolf NA, Swanborg RH (1999) Strain variation in autoimmunity: attempted tolerization of DA rats results in the induction of experimental autoimmune encephalomyelitis. J Immunol. 163:1763-8.

Lépine JP (2002) The epidemiology of anxiety disorders: prevalence and societal costs. J Clin Psychiatry. 63:4-8. Review.

Lesch KP (2001) Mouse anxiety: the power of knockout. Pharmacogenomics J. 1:187-92. Review.

Levine S, Wenk EJ (1961) Studies on the mechanism of altered susceptibility to experimental allergic encephalomyelitis. Am J Pathol. 39:419-41.

Levine S, Wenk EJ (1965) A hyperacute form of allergic encephalomyelitis. Am J Pathol. 47:61-88.

Levine S, Saltzman A (1987) Increased hemagglutinins after intraperitoneal immunization in postinflammatory state. J Immunol Methods. 105:179-82.

Licinio J, Wong ML (1999) The role of inflammatory mediators in the biology of major depression: central nervous system cytokines modulate the biological substrate of depressive symptoms, regulate stress-responsive systems, and contribute to neurotoxicity and neuroprotection. Mol Psychiatry. 4:317-27. Review.

Libert C, Dejager L, Pinheiro I (2010) The X chromosome in immune functions: when a chromosome makes the difference. Nat Rev Immunol. 10:594-604. Review.

Lincoln MR, Montpetit A, Cader MZ, Saarela J, Dyment DA, Tiislar M, Ferretti V, Tienari PJ, Sadovnick AD, Peltonen L, Ebers GC, Hudson TJ (2005) A predominant role for the HLA class II region in the association of the MHC region with multiple sclerosis. Nat Genet. 37:1108-12.

Lindh J (1977) Susceptibility to experimental allergic encephalomyelitis in two strains of rats and their hybrids. Acta Neuropathol. 38:61-6.

Linker RA, Lee DH (2009) Models of autoimmune demyelination in the central nervous system: on the way to translational medicine.Exp Transl Stroke Med. 21:1-5.

Lipton MM, Freund J (1952) Encephalomyelitis in the rat following intracutaneous injection of central nervous system tissue with adjuvant. Proc Soc Exp Biol Med. 81:260-1.

Liva SM, Voskuhl RR (2001) Testosterone acts directly on CD4+ T lymphocytes to increase IL-10 production. J Immunol. 167:2060-7.

Lorentzen JC, Klareskog L (1996) Susceptibility of DA rats to arthritis induced with adjuvant oil or rat collagen is determined by genes both within and outside the major histocompatibility complex.Scand J Immunol.44:592-8.

Lorentzen JC, Andersson M, Issazadeh S, Dahlman I, Luthman H, Weissert R, Olsson T (1997) Genetic analysis of inflammation, cytokine mRNA expression and disease course of relapsing experimental autoimmune encephalomyelitis in DA rats. J Neuroimmunol. 80:31-7.

Lucchinetti C, Brück W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H (1999) A quantitative analysis of oligodendrocytes in multiple sclerosis lesions. A study of 113 cases. Brain. 122:2279-95.

Lupien SJ, Buss C, Schramek TE, Maheu F, Pruessner J (2005) Hormetic influence of glucocorticoids on human memory. Nonlinearity Biol Toxicol Med. 3:23-56.

Lopez-Aumatell R, Guitart-Masip M, Vicens-Costa E, Gimenez-Llort L, Valdar W, Johannesson M, Flint J, Tobeña A, Fernandez-Teruel A (2008) Fearfulness in a large N/Nih genetically heterogeneous rat stock: differential profiles of timidity and defensive flight in males and females. Behav Brain Res. 188:41-55.

López-Aumatell R, Blázquez G, Gil L, Aguilar R, Cañete T, Giménez-Llort L, Tobeña A, Fernández-Teruel A (2009) The Roman High- and Low-Avoidance rat strains differ in fear-potentiated startle and classical aversive conditioning. Psicothema. 21:27-32.

López-Aumatell R, Vicens-Costa E, Guitart-Masip M, Martínez-Membrives E, Valdar W, Johannesson M, Cañete T, Blázquez G, Driscoll P, Flint J, Tobeña A, Fernández-Teruel A (2009) Unlearned anxiety predicts learned fear: a comparison among heterogeneous rats and the Roman rat strains. Behav Brain Res. 202:92-101.

López-Aumatell R, Martínez-Membrives E, Vicens-Costa E, Cañete T, Blázquez G, Mont-Cardona C, Johannesson M, Flint J, Tobeña A, Fernández-Teruel A (2011) Effects of environmental and physiological covariates on sex differences in unconditioned and conditioned anxiety and fear in a large sample of genetically heterogeneous (N/Nih-HS) rats. Behav Brain Funct. 7:48.

Maes M, Lin A, Bosmans E, Vandoolaeghe E, Bonaccorso S, Kenis G, De Jongh R, Verkerk R, Song C, Scharpé S, Neels H (1998) Serotonin-immune interactions in detoxified chronic alcoholic patients without apparent liver disease: activation of the inflammatory response system and lower plasma total tryptophan. Psychiatry Res. 78:151-61.

Maier SF, Watkins LR (1998) Cytokines for psychologists: implications of bidirectional immune-to-brain communication for understanding behavior, mood, and cognition. Psychol Rev. 105:83-107. Review.

Marta M, Stridh P, Becanovic K, Gillett A, Ockinger J, Lorentzen JC, Jagodic M,Olsson T (2010) Multiple loci comprising immune-related genes regulate experimental neuroinflammation. Genes Immun.11:21-36.

Marta M (2007) Gene regulation and immune mechanisms in multiple sclerosis experimental models. Ph.D. Thesis. Karolinska Institutet.

Martínez-Membrives E, Vicens-Costa E, López-Aumatell R,Blázquez G, Cañete T, Tobeña A, Fernández-Teruel A (submitted) Spatial learning in the genetically heterogeneous N/Nih-HS rat stock: relationship with levels of anxiety.

Massella A, D'Intino G, Fernández M, Sivilia S, Lorenzini L, Giatti S, Melcangi RC, Calzà L, Giardino L (2012) Gender effect on neurodegeneration and myelin markers in an animal model for multiple sclerosis. BMC Neurosci. 24;13:12.

Masterman T, Ligers A, Olsson T, Andersson M, Olerup O, Hillert J (2000) HLA-DR15 is associated with lower age at onset in multiple sclerosis. Ann Neurol. 48:211-9.

MacPhee IA, Mason DW (1988) Evidence for an immunosuppressive autoantibody in experimental allergic encephalomyelitis. Ann N Y Acad Sci. ;540:718-9.

MacPhee IA, Antoni FA, Mason DW (1989) Spontaneous recovery of rats from experimental allergic encephalomyelitis is dependent on regulation of the immune system by endogenous adrenal corticosteroids. J Exp Med. 169:431-45.

MacPhee IA, Mason DW (1990) Studies on the refractoriness to reinduction of experimental allergic encephalomyelitis in Lewis rats that have recovered from one episode of the disease. J Neuroimmunol. 27:9-19.

Matza D, Kerem A, Shachar I (2003) Invariant chain, a chain of command. Trends Immunol. 24:264-8.

McDonald WI, Compston A, Edan G, Goodkin D, Hartung HP, Lublin FD, McFarland HF, Paty DW, Polman CH, Reingold SC, Sandberg-Wollheim M, Sibley W, Thompson A, van den Noort S, Weinshenker BY, Wolinsky JS (2001) Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. Ann Neurol. 50:121-7.

McQualter JL, Bernard CC (2007) Multiple sclerosis: a battle between destruction and repair. J Neurochem. 100:295-306.

Mechan AO, Moran PM, Elliott M, Young AJ, Joseph MH, Green R (2002) A comparison between Dark Agouti and Sprague-Dawley rats in their behaviour on the elevated plus-maze, open-field apparatus and activity meters, and their response to diazepam. Psychopharmacology (Berl). 159:188-95

Meaney MJ, Aitken DH, van Berkel C, Bhatnagar S, Sapolsky RM (1988) Effect of neonatal handling on age-related impairments associated with the hippocampus. Science. 239:766-8.

Miller DM, Rudick RA, Cutter G, Baier M, Fischer JS (2000) Clinical significance of the multiple sclerosis functional composite: relationship to patient-reported quality of life. Arch Neurol. 57:1319-24.

Moldovan IR, Cotleur AC, Zamor N, Butler RS, Pelfrey CM (2008) Multiple sclerosis patients show sexual dimorphism in cytokine responses to myelin antigens. J Neuroimmunol. 193:161-9.

Moraska A, Campisi J, Nguyen KT, Maier SF, Watkins LR, Fleshner M (2002) Elevated IL-1beta contributes to antibody suppression produced by stress. J Appl Physiol. 93:207-15.

Moretti M, Colla A, de Oliveira Balen G, dos Santos DB, Budni J, de Freitas AE, Farina M, Severo Rodrigues AL (2012) Ascorbic acid treatment, similarly to fluoxetine, reverses depressive-like behavior and brain oxidative damage induced by chronic unpredictable stress. J Psychiatr Res. 46:331-40.

Morrison LR (1947) Disseminated encephalomyelitis experimentally produced by the use of homologous antigen. Arch Neurol Psychiatry. 58:391-416.

Mott R (2000) Accurate formula for P-values of gapped local sequence and profile alignments. J Mol Biol. 300:649-59.

Mott R, Talbot CJ, Turri MG, Collins AC, Flint J (2000) A method for fine mapping quantitative trait loci in outbred animal stocks. Proc Natl Acad Sci U S A. 97:12649-54.

Mott R, Flint J (2002) Simultaneous detection and fine mapping of quantitative trait loci in mice using heterogeneous stocks. Genetics. 160:1609-18.

Mott RT, Thore CR, Moody DM, Glazier SS, Ellis TL, Brown WR (2009) Reduced ratio of afferent to total vascular density in mesial temporal sclerosis. J Neuropathol Exp Neurol. 68:1147-54.

Motsinger AA, Brassat D, Caillier SJ, Erlich HA, Walker K, Steiner LL, Barcellos LF, Pericak-Vance MA, Schmidt S, Gregory S, Hauser SL, Haines JL, Oksenberg JR, Ritchie MD (2007) Complex gene-gene interactions in multiple sclerosis: a multifactorial approach reveals associations with inflammatory genes. Neurogenetics. 8:11-20.

Musselman DL, Miller AH, Porter MR, Manatunga A, Gao F, Penna S, Pearce BD, Landry J, Glover S, McDaniel JS, Nemeroff CB (2001) Higher than normal plasma interleukin-6 concentrations in cancer patients with depression: preliminary findings. Am J Psychiatry. 158:1252-7.

Mustafa AS, Deggerdal A, Lundin KE, Meloen RM, Shinnick TM, Oftung F (1994) An HLA-DRw53-restricted T-cell epitope from a novel Mycobacterium leprae protein antigen important to the human memory T-cell repertoire against M. leprae. Infect Immun. 62:5595-602.

Núñez JF, Ferré P, Escorihuela RM, Tobeña A, Fernández-Teruel A (1996) Effects of postnatal handling of rats on emotional, HPA-axis, and prolactin reactivity to novelty and conflict. Physiol Behav. 60:1355-9.

O'Connor P; CHAMPS (2003) The effects of intramuscular interferon beta-Ia in patients at high risk for development of multiple sclerosis: a post hoc analysis of data from CHAMPS. Clin Ther. 25:2865-74.

Ockinger J, Serrano-Fernández P, Möller S, Ibrahim SM, Olsson T, Jagodic M (2006) Definition of a 1.06-Mb region linked to neuroinflammation in humans, rats and mice. Genetics. 173:1539-45.

Ockinger J, Stridh P, Beyeen AD, Lundmark F, Seddighzadeh M, Oturai A, Sørensen PS, Lorentzen AR, Celius EG, Leppä V, Koivisto K, Tienari PJ, Alfredsson L, Padyukov L, Hillert J, Kockum I, Jagodic M, Olsson T (2010) Genetic variants of CC chemokine genes in experimental autoimmune encephalomyelitis, multiple sclerosis and rheumatoid arthritis. Genes Immun. 11:142-54.

Olerup O, Hillert J (1991) HLA class II-associated genetic susceptibility in multiple sclerosis: a critical evaluation. Tissue Antigens. 38:1-15. Review.

Olitsky PK, Yager RH (1949) Experimental disseminated encephalomyelitis in white mice. J Exp Med. 90:213-24.

Olsson T (1992) Immunology of multiple sclerosis. Curr Opin Neurol Neurosurg. 5:195-202. Review.

Olsson T, Hillert J (2008) The genetics of multiple sclerosis and its experimental models. Curr Opin Neurol. 21:255-60. Review.

Pähkla R, Kask A, Rägo L (2000) Differential effects of beta-carbolines and antidepressants on rat exploratory activity in the elevated zero-maze. Pharmacol Biochem Behav. 65:737-42.

Panayi GS, Kingsley GH, Lanchbury JS (1992) Immunotherapy of immune-mediated diseases. Q J Med. 83:489-95. Review.

Papini MR (2008) Integrating learning, emotion, behavior theory, development, and neurobiology: the enduring legacy of Abram Amsel (1922-2006). Am J Psychol. 121:661-9.

Peña Y, Prunell M, Rotllant D, Armario A, Escorihuela RM (2009) Enduring effects of environmental enrichment from weaning to adulthood on pituitary-adrenal function, pre-pulse inhibition and learning in male and female rats. Psychoneuroendocrinology. 34:1390-404.

Perlík F, Zídek Z (1974) The susceptibility of several inbred strains of rats to adjuvant-induced arthritis and experimental allergic encephalomyelitis. Z Immunitatsforsch Exp Klin Immunol. 1974 Jun;147(2):191-3.

Petretto E, Sarwar R, Grieve I, Lu H, Kumaran MK, Muckett PJ, Mangion J, Schroen B, Benson M, Punjabi PP, Prasad SK, Pennell DJ, Kiesewetter C, Tasheva ES, Corpuz LM, Webb MD, Conrad GW, Kurtz TW, Kren V, Fischer J, Hubner N, Pinto YM, Pravenec M, Aitman TJ, Cook SA (2008) Integrated genomic approaches implicate osteoglycin (Ogn) in the regulation of left ventricular mass. Nat Genet. 40:546-52.

Pfaff DW (2004) Mechanisms of estrogenic effects on neurobiological functions. Ernst Schering Res Found Workshop. 46:79-88. Review.

Pitychoutis PM, Griva E, Ioannou K, Tsitsilonis OE, Papadopoulou-Daifoti Z (2009) Chronic antidepressant treatment exerts sexually dimorphic immunomodulatory effects in an experimental model of major depression: do females lack an advantage? Int J Neuropsychopharmacol. 12:1157-63.

Pozzilli C, Tomassini V, Marinelli F, Paolillo A, Gasperini C, Bastianello S (2003) 'Gender gap' in multiple sclerosis: magnetic resonance imaging evidence. Eur J Neurol. 10:95-7.

Pravenec M, Petretto E (2008) Insight into the genetics of hypertension, a core component of the metabolic syndrome. Curr Opin Clin Nutr Metab Care. 11:393-7. Review.

Prunell M, Escorihuela RM, Fernández-Teruel A, Núñez JF, Tobeña A (1994) Differential interactions between ethanol and Ro 15-4513 on two anxiety tests in rats. Pharmacol Biochem Behav. 47:147-51.

Prunell M, Escorihuela RM, Fernández-Teruel A, Núñez JF, Tobeña A (1994) Anxiolytic profiles of alprazolam and ethanol in the elevated plus-maze test and the early acquisition of shuttlebox avoidance. Pharmacol Res. 29:37-46.

Pugh CR, Nguyen KT, Gonyea JL, Fleshner M, Wakins LR, Maier SF, Rudy JW (1999) Role of interleukin-1 beta in impairment of contextual fear conditioning caused by social isolation. Behav Brain Res. 106:109-18.

Raison CL, Miller AH (2003) When not enough is too much: the role of insufficient glucocorticoid signaling in the pathophysiology of stress-related disorders. Am J Psychiatry. 160:1554-65. Review.

Ramagopalan SV, Deluca GC, Degenhardt A, Ebers GC (2008) The genetics of clinical outcome in multiple sclerosis. J Neuroimmunol. 201-202:183-99. Review.

Ramos A, Mormède P (1998) Stress and emotionality: a multidimensional and genetic approach. Neurosci Biobehav Rev. 22:33-57. Review.

Raone A, Cassanelli A, Scheggi S, Rauggi R, Danielli B, De Montis MG (2007) Hypothalamus-pituitary-adrenal modifications consequent to chronic stress exposure in an experimental model of depression in rats. Neuroscience. 146:1734-42.

Razafimanalina R, Mormède P, Velley L (1996) Gustatory preference-aversion profiles for saccharin, quinine and alcohol in Roman high- and low-avoidance lines. Behav Pharmacol. 7:78-84.

Remmers EF, Longman RE, Du Y, O'Hare A, Cannon GW, Griffiths MM, Wilder RL (1996) A genome scan localizes five non-MHC loci controlling collagen-induced arthritis in rats. Nat Genet. 14:82-5.

Rice CJ, Sandman CA, Lenjavi MR, Baram TZ (2008) A novel mouse model for acute and long-lasting consequences of early life stress. Endocrinology. 149:4892-900.

Rivers TM, Sprunt DH, Berry GP (1933) Observations on attempts to produce acute encephalomyelitis in monkeys. J Exp Med. 58:39-53.

Rodgers RJ (1997) Animal models of 'anxiety': where next? Behav Pharmacol. 8:477-96, Review.

Rodgers RJ, Dalvi A (1997) Anxiety, defence and the elevated plus-maze. Neurosci Biobehav Rev. 21:801-10. Review.

Roxburgh RH, Seaman SR, Masterman T, Hensiek AE, Sawcer SJ, Vukusic S, Achiti I, Confavreux C, Coustans M, le Page E, Edan G, McDonnell GV, Hawkins S, Trojano M, Liguori M, Cocco E, Marrosu MG, Tesser F, Leone MA, Weber A, Zipp F, Miterski B, Epplen JT, Oturai A, Sørensen PS, Celius EG, Lara NT, Montalban X, Villoslada P, Silva AM, Marta M, Leite I, Dubois B, Rubio J, Butzkueven H, Kilpatrick T, Mycko MP, Selmaj KW, Rio ME, Sá M, Salemi G, Savettieri G, Hillert J, Compston DA (2005) Multiple Sclerosis Severity Score: using disability and disease duration to rate disease severity. Neurology. 64:1144-51.

Rose NR (1975) Differing responses of inbred rat strains in experimental autoimmune thyrioditis. Cell Immunol. 18:360-4.

Roth MP, Viratelle C, Dolbois L, Delverdier M, Borot N, Pelletier L, Druet P, Clanet M, Coppin H (1999) A genome-wide search identifies two susceptibility loci for experimental autoimmune encephalomyelitis on rat chromosomes 4 and 10. J Immunol. 162:1917-22.

Salehi B, Cordero MI, Sandi C (2010) Learning under stress: the inverted-U-shape function revisited. Learn Mem. 17:522-30.

Sandi C and Richter-Levin G (2009) From high anxiety trait to depression: a neurocognitive hypothesis. Trends Neurosci. 32:312-20.

Sandford, J.J., Argyropoulos S.V., and D.J. Nutt (2000) The psychobiology of Anxiolytics drugs Part 1: basic neurobiology, Pharmacol Therapeut. 88, 197-212.

Sapolsky RM (1998) Why Zebras Don't Get Ulcers: An Updated Guide To Stress, Stress Related Diseases, and Coping. 2nd Rev Ed, April 15, 1998. W. H. Freeman ISBN 978-0-7167-3210-5

Savić MM, Obradović DI, Ugresić ND, Cook JM, Sarma PV, Bokonjić DR (2005) Bidirectional effects of benzodiazepine binding site ligands on active avoidance acquisition and retention: differential antagonism by flumazenil and beta-CCt. Psychopharmacology (Berl). 180:455-65.

Savonenko AV, Brush FR, Zieliński K (1999) How do rats cope with the two-way escape problem in a homogeneous shuttle box? Acta Neurobiol Exp (Wars). 59:145-57.

Schmitt U, Hiemke C (1998) Combination of open field and elevated plus-maze: a suitable test battery to assess strain as well as treatment differences in rat behavior. Prog Neuropsychopharmacol Biol Psychiatry. 22:1197-215.

Sawcer S, Ban M, Maranian M, Yeo TW, Compston A, Kirby A, Daly MJ, De Jager PL, Walsh E, Lander ES, Rioux JD, Hafler DA, Ivinson A, Rimmler J, Gregory SG, Schmidt S, Pericak-Vance MA, Akesson E, Hillert J, Datta P, Oturai A, Ryder LP, Harbo HF, Spurkland A, Myhr KM, Laaksonen M, Booth D, Heard R, Stewart G, Lincoln R, Barcellos LF, Hauser SL, Oksenberg JR, Kenealy SJ, Haines JL; International Multiple Sclerosis Genetics Consortium (2005) A high-density screen for linkage in multiple sclerosis. Am J Hum Genet. 77:454-67.

Schauenstein K, Fässler R, Dietrich H, Schwarz S, Krömer G, Wick G (1987) Disturbed immune-endocrine communication in autoimmune disease. Lack of corticosterone response to immune signals in obese strain chickens with spontaneous autoimmune thyroiditis. J Immunol. 139:1830-3.

Schwegler H, Pilz PK, Koch M, Fendt M, Linke R, Driscoll P (1997) The acoustic startle response in inbred Roman high- and low-avoidance rats. Behav Genet. 27:579-82.

Selye, H (1936) A Syndrome produced by diverse nocuous agents. Nature 138: 32.

Segerstrom SC, Miller GE (2004) Psychological stress and the human immune system: a meta-analytic study of 30 years of inquiry. Psychol Bull.130:601-30.

Segura E, Amigorena S, Théry C (2005) Mature dendritic cells secrete exosomes with strong ability to induce antigen-specific effector immune responses. Blood Cells Mol Dis. 35:89-93.

Serrano-Fernández P, Ibrahim SM, Zettl UK, Thiesen HJ, Gödde R, Epplen JT, Möller S (2004) Intergenomic consensus in multifactorial inheritance loci: the case of multiple sclerosis. Genes Immun. 5:615-20.

Shepherd JK, Grewal SS, Fletcher A, Bill DJ, Dourish CT (1994) Behavioural and pharmacological characterisation of the elevated "zero-maze" as an animal model of anxiety. Psychopharmacology (Berl). 116:56-64.

Shors TJ (2006) Stressful experience and learning across the lifespan. Annu Rev Psychol. 57:55-85. Review.

Smith ME, Eller NL, McFarland HF, Racke MK, Raine CS (1999) Age dependence of clinical and pathological manifestations of autoimmune demyelination. Implications for multiple sclerosis. Am J Pathol. 155:1147-61.

Smoller JW, Faraone SV (2008) Genetics of anxiety disorders: complexities and opportunities. Am J Med Genet C Semin Med Genet. 148C:85-8.

Solberg LC, Baum AE, Ahmadiyeh N, Shimomura K, Li R, Turek FW, Takahashi JS, Churchill GA, Redei EE (2006) Genetic analysis of the stress-responsive adrenocortical axis. Physiol Genomics. 27: 362-9.

Solberg LC, Valdar W, Gauguier D, Nunez G, Taylor A, Burnett S, Arboledas-Hita C, Hernandez-Pliego P, Davidson S, Burns P, Bhattacharya S, Hough T, Higgs D, Klenerman P, Cookson WO, Zhang Y, Deacon RM, Rawlins JN, Mott R, Flint J (2006) A protocol for high-throughput phenotyping, suitable for quantitative trait analysis in mice. Mamm Genome. 17:129-46.

Solem JH (1966) Plasma corticosteroids in mice, with special regard to sex difference in adrenocortical responsiveness to exogenous corticotrophin. Scand J Clin Lab Invest Suppl. 93:1-36.

Sorrells SF, Sapolsky RM (2007) An inflammatory review of glucocorticoid actions in the CNS. Brain Behav Immun. 21:259-72.

Sorrells SF, Caso JR, Munhoz CD, Sapolsky RM (2009) The stressed CNS: when glucocorticoids aggravate inflammation. Neuron. 64:33-9. Review.

Sospedra M, Martin R (2005) Immunology of multiple sclerosis. Annu Rev Immunol. 23:683-747. Review.

Stahl SM, Wise DD (2008) The potential role of a corticotropin-releasing factor receptor-1 antagonist inpsychiatric disorders. CNS Spectr. 13:467-83. Review.

Staykova MA, Liñares D, Fordham SA, Paridaen JT, Willenborg DO (2008) The innate immune response to adjuvants dictates the adaptive immune response to autoantigens. J Neuropathol Exp Neurol. 67:543-54.

Stefferl A, Storch MK, Linington C, Stadelmann C, Lassmann H, Pohl T, Holsboer F, Tilders FJ, Reul JM (2001) Disease progression in chronic relapsing experimental allergic encephalomyelitis is associated with reduced inflammation-driven production of corticosterone. Endocrinology. 142:3616-24.

Steimer T, Escorihuela RM, Fernández-Teruel A, Driscoll P (1998) Long-term behavioural and neuroendocrine changes in Roman high-(RHA/Verh) and low-(RLA-Verh) avoidance rats following neonatal handling. Int J Dev Neurosci. 16:165-74.

Steimer T, Driscoll P (2003) Divergent stress responses and coping styles in psychogenetically selected Roman high-(RHA) and low-(RLA) avoidance rats: behavioural, neuroendocrine and developmental aspects. Stress. 6):87-100.

Stefferl A, Linington C, Holsboer F, Reul JM (1999) Susceptibility and resistance to experimental allergic encephalomyelitis: relationship with hypothalamic-pituitary-adrenocortical axis responsiveness in the rat. Endocrinology. 140:4932-8.

Steinman L (1996) Cell. 85:299-302. Review.

Sternberg EM, Young 3rd WS, Bernardini r, Calogero AE, Chrousos GP, Gold PW, Wilder RL (1989) A central nervous system defect in biosynthesis of corticotropinreleasing hormone is associated with susceptibility to

streptococcal cell wall-induced arthritis in Lewis rats. Proc Natl Acad Sci. 86:4771-4775.

Stevens DB, Gold DP, Sercarz EE, Moudgil KD (2002) The Wistar Kyoto (RT1(l)) rat is resistant to myelin basic protein-induced experimental autoimmune encephalomyelitis: comparison with the susceptible Lewis (RT1(l)) strain with regard to the MBP-directed CD4+ T cell repertoire and its regulation. J Neuroimmunol. 126:25-36.

Storch MK, Stefferl A, Brehm U, Weissert R, Wallström E, Kerschensteiner M, Olsson T, Linington C, Lassmann H (1998) Autoimmunity to myelin oligodendrocyte glycoprotein in rats mimics the spectrum of multiple sclerosis pathology. Brain Pathol. 8:681-94.

Storch MK, Weissert R, Steffer A, Birnbacher R, Wallström E, Dahlman I, Ostensson CG, Linington C, Olsson T, Lassmann H (2002) MHC gene related effects on microglia and macrophages in experimental autoimmune encephalomyelitis determine the extent of axonal injury. Brain Pathol. 12:287-99.

Stridh P (2010) Inheritance of autoimmune neuroinflammation. Ph.D. Thesis. Karolinska Institutet.

Stridh P, Thessen Hedreul M, Beyeen AD, Adzemovic MZ, Laaksonen H, Gillett A, Ockinger J, Marta M, Lassmann H, Becanovic K, Jagodic M, Olsson T (2010) Fine-mapping resolves Eae23 into two QTLs and implicates ZEB1 as a candidate gene regulating experimental neuroinflammation in rat. PLoS One. 15:e12716.

Sun D, Whitaker JN, Wilson DB (1999) Regulatory T cells in experimental allergic encephalomyelitis. III. Comparison of disease resistance in Lewis and Fischer 344 rats. Eur J Immunol. 29:1101-6.

Sundvall M, Jirholt J, Yang HT, Jansson L, Engström A, Pettersson U, Holmdahl R (1995) Identification of murine loci associated with susceptibility to chronic experimental autoimmune encephalomyelitis. Nat Genet. 10:313-7.

Swanberg M, Lidman O, Padyukov L, Eriksson P, Akesson E, Jagodic M, Lobell A, Khademi M, Börjesson O, Lindgren CM, Lundman P, Brookes AJ, Kere J, Luthman H, Alfredsson L, Hillert J, Klareskog L, Hamsten A, Piehl F, Olsson T (2005) MHC2TA is associated with differential MHC molecule expression and susceptibility to rheumatoid arthritis, multiple sclerosis and myocardial infarction. Nat Genet. 37:486-94.

Steinman L, Zamvil SS (2006) How to successfully apply animal studies in experimental allergic encephalomyelitis to research on multiple sclerosis. Ann Neurol. 60:12-21. Review.

Theoharides TC, Spanos C, Pang X, Alferes L, Ligris K, Letourneau R, Rozniecki JJ, Webster E, Chrousos GP (1995) Stress-induced intracranial mast cell degranulation: a corticotropin-releasing hormone-mediated effect. Endocrinology. 136:5745-50.

Thessen Hedreul M, Gillett A, Olsson T, Jagodic M, Harris RA (2009) Characterization of Multiple Sclerosis candidate gene expression kinetics in rat experimental autoimmune encephalomyelitis. J Neuroimmunol. 210:30-9.

Takasu N, Komiya I, Nagasawa Y, Asawa T, Yamada T (1999) Exacerbation of autoimmune thyroid dysfunction after unilateral adrenalectomy in patients withCushing's syndrome due to an adrenocortical adenoma. N Engl J Med. 322:1708-12.

Turner BB (1990) Sex difference in glucocorticoid binding in rat pituitary is estrogen dependent. Life Sci. 46:1399-406.

Turner BB (1997) Influence of gonadal steroids on brain corticosteroid receptors: a minireview. Neurochem Res. 22:1375-85. Review.

Ulrich-Lai YM, Herman JP (2009) Neural regulation of endocrine and autonomic stress responses. Nat Rev Neurosci. 10:397-409.

Ueda H, Howson JM, Esposito L, Heward J, Snook H, Chamberlain G, Rainbow DB, Hunter KM, Smith AN, Di Genova G, Herr MH, Dahlman I, Payne F, Smyth D, Lowe C, Twells RC, Howlett S, Healy B, Nutland S, Rance HE, Everett V, Smink LJ, Lam AC, Cordell HJ, Walker NM, Bordin C, Hulme J, Motzo C, Cucca F, Hess JF, Metzker ML, Rogers J, Gregory S, Allahabadia A, Nithiyananthan R, Tuomilehto-Wolf E, Tuomilehto J, Bingley P, Gillespie KM, Undlien DE, Rønningen KS, Guja C, Ionescu-Tîrgovişte C, Savage DA, Maxwell AP, Carson DJ, Patterson CC, Franklyn JA, Clayton DG, Peterson LB, Wicker LS, Todd JA, Gough SC (2003) Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. Nature. 423:506-11.

Vahl TP, Ulrich-Lai YM, Ostrander MM, Dolgas CM, Elfers EE, Seeley RJ, D'Alessio DA, Herman JP (2005) Comparative analysis of ACTH and corticosterone sampling methods in rats. Am J Physiol Endocrinol Metab. 289:E823-8.

Valdar WS, Flint J, Mott R (2003) QTL fine-mapping with recombinant-inbred heterogeneous stocks and in vitro heterogeneous stocks. Mamm Genome. 14:830-8.

Valdar W, Solberg LC, Gauguier D, Cookson WO, Rawlins JN, Mott R, Flint J (2006) Genetic and environmental effects on complex traits in mice. Genetics. 174:959-84.

Valdar W, Solberg LC, Gauguier D, Burnett S, Klenerman P, Cookson WO, Taylor MS, Rawlins JN, Mott R, Flint J (2006) Genome-wide genetic

association of complex traits in heterogeneous stock mice. Nat Genet. 38:879-87.

Vicens-Costa E, Martínez-Membrives E, López-Aumatell R, Guitart-Masip M, Cañete T, Blázquez G, Tobeña A, Fernández-Teruel A (2011) Two-way avoidance acquisition is negatively related to conditioned freezing and positively associated with startle reactions: a dissection of anxiety and fear in genetically heterogeneous rats. Physiol Behav. 103:148-56.

Vyshkina T, Kalman B (2005) Haplotypes within genes of beta-chemokines in 17q11 are associated with multiple sclerosis: a second phase study. Hum Genet. 118:67-75.

Vingsbo C, Sahlstrand P, Brun JG, Jonsson R, Saxne T, Holmdahl R (1996) Pristane-induced arthritis in rats: a new model for rheumatoid arthritis with a chronic disease course influenced by both major histocompatibility complex and non-major histocompatibility complex genes. Am J Pathol. 149:1675-83.

Voigt B, Serikawa T (2009) Pluripotent stem cells and other technologies will eventually open the door for straightforward gene targeting in the rat. Dis Model Mech. 2:341-3. Review.

Wallace DL, Han MH, Graham DL, Green TA, Vialou V, Iñiguez SD, Cao JL, Kirk A, Chakravarty S, Kumar A, Krishnan V, Neve RL, Cooper DC, Bolaños CA, Barrot M, McClung CA, Nestler EJ (2009) CREB regulation of nucleus accumbens excitability mediates social isolation-induced behavioral deficits. Nat Neurosci.12:200-9.

Wallström E OT (2007) Rat models of Experimental Autoimmune Encephalomyelitis. Totowa, NJ: Humana Press Inc, 2007.

Warren, J (2007) Presocratics: Natural Philosophers before Socrates (University of California, Berkeley).

Weiss IC, Feldon J (2001)Environmental animal models for sensorimotor gating deficiencies in schizophrenia: a review. Psychopharmacology (Berl). 156:305-26. Review.

Weissert R, Wallström E, Storch MK, Stefferl A, Lorentzen J, Lassmann H, Linington C, Olsson T (1998) MHC haplotype-dependent regulation of MOG-induced EAE in rats. J Clin Invest. 102:1265-73.

Wigger A, Neumann ID (1999) Periodic maternal deprivation induces gender-dependent alterations in behavioral and neuroendocrine responses to emotional stress in adult rats. Physiol Behav. 66:293-302.

Wilcock J, Fulker DW (1973) Avoidance learning in rats: genetic evidence for two distinct behavioral processes in the shuttle box. J Comp Physiol Psychol. 82:247-53.

Willis-Owen SA, Flint J (2007) Identifying the genetic determinants of emotionality in humans; insights from rodents. Neurosci Biobehav Rev. 31:115-24. Review.

Williams TM, Moolten D, Burlein J, Romano J, Bhaerman R, Godillot A, Mellon M, Rauscher FJ 3rd, Kant JA (1991) Identification of a zinc finger protein that inhibits IL-2 gene expression. Science. 254:1791-4.

Wilder RL, Remmers EF, Kawahito Y, Gulko PS, Cannon GW, Griffiths MM (1999) Genetic factors regulating experimental arthritis in mice and rats. Curr Dir Autoimmun. 1:121-65. Review.

Weber F, Fontaine B, Cournu-Rebeix I, Kroner A, Knop M, Lutz S, Müller-Sarnowski F, Uhr M, Bettecken T, Kohli M, Ripke S, Ising M, Rieckmann P, Brassat D, Semana G, Babron MC, Mrejen S, Gout C, Lyon-Caen O, Yaouanq J, Edan G, Clanet M, Holsboer F, Clerget-Darpoux F, Müller-Myhsok B (2008) IL2RA and IL7RA genes confer susceptibility for multiple sclerosis in two independent European populations. Genes Immun. 9:259-63.

Wolinsky FD, Unverzagt FW, Smith DM, Jones R, Stoddard A, Tennstedt SL (2006) The ACTIVE cognitive training trial and health-related quality of life: protection that lasts for 5 years. J Gerontol A Biol Sci Med Sci61:1324-9.

Yalcin B, Fullerton J, Miller S, Keays DA, Brady S, Bhomra A, Jefferson A, Volpi E, Copley RR, Flint J, Mott R (2004) Unexpected complexity in the haplotypes of commonly used inbred strains of laboratory mice. Proc Natl Acad Sci U S A. 101:9734-9.

Yalcin B, Willis-Owen SA, Fullerton J, Meesaq A, Deacon RM, Rawlins JN, Copley RR, Morris AP, FlintJ, Mott R (2004) Genetic dissection of a behavioral quantitative trait locus shows that Rgs2 modulates anxiety in mice. Nat Genet. 36:1197-202.

Ye Z, Wang Y, Xie HY, Zheng SS (2008) Immunosuppressive effects of rat mesenchymal stem cells: involvement of CD4+CD25+ regulatory T cells. Hepatobiliary Pancreat Dis Int. 7:608-14.

Yasui DH, Genetta T, Kadesch T, Williams TM, Swain SL, Tsui LV, Huber BT (1998) Transcriptional repression of the IL-2 gene in Th cells by ZEB. J Immunol. 160:4433-40.

Yakushiji F, Kita M, Hiroi N, Ueshiba H, Monma I, Miyachi Y (1995) Exacerbation of rheumatoid arthritis after removal of adrenal adenoma in Cushing's syndrome. Endocr J. 42:219-23.