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Role of astrocytic IL-6 and IL-6R in normal physiology and neuroinflammation

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Memoria de tesis presentada por **Maria Erta Cañabate** para optar al grado de Doctor en Neurociencias por la Universitat Autònoma de Barcelona.

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Director de tesis — Doctoranda

Dr. Juan Hidalgo Pareja — Maria Erta Cañabate

Mientras la ciencia a descubrir no alcance
las fuentes de la vida,
y en el mar o en el cielo haya un abismo
que al cálculo resista,
mientras la humanidad siempre avanzando
no sepa a dónde camina,
mientras haya un misterio para el hombre,
¡habrá poesía!

-**Gustavo Adolfo Bécquer**-

ACKNOWLEDGEMENTS

Bernard of Chartres said that “We are like dwarfs on the shoulders of giants, so that we can see more than they, and things at a greater distance, not by virtue of any sharpness on sight on our part, or any physical distinction, but because we are carried high and raised up by their giant size.” [Bernard of Chartres, 1130 AD]

The acknowledgements section of a Thesis is the opportunity to look down, see all those giants pushing us higher and, of course, acknowledge them for their merits and help.

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A tots, moltíssimes gràcies per ser-hi!!

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α-MSH alpha melanocyte-stimulating hormone. 8

Aβ β-amyloide peptide. 14, 35

ACTH adrenocorticotropic hormone. 24

AD Alzheimer's disease. 32

ALS amyotrophic lateral sclerosis. 32

AP anterior pituitary. 24

APCs antigen-presenting cells. 10

APP beta-amyloid precursor protein. 36

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Ast-IL-6R Ast-IL-6R KO mice and their littermate controls. 55

AVP arginin-vasopressin. 24

BB Blocking buffer. 63

BBB blood brain barrier. 9

BDNF brain-derived neurotrophic factor. 11

BMI body-mass index. 22

BrdU Bromodeoxyuridine. 60

BSA Bovine serum albumin. 62

CFA Freund's Complete Adjuvant. 57

CHR cytokine binding homology region. 2

CLC cytokine cardiotrophin-like. 2

CNS Central Nervous System. 8

CREB cAMP response element binding protein. 17

CRH corticotrophin-releasing hormone. 23

CSF cerebrospinal fluid. 10

CT-1 cardiotrophin-1. 2

DAB 3,3-diaminobenzidine-tetrahydrochloride. 63

DG dentate gyrus. 21

DMEM Dulbecco's Modified Eagle Medium. 62

DNase Deoxyribonuclease. 62

DRG dorsal root ganglia. 27

EAE Experimental Autoimmune Encephalomyelitis. 34

ECM extracellular matrix. 11

ELISA Enzyme-linked immunosorbent assay. 63

EPM Elevated Plus-Maze. 51

FGF fibroblast growth factor. 11

FN III fibronectin type III. 2

GABA gamma aminobutyric acid. 12

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HPA hypothalamic-pituitary-adrenal. 22

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KRB Kreps Ringer buffer. 62

LFB Luxol Fast Blue. 63

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LSD Fisher's least significant difference. 66

LTP long term potentiation. 29

MAPK mitogen activated protein kinase. 5

MCAO middle cerebral artery occlusion. 31

MCH melanin concentrating hormone. 24

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NMDA N-methyl-D-aspartate. 21

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NSCs neural stem cells. 21

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NT-3 neurotrophin-3. 11

OSM oncostatin M. 2

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PBS Phosphate buffered saline. 60

PCR polymerase chain reaction. 46

PD Parkinson's disease. 32

PFA Paraformaldehyde. 60

PGs prostaglandins. 17

PI3K phosphatidylinositol 3-kinase. 5

PIAS protein inhibitors of activated STATs. 4

PTSD post-traumatic stress disorder. 38

PVN paraventricular nucleus. 24

qRT-PCR Real-time reverse-transcription PCR. 60

REM rapid-eye-movement. 27

ROS reactive oxygen species. 14

RT Room temperature. 63

sgp130 soluble form of gp130. 6

SHP SH2-containing phosphatases. 4

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SNS somatic nervous system. 24

SOCS suppressors of cytokine signaling proteins. 4

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SZ Schizophrenia. 38

TB Tris buffered solution. 63

TBI Traumatic brain injury. 30

TBS Tris buffered saline solution. 63

TBS-T TBS with 1% triton X-100. 65

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TF transcription factors. 5

TGF-β1 transforming growth factor beta 1. 8

TNF tumor necrosis factor. 2

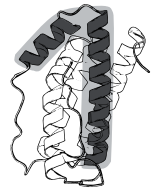
TST Tail suspension test. 52

VCAM-1 vascular cell adhesion molecule-1. 18

VIP vasoactive intestinal peptide. 8

WSXWS tryptophan-serine-X-tryptophan-serine. 2

WT wild-type. 23

CHAPTER 

INTRODUCTION

1.1 Interleukin-6, the founding member of the neuropoietins

Cytokines are small, usually secreted, cell-signaling proteins; initially believed to be exclusive of the immune system, nowadays they have been found to play a much broader role in physiology; regulating cell growth and differentiation, tissue homeostasis and repair, and, of course, many aspects of inflammatory and immune responses [1]. They are usually pleiotropic, redundant and induce production of other cytokines which complicates their understanding. They act mostly in an autocrine/paracrine way although they can enter blood circulation and act far from the site of production in an endocrine way [2].

Interleukin 6 (IL-6) is a cytokine so pleiotropic that has caused confusion to researchers since the very beginning. Tadamitsu Kishimoto, an immunologist critical for our current understanding of the function and structure of IL-6 [3], originally characterized it in 1985 as B-cell differentiation factor (BSF-2)[4] after speculating that T cells must produce certain factors that induce growth and differentiation of B cells into antibody-producing cells [5]. Various other labs were studying what was presumed unrelated growth factors at the time. These factors had several names and functions, interferon $\beta 2$ [6, 7], hybridoma growth factor [8], hepatocyte-stimulatory factor [9], cytotoxic T-cell differentiation factor [10], $\beta 2$-fibrinogen [11], amyloid protein, haptoglobin and hemopexin, to name a few [12]. It was not until 1987 that human IL-6 was cloned [13] (followed by murine and rat IL-6 [14, 15]) and a few years later the protein was purified and utilized in studies to show that these seemingly different factors were the same molecule, hightlighting that IL-6 had various interesting biological activities not limited to B cell immunology; regulation of hepatocytes and liver regeneration, hematopoiesis, the acute phase response, bone remodeling and also actions in the cardiovascular system, the placenta and the nervous and endocrine systems [16]. Furthermore, its expression is dysregulated in many diseases like atherosclerosis and pulmonary diseases; in autoimmune diseases (Crohn's disease, rheumatoid arthritis, diabetes and multiple sclerosis); in some neurological disorders; and in various cancers [17, 18, 19, 20].

Nowadays, the number of known cytokines is enormous. Classification can be made

based in structural analysis grouping these proteins in different classes such as the helical cytokines [21], the trimeric tumor necrosis factor (TNF) family [22], the cysteine knot growth factors [23] and the β2-trefoil growth factors [24]. Cytokines can also be grouped according to the type of receptor they bind, which comprise six major families: class I cytokine receptors (the largest family, also known as the hematopoietin receptors, to which IL-6 belongs), class II cytokine receptors, TNF receptors, tyrosine kinase receptors, and chemokine receptors [25, 26]. Moreover, cytokine families can be named according to other aspects like the sharing of a receptor subunit (i.e. the gp130 family) or its physiological roles (i.e. the neuropoietic family, for its effects on nervous and hematopoietic systems).

IL-6 gene is located in chromosome 7 in humans [27] and in chromosome 5 in mice [28]; in both of them it contains 4 introns and 5 exons [29]. Human IL-6 is a single chain glycoprotein with a molecular weight ranging 21-30 kDa [30], while murine IL-6 is a 22-29 kDa protein [31]. This heterogeneity is because of extensive post-translational modification, due to variations in glycosylation, phosphorylation at multiple serine residues, and sulfation, depending on the cellular source, having little effect on its biological activity [30, 32].

Structurally, IL-6 is a prototypical long-chain class of four-helix bundle cytokine family, arranged in an up-up-down-down topology, that is the founder member of the neuropoietins, a group of cytokines structurally related, which include IL-6, IL-11, IL-27, IL-31, leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin-1 (CT-1), neuropoietin and cytokine cardiotrophin-like (CLC) (also known as new neurotrophin 1 and B cell stimulatory factor-3), and two viral analogs of IL-6 (one from HHV-8 IL-6 and another from the Rhesus macaque rhadinovirus, Rm IL-6) [26].

1.1.1 The IL-6 Receptor complex

Interleukin 6 receptor (IL-6R) is a 468 amino acid protein that binds IL-6 with nanomolar affinity, first isolated soon after IL-6 cloning, in 1988 [33]. However, binding of IL-6 to the IL-6R does not lead to signaling because IL-6R do not have intrinsic enzymatic activity [33].

Interestingly, it was observed that when IL-6 was bound with the 80 kDa IL-6R (also known as gp80 and CD126), it induced the recruitment of another cell surface protein of 130 kDa, and thus was called glycoprotein 130 (gp130), also known as CD130 or IL-6 R β-chain, [34, 35]. Subsequent studies revealed that gp130 functioned as a shared receptor component not only for IL-6, but for all other neuropoietins as well [16], except for IL-31 that binds to a gp130-like receptor [36]. The sharing of gp130 explains in part the redundancy of the actions of these cytokines, besides, it gives name to the family: gp130 family of cytokines [16].

Both gp130 and IL-6R have a modular architecture made of fibronectin type III (FN III) modules of approximately 100 amino acids. The most distant extracellular domain of IL-6R has an immunoglobulin G (Ig)-like domain at the N-terminus followed by the cytokine binding homology region (CHR) [37], which comprises two FN III modules. The closest to the cell membrane has a tryptophan-serine-X-tryptophan-serine (WSXWS) mo-

tif and the intermediate FN III domain carries a set of four conserved cysteine residues. Gp130 has a similar structure but contains three additional FN III modules before the cellular membrane and an intracellular cytoplasmic domain that is involved in signal transduction. The IL-6R contains a short cytoplasmatic domain not implicated in signaling events [16, 38] (see Figure 1.1).

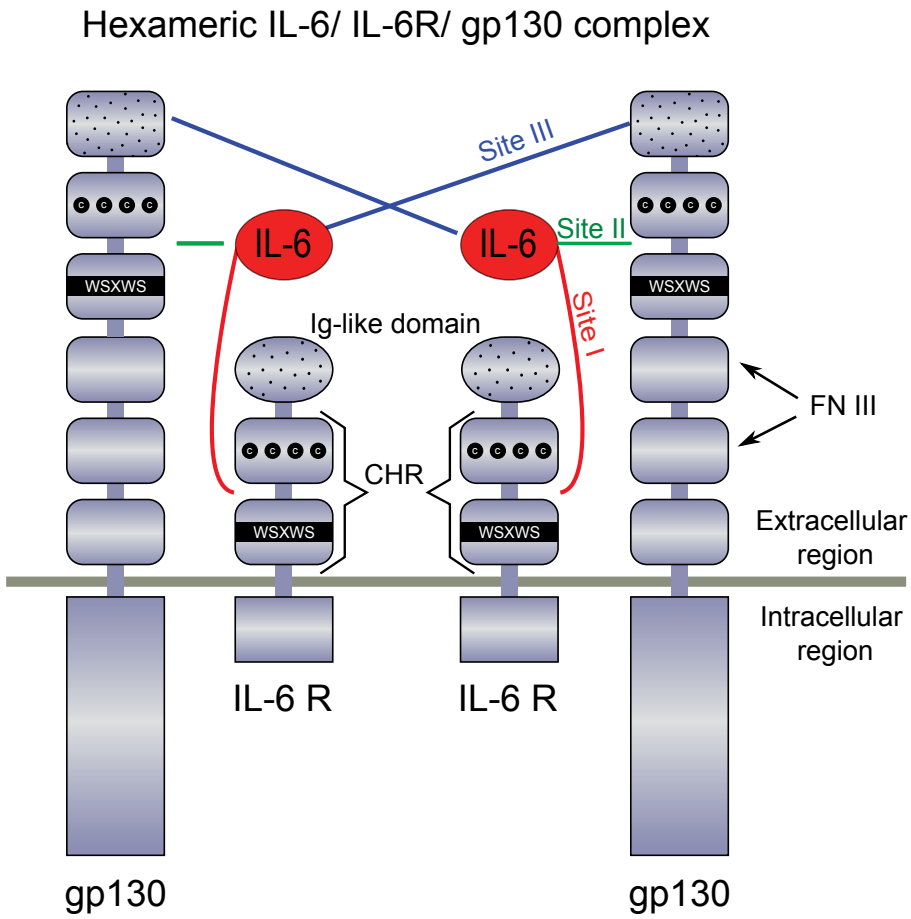


Figure 1.1: Schematic representation of hexameric IL-6 receptor complex. The extracellular region of IL-6R consists of three fibronectin type III modules (FN III), the first one is the Ig-like domain, followed by the cytokine binding homology region (CHR) which is composed of two FN III modules, one carrying four conserved cysteine residues, marked with a "C" and the other carrying WSXWS conserved motif. The extracellular region of gp130 is similar as IL-6R but with three additional FN III modules just before the cellular membrane. Unlike IL-6R, gp130 has a long intracellular domain implicated in signaling events. Finally, two IL-6 molecules completes the hexameric IL-6 receptor complex. These IL-6 bind to IL-6R and gp130 via sites I, II and III as marked in the figure. Based in [16].

The structure of the complex of gp130, IL-6R and IL-6 has been solved by X-ray crystallography and consists of a hexamer of two IL-6, two IL-6R and two gp130 proteins [39, 38] (see Figure 1.1). However, it has been argued that the signaling complex is built of a tetramer of one IL-6/IL-6R complex bound to two gp130 proteins [40, 41]. Functional structure studies have identified three conserved epitopes (sites I, II and III) on IL-

6 molecule important for binding to gp130. Site I on IL-6 N-terminal part between helix A and helix B is involved in binding to IL-6R and gp130 [42]. However, it only binds gp130 in the presence of IL-6R [43]. Site II is formed by residues within helix A and C which interacts with CHR of gp130 [44]. Site III is unique to gp130-cytokines and contains residues in the terminal part of the loop of helix D which binds to immunoglobulin-like activation domain of the other gp130 molecule [44, 45] (see Figure 1.1). Finally, a site 4 determines the assembly of the functional human hexameric structure (2 IL-6, 2 IL-6R, and 2 gp130) of the competent signaling IL-6 receptor complex [46, 38].

1.1.2 Signal transduction of IL-6

The formation of the IL-6-IL-6R-gp130 complex is the initial step in IL-6 intracellular signalling [47]. Once the hexameric complex is formed, conformational changes lead to gp130 activation. However, it is interesting to note that gp130 seems to be also present as a preformed but inactive dimer in the cellular membrane, ready for activation by ligand addition [48]. Gp130 activation leads to activation of associated Janus-tyrosine kinases-1 and 2 (JAK) and phosphorylation of several tyrosine residues of intracellular gp130 portion, providing recruitment sites for Signal Transducer and Activation of Transcription proteins (STATs), molecules containing a src homology 2 (SH2) domain. From all seven known members of STATs, IL-6 mainly activates STAT3 and to a lesser extent STAT1 [49, 47], especially in vivo [50]. Once phosphorylated by JAK, STATs dimerise and translocate into the nucleus where they bind target gene promoters [51]. In addition to JAK/STAT pathway, IL-6 also activates the Ras-Raf-MAPK (mitogen activated protein kinase) pathway through a gp130-associated SHP2-containing protein phosphatase-2 . A third signaling cascade activated by IL-6 is phosphatidylinositol 3-kinase (PI3K) pathway.

To prevent over-stimulation, mechanisms of signal attenuation are necessary in order to ensure an adequate and controlled cellular response to IL-6. Three families of proteins inhibit specific and distinct aspects of this cytokine signal transduction. Firstly the SH2-containing phosphatases (SHP), which are protein tyrosine phosphatases that dephosphorylate receptors and JAKs. Secondly, the protein inhibitors of activated STATs (PIAS); interestingly, some PIAS proteins present small ubiquitin-related modifier (SUMO)-ligase activity which may play a critical role in targeting transcription factors to nuclear bodies and thereby become transcriptionally active or inactive. Finally, there are the suppressors of cytokine signaling proteins (SOCS), (previously named as SSI by Kishimoto's group [54]), which inhibit the kinase activity of JAKs, being rapidly upregulated by IL-6 and creating a negative feedback loop [47, 52]. Furthermore, internalization by the endocytotic machinery and degradation of the receptor-ligand complex also allows IL-6 signal termination [55], as well al the usage of the ubiquitin-proteasome pathway, which causes STATs proteosome-mediated degradation [56].

1.1.3 IL-6 signaling pathways: classic versus trans-signalling

A special feature of IL-6 signaling is the existence of two differents pathways to accomplish it, either by the classic one or by the more recently discovered, trans-signalling. IL-

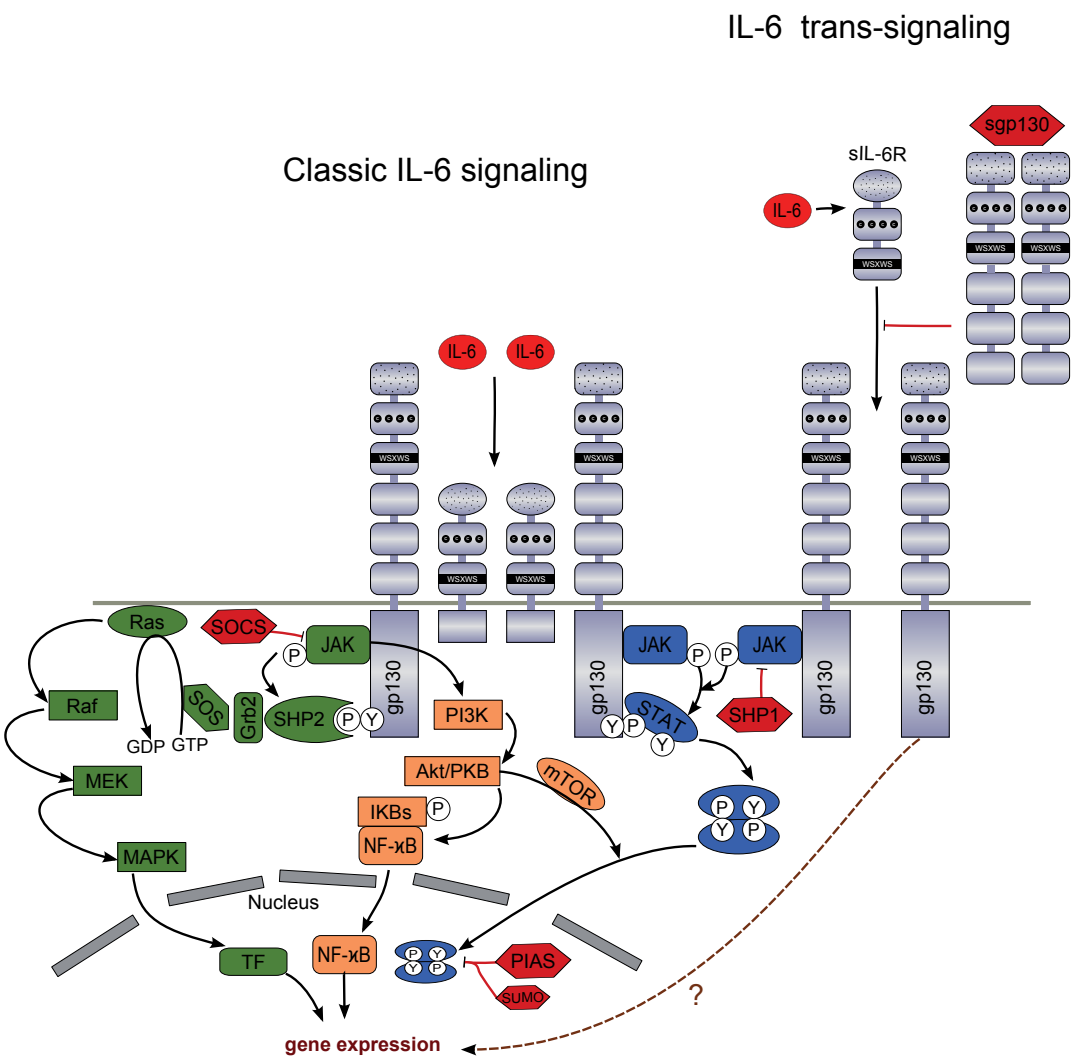


Figure 1.2: Signal transduction pathways activated by IL-6: JAK/STAT pathway (blue), MAPK pathway (green) and PI3K pathway (orange) in cells responding by IL-6 classic signalling (left); or by IL-6 trans-signalling (right). Main inhibitors of the pathways are marked in red hexagons. TF. Based in [47, 52, 53].

6R is found in two forms, a IL-6R membrane bound form (mIL-6R) with a transmembrane and intracellular region and a soluble form of IL-6 receptor (sIL-6R) with only the extracelular region, that can bind IL-6 with a similar affinity as the mIL-6R [57, 58].

Signal transduction of IL-6 via the aforementioned IL-6R membrane complex (see Section 1.1.1) is named classic signaling. In this pathway, IL-6 binds directly to mIL-6R and recruits gp130 signaling subunits, which are ubiquitously expressed [59], whereas the mIL-6R expression is restricted to some cell populations like hepatocytes, neutrophils, monocytes/macrophages and certain other leukocytes [60] as well as some brain regions (see Section 1.3.1). Cells, which only express gp130 do not respond to IL-6 [61]. As a

consequence, IL-6 would only bind to and stimulate cells, which express the IL-6R, which it is not the case.

Signal transduction of IL-6 via trans-signaling involves the binding of IL-6 to sIL-6R in extracellular space and the formation of an active complex with gp130 signaling subunits (see Figure 1.2). Therefore, trans-signaling is a powerful mechanism which confers IL-6 responsiveness to virtually all cells in the body, even the ones lacking IL-6R [62]. This is in contrast to other soluble cytokine receptors which exert inhibitory effects [63].

The sIL-6R was originally found in urine and plasma [64, 65]. Under non-pathological conditions, sIL-6R is present in moderate levels in human plasma around 77 ng/ml, in cerebrospinal fluid at 0.8-1.6 ng/ml and in brain extracts, among others [66, 67], being significantly increased after infections or cerebral trauma [65, 68]. These studies imply a key role of sIL-6R in IL-6 sensitivity in CNS cells that do not express membrane-bound IL-6R (see Section 1.3.1).

It is known that sIL-6R is formed physiologically, either by limited proteolysis of the extracellular domain of mIL-6R by metalloproteases such as ADAM10 and ADAM17 (90%) [69], or by alternative splicing of IL-6R mRNA (10%) [70]. Different stimuli are capable of inducing the proteolytic cleavage of mIL-6R, like bacterial pore-forming toxins [71], c-reactive protein [72], cholesterol depletion [69] and apoptosis [73], among others.

The activity of the IL-6/sIL-6R complex is tightly controlled by a molar excess of a soluble form of gp130 (sgp130) present in human serum at 100-300 ng/ml levels [74]; it is generated only by mRNA splicing [74, 75, 76], at least efficiently [77]. sgp130 is a competitive inhibitor of trans-signaling, but without affecting classic signaling, because of its uncapability to bind neither IL-6 nor IL-6- mIL-6R complex [74, 53]. Despite all IL-6 family cytokines signal throughout gp130, sgp130 is quite specific for IL-6 signaling although it is able to partially inhibit at high concentrations LIF- and OSM-signalling [53].

For a long time there has been controversy between pro- versus anti-inflammatory roles of IL-6 in the body (see Section 1.3.2). Different studies, mostly by Dr Rose-John group, indicate that trans-signaling would be responsible of pro-inflammatory processes and disease models while classic signaling would mostly mediate regenerative actions or the activation of anti-inflammatory pathways [78].

The detailed knowledge of IL-6 signaling pathways has important consequences for therapeutic strategies aimed to block IL-6 effects. Recombinant designed proteins are molecular tools that mimic specific components of IL-6 pathways, helping to distinguish between IL-6 classic and trans-signalling effects both in vitro and in vivo. Firstly, Hyper-IL-6, which consists of a fusion between IL-6 and sIL-6R, much more effective than the combination of these two components unlinked [61]; secondly, there is also the soluble fusion protein sgp130-Fc, which specifically inhibits trans-signaling pathway 10 times more effectively than native sgp130 and seems a promising tool for therapeutic application [53]. Also, there is the L-gp130 protein, in which the entire extracellular portion of gp130 is replaced by the leucine zipper of the Jun protein [79].

1.1.4 IL-6 gene expression

IL-6 is expressed by numerous cell types, by T cells but also by a panoply of cells including macrophages, fibroblasts, synovial cells, endothelial cells, glia cells, keratinocytes among others. Stimuli that regulate the expression of IL-6 are physical injury, heat shock, other cytokines and growth factors, glucocorticoids, PAMPs and toxins [47, 3].

Initial studies of IL-6 gene showed the existence of at least three transcriptional initiation sites [13]. Soon thereafter, a sequence motif in the IL-6 promoter region that conferred IL-1-induced IL-6 expression was identified, as well as a nuclear factor binding to this motif, called NF-IL-6. A cis-sequence was found in IL-6 promotor where NF-IL-6 binds. THis sequence was found to be similar to that of C/EBP site. C/EBP (CCAATT/ enhancer binding protein) is constitutive expressed, whereas NF-IL-6 is only expressed after stimulation with inflammatory signals like LPS (Lipopolysaccharide), IL-1, TNF, and IL-6. These two transcription factors have opposing roles in the control of acute-phase proteins [80].

1.1.5 IL-6 functions

IL-6 is a multifunctional cytokine involved in the regulation of the immune response, inflammation, hematopoiesis, regeneration, metabolism and nervous system functions.

1.1.5.1 Periphery

- Immune responses and inflammation:
  - It is a B cell differentiation factor on activated B cells or B lymphoblastoid cell lines inducing Ig production [4].
  - It is a myeloma and hybridoma-plasmacytoma growth factor [81, 7].
  - It is a costimulant factor for thymocytes and mature T lymphocytes [82] and regulates Th17 differentiation [83].
  - Induces neutrophil differentiation and controls the extent of local or systemic acute inflammatory responses [84].
  - Directs transition from innate to acquired immunity [85].
- Metabolism and regeneration:
  - Its a hepatocyte-stimulating factor and stimulates liver cells to induce acute-phase proteins [9].
  - Promotes hepatic regeneration [86]
  - Stimulates glucose production in liver [87].
  - Inhibits liver glycogen synthesis and insulin receptor signal transduction, all together contributing to insulin resistance [88].
  - Ameliorates fatty liver condition by stimulating hepatic triglyceride secretion and fatty acid oxidation [89].

- In pancreas stimulates α-cell proliferation, prevents apoptosis due to metabolic stress, and regulates glucagon secretion [90].
- Increases adipose tissue lipid metabolism (increasing lipolysis and fat secretion and oxidation) and leptin production, while reduces lipoprotein lipase activity. IL-6 also induces insulin resistance in adipocytes [91, 92, 93].
- IL-6 directly promotes skeletal muscle differentiation and regulates muscle substrate utilization, promoting glycogen synthesis and storage (increasing glucose uptake, expression of glucose transporter-4 and AMP-activated protein kinase) and lipid oxidation [94, 95].

1.1.5.2 CNS

As this is the aim of the thesis it will be developed in more depth in the following sections, first a briefly description of the particularities of CNS immunity (Section 1.2) is made and then specific IL-6 functions in the CNS will be reviewed (Section 1.3).

1.2 CNS immunity

The Central Nervous System (CNS), composed by brain and spinal cord, has been classically considered as an immunologically "privileged" organ because it was thought to be isolated from the immune system and excluded from its surveillance. This idea is based in Medawar's work in late 40s which showed that allograft placed in brain suffered less rejection than in other body part [96]. Medawar explained it due to the lack of lymphatic system in the brain, but later studies demonstrated that this immune "privilege" was due to multiple anatomical, physiologycal and immunoregulating mechanisms [97]. Nowadays, it is accepted that "this privilege" is confined to CNS parenchyma, mainly active and non absolute, as it is no preserved after immunization or age, among other factors (for a review see [98]). It is believed that this different regulation in CNS's immunity is due to the limited capacity for regeneration of this organ, to which potentially damaging molecules, secreted after immune-mediated inflammation, could have devastating consequences [99].

The most important isolation mechanism of the CNS parenchyma is the Blood Brain Barrier, which will be explained in the following subsection, but another important mechanisms of inflammation control are the reduced expression of immune system-activating molecules like major histocompatibility complex (MHC), that mediate cellular lysis by T-lymphocites [100]; and also by expressing factors which impairs the correct function of immunity cells like Fas ligand (a factor inducing apoptosis in T-lymphocytes and neutrophils) [101], transforming growth factor beta 1 (TGF-$\boldsymbol{\beta}$1) [102], vasoactive intestinal peptide (VIP) [103] or the alpha melanocyte-stimulating hormone ($\boldsymbol{\alpha}$-MSH) [104].

However, despite immunological particularities of CNS, it does not escape to immune system surveillance [105]. Most differences are quantitative rather than qualitatives, as it contains a reduced number of macrophages and lymphocytes, as well as resident cells

like astrocytes and microglia which will be able to recognize danger situations for tissue integrity that require immune cells infiltration.

1.2.1 Blood Brain Barrier

The blood brain barrier (BBB) is the interface between blood and brain, protecting the brain against undesirable penetration of compounds or cells and it is the most characteristic feature of CNS immune privilege. It is an structure formed by tight junctions between endothelial cells of the blood vessels, the basal lamina in which pericytes are embedded and, finally, astrocytes end feet covering the vessels, in combination with intra- and extracellular enzymes that represent a metabolic barrier [106].

Despite this apparent shield, cells are able to enter the CNS using the minimal level of attachment molecules that exist, and BBB is able to respond to a number of substances in the circulation; but not only endothelial cells of the CNS vessels can respond to blood substance and become activated upregulating adhesion molecules but, surprinsingly, LPS and proinflammatory cytokines like IFN-γ and TNF-α injected into the circulation, can also activate cells behind the intact BBB [107, 108]. Although the mechanism is not fully understood these molecules act on the brain through neural, humoral and diffusive routes [109]. Endothelial cells of the BBB have polarized sides that respond to cytokines in blood on their luminal side and secrete their own immune molecules into brain parenchyma [110]. Cytokines are also actively transported across the BBB through energy-dependent pumps [111].

BBB can nearly be considered as an organ that protects and maintains the homeostasis of the brain as when diseases with an inflammatory component like multiple sclerosis, meningitis or encephalitis among others change the functionality and/or integrity of the BBB then brain homeostasis also change [106]. It has key functions as keeping the ionic composition of the brain at optimal levels for synaptic signaling functions, tightly controlling neurotransmitters concentration in CNS, allowing brain nutrition and avoiding macromolecules and neurotoxins entering in nervous parenchyma [112].

1.2.2 Resident cells in nervous parenchyma

Glial cells (macro- and microglia) represent 90% of CNS cells. They are included in the term neuroglia or Nervenkitt, as Virchow firstly named it, to describe connective cellular elements from white matter. Later on it has been seen that neuroglial cells have many more roles than being a connective tissue between neurons as they have key functions in immune control, homeostasis and even in synaptic transmission in both physiological or pathological conditions [113, 114].

After a CNS damage, glial cells show phenotypic changes, referred to as reactive gliosis, which is one of the most characteristic features of neuroinflammation [115, 116]. Glial activation is found after traumatic brain injury, ischemia, infections, inflammatory diseases, psychiatric disorders, brain tumors and neurodegenerative diseases, among others [117, 118]

1.2.2.1 Microglia

Microglial cells or microglia represents 10-20% of total glial cells, they were discovered by Del Rio-Hortega using silver impregnation techniques to visualize non-neuronal cells [119]. They are mainly considered the immune cells of the CNS due to observations like their ability to secrete and respond to cytokines (characteristic of immune accessory cells), to serve as antigen-presenting cells (APCs) and the detection of MHC antigens, T- and B- lymphocyte markers and another immune cell antigens in their surface [120, 121].

Microglia have been described as mesodermal, hematomonocytic, or ectodermal origin [122, 123, 124], but the prevailing view is that blood monocytes enter brain during embryonic stage and differentiate into brain resident microglia, sharing many phenotypic markers and effector molecules with peripheral macrophages like CD11b/CD18 complex, IgG receptor (CD16/CD32), ionized calcium-binding adaptor protein-1 (Iba-1) and the MHC among others [125] which support the initial theory of Del Rio-Hortega that developmentally, microglia cells are descendants of the monocytic lineage that invade the CNS in embrionary stage.

In resting conditions, microglia present a ramified morphology characterized by a small soma and ramified processes, with an elaborate tertiary and quaternary branch structure, which allow them to cover between 30-40μm without overlapping and being able to cover and survey the whole CNS parenchyma [126, 127].

"Everything is all right" signals maintain microglia in this ramified state, like the interaction with CD200 glycoprotein present in functional neurons [128] but small changes in the CNS microenvironment (as for example brain injury or pathogens) trigger an immediate, focal and transient activation of microglial cells, resulting in morphology and physiological changes [127] with a different response depending upon the stimulation provided [129, 130]. Key signs of microglia activation are proliferation, shortening of the ramified processes, extending new pseudopodia that enable active migration and swelling the cell soma to an "amoeboid" state with phagocytic capacity for removing dead cells and cell debris [130] (see Figure 1.3).

This morphology change comes with a heightened metabolism with the upregulation of the aforementioned cell surface molecules that make microglia the immune cells of the CNS (e.g. MHC, receptors for cytokines and chemokines, etc.) as well as cytokine production, drastically altering their physiology [125].

1.2.2.2 Astrocytes

Astrocytes are the most abundant cellular type in the CNS, outnumbering neurons by over fivefold [118]. They were first discovered in 1863 by Otto Deiters and initially named Deiters cells, but due to their star-shaped morphology with numerous processess surrounding neighboring neurons and blood vessels, they have been finally called astrocytes. Morphologically, they can be classified as fibrous (with a few but long processes found in white matter) or protoplasmic (with a lot of short branched ramifications located in grey matter) [131]. Astrocytes are, as well, the most abundant type of macroglial cell but not the only: oligodendrocytes (myelin producers in CNS), ependymal cells (cere-

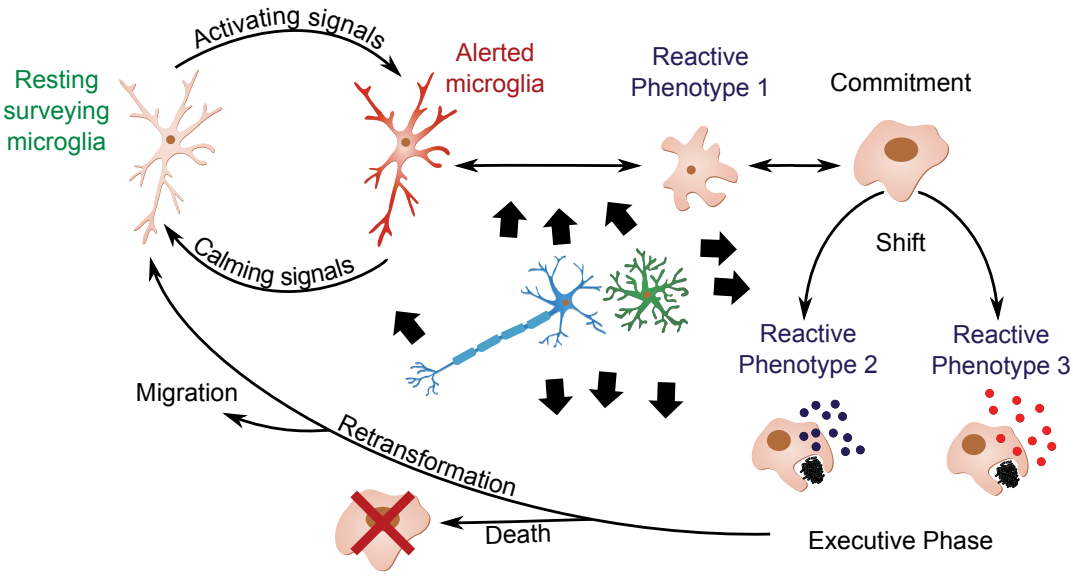


Figure 1.3: Microglia activity states. When "resting" microglia, constantly scanning the nervous parenchyma, are faced with "activating" signals (indicating a threat to the homeostasis) or a loss of "calming" stimulus, they get activated triggering morphologycal changes from ramified state to an "ameboid"-like state shifting into different reactive phenotypes (several cytokines secretion, phagocytic activity, etc.) depending on the cells and media environment and challenging threats. Activated cells could eventually return to a resting state or stay experienced.

brospinal fluid (CSF) producers), radial glia (found in the developing CNS), Schwann cells (myelin producers in perypheral nervous system), satellite cells (found surrounding neurons in sensory, sympathetic and parasympathetic ganglia) and enteric glial cells (found in the intrinsic ganglia of the digestive system) are also considered macroglia.

Astrocytes are the major source of extracellular matrix (ECM) proteins and adhesion molecules in the CNS promoting or inhibiting neurite outgrowth depending on ECM balance and adhesion molecules. They also synthesize and secrete proteolytic enzymes, like matrix metalloproteinases (MMPs) which shape ECM. They are also capable of neuronal maturation and survival by releasing growth factors like nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and fibroblast growth factor (FGF) [132], as well as controlling neuronal differentiation by neurotrophic factor release [133] and promoting synaptogenesis and synapses activity between neurons [134].

In relation with BBB induction and maintenance, it should be known that astrocytes induce angiogenesis, the formation of blood vessels, [135], synthesize laminin [136] and send specialized processes, the endfeet, to form the BBB [137]. Moreover, they regulate the induction of the BBB by tight junction formation and expression of transport systems, among others, [138] which also maintain in the adult brain. Reciprocally, astrocytes are also influenced by endothelial cells [139].

As astrocytes are in contact with both blood vessels and synapses they are able to change local CNS blood flow and blood vessels diameter in response to changes in neu-

ronal activity by releasing molecular mediators [140, 141].

In relation with house-keeping actions, as astrocyte surround synapses they can maintain the fluid, ion, pH, and transmitter homeostasis of the synaptic fluid [142, 143]. During normal neuronal activity, neurotransmission leads to the increase of extracellular K+ which results in hyperexcitability and seizures, if not corrected. Astrocytes networks linked by gap junctions dissipate small molecules such as potassium, gamma aminobutyric acid (GABA) and glutamate [144] and prevent their detrimental accumulation [145]. In addition, glutamate uptaken by astrocytes is critical for the glutamate-glutamine cycle [146] in which astrocytes collect and metabolize glutamate into glutamine to be redistributed to neurons for denovo synthesis of glutamate. Capturing excess ammonia and glutamate and converting them into glutamine also protects neurons against excitotoxicity. As well as the uptake of heavy metals, by metal binding proteins like metallothioneins, contained in astrocytes with both neuroprotective and neuroregenerative properties following injury or CNS disorders [147].

Moreover, astrocytes are the principal storage sites of glycogen in the CNS, being able to sustain neuronal activity during hypoglycemia and during periods of high neuronal activity [148] regulated by glutamate and neuronal activity [149].

Interesting newly-discovered roles of astrocytes showed that these cells actively participate and modulate neurotransmission due to the presence of calcium waves in astrocytes and gliotransmission, suggesting astrocytes as excitable cell capable of rapid cell comunication, especially with neurons (for review [150]).

Another fascinating, newly assessed, characteristic of astrocytes is that Glial fibrillary acid protein (GFAP)-expressing cells can contribute to cell genesis as stem cells in the adult neurogenic zone or as cellular elements of the neurogenic microenvironment (or niche) [151] redefining astrocyte definition, although there is still controversy whether these stem cells can be called astrocyte or not [132, 152]

Finally, astrocytes can serve as a bridge between the CNS and immune system, becoming an effector immune cell and being able to phagocytose cells and act as antigen-presenting cells, helping microglia in their immune tasks. They can present antigens to t-lymphocytes by the MHC or activating myelin basic protein -specific encephalitogenic T-cell lines [153], they also express costimulatory molecules critical for antigen presentation and T-cell activation [116, 154]. Moreover, they express Toll-like receptors, scavenger receptors, the complement system and cytokines and chemokines [154].

Astrocytes respond to CNS insults (like infection, trauma, ischemia or neurodegenerative diseases) by a process referred to as reactive astrogliosis (see Figure 1.4), defined by Sofroniew with four key features as: 1) the spectrum of changes in astrocytes that occur in response to all forms and severities of CNS injury and disease; 2) a variation of the changes undergone by reactive astrocytes with the nature and severity of the insult in gradated alterations of molecular expression, progressive cellular hypertrophy and, in severe cases, proliferation and scar formation; 3) astrogliosis changes regulation in a context-specific manner by specific signaling events able to modify both the nature and degree of those changes; 4) ability of the changes undergone during reactive astrogliosis to alter astrocyte activities both through gain and loss of functions that can impact both beneficially and detrimentally on surrounding neural and non-neural cells [155].

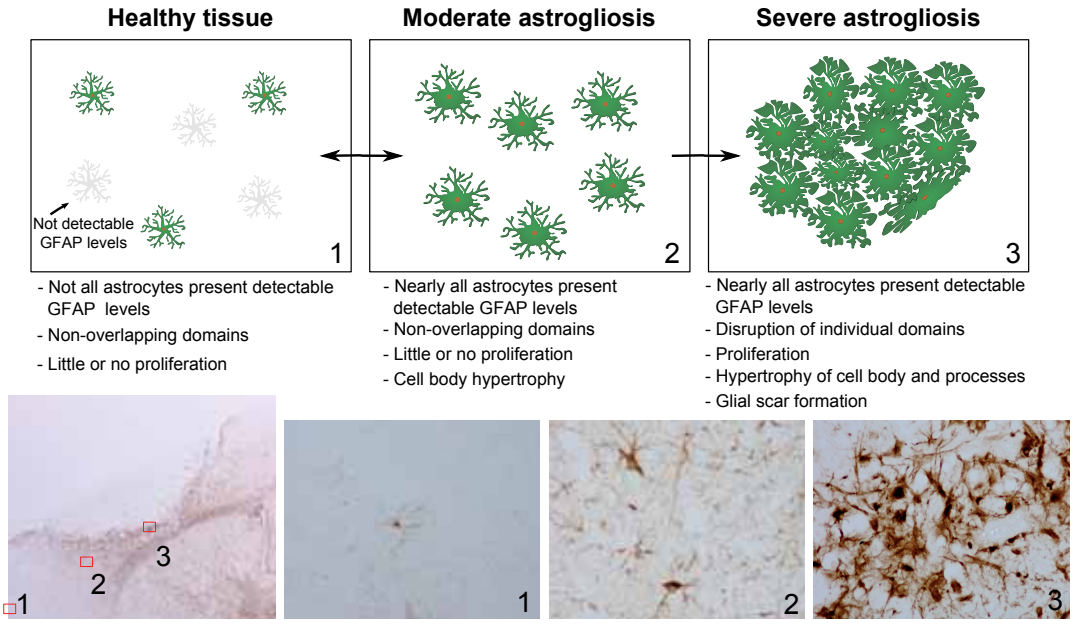


Figure 1.4: Schematic representations summarizing different gradations of reactive astrogliosis. 1. Astrocytes in healthy CNS tissue. 2. Diffuse reactive astrogliosis underlying variable changes in molecular expression and functional activity with variable degrees of cellular hypertrophy. Such changes are found surrounding severe focal lesions, infections or areas responding to chronic neurodegenerative triggers. 3. Severe reactive astrogliosis with compact glial scar formation includes newly proliferated astrocytes and other cell types such as fibromeningeal or other glia cells, as well as deposition of extracellular matrix. Astrocytes have densely overlapping processes acting as barriers protecting healthy tissue from intense inflammation. It occurs along borders to areas with tissue damage and inflammation. Photomicrographs of GFAP IHC from different areas remote (and presumably healthy, 1) and near a traumatic brain injury (2) and in glial scar (3) trying to illustrate astrogliosis characteristics.

The most typical and demonstrated response of reactive astrocytes after an insult is their increase of GFAP expression and other intermediate filament proteins like vimentin and nestin [156]. This increase is mediated by cytokines expression, being induced by IL-6 [157] and TGF-α [158] and being inhibited by TGF-β [159]. Those changes in the cytoskeleton proteins leads to morphology changes. After moderate insults, like axotomy or nerve crush, there is an hypertrophy of the cell body without overlapping neighboring astrocytes, called isomorphic astrogliosis or astrocyte activation, in which astrocyte adopt an stellate form and are able to return to their original state when the insult is removed [160]. Even in moderate astrogliosis, up regulation of GFAP expression in astrocytes that in normal conditions do not express detectable levels of GFAP, lead to the increase of stained cells, giving the false impression of proliferation [118]. In severe insults there is an hypertrophy of both the cell body and processes, with overlapping neighboring astrocytes domains, called anisomorphic astrogliosis or reactive gliosis, which is accompanied with proliferation and migration and results, after gross tissue damage, in the formation of a permanent strongly compacted limiting glial margin, named the glial

scar [161, 162, 155] which also incorporate other glial cells and a deposition of a dense extracellular matrix without the possibility of returning to the previous state, unlike isomorphic astrogliosis [163] (see Figure 1.4). The main role of the glial scar is the physical isolation of the damaged tissue from the healthy one and the reparation of the BBB, acting as neuroprotective barriers [164, 155]. However, although it promotes neuronal survival [165], proteoglycans present in the extracellular matrix also inhibits axonal and cellular migration preventing axonal regeneration [161]. Glial scar formation is induced by BBB breakage [166] being directly proportional the degree of breakage with the leukocyte infiltration. Activated macrophages induce astrocyte migration from the lesion outbreak to the margins helping to glial scar formation and secretion of proteoglycans [161].

Other triggering insults rather than trauma are invasive infections, neoplasm, chronic neurodegeneration and systemically triggered inflammatory challenges. Molecular triggers and regulators include growth factors such as FGF2; cytokines like IL-6, LIF, CNTF, TNF-α, Interferon gamma (INF-γ) and TGF-β; neurotransmitters like glutamate and noradrenalin; purines like ATP and reactive oxygen species (ROS). As external factors we would include innate immunity mediators such as lipopolysaccharide (LPS) and other Toll-like receptor ligands, hypoxia and glucose deprivation, neurodegeneration associated products like β-amyloide peptide (A$\boldsymbol{\beta}$), molecules associated with metabolic toxicity such as NH4; and cell proliferation regulators like endothelin-1 [118].

1.2.3 Circulating cells

Until some years ago, due to the fact that the CNS was considered immunologically privileged, leukocytes present in CNS were thought to always imply pathology, because of their supposed incapacity to cross BBB in healthy conditions. Nowadays it is well known that CNS is not excluded from regular surveillance by the immune system, not only by containing resident immune cells, such as astrocytes, microglia, endothelial cells and pericytes, which are capable of antigen presentation [167]; but also by immune circulating cells surveying CNS.

Leukocyte migration into and through tissues is fundamental to normal physiology, immunopathology and host defence and it also occurs in CNS although restricted in some ways. Two groups of leukocytes: lymphocytes and members of the macrophage/monocyte family must be considered as they appear in the CNS either in the normal state or during various diseases [168].

Lymphocytes, derived from bone marrow stem cells, are the key cells of adaptive immunity. They are small (5-12μm) round-shaped with a big circular nucleus which occupies nearly the whole cell. There are 3 types known: T-lymphocytes, B-lymphocytes and natural killer cells (NK). B-lymphocytes mature in spinal cord and produce antibodies when stimulated by specific antigens or helper T-cells, carrying out the humoral response of the adaptive immunity. Oppositely, T-lymphocytes perform the cellular response, they are produced in the bone marrow but mature in the thymus. They can be grouped between helper T-cells (which express CD4 in the membrane) and cytotoxic T-cells (which express CD8 in the membrane and induce death of pathogen-infected cells by releasing toxic granules). Once activated, T-cells multiply and enter in blood circulation, survey-

ing tissues until finding and recognizing their specific antigen and initiate a complete immune response [2].

Activated CD4 T-lymphocytes possess the ability to cross the CNS parenchyma in search of specific antigen [105] in an apparently random manner and in low numbers in healthy animals [169, 170]. Only activated T-lymphocytes are able to enter the normal CNS, and they leave within a couple of days from entry if fail to encounter antigen [107]. However, if T-cells find CNS parenchyma hostile, they die rapidly via an apoptotic mechanism [171]. B-lymphocytes' ability to patrol CNS is less well understood than T-cells; however, they can enter CNS in pathological processes and differentiate into plasma cells secreting antibodies, but they seem to be also capable to enter CNS in healthy conditions to seek their antigen [172, 173]. It is described that leukocyte migration across the BBB can occur without disruption of the complex tight junctions [174], although there are other routes that bypass the BBB entirely [168].

The macrophage/monocyte family play an important role in immunological surveillance of the healthy CNS, as scavengers, they phagocyte and digest bacteria, cellular debris and other particles and present the antigen (normally a protein found on the surface of the pathogen) to the corresponding helper T-cell. Monocytes circulate in the bloodstream and then typically move into different tissues throughout the body, where they get established and then differentiate into macrophages [2]. Despite promoting inflammation, they also control T-cell responses [175] and in some cases determine T-cells penetration into CNS parenchyma [176]. Few monocytes can enter CNS parenchyma as part of normal physiology, [177] although perivascular cells and macrophages from meninges are continuously being repopulated and thus, under permanent immune surveillance [178]. They are active presenting cells, more efficient than microglia. When macrophages make contact with inflammatory mediators or any other stimulating signal they get activated, increasing their metabolic rate, their mobility and their phagocytosis rate. For this purpose they have numerous receptors for cytokines, chemokines and other factors implicated in inflammation, with great heterogeneity between different macrophage subtypes [179] which will determine preferred target and activation method [180]. However, their fate is controversial, some data suggest that they return to the lymph nodes and spleen, carrying the antigens with them [181].

After a CNS insult or injury, an enhanced entry of leukocytes occurs, mostly neutrophils, macrophages and lymphocytes, which differ in its number and extension proportionally to the severity of the brain pathology [126]. Cytokines, such as TNF-α, IL-1 and IL-6, secreted by astrocytes and microglia under inflammatory conditions, upregulate adhesion molecules expression and chemokines in endothelial cells facilitating leukocytary infiltration [182]. These chemokines (a chemotactic type of cytokines) and their receptors are constitutively expressed at low or negligible levels in various cell types in the brain but, their expression is rapidly induced by inflammatory stimulus. Chemokines have a crucial role in directing inflammatory-cell recruitment in host defense, generating the adaptive immune response and contributing to the pathogenesis of many diseases; as well as maintenance of CNS homeostasis and potential mediators of neuroinflammation. There are about 50 different chemokines classified into four families depending on differences in structure and function. The largest families are CC

chemokines, such as monocyte chemoattractant protein 1 (MCP-1) (also called CCL2), which is a potent agonist for monocytes, dendritic cells, memory T-cells, and basophils; and CXC family, like interleukin-8 (CXCL8), which attracts polymorphonuclear leukocytes and activates monocytes [183].

Neutrophils enter the inflammation site, mostly induced by CXCL8, CXCL1 and CXCL2 [184], and degradate tissues due to their important phagocytic capacity and secretion of the matrix metallopeptidase 9 (MMP-9) [185], an enzyme able to degrade BBB [186]. They also regulate T-cell responses [187] and produce other chemokines like CCL2 and CCL3 which induce following monocytes and macrophages migration [188]. Macrophages arrival in the first stage is beneficial as they phagocyte cellular debris allowing injured area remodelation and regeneration, but, in CNS, both the number of infiltrated macrophages and their phagocytic activity, are diminished in comparison with other tissues [189]. However, macrophages have a dual role in neuroinflammation and also produce a bunch of factors potentially neurotoxic when released in big amounts, like free radicals as reactive oxygen species or nitric oxide (NO) (also due to activation of inducible nitric oxide synthases (iNOS)), hydroxyl radicals, proteases and glutamate [190].

1.3 IL-6 role in the CNS. Data gathered from humans and animal models

The discovery that IL-6 was expressed in astrocytes led to speculation that it could play a role in the CNS [13]. When 10 years later it was suggested that IL-6 could function as a neurotrophic factor [191], it's role in CNS really emerged.

1.3.1 Expression

After IL-6 discovery, it was demonstrated that some astrocytoma and glioma lines expressed IL-6 when stimulated with IL-1β, which pointed out that IL-6 could have a role in the CNS [13]. The same group demonstrated that IL-6 was also capable of inducing neuronal differentiation of the rat pheochromocytoma PC12 cell line, in a similar way to the prototypical neurotrophin NGF [192]. It was therefore not surprising the finding that both glial and neuronal cells expressed IL-6 and IL-6R to various degrees throughout the brain [193, 194, 195, 196, 197, 198]. In vitro, microglia, astrocytes and the neuronal line N18, but not oligodendrocytes, expressed IL-6R [199]; nevertheless, in vivo, oligodendrocytes may express IL-6 and IL-6R [200]. It is worth to take into account that despite IL-6R expression in astrocytes, they seem to have low level expression of the membrane-bound receptor, since they can modestly respond to IL-6 alone, needing sIL-6R for optimal responsiveness [201]. Also, IL-6 and IL-6R were expressed in sympathetic and sensory ganglia, predominantly in neurons [202], as well as in adrenal chromaffin cells [203], of adult rats. In line with these results, in vitro studies demonstrated that dissociated sympathetic neurons and PC12 cells expressed IL-6 and the two receptors, IL-6R and gp130 [204, 205].

Besides neurons and glial cells, endothelial cells produce copious amounts of IL-6, which can act on surrounding cells but also in an autocrine manner regulating a number

of adhesion proteins and IL-6 synthesis, particularly in the presence of sIL-6R [206, 207]. Moreover, trans-signaling confers IL-6 responsiveness to all cells in CNS, even the ones lacking IL-6R [208, 62, 209].

Many cytokines and inflammatory factors, as well as neurotranmitters and neuropeptides, have been shown to affect IL-6 regulation in brain cells; some of them studied in in vitro assays will be commented here. IL-1β and TNFα induced IL-6 in cultured cortical neurons [210] and astrocytes [211, 212], in the latter involving NFκB [213] and the PKC pathway [214]. The AMPc-PKA pathway may also induce astrocytic IL-6 [214, 215]. It is likely that membrane depolarization is one of the main mechanisms for neuronal upregulation of IL-6, where Ca2+ currents (such as those elicited by the glutamate agonist NMDA) and Ca2+/calmodulin-dependent kinases are critical factors [216]. The major bacterial pathogen, LPS, normally induces IL-6 in both astrocytes and microglia [217], but TNF-α induces IL-6 in astrocytes but not microglia [218]. There may be species-specific effects since in human cells in vitro, LPS mostly affect microglia rather than astrocytes; regarding TNF-α, IL-1β and IL-6 production, although IL-1β is a potent stimulator of IL-6 production in astrocytes, in microglia the three cytokines are upregulated [217, 219]. GM-CSF stimulates microglial IL-6 but not that of astrocyte [220], whereas IFN-γ induces IL-6 (and NO) in the murine microglial cell line 6-3 [221]; this cytokine does not induce IL-6 in astrocytes unless it is coincubated with IL-1β [211]. Interestingly, adult human astrocyte cultures subjected to mechanical injury upregulated IL-6 [222]. IL-6 production by astrocytes is subjected to autocrine regulation by IL-6, and the addition of sIL-6R synergizes dramatically with IL-1β and TNF-α to induce IL-6 [67]. OSM induced IL-6 alone and synergized with TNF-α to induce IL-6 [67]. IL-17 functioned in a synergistic manner with IL-6 (+ sIL-6R) to induce IL-6 expression in astrocytes [223].

Norepinephrine, VIP and pituitary adenylate cyclase activating polypeptide (PACAP38) stimulate IL-6 in astrocytes, and may synergize with IL-1β and TNF-α [224, 225, 226]; in contrast any of these factors have a major effect in microglia [224]. VIP may induce IL-6 through the PKA pathway and independently of prostaglandins (PGs) [227]. Prostaglandin E1 (PGE1) and PGE2, but not PGD2 and PGF2 alphaE2, induce IL-6 in human astrocytoma cells; PGE2 potentiates IL-1β induction of IL-6 [228, 229]. The synthetic ceramides C2- and C6-ceramide as well as the enzyme sphingomyelinase were able to induce IL-6 in astrocytes [230]. IL-1β, substance P and histamine induced IL-6 expression in human astrocytoma cells [215, 231], through NF-κB for IL-1β and through NF-IL-6 for SP and histamine [232]. Bradykinin stimulates IL-6 expression through activation of NF-κB in murine astrocytes [233]. Serotonin and adenosine agonists are also effective inducers in human astrocytoma cells [234, 235]; in mouse astrocytes, adenosine induces IL-6 through activation of PKA and NF-IL-6 [236]. TGF-β inhibits microglia proliferation and activation, including IL-6 production [237]; in contrast, it stimulates IL-6 production in astrocytes [238].

The main transcription factors regulating IL-6 expression, both in the periphery and the CNS, are nuclear factor κB (NF-$\boldsymbol{\kappa}$B), activator protein 1 (AP-1), cAMP response element binding protein (CREB) and CCAAT/enhancer-binding protein β (NF-IL-6) [239].

1.3.2 Role of IL-6 as a pro-inflammatory and anti-inflammatory cytokine

Inflammation consists on a strictly orchestrated process designed to combat infection or tissue injury. When effective, it ensures successful resolution of the damage and restoration of normal tissue architecture. However, inappropriate control of this defense mechanism ultimately contributes to disease worsening [85, 240]. A key event in the resolution of any inflammatory episode is the transition from innate to acquired immunity [241] and, IL-6 has arisen as an essential regulator of this immunological switch by means of differential control of leukocyte recruitment, activation and apoptosis [85]. Moreover, IL-6 also influences other vital branches of neuroinflammation process, affecting microgliosis, astrogliosis and BBB integrity (for neuroinflammation hallmarks see Section 1.2).

Astrocytes are major responders to IL-6, which is able to affect them in many ways: inducing their proliferation and chemotaxis by different mechanisms [242, 243]; inducing astrocyte secretion of inflammatory mediators (like PGs, cytokines, chemokines or acute phase proteins) [244, 245, 246] and, finally, by driving astrogliogenesis (see sections 1.3.3.2) in both the developing and the mature CNS [247, 248]. Microglia is also stimulated by IL-6, which induces its proliferation [249], morphological changes and upregulation of MHC expression and phagocytic activity [250].

Astrocytes are also considered to be the main CNS producers of IL-6 (followed by neurons [249]) with lots of stimulus leading to astrocitary IL-6 production such as neurotransmitters, neuropeptides, inflammatory cytokines (like IL-6 itself, TNF-α and IL-1β) and viral and bacterial pathogens [251, 182, 67].

The key role of IL-6 in astrogliosis and microgliosis has been assessed in several in vivo models. Transgenic mice with chronic expression of pathophysiological levels of IL-6 under the astrocytary promoter GFAP (GFAP-IL6) or the neuronal promoter NSE (NSE-IL6), overexpressing IL-6 in astrocytes and neurons respectively, showed an important brain astrogliosis and microgliosis [252, 157, 253] although, in opposite with GFAP-IL6 mice, NSE-IL6 do not show histological or behavioural signs of neuronal damage [252, 253]. A double transgenic mice with systemic overexpression of both IL-6 and sIL-6Rα (IL-6/sIL-6Rα mice) also present astrogliosis and microgliosis [254]. Moreover, both direct infusion of IL-6 in brain and indirectly in the striatum produce massive astrogliosis [255, 256]. By the opposite aproximation, animals without IL-6 (IL-6 KO) present a severe decrease in astrocytic and neuroglial activation after injury [257] and reduced early microglial activation [258]. In addition, rats intracerebrally injected with anti-IL-6 antibody presented reduced astrogliosis and microgliosis after LPS induction [259].

Astrocytic hypertrophy and enhanced expression of GFAP protein are linked to activation of the JAK2/STAT3 signaling pathway [260]; thus STAT3 pathway is considered a critical regulator of astrogliosis and have a considerable role in the reparation of injured tissue and the recovery of motor function after injury [261]; again pointing out the fact that astrogliosis is not detrimental per se. In line with this dual effect, IL-6 can induce both neuroprotective [262] and neurotoxic [263] microglial responses. IL-6 alone has direct effects on microglia by activating STAT3 and kinase pathways. Those different responses of microglia to IL-6 are context-dependent, specifically on the presence or absence of sIL-6R, IFNγ or granulocyte macrophage colony stimulating factor (GMCSF)

which will alter the type and amplitude of microglial response [264].

The last vital branch of neuroinflammation processes altered by IL-6 is BBB degradation. IL-6 performs a vital role maintaining its integrity influencing both astrocytes and endothelial cells by inhibiting vascular cell adhesion molecule-1 (VCAM-1), but not intracellular adhesion molecule-1 (ICAM-1) upregulation; this unexpected negative effect on adhesion molecule expression, appear to function as an anti-inflammatory cytokine, preventing leukocytes infiltration and helping to maintain BBB integrity in neuroinflammatory conditions [265]. GFAP-IL6 mice also presented elevated β4 and α5 integrin subunits expression in astrocytes and increased laminin and fibronectin expression in endothelial cells, the authors proposed that during neuroinflammation, astrocytes attempt to increase adhesive interactions at the BBB in order to increase barrier integrity [266]. However, in GFAP-IL6 mice, the BBB does not fully develop, presenting an extensive breakdown with massive vascular proliferation that could cancel the previous action on integrins, leading to parenchymal inflammation, gliosis, spongiform change, axonal degeneration and macrophage accumulation [252, 267]. Those findings suggest that IL-6 increase above physiological levels can influence the development and physiologic function of the BBB and contribute to CNS injury by abnormal iron metabolism [268]. Curiously, mice with systemic IL-6/sIL-6R overexpression did not present either vascular proliferation or inflammatory infiltration and, although BBB presented some changes manifested by hydropic astrocytic end-feet, there was no major leakage and the BBB remained intact [254].

In conclusion, based on its major influence on directing astrogliosis, microgliosis, BBB integrity and leukocyte infiltration, IL-6 can be considered one of the main orchestrators of the inflammatory response in the brain (together with TNF-α and IL-1β) and in the whole body, as it can be seen in IL-6 deficient mice presenting an incomplete inflammatory response against infections [269], yet, under certain circumstances IL-6 can also act as an anti-inflammatory cytokine [270, 84] by inhibiting TNF-α expression [271] and inducing the expression of soluble TNF-α receptors and IL-1β receptor antagonist [270]. However, directing neuroinflammation has marked IL-6 as detrimental, although those inflammatory processes, as already said, can be beneficial and help to return to normal homeostasis after injury [155].

1.3.3 Role of IL-6 in development and normal physiology

In basal conditions, IL-6 levels are so low in CNS that they cannot be measured by traditional ways [198] and led to the initial conclusion that IL-6 would not be involved in the daily "housekeeping" functions of the body but only to orchestrate host defenses against infections or damage, stated in the previous section. However, this has not been supported by further studies, and it is now widely accepted that both IL-6 and its receptor are found in several brain regions in basal conditions (see Section 1.3.1); thus indicating a role of IL-6 in physiological processes [272].

As explained in this section, IL-6 is involved in the control of body weight and metabolism, pain perception, body temperature, stress response, synaptic plasticity, learning,

sleep and nervous system development; being eligible to be classified as a neurotrophic factor as well.

1.3.3.1 Differentiation, survival and regeneration of neurons

Soon after its discovery, IL-6 was shown to induce the neuronal differentiation of PC12 cells [192]. Both NGF and IL-6/sIL-6R synergise and induce the differentiation of PC12 but by independent pathways [273]. Moreover, NGF induces IL-6R expression, creating a positive feedback loop [274]. IL-6 also stimulates differentiation of primary dorsal root ganglion neurons and Schwann cells [275, 276].

Besides inducing differentiation, IL-6 also promote basal survival of catecholaminergic, cholinergic and dopaminergic neurons [277, 278], acetylcholinesterase-positive neurons [279], enteric neurons [280], sympathetic neurons [205], sensory neurons [281] and oligodendrocytes [282]; those neuron and oligodendrocytes responses are strongly dependent on IL-6R or sIL-6R presence [205, 280, 281, 282] and IL-6 concentration [283]. It has been suggested that a survival mechanism could be the inhibition of neuronal activity and release of glutamate [284].

Moreover, IL-6 promotes neuronal survival in the presence of oxidative stress [285], hypoxia [286], ionizing radiation [287], serum or calcium deprivation [288] and after injury. IL-6 increases with development but levels are low in CNS; however, after an injury like drop weight model of cortex lesion (closed skull) or axotomy its expression increases dramatically, mainly in neurons [289], which facilitates its role in survival depending on the type of neuron. After axotomy, IL-6 increases neuronal survival [290]. In the sciatic model, IL-6 expression increases potently within large and medium-sized axotomized neurons [289], whose survival is decreased by 50% in IL-6 KO mice [291]. IL-6 probably promotes survival through inducing BDNF, and indeed, in vivo IL-6 KO mice do not upregulate this neurotrophin in dorsal root ganglia (DRG) following nerve injury, presenting increased neuronal death [291]. In contrast, in the facial nerve axotomy model no differences in motoneuronal survival were observed [258], whereas IL-6 was shown to be detrimental in a model of optic nerve injury [292] and in a spinal cord injury model delivering Hyper-IL-6 [293]. Moreover, acute administration of an anti-IL-6 antibody after spinal cord injury favors regeneration; however, total absence of IL-6 in IL-6 deficient mice do not show these regenerative effects [294]. IL-6 effects are greatly dependent on the type of cell and microenvironment context, with huge differences among regions, as for example IL-6 administration induces spinal cord injury, via a JAK–STAT induction of iNOS in microglia, but it is protective in hippocampus and cortex, potentially due to increased expression of sIL-6R in the brain relative to the spinal cord that may antagonize IL-6 signaling in this context [295].

IL-6 also affect neuronal functionality, such as the electrical properties of Purkinje [296] and enteric neurons [297]; additionally, it modulates synaptic release in neurons [298] and suppresses presynaptic release of norepinephrine in sympathetic enteric neurons [297]. Finally, it induces the cholinergic phenotype of sympathetic neurons [205]. Sensory neurons are particularly affected by IL-6 deficiency, since in normal conditions

IL-6 KO mice show a 60% reduction of the compound action potential of the sensory branch of the sciatic nerve [299].

Finally, IL-6 plays an important role in neuronal regeneration after peripheral nerve lesion. Experiments after injury of the hypoglossal nerve in mice demonstrated this nerve regenerating role of IL-6 [275]. Sensory branch of the sciatic nerve neurons are also highly dependent on IL-6 for functional recovery following injury, being retarded in IL-6 KO [299], possibly due to the absence of STAT-3-mediated GFAP induction in Schwann cells [300]. Results with IL-6 KO mice imply a role for IL-6 on sympathetic sprouting induced by nerve injury [301, 302]. It also promotes sprouting and functional recovery of organotypical cultures of hippocampus [303]. Moreover, Hyper-IL-6 promotes nerve remyelination after sciatic nerve injury [304] while IL-6 KO mice show reduced regeneration of the injured facial nerve [258].

IL-6 is sufficient, but not necessary, to mimic both cAMP effect and peripheral conditioning lesion responses (a first peripheral lesion promote the regeneration of a central second lesion [305]) on axonal growth; as it can be observed that intrathecal IL-6 allow neuronal growing despite the presence of inhibitors of regeneration found in myelin, although there were no differences in conditioning lesion responses between IL-6 KO and wild-type mice [306]. However, another study showed that IL-6 KO mice did not present axonal conditioned lesion-induced regeneration [307].

Despite IL-6, as already said, does not increase motoneurons survival of adult neurons, it does induce microgliosis and neuronal regeneration after facial axotomy [249, 258] and enhances neurite regeneration from transected nerve terminals [308]. IL-6 in conjunction with sIL-6R, also delay degeneration in a mouse model of wobbler motor neuron disease [309].

1.3.3.2 Neurogenesis, astrogliogenesis and oligodendrogenesis

Adult neurogenesis, the process of creating new neurons and glial cells from neural stem cells (NSCs) in the developed brain, was considered imposible until its discovery almost 50 years ago [310]. The most active neurogenic regions are the dentate gyrus (DG) of the hippocampus, the subventricular zone of the lateral ventricles and the olfactory bulb [311].

Neurogenesis is now known to be dramatically affected by a myriad of factors such as exercise, environmental enrichment, stress, reproductive behaviour or aging, among others; as well as in many neuropathological situations like stroke, status epilepticus, mechanical damage, and Alzheimer, Parkinson and Huntington diseases (for review see [312]); in all these cases a detrimental role of inflammation has usually been suggested [313], and thus, IL-6 will be upregulated and could have a detrimental role on neurogenesis.

Indeed, GFAP-IL6 mice show a diminished hippocampal neurogenesis [314], and in vitro IL-6 clearly decreases the differentiation of neural stem/progenitor cells into neurons [315, 316]. In contrast, IL-6 is involved in oligodendrogliogenesis via JAK/STAT pathway [282] and astrogliogenesis [316]. Yet, other studies claimed that IL-6 fused with IL-6R-

α promotes both gliogenesis (through the STAT-3 pathway) and neurogenesis (through MAPK/CREB pathway) [317].

1.3.3.3 Regulation of Excitatory and Inhibitory Transmission

Neurons communicate through chemical synapses in which information flows by means of electrochemical codes translated into neurotransmitters release. Glutamic and GABA acids are neurotransmitters that are able to facilitate or difficult the transmission of information through synapses. A balance between glutamate's excitatory actions and GABA's inhibitory actions determines whether information will circulate through CNS circuits [318].

Cytokines affect neuronal synaptic communication modulating GABA and glutamate-mediated neuronal transmission. Glutamatergic agonists such as N-methyl-D-aspartate (NMDA) and kainic acid have been used extensively to investigate neurodegeneration resulting from excessive glutamatergic excitation. GFAP-IL6 mice are more sensitive to NMDA, but not to pilocarpine-induced seizures, and they are also sensitive to low doses of kainic acid, which provoke to them severe tonic-clonic seizures and death [319]. IL-6 stimulates GABA release from both hypothalamus and posterior pituitary by a mechanism mediated by PGs, while TNF-α inhibits GABA release from both tissues [298]. This effect is mediated by PGs and is abolished by indomethacin [298]. The effects of IL-6 on neuronal functioning are not restricted to the CNS, as it also inhibits nociceptive fiber responses to heat both in vivo and in vitro (see Section 1.3.3.8); by being administered systemically to anesthetized rats it inhibits all naturally evoked neuronal responses, but, only animals with nerve ligation presented heat responses, while intraplantar IL-6 injection leads to ipsilateral thermal hypoalgesia. In conclusion, interleukin-6 inhibits normal peripheral nociceptive transmission, yet such anti-nociceptive effects are attenuated following nerve injury in a modality-specific manner [320, 321].

1.3.3.4 Control of body weight

The mechanisms that balance food intake and energy expenditure determine who will be obese and who will be lean. Insulin, a pancreatic hormone, was the first hormone known to control body weight in CNS entering from the periphery [322] and controlling energy expenditure. Soon after, leptin (or the product of Obese gene), an hormone secreted mainly by adipocytes, was described to be able to enter to CNS and control food intake [323] by exerting an inhibitory feedback effect on fat mass by acting on hypothalamic nuclei that express its signal-transducing receptor, ObRb [324]. Both hormones are considered adiposity signals [325] as they circulate at levels proportional to body fat content [326] and enter the CNS in proportion to their plasma level [327]. Receptors of both leptin and insulin are expresssed by brain neurons involved in energy intake [328, 329]; consequently, administration of these peptides directly into the brain reduces food intake [330, 331], whereas the deficiency of these hormones increases it [323, 332].

IL-6, like leptin, is also secreted by adipose tissue during non-inflammatory conditions and its serum levels are correlated with body-mass index (BMI) and percent body fat

[333]. However, newer studies shown that IL-6 levels correlated negatively with total body weight and total body fat [334]. Unlike leptin, IL-6 and its receptor are also expressed in neurons of hypothalamic nuclei that regulate BMI [195] as well as in glial and brain endothelial cells (see Section 1.3.1) being produced both peripherally and centrally during non-inflammatry conditions. Circulating IL-6 levels also correlate with other metabolic parameters like insulin sensitivity [335].

IL-6 inhibit lipoprotein lipase (LPL) activity and decrease its production in murine adipocyte cell lines [336] as well as increase lipolysis [337] and acutely enhance circulating levels of triglycerides and glucose [338, 339], thus helping to limit obesity [340]. TNFα and IL-6 cause weight loss in mice, which is inhibited by pretreatment with either anti-TNFα or anti-IL-6 monoclonal antibodies, respectively [341]. However, IL-6 can also stimulate the hypothalamic-pituitary-adrenal (HPA) axis (see Section 1.3.3.5) whose over-activity may be important for obesity-associated morbidity [342]. Additionally, some studies indicate that high circulating IL-6 levels may exert pathogenic functions in obesity and atherosclerosis, and that suppression of IL-6 would be beneficial [343, 344].

Studies with deficient IL-6 mice showed that endogenous IL-6 exerts a tonic suppress of fat mass, as IL-6 KO mice developed obesity, that was partly reversed by IL-6 replacement at low doses, which did not affect fat mass in wild-type (WT) mice or produce acute-phase reaction, arguing against a pharmacological effect [345]. These mice also shown decreased glucose tolerance and increased circulating triglyceride levels in females, as well as increased leptin levels and leptin insensitivity in older mice of both sexes [345]. Those metabolic changes might be secondary to obesity or due to enhanced leptin effects by IL-6 interaction with leptin signalling like IL-1β does [346], showing that total depletion of IL-6 causes adverse rather than beneficial effects on fat and glucose metabolism. This anti-obesity effect of IL-6 is believed to be mainly exerted at the CNS level, specifically in the hypothalamus [195, 345] as intracerebroventricular (icv) administration of IL-6 increased energy expenditure at a dose without effects when given peripherally, which required considerably higher dose to decrease body weight [347, 345]. Moreover, chronic icv treatment with IL-6 can suppress body fat mass in rats decreasing body weight by 8.4% and decreasing the relative weight of mesenteric and retroperitoneal fat pads [348].

However, some authors claim that IL-6 deficient mice did not show an increase in obesity or in total body fat with aging, even when they were fed a high-fat diet, also, not presented consistent changes suggesting insulin resistance [349]. Moreover, whether or not IL-6 has a beneficial role in insulin sensitivity and glucose homeostasis is a hot debate [350, 351].

In line with Wallenius et al. results, GFAP-IL6 transgenic mice expressing IL-6 under the GFAP promoter are resistant to both control and high-fat diet-induced obesity in a gender-specific manner; showing GFAP-IL6 a significantly smaller body weight gain, with a IL-6 role more prominent during obesity. This study also suggest that in GFAP-IL6 animals insulin signalling is normal, while insulin regulation is not, as they respond normally to exogenous insulin but presented an altered control of blood glucemia [352].

The role of IL-6 in food intake is controversial. Some authors claimed that IL-6 acts in the CNS provoking anorexia [348] while others described no change [353, 352]. It may be due to the need of higher levels than the ones necessary for body weight control [354].

IL-1β, IL-6, IL-8, and TNF-α induced acute anorexia by direct action in CNS at estimated pathophysiological concentrations in the CSF [355]. IL-6 injected icv decreased food intake, but it did not affect water consumption [58]. When analysing effect duration it was seen that single IL-6 icv injection can suppress 2h food intake [356], but not 24h food intake following chronic icv treatment [355] although others have described a reduction in food intake during a two weeks period with daily icv injections [348]. In line with these results, obese IL-6 KO mice had increased absolute food intake [345] but GFAP-IL6 mice did not present altered food intake in either control or high-fat diet [352].

The mechanism explaining food intake increase in IL-6 deficient mice may be a decreased expression of corticotrophin-releasing hormone (CRH) mRNA in the paraventricular nucleus of the hypothalamus, which is known to be important in the control of food intake and energy expenditure, playing a role for the late onset obesity observed in these IL-6 KO mice. Moreover, they also have decreased mRNA expression of the anorexigenic and obesity-suppressing peptide oxytocin. Co-localization of IL-6Rα with CRH, oxytocin and arginine-vasopressin means that IL-6 could stimulate the expression of these peptides directly increasing energy expenditure and decreasing food intake [357]. Also, in CRH-deficient animals, both baseline and LPS-stimulated hypothalamic IL-6 mRNA levels were elevated, as a compensatory effect [358].

Moreover, IL-6Rα is also co-localized in the same neurons as the neuropeptide, melanin concentrating hormone (MCH) in the mouse hypothalamus [359] which is known to have profound impact in food intake as mice lacking MCH are hypophagic and lean [360] while MCH overexpression leads to increased food intake, obesity and insulin resistance [361].

IL-6 prevents obesity not only by food intake suppression but also by increasing the energy expenditure. It has been demonstrated that IL-6 injected icv, but not peripherally, increases oxygen consumption, carbon dioxide production and thermogenesis, likely through the autonomic system [347, 353, 345]. The results in IL-6 KO mice are consistent with that assumption, showing that lack of endogenous IL-6 is associated with a decreased fat utilization during baseline conditions at room temperature and a blunted increase in energy expenditure during exposure to new-cage stress and cold ambient temperature in young IL-6 KO mice, which presented lower temperature levels even in basal conditions [362]. These findings seem to corroborate the role of endogenous IL-6 in stimulation of the somatic nervous system (SNS) in mice [205, 353, 362] although there are some discrepancies [363]. In addition, GFAP-IL6 mice showed an impaired thermogenesis rather than the opposite, not supporting an increased sympathetic tone [352], as it is essential for thermogenesis control [364]. Regardless of the type of diet, GFAP-IL6 mice showed a decreased liver weight similar to IL-6 KO mice, but that might have to do with hepatic local role of IL-6 rather than a CNS mechanism [365, 352].

Moreover, IL-6 KO mice have decreased endurance and energy expenditure during exercise, suggesting that it is necessary for normal exercise capacity [366]. IL-6 is released from skeletal muscle, specially during exercise, mediating effects of physical exercise on metabolism [367] and also from adipose tissue [368], being necessary to achieve brain-specific IL-6 KO mice to rule out peripheral effects of IL-6 and help to understand the roles of this pleiotropic cytokine.

1.3.3.5 HPA axis stimulation

The hypothalamic–pituitary–adrenal (HPA) axis is a complex neuroendocrine system involved in several central and peripheral physiological functions, most of them related to appropriate adaptation to stressful situations. Its activation is the consequence of the convergence of stimulatory inputs from different brain regions into the paraventricular nucleus (PVN) of the hypothalamus, where corticotrophin-releasing factor/hormone (CRF or CRH) and arginin-vasopressin (AVP), the most important neuropeptides for HPA axis regulation, are formed. Their reach to the anterior pituitary (AP) activates adrenocorticotropic hormone (ACTH) release by corticotrope cells, activating the synthesis and secretion of glucocorticoids (GC) in the adrenal cortex [369].

The concept of bidirectional communication between immune and neuroendocrine systems is quite old. Thomas Addison documented that a patient with adrenal insufficiency had an increase of circuting lymphocytes [370]; adrenal gland removal was found to produce hypertrophy of the thymus (the organ responsible of lymphocyte production) [371] and Hans Selye found that animal's response to stress was an adrenal gland hypertrophy and involution of the thymus [372]. The isolation of GC from adrenal cortex by kendall and Reichstein and the assesment of their anti-inflammatory actions noted that inflammatory symptoms were alleviated when there was an increase in steroids in the body [373]. Besedovsky and Munck demonstrated a physiological role for GC in preventing over-activity of immune/inflammatory reactions [374, 375] and finally, it was suggested that additionally to the fact that GC had an sizeable impact on immune activity, in turn, immune activity did influence GC secretion [376] giving name to the concept of a neurocrine-immune regulatory loop (reviewed in [377]).

Cytokines, specially IL-1, IL-6 and TNF-α are well known to affect and stimulate HPA axis acting synergistically and driving the release of ACTH and corticosterone by potentiating each other's actions [378, 379]. The HPA response to Intraperitoneal (ip) IL-6 is less potent, fast and short-lived compared to that to IL-1, requiring higher doses and causing lower maximum responses [380] but it acts in a synergistic way enhancing IL-1 effects [381, 382].

IL-6 initiates the release of CRF and AVP from isolated hypothalamic tissue and administration of IL-6 into the third ventricle causes a dose-dependent hypersecretion of ACTH and corticosterone [383]. IL-6 also have a direct stimulatory effect on ACTH and corticosterone secretion from the pituitary [384].

1.3.3.6 Control of body temperature

IL-6 meets all the criteria necessary to be an important endogenous pyrogen [347, 385], regulating the acute phase reaction in peripheral tissues and governing its central components including fever, anorexia, and apathy [386, 387]. On the other hand, TNF-α acted as an endogenous antipyretic, as mice deficient in TNF receptors developed exacerbated fevers after LPS injection [388]. Local production of IL-6 in medial preoptic nucleus and the medial hypothalamus activates the HPA axis (see Section 1.3.3.5) and regulates a variety of central effects such as sleep, food intake and fever; and all those IL-6 central actions

are potentiated by sIL-6R administration [58], specifically in fever, where trans-signaling seems to be of major interest [389]. The essential role of IL-6 in controlling the fever response was shown in IL-6 deficient mice in which LPS and IL-lβ failed to induce fever [386]. Moreover, those IL-6 KO mice presented lower temperature levels at thermoneutrality (30°C) conditions respect control animals [362]. Unexpectedly, GFAP-IL6 mice also showed, if anything, an impaired thermogenesis in a cold environment [352] pointing that there is still a lot of work to do.

Psychological stress, which modulates the thermoregulatory centre through a reversible IL-6-dependent pathway, induces an increase in both body temperature [390] and plasma IL-6 levels [391]. Normalization of the increased body temperature after 2 h of psychological stress in mice is accelerated after injecting antibodies against IL-6, indicating that IL-6 is a thermoregulatory factor during psychological stress [392]. IL-6 may cross the BBB [393] activating neurons of the thermoregulatory centre directly [394] or indirectly, by local production of thermogenic substances by means of activating endothelial cells and monocytes among others, to produce these thermogenic substances, such as PGs [395] or NO [396].

1.3.3.7 Sickness behaviour

Sickness behavior refers to the non-specific symptoms (anorexia, depressed activity and fatigue, coldness, loss of interest in usual activities, disappearance of body-care activities) that accompany the response to infection [397]. Despite their negative impact on well-being, on the past years, these symptoms of sickness had usually been ignored, perceived as uncomfortable but banal components of infections [398]. However, this view has turned out to be incorrect as the psychological and behavioural components of sickness represent, combined with the fever response and the associated neuroendocrine changes, a highly organized strategy of the organism to fight infection. That changes are triggered by pro-inflammatory cytokines, produced at the site of infection by activated accessory immune cells, such as IL-1 (α and β), TNF-α and IL-6; which coordinate together the local and systemic inflammatory response to microbial pathogens [109]. Those peripherally produced cytokines can act on the brain, causing the behavioural symptoms of sickness, by two communication pathways: a neural route represented by the primary afferent neurones innervating the body site where the infection takes place; and a humoral pathway involving cytokine diffusion to brain target areas and its possible relay by PG intermediates [399].

Systemic or central infusion of IL-1β or TNF-α induces the full spectrum of behavioral signs of sickness (decreased locomotor activity and exploration of their physical and social environment, reduced food and water intake, and impaired learning and memory) in a dose and time-dependent manner [400, 401, 402]. The same effects are obtained in response to the administration of molecules that induce the synthesis of endogenous cytokines, like LPS, which induces the expression of pro-inflammatory cytokine in the brain at subseptic doses [403].

In contrast to IL-1β and TNF-α, IL-6 administered systemically or centrally is not able to induce complete sickness behaviour effect despite its ability to induce a fever response

[404, 402] but it contributes to the expression of brain cytokines in response to immune stimuli as LPS-induced sickness behaviour and cognitive impairment were less noticeable in IL-6 KO mice respect controls [387, 405]. However, after a turpentine abscess, lack of IL-6 completely prevented fever, anorexia, and cachexia and, although it did not prevent lethargy, IL-6 KO mice recovered to normal motor activity levels sooner than wild-type mice [406]. During influenza virus infection in IL-6 KO mice, symptoms of sickness were only slightly modified indicating that IL-6 seems more critical in induction of the symptoms of sickness behavior during sterile tissue abscess than during influenza infection although it is induced in both cases [406].

Anti-inflammatory cytokines, such as IL-10, regulate the intensity and duration of sickness behaviour, possibly by decreasing pro-inflammatory cytokines production and signalling, such as IL-6 [407].

1.3.3.8 Pain

IL-6-like immunoreactivity was found in the peripheral nerves in normal and inflamed human skin [408] and the peripheral nociceptors lack IL-6R but constitutively express gp130 [409]. A correlation between the number of IL-6-positive cells and the degree of allodynia (pain due to a stimulus which does not normally provoke pain) was found [410]; indicating that IL-6 could modulate the development of neuropathic pain. IL-6 interferences with pain are greatly reviewed in Jongh et al [411].

Some authors claimed that an IL-6 increase in plasma produced analgesia due to a local release of endogenous opioid peptides by immune cells [412] whereas others described an hyperalgesic effect [413]. If injected in combination with IL-6R, it could sensitise nociceptors to heat [414]. Injection of IL-6 in a rat hind paw induced dose-dependent mechanical hyperalgesia (mechanoallodynia) in both hind paws and pretreatment with anti-IL-6 antibodies reduced the hyperalgesia [413] although anti-IL-6 antibodies alone had no effect on nociception [412, 413]. In the same line, blocking IL-6 trans-signaling in periphery decreased heat sensitivity in transgenic mice [415] and IL-6 KO mice also presented a lesser degree of thermal hyperalgesia and mechanoallodynia via the PGs pathway only when genetically related wild-type mice were used [302, 416].

Regarding dorsal root ganglia (DRG), it was found a decrease in both sympathetic invasion of the fiber tract and cell layer of the DRG after sciatic nerve ligation in IL-6 deficient mice respect controls [301] with a delayed mechanoallodynia but no differences in thermal allodynia [301, 417]. Intrathecal IL-6 produced tactile allodynia in normal rats and thermal hyperalgesia in rats previously lesioned in sciatic nerve [418] while intrathecal administration of anti-IL-6 antibodies decreased mechanical allodynia [419]. Also, icv injection of IL-6 induced thermal hyperalgesia in rats [420].

However, IL-6 deficient mice showed a reduced analgesic response to restraint stress or to the administration of morphine [421] as immune cells found in inflamed tissue synthesize β-endorphins [422] and, in tissue undergoing inflammation processes, IL-6 administration is analgesic as it recruits inflammatory cells and induces their endorphin or enkephalin release [412].

1.3.3.9 Sleep-wake behaviour

Sleep is a fundamental physiologic brain process for health and well-being; it has recovery and restorative functions including cellular, endocrine, immune, metabolic, thermoregulatory and behavioural processes like learning and synaptic plasticity [423]. Two stages of sleep can be identified: non-rapid-eye-movement sleep (non-REM sleep; subdivided into light and deep sleep) and rapid-eye-movement (REM) sleep [424].

Homeostatic, circadian, neuronal and humoral processes are linked in a very complex network regulating sleep-wake behaviour. The circadian control by the clock located in the suprachiasmatic nucleus of the hypothalamus receives retinal input, and coordinates the timing of the sleep-wake cycle [425]. Hormones of the HPA axis like ACTH, CRH and cortisol regulate interaction between sleep and stress system [426]. As already stated, IL-6 stimulate the HPA-axis (see Section 1.3.3.5) having a role in sleep-wake behavior. Moreover, cytokines are implicated in the humoral link of the interaction between immune and CNS; interacting with other cytokines, neurotransmitters and hormones affecting sleep regulation in a circadian phase- and dose-dependent way and also being cytokine expression affected by sleep itself (greatly reviewed in [427]).

IL-6 is a sleep modulatory factor [428]. It is secreted in a circadian rhythm and correlates with the sleep-wake cycle, being increased during the major sleeping period (the light cycle in rodents) in brain (cortex, hippocampus and hypothalamus), adipose tissue, and blood; and also after sleep deprivation [429].

Central administration of rat IL-6 to rats altered non-REM sleep pattern and fragmented sleep in a dose-dependent manner without changes in total REM sleep amount [430]. However, central administration of anti-rat IL-6 antibodies did not alter any of the parameters studied, which may indicate that although IL-6 possesses sleep modulatory properties it may not be involved in the regulation of spontaneous sleep in healthy animals [430]. IL-6 deficient mice showed normal non-REM sleep but 30% more REM sleep and, following sleep deprivation, they required 6 h longer to accumulate the additional sleep amount than WT mice did [431]. Hypersecretion of IL-6 together with high cortisol levels led to poor sleep and fatigue [432] but elevated IL-6 and low cortisol led to sleep and sleepiness [433]. Also, disorders and pathologies associated with excessive daytime sleepiness (like insomnia, narcolepsy, sleep apnea, obesity or depression) are related with elevated IL-6 levels (reviewed in [434].

Regarding IL-6 receptors, sleep, compared with continuous wakefulness, increased about 70% concentrations of sIL-6R in the period of predominant REM sleep [435]. Increases in circulating sIL-6R were completely dependent on sleep, in contrast to mIL-6R, implicating that sleep enhances the capacity for IL-6 trans-signaling, enabling the activation of cell types that normally would remain unresponsive to IL-6 [435].

1.3.3.10 Emotional reactivity and other behavioural traits

When exposed to an unknown environment, animals have a motivation to explore it, but they also experience fear, which tends to increase immobility (freezing) and to reduce exploratory activity in a novel environment. Low activity levels, a high defecation rate,

and low exploratory activity in novel environments, like in the hole-board and plus-maze apparatus, have been considered sings of fear in rodents [436, 437, 438]. Thus, the exploratory activity is reduced in emotional animals.

Regarding the role of IL-6 in behavioral parameters, there is a lot of controversy. Our group previously demonstrated that total IL-6 KO mice had an increased emotional reactivity in novel environments (Hole-board and Plus-maze test), exhibiting less deambulation, rearings and head-dipping in the Hole-Board test and less exploration of the open arms (thus increased anxiety) in the elevated plus-maze test [439]. However, using the same IL-6 KO mice, other authors confirmed the decrease in rearings and the increase in anxiety, but regarding locomotion they found instead an increase (although using an Open Field apparatus or activity-meter instead of Hole-Board) [440, 441]. To complicate things further, other authors observed altered exploration and anxiety levels of IL-6 KO mice only in specifics settings [442, 440], or not at all in a different genetic background [443] or with a different mouse line [444, 445].

Trangenic expression of astrocitic IL-6 in mice has been shown to cause severe neurological disease characterized by runting, tremor, ataxia and seizure [252] as well as progressive decline in avoidance learning [446] while transgenic expression of IL-6 under neuronal promoter NSE produced no neuronal deficits [253] although alterations in social behavior were described [444]. Those NSE-IL6 animals showed a greater amount of affiliative social interactions respect controls while IL-6 KO mice presented a higher frequency and longer duration of offensive-type behavioral events [444].

Other behavioral parameters that are not exempt of controversy are those related to learning and memory. Exogenous administration of IL-6 led to impaired spatial learning in rats [447], and, as already said, GFAP-IL6 mice exhibited a decrease in avoidance learning [446]. Also, increased IL-6 was suggested to be a biomarker for the risk of future cognitive decline [448]. Specifically, IL-6 influences long term potentiation (LTP), which is a paradigm for the cellular mechanisms explaining learning and memory. LTP upregulates IL-6 expression in an NMDAR-dependent manner [449] and neutralization of IL-6 with an anti-IL-6 antibody prolongs LTP and improves long term memory. Moreover, GFAP-IL6 mice also have a reduced LTP in the dentate gyrus [450]. These observations showing a detrimental role of excessive IL-6 in learning and memory would predict that the absence of IL-6 would lead to improved memory. Indeed, in one study it was reported that IL-6 KO mice exhibited a facilitation of radial maze learning, in terms of a lower number of working memory errors and a higher percentage of animals reaching the criterion as compared with WT animals [445]; but, it should be commented that in that study it could be criticized the fact that the radial maze measures spatial learning only when transparent walls are used [451], which was not the situation. In any case, other behavioural studies found no change in spatial learning [405], or saw impaired novel object recognition in IL-6 KO [452], or found impaired memory processes for both hippocampus-independent (novel object recognition memory test) and -dependent learning (Morris Water Maze test) in IL-6 KO mice, when compared to controls [442].

Regarding despair behaviour, GFAP IL-6 mice demonstrated increased glucocorticoid levels after stress [453], suggesting an increased stress sensitivity with a potential vulnerability for the development of depressive-like behavior. Consequently, IL-6 deficient mice

showed reduced despair behavior in the forced swim test [442, 454] and in the tail suspension test, as well as enhanced hedonic behavior (seen by the increased sensitivity to the reinforcing properties of sucrose) [454]. Moreover, IL-6 KO mice presented a strong resistance to develop learned helplessness following intense stress consisting of two consecutive days of inescapable and unpredictable footshocks [454]. Further evidence relating this resistance to the lack of IL-6 is found by the fact that in WT mice, the stress preceding the helplessness procedure significantly increases IL-6 levels in the hippocampus, thought to be involved in murine depression-like behaviors [455, 456]. It is worth to note that increased anxiety in IL-6 KO as a consequence of stress is independent from depressive-like behaviour [457, 458] not being contradictory with the fact that IL-6 KO mice showed this reduced despair behaviour in the force swimming test but an increased anxiety in novel environments.

In summary, our knowledge of the roles of IL-6 on behavior obtained by using total IL-6 KO mice is very limited. There are many putative reasons for the stated discrepancies, but undoubtedly the fact that IL-6 can eventually be produced by neurons, astrocytes, microglia and endothelial cells, and that in turn these cells can respond to IL-6, may be a significant factor. To give some insight into this problem, it is mandatory to study the role of IL-6 in a cell-specific fashion.

1.3.4 Role of IL-6 in pathology

Due to its importance, it is worth to point out that injuries to the CNS, including the brain and spinal cord, are major health problems around the globe.

Traumatic brain injury (TBI) is the leading cause of death and severe disability in people under 45 years in Western industrialized countries, mainly due to motor vehicle accidents; which causes a major worldwide social, economic, and health problem, being responsible in Europe for more years of disability than any other cause [459, 460].

Stroke or cerebrovascular accident, which is the the sudden death of brain cells in a localized area caused by inadequate blood flow due to ischemia after a vessel blockage or haemorrhage; it is the third leading cause of death in the Western world and also a leading cause of permanent disability [461].

Autoimmune diseases are one of the leading causes of death among young and middle-aged women in the United States, resulting in a significant impact in terms of direct and indirect economic costs, and quality of life. Incidence rates vary among the autoimmune diseases, with estimates ranging from less than one newly-diagnosed case of systemic sclerosis to more than 20 cases of adult-onset rheumatoid arthritis per 100 000 person, per years [462].

As explained before (see Section 1.3.2), IL-6 is a critical molecule during a CNS injury/inflammation/autoimmune diseases with a very complex role due to its plurifunctionality and outcome's duality. Thus, it is mandatory the study of IL-6 role during the inflammatory response after a CNS injury or disease and the mechanism of action that will ultimately determine either a beneficial or a detrimental role of this cytokine to the final output.

1.3.4.1 Traumatic brain injury

IL-6 levels in CSF were significantly higher than plasma levels in patients who had suffered TBI [463], and recently, an IL-6 polymorphism (-174C/G) has been associated with fatal outcome in patients with severe traumatic brain injury [464]. As expected, IL-6 is upregulated in several animal models of brain injury [465, 466, 467] and shows a myriad of actions as suggested by studies in GFAP-IL6 and IL-6 KO mice.

GFAP-IL6 mice showed more rapid healing and recovery after TBI [468, 469] because of extensive revascularization [468] and an early upregulation of inflammatory cells recruitment [469]. Also, anti-oxidant and neuroprotective proteins, metallothioneins I and II, are upregulated in GFAP-IL6 mice together with a decreased oxidative stress and apoptosis [470, 469]. Consequently, IL-6 KO mice presented a compromised inflammatory response [471, 472], increased oxidative stress [472], impaired neuroglial activation [257], decreased lymphocyte recruitment [258] and a slower rate of recovery and healing, due to the slower revascularization and loss of integrity of the BBB [468] (see Figure 1.5).

The transcriptomic analysis of IL-6 KO mice versus WT mice [473] and that of GFAP-IL6 mice [474] in a model of brain cortex cryoinjury revealed that IL-6 modulates the expression of many genes involved in inflammation, apoptosis, oxidative stress and synaptic activity among others. Although we could conclude that IL-6 seems to improve recovery in vivo, it is worth pointing out that other studies reported that icv administration of anti IL-6 antibody did not influence the edema or the acute cognitive and neurological motor deficits after injury [475] and one study showed that IL-6 KO mice showed no changes compared to WT after TBI in BBB integrity, neurological impairment and inflammatory cell recruitment [476]. However, clinically, IL-6 levels in CSF and brain parenchyma correlate with improved outcome after severe TBI in humans [477, 478] confirming IL-6 neuroprotective effects after TBI. NGF appeared in the CSF, correlating with the severity of the injury, when IL-6 levels reached high concentrations; simultaneously with or following an IL-6 peak. Also, NGF induction was completely inhibited by anti-IL-6 antibodies, suggesting that IL-6 neuroprotective effects could be mediated by the release of neurotrophic factors by astrocytes [479].

1.3.4.2 Ischemia

Ischemic brain injury involves inflammation, excitotoxicity, oxidative damage and apoptosis, and thus to some extent it is a similar scenario as that following traumatic brain injury; it is therefore not surprising that IL-6 may be an important factor orchestrating the responses elicited by stroke. IL-6 seems to be associated with the occurrence of stroke, as IL-6 elevations in plasma correlate with short-term adverse prognosis reflecting high prevalence of coronary instability [480, 481]. Moreover, higher serum IL-6 levels appear to be associated with unstable carotid plaques, suggesting a potential risk for stroke [482]. The IL-6 polymorphism (-174G/C) is also associated with carotid artery atherosclerosis and coronary heart disease (reviewed in [483]). Stroke patients also show significant elevations of CSF and serum IL-6 shortly after the ischemic event that correlate with brain infarct volume, and IL-6 haplotype affects both infarct size and IL-6 levels [484, 485]. The

authors showed that the increase in plasma IL-6 started within 24 h and peaked between 2 and 4 days after the onset of stroke.

In animal models of cerebral ischemia, it has been a consistent finding the upregulation of IL-6, mostly in neurons but also in glial cells and vascular endothelium [486, 487]. Also, systemically produced IL-6 was thought to be potentially able to enter into CSF through the damaged BBB, but, significantly lower levels of serum IL-6 compared to CSF levels were reported during the first week after the onset of the stroke, thus indicating that CNS is the main source of IL-6 after a stroke [488].

Exogenously administered IL-6 in lateral ventricle of gerbils subjected to transient forebrain ischemia prevented learning disabilities and delayed neuronal loss [489]; also icv administration of IL-6 in rats with permanent middle cerebral artery occlusion (MCAO) reduced ischemic brain damage 24 h after the insult [486]. These, and many other [490, 491] results from different experimental models show the neuroprotective effects of IL-6 on cerebral ischemia; controlling oxidative stress [492] and angiogenesis [493] are among the attributed functions of IL-6 during stroke. In accordance with those results, anti-mouse IL-6 receptor antibody injection aggravates ischemic cerebral damage in mice by blocking Stat3 activation pathway [494]. Surprisingly, an study with IL-6 KO mice resulted in no differences in neither the infarct size nor neurologic function between IL-6 deficient mice and WT after cerebral ischemia [495]. However, as explained before (see Section 3.5), IL-6 is a major regulator of body temperature and postsurgical hypothermia in IL-6-deficient mice was being ignored; when body temperature was controlled by external warming after MCAO, IL-6 KO mice showed reduced survival, worse neurologic status, and larger infarcts than WT animals [491, 493]; thus, the dual role of IL-6 in ischemia is highlighted, with not only beneficial effects (see Figure 1.5).

1.3.4.3 Exitotoxicity and neurotoxicity

Excitotoxicity is the pathological process by which neuronal cells are damaged or killed by excessive stimulation by neurotransmitters such as glutamate. Its receptors (like NMDA and AMPA), become over-activated, high levels of calcium ions are allowed to enter the cell and, together with the ROS production, are the main mechanisms by which it damages cells. Excitotoxicity is involved in stroke, CNS injury, neurodegenerative diseases like Multiple sclerosis (MS), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), alcoholism or alcohol withdrawal, epilepsy and Huntington's disease (HuD) [497, 498]. It is a major contributor to neuronal cell death in those conditions.

Excitotoxicity is a potent inducer of IL-6 production in the brain [499, 500, 490] and it is thought to be an endogenous protective mechanism in cerebral ischemia [490]. IL-6 protects against excitotoxicity in different neuronal populations (see Section 1.3.3.1). Epileptic seizures often involve excitotoxicity, and thus it was expected to find that patients suffering of epilepsy show increased CSF levels of IL-6 after seizures [501], and that well-known animal models of epilepsy such as the glutamate analog kainic acid (KA) upregulate CNS IL-6 [499]. IL-6 KO mice are more susceptible to various convulsant stimuli including several glutamate analogs and show clear signs of increased hippocampal dam-

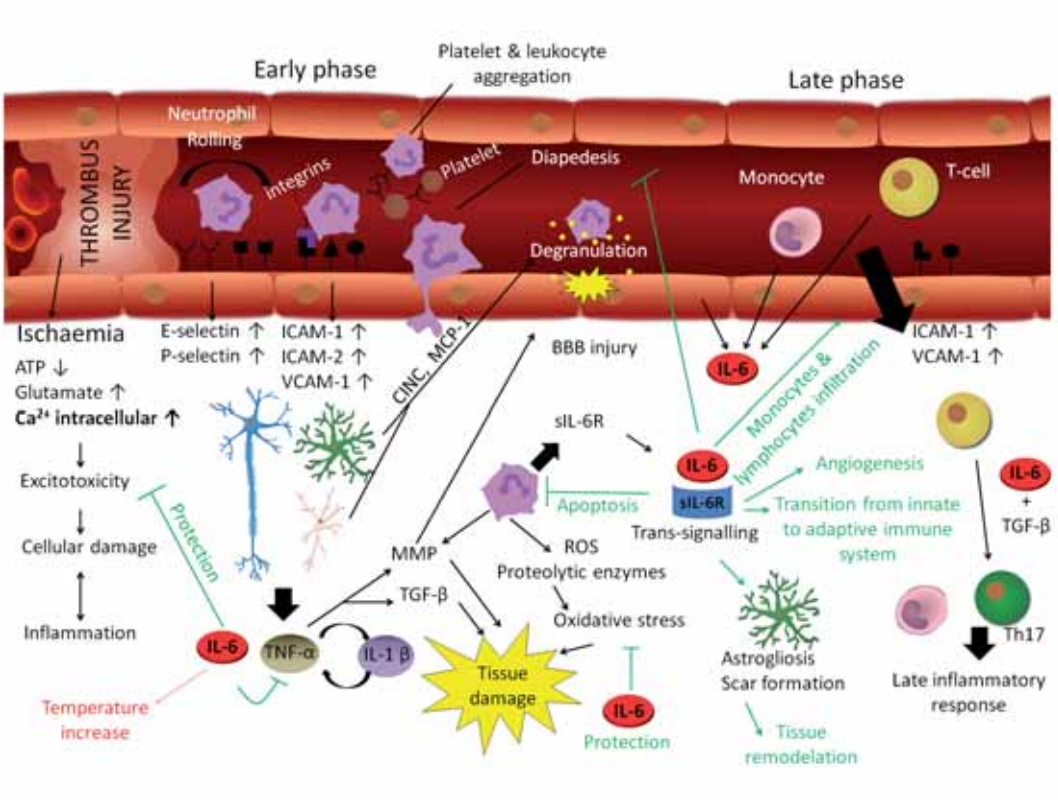


Figure 1.5: IL-6 has a major role in the response of the brain to injury. To some extent the response of the brain to trauma and stroke is similar. Stroke may be caused by an embolus/thrombus occlusion, an hemorrhage or a vasospasm, resulting in ischemia. Hypoxia initiates a biochemical cascade leading to cell death, involving excitotoxicity, oxidative stress and apoptosis, in where IL-6 has a protective effect. In the early response, neutrophils extravasate to nervous parenchyma involving a process of rolling, activation and transmigration due to an upregulation of P and E-selectin, followed by an upregulation of ICAM and VCAM. Neutrophils are a rich source of sIL-6R, and damaged resident cells produce IL-6, TNF-α, IL-1β and chemokines, enhancing leukocyte migration to parenchyma. TNF-α and IL-1β lead to neutrophil degranulation and tissue destruction by means of MMP and TGF-β, while IL-6 inhibits TNF-α and neutrophils' diapedesis. Moreover, it induces apoptosis in neutrophils in a negative feedback loop. In the later phase, IL-6 orchestrates the transition between innate and adaptive immune response, not only inhibiting neutrophils but recruiting monocytes and T-cells for a late inflammatory response. Besides, it induces astrogliosis and angiogenesis needed for the tissue remodelation and recovering. However, IL-6 exhibits a detrimental effect in relation with body temperature increase, critical in the patient outcome. If deregulated, chronic IL-6 may cause significant brain damage. Figure reprinted from [496].

age [502, 503]. Yet, this is a complex system, since intranasal administration of IL-6 to rats [504] and transgenic IL-6 expression in the brain [319] are pro convulsive. Some authors also claim a damaging role of IL-6 on neuron development in culture and on the response to NMDA [505, 506]. There could be several reasons for such dual effects of IL-6, probably the context has a dramatic effect, as often is the case with growth factors, which is influenced by either the dosage of both IL-6 and NMDA [507], the presence of both IL-6R and

sIL-6R [508, 280], the timing of IL-6 administration [505], the physiological state of tissue and the brain region or the cell type, among others [505].

IL-6 would enhance excitotoxicity, on the one hand, via its enhancement of the Ca^{2+} currents in some brain areas [509] and protect against excitotoxicity on the other hand. This protection would be reached via induction of growth factors; suppression and earlier termination of calcium elevations [507, 510], by both the gp130/JAK/STAT-3 and the gp130/Ras/ERK pathway [511]; induction of the adenosine A1 receptor [512]; and suppression of the production of ROS [502]. Protection against NMDA-induced excitotoxicity conferred by IL-6 can be blocked by an IL-1R antagonist and partially blocked by an anti-NGF antibody [513]. STAT-3 and ERK activation were also suggested to be involved in the protective effect of IL-6 [284, 508], but up to now, the exact molecular mechanism involved in IL-6 actions regarding excitotoxicity are not completely understood.

IL-6 also protects against other neurotoxic insults induced by a wide array of stimuli. It reversed the chemotherapy-induced neuropathy in different animal models [514]. IL-6 also decreased diabetes-related neuropathy (which is the most severe complication of diabetes) [515]. Moreover, in GFAP-IL6 mice, the cytotoxicity due to 6-aminonicotinamide (6-AN), an antimetabolite that induces astroglial toxicity, is relieved, showing increased angiogenesis and a reduction of oxidative stress and cell death [469]. However, IL-6 KO mice are more resistant to methamphetamine-induced neurotoxicity [516], having IL-6, in this case, a detrimental effect.

1.3.4.4 Multiple Sclerosis

Multiple sclerosis (MS) is an inflammatory demyelinating autoimmune disease of the CNS mediated by $CD4^+$ T cells and soluble inflammatory mediators, in which IL-6 has an important role. While there is significant controversy on whether or not MS is correlated with either plasma or CSF IL-6, sIL6R and sgp130 levels [517, 518, 519, 520], it has been demonstrated the presence of IL-6 in acute and chronic active plaques of MS patients, mainly associated with astrocytes rather than macrophages or mononuclear infiltrating cells [521]. Yet, the association of MS with IL-6 polymorphisms is again not conclusive [522].

One of the most common animal model of MS is Experimental Autoimmune Encephalomyelitis (EAE) [523]. IL-6 is upregulated in CNS during EAE [524], and different approaches have demonstrated a major role of this cytokine. Thus, it was soon established that neutralization of IL-6 with antibodies led to a reduced disease [525] (although another group found no effect [526]), which later on was seen to be due to the suppression of the MOG-induced differentiation of naive T-cells into Th17 and Th1 cells [527]. However, some controversy arise when EAE is induced by a virus instead of myelin antigen in which recombinant IL-6 reduced demyelination and inflammation in spinal cord [528].

Another approach was using IL-6 deficient mice, early studies demonstrated that these mice were resistant to EAE [529, 530, 531, 532, 533], highlighting the essential role of this cytokine. Absence of infiltrating cells in the CNS, reduction of lymphocyte proliferation, blockage of the activation and differentiation of T-cells, change of the cytokine profile and

failure to stimulate endothelial VCAM-1 were some of the reasons suggested to be responsible for resistance to EAE at the time. Trans-signaling is also crucial for EAE induction as its blockade with gp130-Fc fusion protein delayed the onset of adoptively transferred EAE compared to controls due to a reduction in VCAM-1 expression on spinal cord microvessels [534]. Since the discovery of two novel subsets of T helper cells, named Treg and Th17 cells, and its importance in MS [535, 536], it has been seen that a balance between Th17 and Treg is crucial for immune homeostasis, and TGF-β is required for both Th17 and Treg differentiation being able to induce both Foxp3 and RORγt expression [537].

IL-6 has also a major role in Th17 cell differentiation from naive $CD4^+$ T-cells (reviewed in [538]), particularly in the EAE model [527, 83, 539]. Th17 cells produce IL-17 (among other cytokines) which enhances IL-6 production by astrocytes, which in turn induces differentiation of Th17 cells in a positive feedback loop between IL-17 and IL-6 via activation of NF-κB and STAT-3 [223, 540]. Oppositely, TGF-β1 can promote Treg differentiation, suppresing adaptive T-cell responses and preventing autoimmunity [541, 542]. Although initial reports claimed that IL-23 is required for the generation of Th17 cells from naive T-cells [543, 544], it was demonstrated that IL-23R is not expressed on this naive T-cells [545]. Instead, IL-23 and TNF-α, act as a survival signals for Th17 cells [546, 544]. But, it seems clear that IL-6 induces Th17 differentiation together with TGF-β [83, 545] (although IL-21 can also initiate Th17 differentiation combined with TGF-β [547] in an alternative pathway); also, additional signals may be required to induce fully functional Th17 cells in vivo [548]. Although IL-6 activates both STAT3 and STAT1, it has been demonstrated that STAT3 activation is maintained while STAT1 activation is suppressed in Th17 cells [549]. EAE-resistant IL-6 KO mice demonstrated a deficiency in Th17 cells infiltrated in the CNS [83]. When responsiveness to IL-6 is eliminated only in T helper cells there is resistance to EAE, as IL-21 pathway is intact but not active in the absence of IL-6 [550].

Although EAE is considered a disease mostly induced peripherally, the fact is that the CNS local milieu seems to have dramatic effects (see Figure 1.6).

IL-6 production by astrocytes after contact with T-cells was suggested to be mediated by a different pathway, involving integrin heterodimers and NF-κB [551]. The importance of glial production of IL-6 have been studied by our group in GFAP-IL6 mouse model of chronic transgenic IL-6 expression, which presents an atypical EAE due to a retargeting of the immune attack, with no signs of spinal cord damage while showing a prominent cerebellar damage [552]. The mechanisms responsible for these responses to IL-6 in the cerebellum are complex and likely to include an increased vascular activation, loss of integrity of the BBB and the induction of specific cytokines and chemokines. Consequently, targeted inhibition of NF-κB in astrocytes improves the outcome of EAE by suppressing chronic CNS inflammation [553]. On the other hand, neuronal deletion of the inhibitor of NF-κB induces a more severe outcome of EAE [554]. We also have generated mice producing IL-6 essentially only in the brain (by crossing the GFAP-IL6 mice with IL-6 KO mice) which showed a milder but almost identical phenotype as the GFAP-IL6 mice, indicating that not only is cerebellar IL-6 production and eventual leakage into the peripheral compartment the dominating factor controlling this type of EAE, but that it can also facilitate induction of autoimmunity in the absence of normal systemic IL-6 production

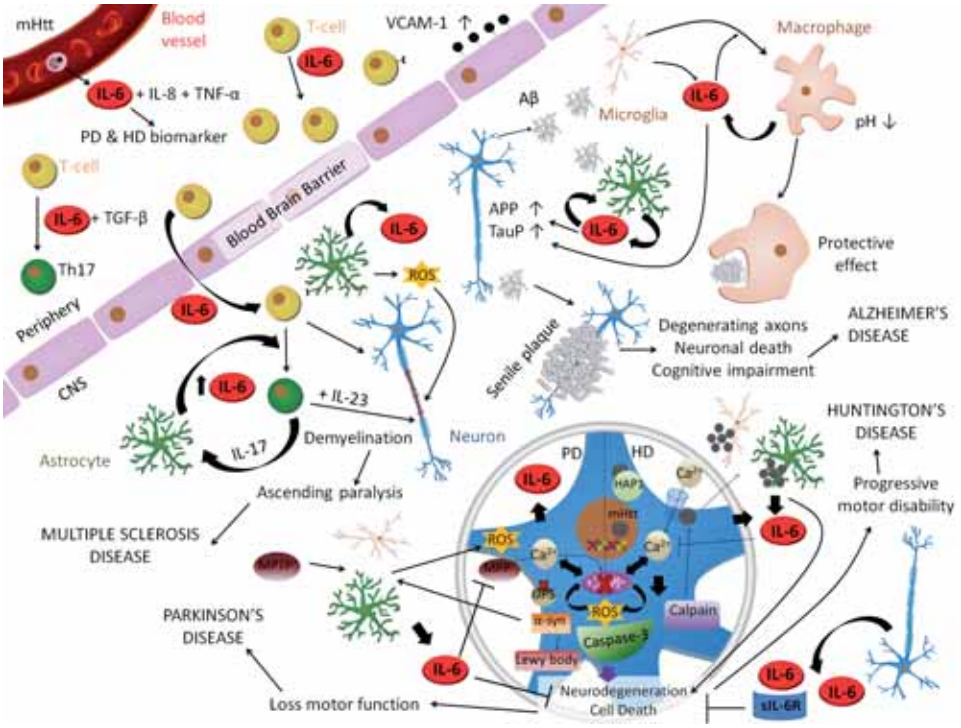


Figure 1.6: IL-6 is related to many brain diseases. In MS, IL-6 influences T-cell function inducing its proliferation and infiltration into CNS by upregulation of VCAM-1 on the vascular endothelial cells. In the presence of TGF-β, it also induces T-cell differentiation into Th17 cells, which secrete IL-17 that stimulates IL-6 production in astrocytes in a positive feedback loop. Besides, T-cell direct contact induces production of IL-6, ROS and NO in astrocytes, contributing to damage myelin sheath and neurons leading to ascending paralysis and, if IL-23 is present, the fully development of MS. In AD, Aβ peptide produced by cleavage of APP, induces microgliosis, astrogliosis and triggers IL-6 production in both types of cells upregulating APP and hyperphosphorylating tau in neurons. Aβ is accumulated in the extracellular space forming senile plaques and inducing neuronal death. However, IL-6 can play a protective role differentiating microglia into phagocytic macrophages capable of degrading Aβ. Mutant Huntingtin (mHtt), associated with HuD, is a CAG expansion translated into intracellular polyglutamine inclusions which are toxic for the cell due to different pathways: increase in intracellular Ca^{2+} (iCa^{2+}) due to NMDA receptor binding, increase mitochondrial dysfunction with ROS production, and axonal transport disruption due to mHtt/HAP1 complexes. Elevated iCa^{2+} activates caspases and calpains, cleaving mHtt into toxic N-terminal fragments and triggering apoptosis in a positive feed-back loop. Also, calpain causes autophagy inhibition resulting in high levels of mHtt in another loop. Moreover, microglia expressing mHtt contribute to neuronal cells degeneration. PD is considered a synucleinopathy due to an abnormal intracellular accumulation of insoluble α-synuclein aggregations in the form of Lewy bodies in dopaminergic neurons. Neuronal death is thought to be as a result of mitochondrial dysfunction with ROS production, an increase of iCa^{2+}, oxidative stress and alterations in the ubiquitinproteasomal system that become incapable to degrade α-synuclein, triggering microglia to produce ROS. All together produce neurodegeneration and PD symptomatology. MPTP is metabolized in MPP+ by glial cells affecting dopaminergic neurons by interfering with mitochondrial metabolism, producing toxic PD in animals. IL-6 protects against Ca^{2+} and ROS excitotoxicity decreasing neuronal death. Figure reprinted from [496].

[555].

1.3.4.5 Alzheimer's disease

Alzheimer disease is an age-dependent neurodegenerative disorder that results in progressive loss of cognitive function. It is characterized by the accumulation of the A$\boldsymbol{\beta}$ peptide into amyloid plaques in the extracellular brain parenchyma [556] and the formation of tangles inside neurons as a result of abnormal phosphorylation of the microtubule-associated protein tau [557].

IL-6 expression is altered in the brains of AD patients, being increased in general, around amyloid plaques (even preceding neuropathological changes [558]) and in cerebrospinal fluid [559, 560, 561], as well as in several AD animal models [562, 563]. Transgenic animals evidenced a connection between IL-6 and AD, GFAP-IL6 mice showed neurodegeneration and cognitive impairment but NSE-IL6 mice did not present behavioral abnormalities (see Section 1.3.3.10), emphasizing once more the importance of IL-6 cellular source of production. In humans, attempts to find important interactions between polymorphisms in specific alleles, such as the IL-6-174 G/C promoter allele, and genotype frequencies, are not conclusive [564, 565]. The -572C/G polymorphism of IL-6 gene promoter region, is another polymorphism that might be associated with AD [566].

Both IL-6 and sIL-6R/IL-6 complex stimulated the synthesis of the AD beta-amyloid precursor protein (APP) [567, 568] and, conversely, IL-6 was upregulated in cultured glial cells upon stimulation with the carboxy-terminal 105 amino acids of APP [569] and in brain cells upon induction of Aβ [569]. Moreover, IL-6 also enhances neuronal damage induced by Aβ peptide in cultured rat cortical neurons [506] and influences APP processing [570]. In addition, IL-6 cause an increase in tau hyperphosphorylation due to JAK-STAT pathway [571].

Despite this detrimental role of IL-6 seen in vitro, in vivo studies with AD transgenic mouse models rather show a beneficial role of IL-6, in principle due to a massive gliosis which attenuated beta-amyloid peptide deposition and enhanced plaque clearance [572]. Thus, treatment with IL-6 leads to acidification of the lysosomes and microglia activation to a macrophage-like state, enabling them to degrade fibrillar Aβ [573]. This is not unexpected, as activated microglia can efficiently phagocytize Aβ peptide and delay pathology course in transgenic models [574, 575, 576]. Astrocytes also may be involved in the clearance of Aβ peptide [577], also by regulating microglial phagocytosis [578] (see Figure 1.6). Regarding the influence of IL-6 on learning and memory see Section 1.3.3.10.

1.3.4.6 Parkinson's disease

Parkinson's disease (PD) is a degenerative disorder of the CNS with severe motor impairment, like shaking or rigidity among many others, due to the death of dopaminergic neurons in the substantia nigra. IL-6 is upregulated in PD [579] and IL-6 levels in the CSF of PD patients are elevated [580], although an inverse correlation between severity of PD and IL-6 levels is observed [581]. IL-6 induces the expression, and changes the distribution, of the Parkinson-associated proteins ubiquitin, α-synuclein and tau in cultured glial cells [582]. Also, mutant forms of α-synuclein, that are associated with autosomal PD, induce IL-6 expression in astrocytes [583]. IL-6 KO mice showed increased vulnerability to

MPTP, a molecule that is metabolized into the complex I inhibitor MPP+ by astrocytes and is a selective dopaminergic toxin [584], whereas, in vitro, IL-6 is capable of protecting rat dopaminergic neurons from the neurotoxicity of MPP+ [585]. Altogether, the results with these animal models of PD suggest that IL-6 could be exerting a neuroprotective role during PD (see Figure 1.6).

1.3.4.7 Huntington's disease

Huntington's disease (HuD) is an inherited progressive neurodegenerative disorder with both psychiatric, cognitive and motor disturbances, due to the progressive loss of striatal GABAergic neurons and atrophy of the neocortex, it is characterized by abnormalities in the huntingtin gene [586]. IL-6 expression is dramatically elevated in the striatum of HuD patients, and in general these patients showed clear signs of abnormal immune activation [587]. Lentiviral administration of IL-6 and, to a higher extent a chimeric molecule of IL-6/sIL-6R, rescued striatal neurons from a quinolonic acid induced model of degeneration [588], showing a neuroprotective role of IL-6 in HuD (see Figure 1.6).

1.3.4.8 Mental disorders

IL-6 has also been related to some psychiatric disorders. IL-6 in plasma is elevated and correlated with major depression [20]. Also, IL-6 levels are modulated by anti-depressants [589, 590]. Some studies have pointed out that IL-6 levels in CSF but not in plasma were increased in combat veterans with post-traumatic stress disorder (PTSD) in comparison with those of controls [591], and that IL-6 and/or sIL-6R levels in plasma but not sgp130 were significantly higher in PTSD patients respect to controls, and higher in PTSD patients with concurrent major depression than in other PTSD patients or controls [592]. The link between IL-6 and depression seems to be both the stimulation of the activity of the HPA-axis and the modulation of tryptophan and serotonin metabolism [592, 380, 109]. Studies with IL-6 KO mice have been contradictory, while some stated that IL-6 deficient mice were resistant to stress and less prone to depression-like behavior [454], others reported no enhanced depression-related behavior or enhanced susceptibility to stress [443].

IL-6 has also been related with Schizophrenia (SZ), a complex neurological disorder characterized by a breakdown of thought processes and by poor emotional responsiveness, commonly manifested with hallucinations, delusions, paranoid and mental deterioration [593, 594]. There is a significant increase of IL-6 but not in sIL-6R levels in SZ patients [593]. IL-6 production due to exposure to Ketamine (a NMDA receptor antagonist and model of SZ) is responsible for the activation of NADPH-oxidase in brain leading to dysfunction of a subset of fast-spiking inhibitory interneurons [595]. Also, a single maternal injection of IL-6 during pregnancy causes schizophrenia-like behavioral abnormalities in WT mice but not in IL-6 KO mice [596]. Although IL-6 levels in SZ are dysregulated transmitting abnormal inflammatory signals to brain tissue in the so-called cytokine hypothesis of schizophrenia, the exact contribution of IL-6 to the pathogenesis of this disease still remains unclear [597].

Moreover, IL-6 has been found to be increased in the cerebellum of autistic brain [598] and has been suggested to mediate autism-like behaviors [599].

Inflammation and IL-6 undoubtedly contributes to some other chronic CNS disorders, not to talk about the periferical diseases, which are not visited in this thesis and have been reviewed elsewhere [600].

1.3.4.9 Viral infections

Encephalic viral infections with leukemia virus [601], lymphocytic choriomeningitis virus [602], human immune deficiency virus (HIV) [603, 604] and Theiler's virus [605], among others [272], increases IL-6 expression in the brain. Also, IL-6 KO mice infected with Theiler's virus show severe neurological deficits and mortality in comparison to WT mice, showing a neuroprotective effect of IL-6 on the survival of spinal motor neurons [605]. Regarding herpes simplex virus, lack of IL-6 enhanced susceptibility to infection but did not alter latency or reactivation of the virus in IL-6 KO mice [606].

1.3.4.10 Cancer

As first suggested by Virchow, inflammation seems a critical component of tumor progression [607, 608]. However, molecular mechanisms connecting inflammation and cancer are just beginning to emerge, like NF-κB critical role in in cancer development and progression [609]. At least 15–20% of cancers are attributed to infections or inflammation [610]. The start of a neoplasia requires several elements like self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, tissue invasion/metastasis and sustained angiogenesis [611]. Most of these functions occur through activation of NF-κB [612]. In chronic inflammation, NF-κB prevents apoptosis and initiates signals enhancing the inflammatory process [613]. IL-6 blocks apoptosis and is one of the effector signals of activated NF-κB in the promotion of neoplasia; IL-6 signaling through STAT3 also activate diverse cancers [614] by regulating genes mediating cell proliferation, promoting angiogenesis and suppressing apoptosis [615].

The more obvious cancer in which IL-6 involvement is found is multiple myeloma, which is a neoplasm, dependent on IL-6, of terminally differentiated B-cells [616]. Also, IL-6 KO mice were resistant to the development of murine plasmacytomas [617]. IL-6 trans-signaling, instead of the classical pathway, seems to be the mechanism by which IL-6 promotes colon cancer as TGF-β suppressed the formation of cancer by inhibiting IL-6 trans-signaling [618], and IL-6R is down-regulated in inflamed and tumorous colon samples [619]. IL-6 receptor antagonism slowed tumor growth but did not change the number of tumors that developed suggesting that IL-6 has its major effect on tumor promotion and progression rather than initiation [618].

Finally, in an experiment involving IL-6 floxed mice generated by our group, it was demonstrated that in hepatocellular carcinoma, premalignant lesions contained tumor progenitors cells that were yet to become fully malignant needing a damaged liver which had activated STAT3 and IL-6, among others [620]. Also IL-6 expression was elevated

in isolated hepatocellular carcinoma progenitor cells and in fully transformed malignant cells and IL-6 was important for malignant progression and for tumorigenic growth [620].

1.3.4.11 Aging

Both IL-6 plasma levels [621] and IL-6 expression in the brain increases with age [622, 343], and microglia from aged mice express higher levels of IL-6 both basally and in response to LPS treatment [622, 623]. Those age-related increments in IL-6 are not explained by differential prevalence of IL-6 gene polymorphisms [624], although the excessive production or reduced clearance of oxygen free radicals, which stimulate IL-6 production, may be important [625]. Moreover, sIL-6R and sgp130 also seem to increase with age, followed then by a gradual decline [626].

There is an exaggerated brain IL-6 response during infection in aged animals because there is excessive cytokine production in the brain in response to a peripheral immune stimulus [627]. This pathological production of cytokines are coupled with an increase of behavioral deficits [628]. Excessive pro-inflammatory IL-6 in the brain plays a critical role in the development of these infection-related alterations in sickness behavior and cognition [387]. Another study showed that aged microglia expressed a higher level of IL-6R and the aged brain expressed higher ADAM17 sheddase respect to adult animals, and co-administration with sgp130 attenuated LPS-induced exaggerations in sickness and IL-6 protein production in microglia and astrocytes of aged animals [629].

CHAPTER 2

HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

IL-6 is a highly plurifunctional cytokine, with many pleitropic actions and considered one of the main cytokines controlling the immune system and coordinating it with the nervous and endocrine systems. It is produced in multiple cell types in the CNS, and in turn, many cells do respond to it. Astrocytes are major responders to IL-6 as well as one of its main CNS producers. IL-6 signals via binding to either the membrane bound IL-6Rα (classical signaling) or soluble sIL-6Ra (trans-signaling), being able to signal to virtually all cells in the body. Due to the enormous controversy regarding its context-dependent effects it is therefore important to ascertain which is the contribution of each cell type and major pathways in the overall role of IL-6 during both physiological and pathological conditions.

Our hypothesis is that, being astrocytes major producers of and responders to IL-6 in CNS, the suppression of either astrocyte-derived IL-6, astrocyte IL-6 receptor or central IL-6 trans-signaling, could have important consequences in both normal physiological states and during neuroinflammation or CNS injury.

2.2 Objectives

To test our hypothesis, we propose the following objectives:

2.2.1 Objective 1

To obtain mice lacking astrocyte-derived IL-6 and proper littermate controls and phenotype them in several physiological and behavioural traits; response to LPS administration and to cold exposure, as well as phenotyping them in neuroinflammatory conditions such as EAE and traumatic brain injury.

2.2.2 Objective 2

To obtain mice lacking astrocyte IL-6 receptor and proper littermate controls and phenotype them in several physiological and behavioural traits, as well as phenotyping them in neuroinflammatory conditions such as EAE and traumatic brain injury.

2.2.3 Objective 3

To obtain mice with impaired IL-6 trans-signaling pathway in the CNS and phenotype them for several traits (neurogenesis, neurodegeneration, vasogenesis and gliosis) in the transgenic neuroinflammatory model of astrocyte-targeted production of IL-6.

CHAPTER 3

MATERIALS AND METHODS

3.1 Animals

Mice were maintained with food (Harlan global diet 2918) and water available *ad libitum* under temperature controlled conditions with a 12:12 light-dark cycle. All experiments performed were approved by either the UAB Animal Care Committee or the University of Sydney Animal Care and Ethics Committee.

3.1.1 IL-6 floxed mice

Il-6$^{lox/lox}$ (floxed) mice were generated by gene targeting technology in our group, as described by Quintana et al [630]. During the generation of IL-6 floxed mice they were intercrossed with C57BL/6 background up to ten times.

3.1.2 IL-6R α-chain floxed mice

IL-6R floxed mice were generously provided by Dr. Angela Drew, and were generated as described elsewhere [631]. These mutant mice possess two loxP sites flanking exons 4–6 of the interleukin 6 receptor alpha chain (IL-6Rα) gene in C57BL/6 genetic background.

3.1.3 GFAP promoter-specific Cre recombinase expressing mice (GFAP-Cre)

GFAP-Cre mice (01XN3, C57BL/6-Tg(GFAP-Cre)8Gtm) were obtained from the National Cancer Institute Mouse Models of Human Cancers Consortium (MMHCC) at Frederick, MD 21702, USA. This mouse line expresses Cre recombinase and beta-galactosidase (LacZ) driven by the human glial fibrillary acidic protein (GFAP) promoter, showing a robust expression in astrocytes but not in MAP-positive neurons or APC-positive oligodendrocytes [632].

3.1.4 CD-1 mice

Three month-old CD-1 Swiss male mice (N = 16) purchased from Harland by means of UAB estabulari were kindly gifted by Cristina Rabasa and Antonio Armario after a restraint experiment.

3.1.5 Generation of Astrocyte IL-6 KO (Ast-IL-6 KO) and Astrocyte IL-6 Receptor KO (Ast-IL-6R KO) mice

To obtain conditional KO mice where either IL-6 or IL-6 receptor expression were blunted in astrocytes (see figure 3.1) together with appropriated controls, we proceeded as follows: heterozygous GFAP-Cre mice were first crossed with each of the two floxed mice, and from the offspring GFAP-Cre positive animals were selected and crossed again with a proper floxed mice (for either IL-6 or IL-6 receptor) to get the subjects to be studied. With this strategy, four possible genotypes are obtained which are littermate and thus assuring genetic homogeneity: GFAP-Cre$^{+/-}$ IL-6 (or IL-6R)$^{lox/lox}$, which will lack either IL-6 or IL-6R expression in astrocytes (GFAP-Cre$^{+/-}$ IL-6$^{\Delta/\Delta}$ or GFAP-Cre$^{+/-}$ IL-6R$^{\Delta/\Delta}$ mice); GFAP-Cre$^{+/-}$ IL-6 (or IL-6R)$^{lox/+}$ which will be heterozygous for the conditional deletion in astrocytes (GFAP-Cre$^{+/-}$ IL-6$^{\Delta/+}$ or GFAP-Cre$^{+/-}$ IL-6R$^{\Delta/+}$); GFAP-Cre$^{-/-}$ IL-6 (or IL-6R)$^{lox/lox}$; and GFAP-Cre$^{-/-}$ IL-6 (or IL-6R)$^{lox/+}$. For the sake of simplicity, we will call these groups Ast-IL-6 KO/Ast-IL-6R KO, heterozygous, floxed and wild-type mice, respectively. Although all genotypes were studied, emphasis will be given to the comparisons between Ast-IL-6 KO and Ast-IL-6R KO with their respective floxed mice as the proper controls due to the fact that conditional KOs present the target gene floxed and knocked in the desired cell population but, in the rest of the cells, the gene is still floxed, instead of WT (see figure 3.1), being eventually able to alter the normal regulation of the gene, making floxed mice a more reliable control.

Ast-IL-6 KO animals and their littermate controls were initially phenotyped in partial C57BL/6 background (5th backcrossing group) at both physiological and under neuroinflammatory conditions. Also, IL-6 floxed mice were backcrossed with C57BL/6 mice 10 times and the same crossing strategy as aforementioned was followed, although in this case, heterozygous mice were not used. Ast-IL-6 KO animals and their littermate controls in complete C57BL/6 background (10th backcrossing group) were also deeper phenotyped at both physiological and under neuroinflammatory conditions.

3.1.6 GFAP-IL6/sgp130 bigenic mice

GFAP-IL6 transgenic mice, previously generated and characterized by Dr Campbell [252]. GFAP-sgp130 transgenic mice were generated by Dr. Scheller [633]. GFAP-IL6/sgp130 bigenic mice heterozygous for each transgene, GFAP-IL6 and GFAP-sgp130 single transgenic and WT mice were derived by interbreeding of the GFAP-IL6 and GFAP-sgp130 parental lines. All mice used in this study were fed ad libitum and were maintained under SPF conditions in the University of Sydney. Different age groups comprised from 1.5 to 9 month-old were used.

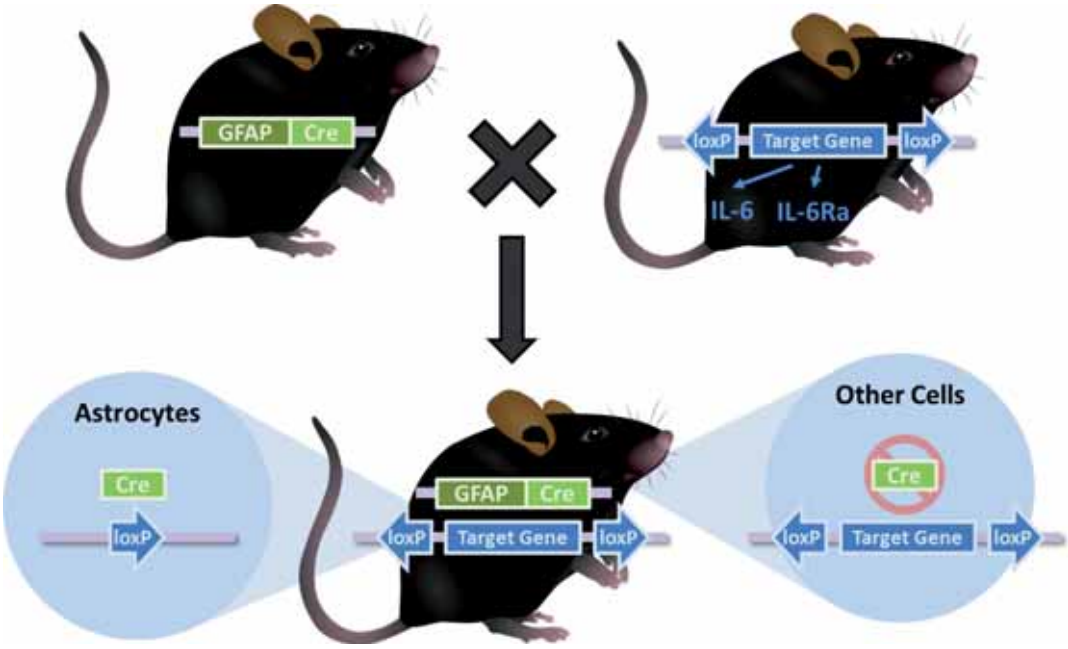


Figure 3.1: Generation of conditional Knock-out mice for astrocytary IL-6/IL-6R. In order to obtain a mouse presenting a lack of IL-6 or IL-6R expression in a given cell type it is necessary the generation of a mouse presenting the gene of interest flanked by two loxP sequences and another one with a targeted Cre recombinase expression in the cell type of interest. When Cre recombinase recognizes loxP sites in the desired cell population, the sequence comprised between them will be recombined, thus knocking-out critical exons from the targeted genes, exon 2 in IL-6 gene and exons 4-6 in IL-6Rα.

3.2 Tail DNA extraction and genotyping

At three weeks of age (weaning), mice were separated from their parents, sexed by measuring anogenital distance, identified by ear perforation, weighed and housed separately by gender (see figure 3.2). Moreover, a small fragment of tail was cut, in order to genotype the mouse, and the tail was cauterized with heat for a few seconds to avoid bleeding.

To obtain the DNA, a variation of the Sodium hydroxide (NaOH) extraction method was used [634]. Although this extraction method has a lower DNA quality than that obtained with other extraction methods, it is faster and adequate for mice screening. Briefly, a small section of mouse tail (<1mm) was digested in 100 μl of 50 mM NaOH by being boiled for 10 minutes (or 30 min in case of newborn animals genotyping, see section 3.11) and placed on ice immediately afterwards. Samples were directly used for Polymerase chain reaction (PCR) or stored at -20 ^{o}C.

Mice genotype screening was done by PCR in a Stratagen apparatus (RoboCycler Stratagene gradient 96). Two different PCR reactions were needed to confirm the genotype of each mouse, one for the presence of Cre-recombinase and the other for the presence of floxed alleles. Also, once the animal was killed, PCR for recombination in specific tissues was also performed.

Figure 3.2: Gender identification and ear marking. Mice are sexed on the basis of their anogenital distance, which is the distance between the anus and genital papilla. The anogenital distance is smaller in females (A) than in males (B) at all ages (newborns, juveniles and adults). Image C illustrates how ears are marked.

After amplification, samples were maintained at 4 ºC until 3 μl of loading buffer (0.25% of bromophenol blue and 40% of sucrose in milliQ water plus a bit of sodium azide to avoid bacterial contamination) were added to each sample. Then samples were loaded and ran in 2 or 2.8% agarose gels depending on the expected distance between bands. Finally, gels were stained (SYBR Safe DNA gel stain, Invitrogen) and bands observed with a uv transiluminator (Safe Imager 2.0, Invitrogen).

3.2.1 Cre recombinase PCR

In order to identify mice containing a Cre recombinase gene under the control of an astrocyte-specific human GFAP promoter element, the chosen genotyping strategy was to amplify the hGFAP/Cre junction using a primer combination G001/G002 (see table 3.1) with a product size of 600 bp. Also, as it is a band/non-band PCR it is useful to have a PCR internal control to know that the PCR has worked correctly, specially in animals without the transgenic band. For this purpose, primers for Metallothionein (MT) exon 1 detection, p8/p9, a constitutive gene [635], were added to the PCR mix (see table 3.1) and therefore a band of 178 bp was seen in all samples if the PCR worked well (see figure 3.3).

3.2.2 IL-6 Floxed PCR

In order to identify mice containing loxP sequences surrounding exon 2 of IL-6 gene, the chosen genotyping strategy is to amplify the exon 1/exon 2 junction, containing a loxP sequence, using a primer combination Exon 1F/Exon 2R (see table 3.1) with a product size of 317 bp in WT alleles and 420 bp in floxed alleles (see figure 3.4).

3.2.3 IL-6Rα Floxed PCR

In order to identify mice containing loxP sequences flanking exons 4-6 of IL-6Rα gene, the chosen genotyping strategy is to amplify the intronic sequence where loxP is located using a primer combination IL-6RaF/ IL-6RaR (see table 3.1) with a product size of 527 bp in WT alleles and 671 bp in floxed alleles (see figure 3.5).

A

B

Cycling Conditions *(Time in minutes:seconds)*:

Temperature	Time	Nº cycles
94°C	03:00	1x
94°C	00:30	35x
60°C	00:30	
72°C	00:30	
72°C	03:00	1x
4°C	Hold	

C

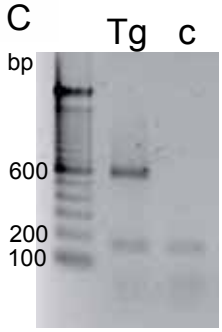
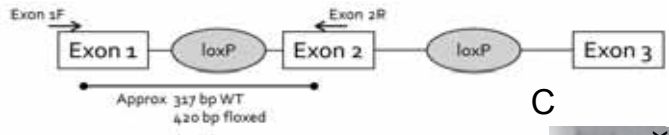


Figure 3.3: GFAP-Cre mice PCR. A. This scheme illustrates the genotyping strategy, which is to amplify the hGFAP/Cre junction. B. Optimal cycling conditions for the PCR. C. Photograph of a 2% agarose gel showing a transgenic animal with GFAP-Cre recombinase expression (Tg) and thus, a band of 600 bp, and a non-transgenic animal (c) without the recombinase expression and therefore without the 600 bp band. Both animals present a PCR control band for MT exon 1 gene of 178 bp.

A



B

Cycling Conditions *(Time in minutes:seconds)*:

Temperature	Time	Nº cycles
94°C	01:00	33x
57°C	01:00	
72°C	01:10	
4°C	Hold	

C

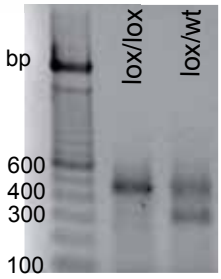
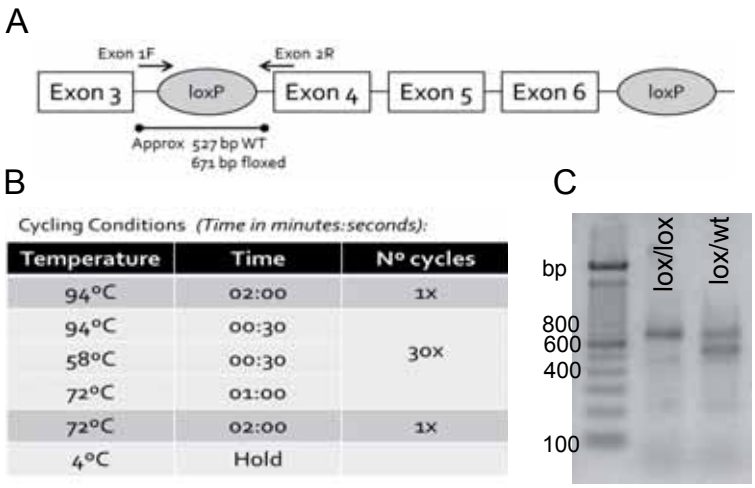


Figure 3.4: IL-6 floxed mice PCR. A. This scheme illustrates the genotyping strategy, which is to amplify the exon 1/exon 2 junction containing a loxP sequence in floxed alleles but not in WT alleles. B. Optimal cycling conditions for the PCR. C. Photograph of a 2% agarose gel showing a IL-6 floxed animal for both alleles presenting a band only at 420 bp (lox/lox) and an animal with a floxed and a WT allele of 420 and 317 bp respectively (lox/wt).

3.2.4 Gene recombination PCR

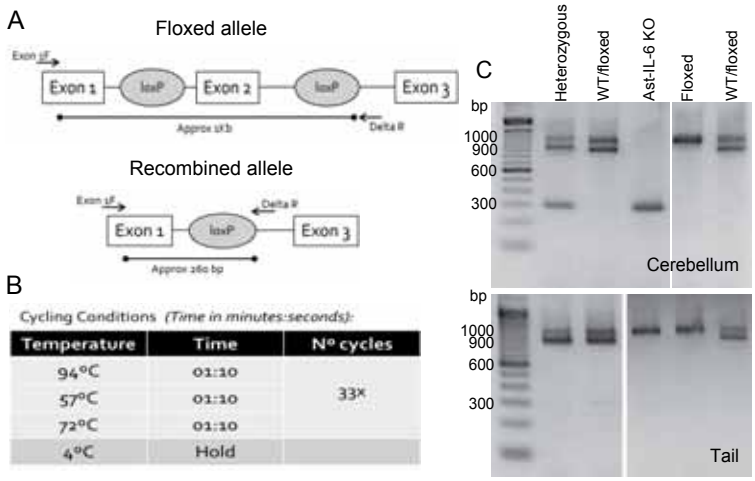
Several primers were designed for identifying the deletion of the exon 2 upon Cre recombination. By using primers Exon 1F/Delta R (see table 3.1) in CNS tissues (see section 3.9), the PCR products were aproximately 900, 1000, and 260 bp for the WT, floxed and recombined alleles, respectively. Recombination was observed in brain and spinal cord but not in spleen, tail or muscle (see figure 3.6).



Cycling Conditions *(Time in minutes:seconds):*

Temperature	Time	Nº cycles
94ºC	02:00	1x
94ºC	00:30	30x
58ºC	00:30	
72ºC	01:00	
72ºC	02:00	1x
4ºC	Hold	

Figure 3.5: IL-6Ra floxed mice PCR. A. This scheme illustrates the genotyping strategy, amplifying the loxP sequence. B. Optimal cycling conditions for the PCR. C. Photograph from a 2.8% agarose gel showing a IL-6Ra floxed animal for both alleles presenting a band only at 671 bp (lox/lox) and an animal with a floxed and a WT allele of 671 and 527 bp respectively (lox/wt).



Cycling Conditions *(Time in minutes:seconds):*

Temperature	Time	Nº cycles
94ºC	01:10	33x
57ºC	01:10	
72ºC	01:10	
4ºC	Hold	

Figure 3.6: Astrocytary IL-6 recombination PCR. A. This scheme illustrates the genotyping strategy, amplifying the sequence between exon 1 and exon 3 alone (WT allele; 900 bp) or flanked by loxP sequences (floxed allele; 1000 bp) or evidence of recombination (knock out allele; 260 bp). B. Optimal cycling conditions for the PCR. C (Top). Photograph of a 2.8% agarose gel showing cerebellum tissue samples from left to right of an heterozygous mice containing both alleles WT and floxed plus the recombination band; a WT/floxed animal containing both alleles WT and floxed without recombination; an Ast-IL-6 KO animal showing a strong recombination band (which in cerebellum frequently overshadows the floxed 1000 bp band); a floxed animal presenting only the 1000 bp band; and another WT/floxed mice. No recombination band is seen in the same genotypes in tail (Bottom).

Reagent	GFAP-Cre (µl/sample)	IL-6 floxed (µl/sample)	IL-6R floxed (µl/sample)	Gene recombination (µl/sample)
DNA extraction	1	1	1	1
Polymerase Buffer 10x (Biotools)	1,5	1,5	1,5	2
MgCl2 25 mM (Biotools)	1,2	1,5	2	1
dNTPs 2 mM mix (GeneCraft/ Sigma) (dGTP, dATP, dTTP and dCTP)	1,5	1,5	1,5	2
***Taq* Polymerase 1 u/µl** (Biotools)	0,375	1,25	1	0,5
***MilliQ* quality water**	6,925	6,25	6	12,5
Primers Goo1/ Goo2 (10 pmol/µl) Goo1: 5'-cat cgc cag tct agc cca ct -3' Goo2: 5'-cac gtt cac cgg cat caa c -3'	0,75 /0,75	-	-	-
Primers p8/ p9 (10 pmol/µl) p8: 5'-tca cca gat ctc gga atg g-3' p9: 5'-aag aac cgg aat gaa tcg c-3'	1 /1	-	-	-
Primers Exon 1F/ Exon 2R (10 pmol/µl) Exon 1F: 5'-ccc acc aag aacgat agt ca-3' Exon 2R: 5'-ggt atc ctc tgt gaa gtc ctc-3'	-	1,5 / 1,5	-	-
Primers p1F/ p2R (10 pmol/µl) IL-6RaF: 5'-gaa gga gga gct tga cct tgg-3' IL-6RaR: 5'-aac cat gcc tat cat cct ttg g	-	-	1,5 / 1,5	-
Primers Exon 1F/ Delta R (10 pmol/µl) Exon 1F: 5'-ccc acc aag aacgat agt ca-3' DeltaR: 5'-atg ccc agc cta atc tag gt-3'	-	-	-	0,67 / 1,33
Mineral Oil	1 drop	1 drop	1 drop	1 drop

Table 3.1: Reagents and proportions per DNA sample for PCR reaction. For each PCR reaction, a mix of common different reagents and specific primers are added to 1μl DNA sample and finally a drop of mineral oil is added to the tube to avoid evaporation. Samples are then placed in an automatic thermocycler apparatus from Stratagene with specific amplification conditions for each PCR.

3.3 Behavioural tests battery

Prior to starting the behavioral test battery, animals were transferred from the colony room to the colony testing room, at least one week before starting any behavioral test, in order to habituate to the new location (see figure 3.7). During this habituation period, mice were moved daily in the home cages to the behavioral testing rooms (located in front of the colony testing room) and were handled during the first part of the light cycle (8 to 12 a.m.) for 3 or 7 days (depending on the experiment). Handling consisted not only in the habituation to the behavioral testing room for 15-30 min but also in experiencing the same procedure routine as they were going to experience on test day, as well as being exposed to the sound of timers, stopwatches and other mice. Between different behavioral tests mice remained in the colony testing room.

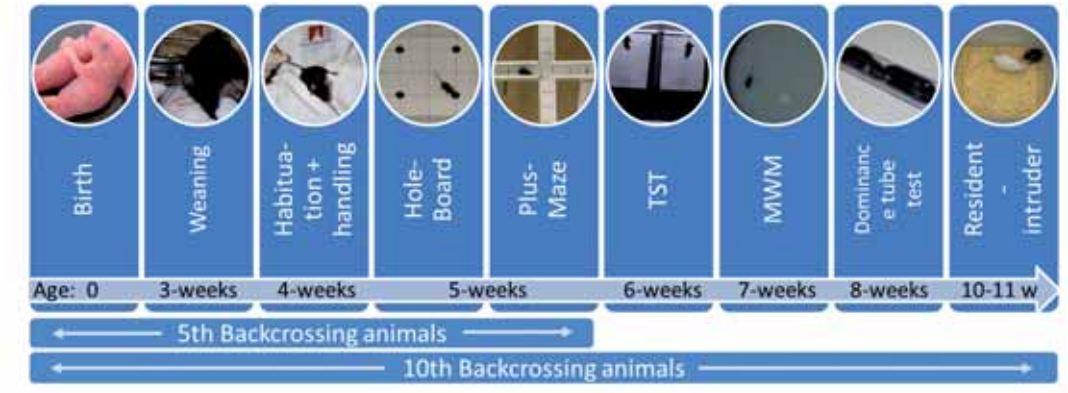


Figure 3.7: Schematic chronology for behavioral tests. Animals from 5th backcrossing Ast-IL-6 group only performed HB and PM tests with a separation of 2-days between both tests. In Ast-IL-6 receptor group, animals followed this same scheme. In Ast-IL-6 group from 10th backcrossing, both young and adults/old were tested. Young animals were tested as in the scheme. Adult animals group were weaned as normal and then kept in the colony room being weighed once a week until 8 month-old, when they were transferred to the colony testing room for handling and habituation. The day after the last handling session they started this same behavioral battery but with all tests separated one week from each other. In all cases only males performed Resident-intruder test.

Behavioral testing was not performed the same day or the immediate following days when home cages were scheduled for bed changing, as it causes an increase in general activity and stress levels which could alter test results. Mice were brought to the testing room in their home cages at least 30 minutes prior to the start of behavioral testing. Individual mice were then transported into the testing apparatus. Test room lighting, temperature, and noise levels were consistent for all subjects. Prior to test each mouse, apparatus were cleaned with 5% ethanol and completely dried.

All behavioral tests were carried out and analyzed blindly. The order of animals was counterbalanced across genotypes and sexes.

3.3.1 Hole-Board

Hole-Board (HB) was originally devised by Boissier and Simon [636], it allows to separately assess locomotion and exploration (measured by Head-dippings (HD)) in mice confronted with a new environment; it is also a generally used method for screening the potential anxiolytic character of drugs. HB apparatus consists of a white box (40 x 40 x 20 cm) with four 3-cm diameter equidistant holes on the floor. The floor is divided in 16 squares of 10 x 10 cm marked with black lines, conforming 12 external squares (around the walls) and 4 internal squares (see figure 3.8). A video camera recorder (Everio G camera, JVC) was set up to allow recording of the whole session. This test was performed one day after the final handling session, during the first part of the light cycle (8 to 12 a.m.).

On the test day, animals were taken to the behavioural room in the same manner as in the previous handling days, and mice remained in the room for 30 min. Mice were placed

Figure 3.8: Hole-board (left) and Plus-maze (right) test apparatus.

in a corner of the box. Animals were tested during 5 min; defecations and frequency and duration of head-dipping events (considered as such when an animal inserted the head into the holes at least to its ear level) were assessed in situ by the investigator. Locomotion (number of areas crossed) and the number of rearings (considered as such when an animal stood completely erect on their hind legs and sniffing) were measured by visualizing the recorded video with a self developed Excel program (found in supplementary material A.1).

3.3.2 Elevated Plus-Maze

Elevated Plus-Maze (EPM) test, originally devised by File and co-workers [637], is a widely used rodent model for assessing putative anxiolytic drugs and to characterize genetic and environmental influences on anxiety [638]. It relays on the preference of rodents for closed spaces instead of open ones. EPM apparatus consists of four arms (20 cm long and 5 cm wide; divided in 3 rectangles of 6 x 5 cm) connected by a central square of 5 x 5 cm. The apparatus is elevated 50 cm above the floor by two metallic legs. Two opposite arms have walls of 15 cm tall (closed arms), whereas the other two only have a small ridge to prevent slipping of the mice (open arms) (see figure 3.8).

Two, three or seven days (depending on the experiment) after performing the HB test, animals were subjected to the EPM, which was carried out in the same room and with the same habituation time, during the first part of the light cycle (8 to 12 a.m.). Two animals were simultaneously tested in two different EPM separated with a black screen to prevent sight between mice. The entire procedure (which lasted 5 min) was recorded by cameras placed above each EPM apparatus.

Mice were gently placed in the center square oriented towards a closed arm to avoid confounding flight response as open arm exploration [638]. Like in the HB test, the investigator was in the test room during all tests. Different behavioral traits were analyzed using a self-developed application for Excel software (found in supplementary materials of A.1) from recorded data. Parameters analyzed were: number of entries and time spent in both open and closed arms (both absolute and as a percentage of total arm entries or total time spent in the arms); time spent in the central square, latency of central square

first escape, locomotion (crossed squares), number of defecations, number of rearings and number of protected, unprotected and total head-dips, a valuable ethological defensive behavior [639]. Head-dipping behaviour is considered when animal's head and shoulders are over and below the ridge present in the open arms, in an attempt to explore under the maze. Protected HD are recorded when the animal makes a head-dip from the centre or an adjacent close square of the maze around the edge of a closed arm wall and unprotected HD are those made by mice staying in open arms (open HD).

3.3.3 Tail Suspension test

The Tail suspension test (TST) is a simple screening test for either the behavioral effects of antidepressants or the individual differences in stress responses in rodents. It is based on the observation that after initial escape-oriented movements, mice develop an immobile posture when placed in an inescapable stressful situation (mainly is the haemodynamic stress of being hung by their tail) [640].

This behavioral test was performed between 2:00 and 6:00 p.m. Mice were suspended by the tail (with adhesive tape) in the TST apparatus. The test lasted 6 min and all process was recorded; results were analyzed in three 2 minutes periods. Up to five simultaneous mice were performed at the same time without visualization between them (see figure 3.9).

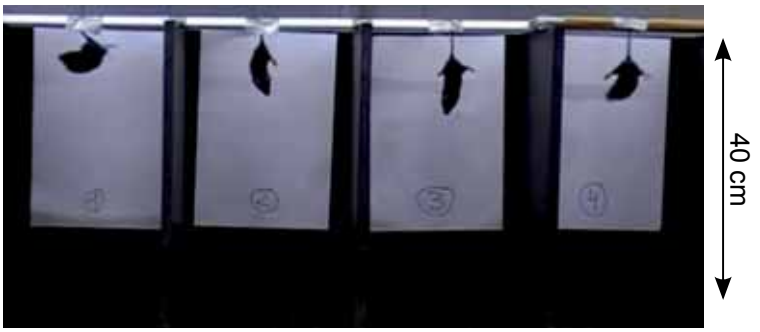


Figure 3.9: Tail Suspension test.

3.3.4 Morris Water Maze

The Morris water maze (MWM) test is one of the most widely used tasks in behavioural neuroscience for studying spatial memory and learning in rodents, relying on distal cues to navigate from random start locations around the perimeter of an open swimming area to locate a submerged escape platform. Spatial learning is assessed across repeated trials and reference memory is determined by preference for the platform area when the platform is absent. Different mechanisms of navigation, learning and memory can be tested in this maze using different training protocols [641].

The MWM tank consists of a propylene round pool (120 cm diameter x 60 cm deep), which is virtually divided in 4 equal quadrants, with a removable platform that can be

adjusted at different heights (8 cm diameter). It contains water at 22 ºC ±1 made opaque by the addition of approximately 1cl/l non-toxic liquid latex (Látex Compound Española S.A). Four black cues (12x12 cm) and four white cues (24x24 cm) were placed in the pool wall of each quadrant and in a black curtain surrounding the pool, respectively (see figure 3.10). A video camera was placed above the the pool to record the test. It was performed between 2:00 and 6:00 p.m.

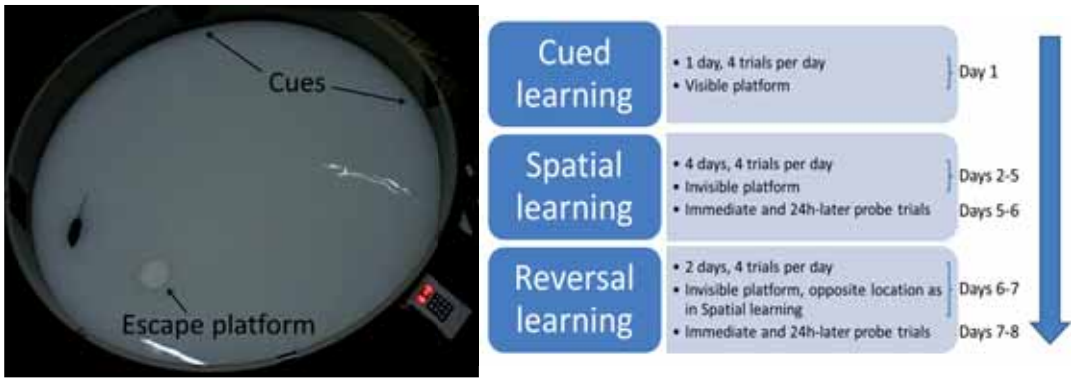


Figure 3.10: Morris Water Maze test. Spatial learning acquisition

3.3.4.1 Visible platform Test/ Cued learning test (Day 1)

It is a control condition frequently used in the MWM which consist in testing the ability to learn to swim to a cued goal. The platform was the same as in the hidden platform test, except that it was elevated above the water surface and a visible black 'flag' was mounted on it making it easily identifiable from across the pool. The 'cue' is designed to allow animals a direct line-of-sight to the platform. To ensure that animals were using this proximal cue to locate the platform, both the location of the goal and the starting point were moved to new positions in each trial. Mice were given 60 seconds to swim and reach the visible platform and then allowed to remain on it for 15 seconds. Mice unable to find or reach the platform in that time, were guided to it and/or helped to climbed it and allowed to remain on it for 15 seconds.

In this test, the only cue that reliably indicates the location of the platform relative to the start is the cue that is attached to the platform. If subjects are impaired in cued learning, there could be spatial deficits as well, because cued learning requires the same basic abilities (good eyesight, swimming, climbing on the platform, etc.) and the same motivation (escape from water) as in the spatial learning test. This test can be performed before or after the spatial test, but doing it before allows the identification of those animals not acquiring the appropriate subordinate skills (swimming ability, climbing the platform, not jumping from it to find another escape route, etc.) before they are presented with the hidden platform test. Therefore, it was performed the first day (only one day) with 4 trials

per day separated approximately one hour. Latency to reach the platform was measured in each trial.

3.3.4.2 Hidden platform test (Days 2-5)

The following day to the visible platform test, the hidden test was initiated. In this phase, mice were challenged to escape from the pool by finding a hidden platform (submerged 1 cm below opaque water) using distal cues as spatial references. It is the most basic MWM procedure. The animal must learn to use distal cues to find the hidden platform.

The platform was in a fixed location relative to the cues across days and mice were placed into the water facing the edge of the pool at random start positions across trials (4 trials per day). Animals were allowed to freely explore the pool for 60 seconds and after that, in case they failed to find the hidden platform, they were guided to it and required to remain on it for 15 seconds in all cases. Trials were separated by 60-80 min; during intervals mice were dried with paper and kept into their cages in the test room. Animals were trained for 4 consecutive days and the latency to find the hidden platform in each trial was recorded.

3.3.4.3 Probe trial test (Days 5-6)

To assess reference memory at the end of learning, a probe trial (without the platform) is given. The most common method is to administer one probe trial 24 h after the last acquisition day. However, in order to be able to differentiate between short- and long-term memory, we gave each animal two probe trials, one immediately after the last acquisition trial and the other 24 h later [641].

During probe trials, animals were placed in the tank for thirty seconds and then immediately removed from water.

3.3.4.4 Spatial reversal test (Days 6-7)

To relocate the platform to another quadrant (usually the opposite one) and subject mice to another set of trials after the spatial learning test is called reversal learning. It reveals whether or not animals can extinguish their initial learning of the platform's position and acquire a direct path to the new goal position (i.e., cognitive learning flexibility).

In this phase, the protocol was basically the same used in the hidden platform phase but the platform was relocated to the opposite quadrant of the pool. Mice were trained on this task only for 2 consecutive days with 4 trials per day (1 min each one) and escape latency was measured in each trial.

3.3.4.5 Spatial reversal probe trial test (Days 7-8)

As in the hidden platform test, at the end of the reversal test, a 30 seconds reversal probe trial test was given immediately after the last acquisition trial and 24 h later.

3.3.5 Dominance tube test

As an initial screen for normal social behaviour, mice were tested, between 2:00 and 6:00 p.m., in a tube test for social dominance adapted from Lindzey et al. [642] without previous food deprivation nor reward. In this test, two mice of different genotypes (Ast-IL-6 KO, floxed or WT) but the same gender were placed head first at opposite ends of a Plexiglas transparent plastic tube (3 cm inner diameter, 30 cm in length; a size just sufficient to permit one mouse to pass through without reversing the direction) and released simultaneously (see figure 3.11). The test ended when one of the two mice completely retreated from the tube. The mouse remaining in the tube was designated as the winner (score = 1), and the retreating mouse the loser (score = 0). In some cases neither mouse was dominant or subordinate, and both mice were considered equivalent. Tests in which one mouse backed up and left the tube without being pushed by the other were not scored. With at least 15 minutes between tests, animals were faced with another opponent in a counterbalanced order in a maximum of five different encounters per animal and the percentage of victories over the other two genotypes is assessed for each animal.

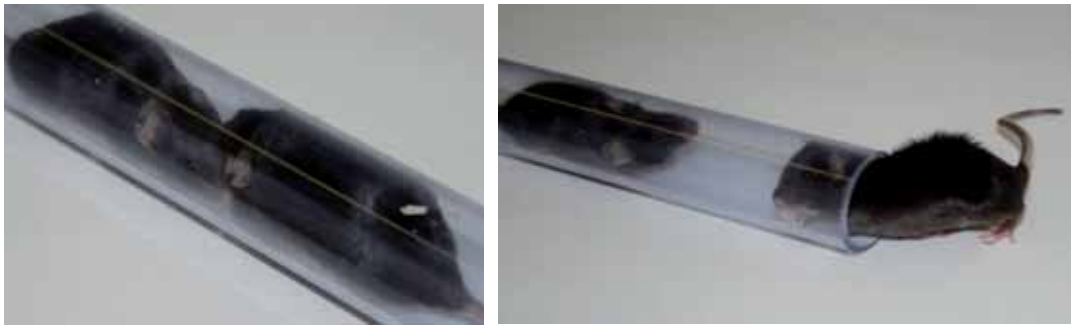


Figure 3.11: Dominance tube test. Two animals are released at the same time at opposite ends of the tube, they meet in the center and the dominant animal push back the other until the submissive animal is completely retreated from the tube.

3.3.6 Resident-Intruder test

In the resident-intruder test, two rodents are introduced to each other in the home cage of one of the animals (the resident) and then allowed to interact for a set period of time with the new animal (the intrudent). The resident-intruder paradigm can be used to study offensive aggression, defensive behavior, violence and social stress in rodents [643].

For this test only male subjects were employed. They were all individually housed for 15 days before the first aggressive encounter. Body weight and food intake was monitored during this two weeks individualised. Bedding was not changed the week before testing in order to left olfactory cues from the resident allowing him to establish its own territory. All encounters were recorded and had a duration of 5 minutes. This test was performed between 2:00 and 6:00 p.m. (see figure 3.12).

In the first encounter, Ast-IL-6 KO mice and their littermate controls (Ast-IL-6) and Ast-IL-6R KO mice and their littermate controls (Ast-IL-6R) mice acted as residents and a CD1 mouse was introduced as an intruder to the same cage and left with the resident mouse for 5 min. In a second encounter one week later, Ast-IL-6 and Ast-IL-6R mice acted, this time, as intruders and were placed with a CD1 male mouse in the cage of the later. The second CD1 mice confronted was always different to the one from the first encounter.

Behavior observed was classified between aggressive (latency to the first attack, number of attacks, number of chases, number of tail rattles and number of upright and crouched postures) and affiliative (number of anogenital/body/nose sniffs, number of allogroomings (grooming the partner) and crawl-under behavior were assessed) behaviours.

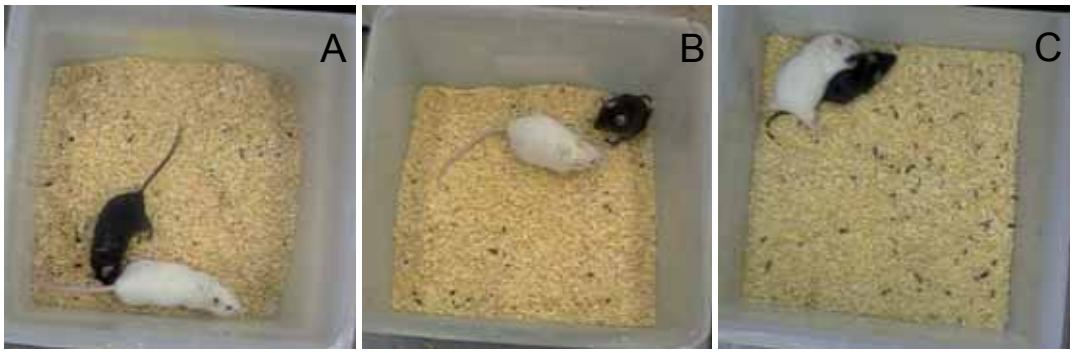


Figure 3.12: Resident-Intruder test. Different behaviours performed can be observed. A. Anogenital sniffing, B. Upright posture, C. Attack.

3.4 Body weight

Regarding body weight, mice from both genders were weighed at weaning and once a week thereafter (at the same day and time) until they were sacrificed.

Regarding Ast-IL-6 mice, two experiments were performed. The first one with animals from the fifth back-crossing which were weighed until 15 weeks (N = 45-91 at 3 week-old and N = 6-18 at 15 week-old). The number of animals per group decreases along time as they were used in EAE and other experiments, specially around week 10. Also, all animals were transferred from colony room to the colony testing room at 5 week-old; they also received handling once a day at 6 week-old and performed Hole-board and Plus-Maze tests at 6 and 7 week-old, respectively.

In a second set of animals from the tenth back-crossing group, they were weighed until 25 week-old (N = 15-39), maintaining all animals in the same room along time. In this case, animals were not subjected to any location change or any manipulation other than cage cleaning and weighing handling. When 25 week-old those mice underwent different behavioural tests (Hole-board, Plus-maze, tail-suspension, Morris water maze, dominance tube test and, just in males, resident-intruder test) and were not weighed

during this period. After 2-week rest, when they were 35 week-old, they were weighed again before they underwent further experiments. This weight is also added to the study.

Regarding Ast-IL-6R mice, animals were weighed until they were 15 week-old in the same conditions as for 5th backcrossing Ast-IL-6 mice (N = 21-48 at 3 week-old and N = 3-15 at 15 week-old).

3.5 Body temperature

Core body temperature was measured by a lubricated rectal probe (Cibertec) in various conditions in Ast-IL-6 mice from tenth backcrossing (see figure 3.13). In the first experiment it was measured, after proper handling, in seven month-old mice in basal conditions between 03:00 to 06:00 p.m., at room temperature (23 ºC ±1)(N = 14-33 per genotype and gender). At 9 months of age, some of these animals (N = 8-10, only males) were kept individualized in a cold room (8±1 ºC) starting at 9:00 a.m. with no access to water and food and their temperature was recorded 2, 4 and 6 hours later.

Moreover, other 9 month-old animals (N = 3-8 per genotype and gender) were injected ip with either 1 mg/kg LPS (L2880, Sigma) in the morning or saline solution in the afternoon, and temperature was measured just before the injection and 3 hours later. In another group of 4-6 month-old animals (N = 3-8 per genotype and gender), their temperature in basal conditions was recorded every 3-hours in a 24-hour period (except for the 3 a.m. measure). They were also injected with 10 μg/kg LPS ip in the morning (8 a.m.) of a different day, and temperature was measured 1, 2 and 3 hours later. Animals were always kept at room temperature.

Figure 3.13: Body temperature assessment in light and dark conditions.

3.6 Experimental autoimmune encephalomyelitis (EAE)

For the induction of EAE, an animal model of multiple sclerosis, two month-old male and female mice from Ast-IL-6 5th backcrossing group (N = 11-28, as a summatory of 3

separate experiments), three month-old male and female mice from Ast-IL-6 10th backcrossing group (N = 9-19) and three month-old Ast-IL-6R male and female mice (N = 12-14) were used. EAE was induced by active immunization with Myelin Oligodendrocyte Glycoprotein fragment 35-55 (MOG_{35-55}) peptide. On day 0, all mice, under isoflurane anesthesia, were injected subcutaneously into the hind flanks with an emulsion of 100 μl MOG_{35-55} (3 mg/ml) and 100 μl Freund's Complete Adjuvant (CFA) (Sigma-Aldrich, St. Louis, MO) supplemented with 4 mg/ml *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI). In addition, animals received an ip injection of 500ng pertussis toxin (Native Antigen Company, PT-TNL-100), which was repeated two days after immunization (see Figure 3.14).

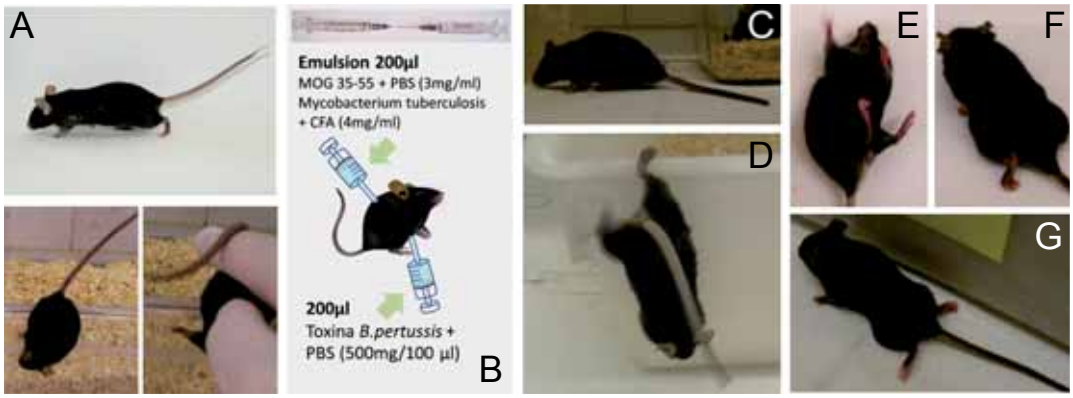


Figure 3.14: EAE induction and symptomatology assessment. A. Examples of non-induced mice performance: tail from healthy mice is elevated when walking, they have enough strength in the hind legs to support their body and also tail is responsive when a finger is approach in order to avoid falling. B. Scheme of EAE induction showing subcutaneous injections of MOG and *M.tuberculosis* emulsion (made by repeatedly passing by two syringes or by placing the solution in a sonicator until ready). C-G. Example of different clinical symptoms and the clinical score of MOG-induced mice: C. Tail paralysis (1); D. Moderate hind limb paraparesis (2); E. Severe hind limb paraparesis (2.5); F. Partial hind limb paralysis (3); G. hind limb paralysis (3.5).

After immunization, mice were examined daily, weighed and scored for EAE according to the following clinical criteria: 0 = no signs of disease, 0.5 = partial loss of tail tonus, 1 = loss of tail tonus, 2 = moderate hind limb paraparesis, 2.5 = severe hind limb paraparesis, 3 = partial hind limb paralysis, 3.5 = hind limb paralysis, 4 = hind limb paralysis plus partial front leg paralysis, 4.5 = moribund and 5 = death (see Figure 3.14). Finally, for each animal we determined the time to disease onset (clinical score or ataxia $\geq$ 1), time to peak disease, peak-score, cumulative score (sum of all scores from disease onset to day 20 or 46), outcome (final score) and grade of remission (difference between peak score and outcome).

3.7 Cryolesion

Two month-old male and female mice from 5th backcrossing group (N = 19-25) and three month-old male and female mice from 10th backcrossing group (N = 20-21) of Ast-IL-6 KO animals plus controls and four month-old Ast-IL-6R KO plus controls (N = 13-24) were analyzed after being lesioned under isoflurane anaesthesia (both genders considered together). Cortical cryogenic injury was performed as previously described [644]. Basically, the skull over the right fronto-parietal cortex was exposed and a cryoinjury produced on the surface of the brain (without craniotomy) by placing a size-controlled pellet of dry ice onto the skull during 60 seconds. The skin incision was then sutured and treated with iodine solution and animals monitored until they were completely awaken from surgery. Non-lesioned controls were anaesthetized and their skulls exposed but cryoinjury was not performed.

To study the volume of brain lesion, damaged area was cut from the beginning to the end in sagittal 8 μm-wide sections in the microtome and one of every 25 sections was mounted in superfrost slides. Dry sections without staining were examined in a bright field microscope (Nikon Eclipse 90i) and images were acquired using a Nikon Digital camera DXM 1200F and Nikon Act-1 v. 2,70 software. Injured area was measured with the software ImageJ (NIH) in 20X images (Rasband, 1997). The summation of the injured area, in millimeters, of all sections of an animal was performed and multiplied by 0.008 (tissue width in mm) and by 25 (number of sections cut between two sections analyzed) to obtain a total injury volume estimation (see figure 3.15).

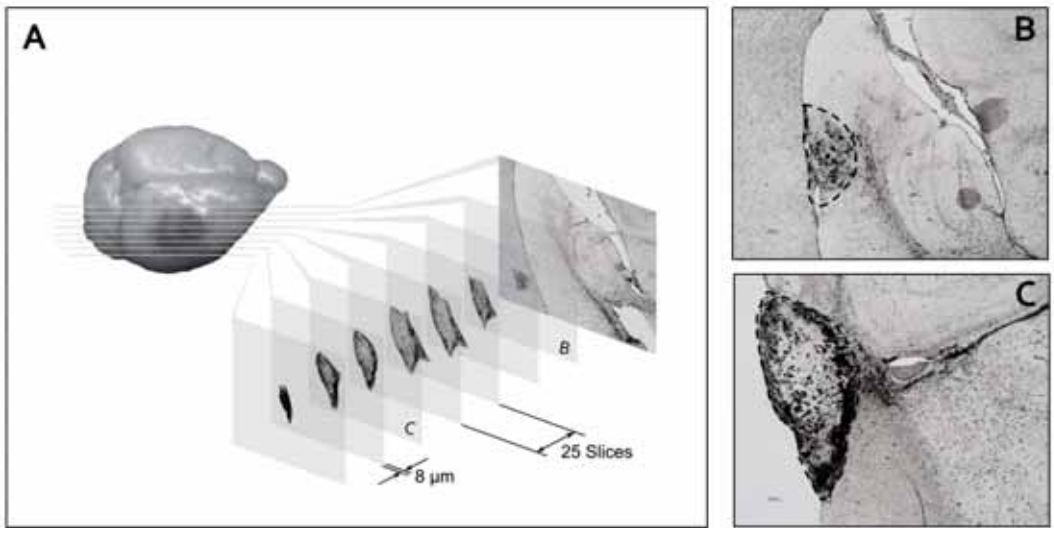


Figure 3.15: Injured volume assessment. A. Damaged area was cut from the beginning to the end in 8 μm-thick sagittal sections in the microtome and one of every 25 sections was mounted in Superfrost slides. B-C. The lesion of the cortex is clearly identifiable at the microscope without the need of staining and the area is measured with ImageJ software.

3.8 LPS response

Ast-IL-6 males, from 5th backcrossing (N = 5-9) were injected ip with 1 mg/kg LPS from Escherichia coli 055:B5 (L2880, Sigma) and killed 1 hour later. IL-6 levels in serum and IL-6 mRNA in brain were assessed by ELISA (see 3.12) and RT-PCR (see 3.10) respectively.

3.9 Animal sacrifice and sample storage

For EAE experiments, mice were sacrificed at days 19, 20, 22 or 46 days post-immunization (dpi) and cryoinjured mice were killed at 3 or 10 days post-lesion (dpl). All mice were sacrificed by rapid decapitation and, if necessary, trunk blood was collected in cold 2 ml eppendorfs, centrifuged and serum stored at -80 ºC. Upon brain dissection, the right hemisphere of all mice was fixed for 24h in 4% Paraformaldehyde (PFA) in PBS at 4 ºC, then washed with Phosphate buffered saline (PBS) 1x and stored in 70% ethanol at 4 ºC until dehydration and paraffin embedding for Immunohistochemistry (IHC) and Histochemistry (HC) analyses; while the left hemisphere was frozen with liquid nitrogen and stored at -80 ºC. Spinal cord was also removed together with the vertebral column and fixed in PFA for 24h, then it was dissected from the column and fixed for 24h more in new PFA and finally washed with PBS and stored in 70% ethanol as in brain storage. In some experiments spleen and liver were also extracted and frozen with liquid nitrogen and stored at -80 ºC.

Regarding the Australian experiment, six days after an ip Bromodeoxyuridine (BrdU) injection (100 mg/kg in 0.9% saline solution, every 2 h over a 24 h period; Sigma-Aldrich) mice were anaesthetized with xylazine/ketamine and subjected to transcardial perfusion with 4% PFA in PBS for 10 min. Brain, spinal cord, spleen, liver and a fragment of gut were resected and post-fixed in the same fixative for four hours, then rinsed with 0.1 M Phosphate buffer (PB) and placed in 30% sucrose solution in 0.1 M PB for 48 h. Following sucrose equilibration tissues were frozen with 2-methylbutane on dry ice and stored at -80 ºC pending the preparation of frozen sections (30 μm) using a cryomicrotome (Leica). Sections were then placed in OLMOS anti-freezing solution (20% sucrose, 1% polyvinylpyrroline, 30% ethylene glycol in 0.2 M phosphate buffer) and stored at -20 ºC.

3.10 RNA extraccion and Real Time PCR

Total RNA was isolated from frozen cerebellum and spleen tissue of 7 week-old male and female mice in basal conditions (only subjected to normal handling procedures) using Tripure reagent (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer's instructions.

Briefly, 90 mg of frozen tissue were homogenized by being placed in a tube containing 2 ml of Tripure reagent together with two 3 mm stainless steel balls. High speed oscillation was carried out for a total of 4 min in brains and 6 min in spleens, comprising 30 Hz cycles of 2 min separated by a 60 s recovery period in a MM400 (Retsch, Germany). Then, the samples were centrifuged at 12000xg for 10 min and supernatants were ex-

Gene	Forward primer	Reverse primer
IL-6	5'-GCTTAATTACACATGTTCTCTGGGAAA-3'	5'-CAAGTGCATCATCGTTGTTCATAC-3'
TNF-α	5'-ATGGCCCAGACCCTCACA-3'	5'-TTGCTACGACGTGGGCTACA-3'
IL-1β	5'-GGGCTGCTTCCAAACCTTTG-3'	5'-TGATACTGCCTGCCTGAAGCTC-3'
β-actin	5'-TACCACAGGCATTGTGATGG-3'	5'-TTTGATGTCACGCACGATTT-3'

Table 3.2: Primer sequences for real time PCR.

tracted with chloroform. RNA was recovered by isopropanol precipitation, washed in 75% ethanol and dissolved in DEPC treated water. Concentration of RNA was measured spectrophotometrically with a NanoDrop 1000 (Thermo Scientific, CA) and stored at -80 ºC until used. Then, 2.5 μg of total RNA was reverse transcribed into cDNA in a 19 μl reaction without RNase Inhibitor using a first-strand cDNA synthesis kit, SuperScript III Reverse transcriptase (Invitrogen Corp., Carlsbad, CA) following manufacturer's directions and stored at -20 ºC. Correct generation of cDNA was assessed by conventional PCR ran in a 1% agarose gel with reference gene's primers (β-actin) and was diluted fivefold prior to Real-time reverse-transcription PCR (qRT-PCR) amplification.

Analysis of IL-6 expression was carried out in the cerebellum and in the spleen; Table 3.2 shows the primer sequences used. Possible compensatory effects of IL-6 deficiency on other cytokines were analyzed by measuring the expression of IL-1β and TNFα. RT-qPCR for IL-6 and β-actin genes was performed in 96-well plates in a final volume of 20 μl containing 131 ng cDNA (5 μl), SYBR Green Master Mix (10 μl)(Bio Rad), 500 nM of forward and reverse primers (Sigma–Aldrich) and sterile distilled water. Reactions were run in the iCycler MyIQ real-time PCR detection system (Bio-Rad Laboratories, Inc., CA). Subsequent RT-qPCR for TNF-α, IL-1β and β-actin in the cerebellum were performed in 384-well plates in a final mix volume of 10 μl containing the exact half proportions of the above. Reactions were run in CFX384 real-time PCR detection system (Bio-Rad Laboratories, INc.,CA). The cycling conditions comprised a first stage of 5 min at 95 ºC with optics off, a second stage of 40 cycles at 95 ºC for 10 sec (optics off) and 60 ºC for 30 sec (optics on) and a melt curve of 81 repeats of 10 sec starting from 55 ºC with 0.5 ºC temperature increments (optics on). Samples were run in duplicates and non-template controls were included in each assay. PCR efficiency for both groups of primers and tissues was assessed to be near 100% by a duplicated standard curve of serial dilutions of cDNA.

To confirm amplification specificity the PCR products from each primer pair were subjected to agarose gel electrophoresis and resulted in a single product with the desired length (β-actin, 200 bp; IL-6, 100 bp). Moreover, a melting curve analysis was performed which resulted in single product specific melting temperatures (β-actin, 88 ºC; IL-6, 78.4 ºC; IL-1β, 83.5 ºC; TNFα, 81 ºC) and no primer-dimers generation. RT-qPCR data was obtained and analyzed with the Bio-Rad iQ5 v2.1 software for IL-6 and with Bio-Rad CFX Manager 2.1 software for IL-1β and TNFα which automatically determined baseline and threshold values and used the measured expression level of our reference gene (β-actin) as a normalization factor for cytokine genes; the lower expressor was given a value of 1 and the rest of values worked out as a fold-change of that reference.

3.11 Cell cultures

Astrocyte-enriched cultures were carried out in the Servei de cultius cel·lulars de l'Institut de Neurociències de la UAB.

3.11.1 Astrocyte-enriched cultures from cerebellum

After proper identification and genotyping (Ast-IL-6 KO, Floxed and WT), neonatal (7 day-old) animals were killed by decapitation, and the scalp and the entire dorsal portion of the cranium were removed (N = 8). The cerebellum was isolated from the rest of the brain and put in a petri dish with PBS 1x, and the meninges and pia mater were stripped and discarded, and the remaining tissue was mechanically and enzymatically (trypsin) dissociated by being incubated 10 min at 37 ºC in agitation in a solution with 0.25 mg/ml trypsin in Kreps Ringer buffer (KRB) with 0.3% Bovine serum albumin (BSA). Then the reaction was stopped by adding KRB with 0.3% BSA containing 0.083 mg/ml of trypsin inhibitor and 0.013 mg/ml of Deoxyribonuclease (DNase). Next, the cells were pelleted by centrifuging in a quick spin and the supernatant discarted. Then, the pellet was disgregated mechanically in a KRB plus BSA solution with 0.52 mg/ml of trypsin inhibitor and 0.08 mg/ml of DNase, and finally resuspended in 10 ml Dulbecco's Modified Eagle Medium (DMEM) culture medium (Sigma, D-5796) with 1% penicillin/ streptomycin, 1% glutamine 200 mM and 10% fetal bovine serum. To estimate the number of cells per ml and total cell population, a 10 μl aliquot from the culture was taken, mixed with trypan blue and counted in a Neubauer chamber. Then cell concentration was diluted (in the same DMEM media) to the desired cell concentration, seeded and placed in 5% CO_2 at 37 ºC; the density of cells used in our cultures was $6 * 10^5$ cells/ml.

Two hours later the culture medium was renewed in order to prevent growth of microglia and olygodendrocytes. Two days later, cells were trypsinized and washed, following the aforementioned protocol, to enrich astrocyte-cell population and the medium was renewed again one week after seeding. Cells were checked frequently under the microscope.

3.11.2 Astrocyte-enriched cultures from cortex

After proper identification and genotyping (Ast-IL-6 KO, Floxed and WT), neonatal (24 hour-old) animals were killed by decapitation, and the scalp and the entire dorsal portion of the cranium were removed (N = 8). The cerebral cortex was isolated and the same protocol as for obtaining cerebellar astrocytes was followed but the density of cells used in the culture was $3 * 10^5$ cells/ml.

Cortex astrocytes cultures were stimulated, once they reached confluency (two weeks after seeding), with 10 ng/ml of LPS and 10 ng/ml of INFγ. For each genotype we cultured a 6-wells plate (2 wells as controls, 2 wells after 2 hours of stimulation and 2 wells after 24 h of stimulation) and a 24-wells plate (8 wells for each condition) with sterilized coverslips for immunocytochemistry purposes. Cells were stained with GFAP and tomato

lectin with the same products and concentrations as for embedded paraffin tissues (see section 3.13).

3.11.3 Splenocytes cultures

Spleens from EAE animals (see section 3.6) were mechanically dissociated and cells were flushed out from the spleens gently using a syringe plunger and a 21G needle with sterile Dulbecco's PBS 1x with 2% antibiotics (penicillin/ streptomycin) and centrifuged at 4 ºC. The pellet was resuspended and left in RBC lysis buffer (17 mM Tris, 140 mM NH_4Cl) to remove erythrocytes. After washing with PBS and further centrifuged, cells were resuspended in complete medium (RPMI with L-glutamine supplemented with 10% fetal calf serum, 1% antibiotics, 1% sodium piruvate and 0.1% β-mercaptoethanol).

Splenocytes were seeded in 24-well plates at a density of $4 * 10^6$ cells/ml and some wells were exposed to MOG_{35-55} (10 μg/ml, 72 h) or LPS (10 μg/ml, 24 h). A colorimetric assay to measure the levels of nitrites in splenocytes culture media was performed using Griess reagent kit (G-7921, Molecular Probes) following manufacturer's instructions.

3.12 ELISA

Enzyme-linked immunosorbent assay (ELISA) was used to quantify IL-6 levels in astrocyte cultures and blood serum samples from mice.

IL-6 determination was done using a commercial murine IL-6 Sandwich ELISA kit (Diaclone) according to manufacturer's instructions. Absorbance at 450 nm was measured with Labsystems Multiskan Bichromatic microtiter plate reader.

3.13 Histochemistry (HC) and Immunohistochemistry (IHC)

Ast-IL-6 and Ast-IL-6R mice

Embedded paraffin tissues were cut in 8 μm-thick sections in a microtome (Leyca, Germany) and mounted in superfrost slides (Thermo scientific).

Before proceeding with the staining protocol, the slides were deparaffinized with xylene and passed through a series of decreasing ethanol concentrations until placed in Tris buffered saline solution (TBS).

For both cryolesioned and EAE-induced mice, microglia was demonstrated by lectin HC (biotinylated tomato lectin from Lycopersicon esculentum, Sigma–Aldrich 1:500 in TBS with 0.5% triton X-100 (TBS-t)). Astrocytes were demonstrated by GFAP IHC (rabbit anti-Glial Fibrillary Acidic Protein from DakoCytomation Denmark, 1:1200 in Blocking buffer (BB) (0.5% BSA in TBS-t)). Lymphocytes were demonstrated by CD3 IHC (rabbit anti-human CD3, Dako A0452, 1:100 in BB). Spinal cord sections from the EAE experiment mice were also stained with Hematoxylin-Eosin and with Luxol Fast Blue (LFB) solution (0.1%, overnight at 37 ºC) counterstained with Cresyl violet (0.1%, 1 min) in order to assess the number of cellular infiltrates and demyelination in white matter.

Sections for IHC and HC were preincubated for 1 h with the BB, and then incubated with the primary antibodies (GFAP, CD3) or tomato lectin overnight at 4 ºC, followed by 1 h at Room temperature (RT) (GFAP, CD3) or at 37 ºC (lectin). For CD3 IHC, a previous antigen retrieval step was performed with protease type XIV (Sigma P5147) 0.1% in TBS at 38 ºC for 10 min. After washing in TBS, sections were incubated with either horseradish peroxidase-coupled streptavidin (Vector Labs, SA-5004, 1:600, 1 h at RT) for HC or biotinylated secondary antibody (Vector Labs, BA-1000, 1:300, 1 h at RT) followed by washes and horseradish peroxidase-coupled streptavidin (Vector Labs, SA-5004, 1:600, 1 h at RT) for IHC. The immunoreactivity was visualized by using 0.033% H_2O_2 in 0.5 mg/ml 3,3-diaminobenzidine-tetrahydrochloride (DAB) in Tris buffered solution (TB) 0.05 M, pH 7.4 for 4–30 min at RT. Reaction was stopped with TB, washed, dehydrated and mounted in DPX (Sigma, USA). Images at 100x (GFAP) or 200x (Lectin, CD3) were taken in a bright field Nikon Eclipse 90i microscope and acquired with Nikon digital camera DXm1200F and Nikon Act-1 v. 2,70 software from different brain areas and spinal cord. Finally, to quantify staining areas, intensity and number of cells, images were analyzed using ImageJ software (NIH) [645]. Histological analyses were performed on at least two sections per mouse. Control sections for non-specific binding analysis (where primary antibody or tomato lectin was not used) were included routinely. For GFAP IHC, percentage of stained area or stain quantity was measured in different areas of encephalon (cerebellum, cortex and hippocampus) from cryolesioned and EAE-induced mice at 100X, between 6 and 12 images per animal depending on the area of study. In the later, white and grey matter of spinal cords were also analyzed (12 images per animal and area). Threshold in each area was adapted to better define cells from tissue background. Tomato lectin HC stains both microglia and vessels, so quantification of staining must be complemented with manual countings of basal (ramified), reactive (protacted cell processes) and fully activated (round) microglia and vessels at 200X.

Also, total numbers of microgliosis/ macrophages infiltration areas in spinal cord were recorded and analyzed. For CD3 IHC percentage of stained area was measured in brain and spinal cord (10 images per area and animal). Moreover, in cryolesioned mice the number of infiltrating lymphocytes around the lesion was manually counted in two brain sections using a bright field microscope (Nikon 50i) and for EAE-induced mice, total numbers of infiltrates in the white matter of spinal cord were also assessed in two replicates. In LFB/cresyl violet staining, total numbers of cellular infiltrates in white matter of spinal cord were counted and afterwards, a color deconvolution plugin developed for ImageJ software [646] was used in order to separate colors from LFB and Cresyl Violet to be able to quantify percentage of LFB staining in spinal cord (see figure 3.16).

GFAP-IL6/sgp130 mice

For IHC and HC, free-floating cryosections (30μm) stored in OLMOS solution, were washed, endogenous peroxidase inhibited by 10 min incubation in 70% methanol plus 2% hydrogen peroxide, washed and pre-incubated with BB (10% fetal bovine serum and 0.3% bovine serum albumin in TBS with 1% triton X-100 (TBS-T) for one hour at RT followed by incubation with rabbit anti-GFAP antibody (Abcam) at 1:2000 dilution in BB overnight

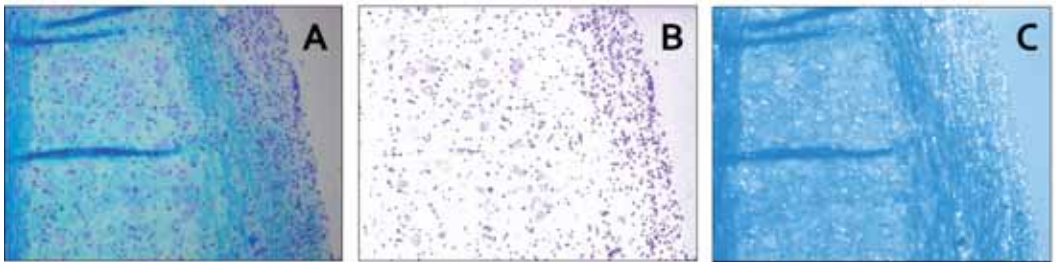


Figure 3.16: Demyelination assessment. A. Example of a spinal cord section from a EAE-induced mice stained with Luxol Fast Blue and counterstained with Cresyl violet at 100x. Deconvolution plugin [646] is applied in order to separate cresyl violet dye (B) from LFB color (C). After separation, a threshold is applied to LFB staining and percentage of occupied area is measured in white matter.

at 4 ºC followed by 1 h at RT. After washing, samples were incubated with a biotinylated anti-rabbit IgG secondary antibody (Vector Laboratories) at 1:500 dilution in BB for 1 h at RT, washed again and incubated with streptavidin-peroxidase (Vector Laboratories) at 1:500 in BB for 1 h at RT. For visualization of GFAP IHC, sections were incubated with DAB solution kit for 3 min according to the Manufacturer's directions (Vector Laboratories). Following washing, the sections were dehydrated and mounted onto slides with Eukitt mounting media (Fluka). For BrdU/doublecortin double staining, a similar IHC protocol was used, with incubation with doublecortin antibody (Abcam) at 1:8000 in BB and DAB incubation, but followed by BrdU IHC. The latter included an initial DNA denaturation step, with the sections being treated with 1N HCl for 10 min on ice followed by 30 min in 2N HCl at 37 ºC, and finally with 0.1M borate buffer (pH 8.5) for 12 min at RT to neutralize the HCl. After washing, sections were incubated with rat anti-BrdU antibody (Oxford Biotechnology) at 1:800 in BB overnight at 4 ºC followed by 1 h at RT and, after washing, incubated with a biotinylated anti-rat IgG secondary antibody (Vector Laboratories) at 1:500 in BB for 1 h at RT. After incubation with streptavidin-peroxidase, the sections were developed using nickel-DAB following the manufacturer directions (Vector Laboratories), and mounted onto slides, as above. For lectin HC a similar protocol was followed but without BB; samples were incubated with biotin-labelled tomato lectin (1:150 in TBS-T) overnight at 4 ºC followed by 1 h at RT, and visualized as above. After mounting onto slides, these lectin samples were counterstained with Nissl staining.

Stained sections were examined in UAB with a bright field microscope (Nikon Eclipse 90i) and images were acquired using a Nikon Digital camera DXM 1200F and Nikon Act-1 v. 2,70 software. GFAP and Nissl staining were quantified with the NIH ImageJ (NIH) analysis software. The number of lectin-positive, doublecortin-positive, and BrdU-positive cells, as well as that of blood vessels, was manually counted in specific CNS areas in a blinded manner. In all cases at least three tissue sections per animal were used. For staining quantifications, several images (3-5) per section were taken depending on the brain area. Since a double staining for lectin and Nissl was carried out, a color deconvolution plugin for ImageJ [646] was used with all images to separate lectin (DAB) staining from that of Nissl.

3.14 Statistical analysis

Statistical calculations were performed using the Statistical Package for Social Sciences (SPSS) v. 17.0 for Windows (SPSS Inc).

To analyze genotype frequency at weaning, we performed a Chi-Square test. In all other analysis and for the sake of simplicity, only floxed and knock-out animals were included in the statistical analysis. However, WT animals are also showed in graphical data.

Data was analysed using a Generalized Linear Model (GzLM) [647] and for the analysis of repeated measurements, such as clinical evaluation, body weight changes, cold resistance or MWM learning acquisition (among others), a GzLM test for repeated measures, the Generalized Estimating Equations (GEE) [648] test was used. In both cases, genotype, gender and age or sacrifice day (when applicable) were used as main factors. In addition, in GEE analysis effect of time (day) was used as within-subject factor. GzLM and GEE tests are a more flexible statistical tool than the standard general lineal model (GLM) because several types of data distribution can be chosen; the analysis can run even if there are missing data in the repeated measures; and they do not need homogeneity of variance.

In all cases, if interaction between any factor was found significant, it was decomposed pairwise and corrected by Sequential Bonferroni for multiple comparisons [649]. In few cases where either gender or age factors were found significant without any interaction, then data was split for this factor and GzLM or GEE were performed separately in each group. In all cases Ast-IL-6 and Ast-IL-6R groups were analysed separately and, in Ast-IL-6 mice, 5th and 10th backcrossing groups were also analysed independently.

To validate the GzLM, several parameters were also analyzed using ANOVA and Student's t test, with similar results obtained. Occasionally, results after sequential Bonferroni post-hoc analysis were compared with those obtained with Fisher's least significant difference (LSD) post-hoc test.

Values in text and figures shown are mean ± standard error of the mean (SEM) and the number of animals per group (N). Statistical significance was defined as $P \leq 0.05$ and marginal significance as $P \leq 0.1$. Data presentation was carried out using Graph Pad Prism Software (version 5). In figures, for the sake of simplicity, we show the most relevant data, indicating only differences with the appropriate control for genotype (★), age (▲) and day of sacrifice (⧫) factors, as well as the decomposition of significant interactions with genotype (✻).

CHAPTER 4

CHARACTERIZATION OF ASTROCYTE-DERIVED IL-6 DEFICIENCY

4.1 Gene recombination and IL-6 brain levels

Recombination of IL-6 exon 2 gene (see section 3.2.4) was observed in CNS tissues, brain and spinal cord, of Ast-IL-6 KO and heterozygous mice from both 5th and 10th C57BL/6 backcrossings, but not in spleen, tail or muscle.

Additionally, analysis of IL-6 expression by RT-qPCR were carried out in the cerebellum and in the spleen of young naive animals from 5th backcrossing; Table 3.2 shows the primer sequences used. We observed a decrease of IL-6 expression in the cerebellum but not in the spleen of Ast-IL6 KO mice, although this was statistically significant only in comparison with WT mice (4.1). Possible compensatory effects of IL-6 deficiency on other cytokines were analyzed by measuring the expression of IL-1β and TNFα in both cerebellum and spleen; there was a non-significant tendency for both cytokines to be up-regulated in the cerebellum of Ast-IL-6 KO mice and a significant increase of TNFα in spleen of Ast-IL-6 KO respect floxed mice.

IL-6 levels in brain and spinal cord were also intended to examine by IL-6 IHC (sc-1265-R, Santa Cruz Biotechnology) in both paraffin and free-floating sections without any success.

4.2 Frequencies at weaning

A total of 587 and 517 Ast-IL-6 mice from fifth and tenth backcrossings, respectively, were produced and genotyped (Table 4.1). There were no abnormalities in the distribution of sexes, but the distribution of genotypes did not follow a Mendelian ratio, being Ast IL-6 KO and heterozygous mice clearly under-represented at the moment of weaning at both 5th and 10th backcrossings ($P \leq 0.01$).

In general, each breeding pair had 5–7 offspring. Mortality between birth and weaning, assessed in the 10th backcrossing group, was equally distributed among genotypes (~10%). After weaning and up to four or ten months of age, very few deaths were ob-

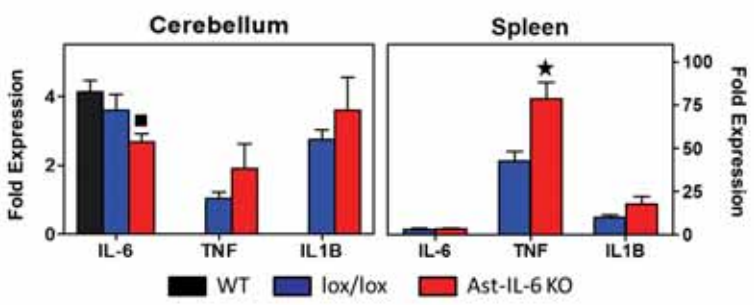


Figure 4.1: Characterization of IL-6 brain and spleen levels in Ast-IL-6 KO. Ast-IL-6 KO mice from both sexes showed a decrease of cerebellar IL-6 mRNA levels as measured by quantitative RT-PCR (■ $P < 0.01$ vs WT mice); in contrast, this was not seen in the spleen. TNFα and IL-1β tended to be upregulated in Ast-IL-6 KO mice in both tissues but only significantly for spleen TNFα. ★ P at least < 0.01 vs floxed mice. Results are mean ± SEM (N = 7–17).

	Ast-IL-6 5th backcrossing			Ast-IL-6 10th backcrossing		
	Observed N	Expected N	Residual	Observed N	Expected N	Residual
Genotype						
WT	169	146.8	22.3	163	129.3	33.8
Floxed	164	146.8	17.3	133	129.3	3.8
Het.	141	146.8	-5.8	117	129.3	-12.3
KO	113	146.8	-33.8	104	129.3	-25.3
Total	587			517		
Sex						
Females	308	296	12	248	258.5	-10.5
Males	284	296	-12	269	258.5	10.5
Total	592			517		

Table 4.1: Genotype and sex frequencies at weaning.

At weaning Ast IL-6 KO mice were underrepresented at both fifth ($\chi(3, N = 587) = 13.388$, $P = 0.004$) and tenth ($\chi(3, N = 517) = 15.015$, $P = 0.002$) backcrossing, demonstrating a prosurvival role of astrocyte IL-6 release. Het. stands for Heterozygous. Sex distribution was as expected at both fifth ($\chi(1, N = 592) = 0.973$, $P = 0.324$) and tenth ($\chi(1, N = 517) = 0.835$, $P = 0.356$) backcrossing.

served, normally caused by eventual injuries because of fights between males or dental problems.

4.3 Body weight

Male Ast-IL-6 KO mice from 5th backcrossing presented progressively an increased body weight compared to their floxed controls (4.2). This was not observed in Ast-IL-6 KO females. Regarding animals from 10th backcrossing, there were no body weight differences

between genotypes in females and, in males, the effect of IL-6 deficiency was weaker and occurred later, from week 23 onward.

The GEE statistical analysis for floxed and Ast-IL-6 KO mice revealed that both time and sex had a very significant effect in both backcrossing groups ($P \leq 0.001$ in all cases, increasing weight with age and weighting males more than females). Genotype effect was only significant in fifth backcrossing group ($P \leq 0.001$) but interaction between genotype, age and time was significant in both 5th and 10th backcrossings ($P \leq 0.01$ and 0.001, respectively). After decomposing the genotype and time interaction in 5th backcrossing group, we can observe that differences between Ast-IL-6 KO and floxed mice are only statistically significant from week 12 onwards in the sequential bonferroni test (4.2, for the sake of clarity, significant genotype effects have not been marked in the figure). However, if a less rigid test like LSD post-hoc is applied, significant differences start from week 8 onwards. When decomposing the genotype per time interaction in the 10th backcrossing group, there are no significant differences regardless the post-hoc test used, presumably because differences are only in the latter age while all other weeks body weights are identical. A GEE test considering body weight only from week 23 onwards is performed in males, resulting in a significant effect of genoype ($P \leq 0.05$) (4.2).

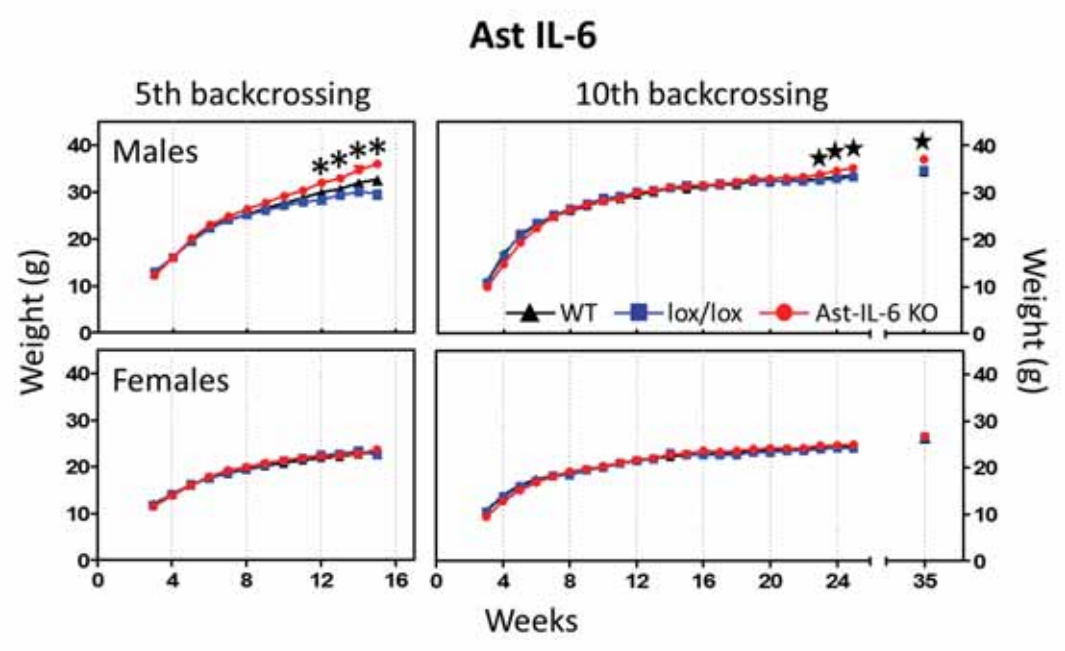


Figure 4.2: Body weight growth is affected by astrocyte IL-6 deficiency in a sex-dependent manner. Mice were weighed weekly (N = 49–91 at 3 weeks and N = 6–18 at 15 weeks in the fifth backcrossing, and N = 15-39 at tenth backcrossing). ✻ P at least ≤ 0.05 vs floxed mice after decomposition of genotype and time interaction with sequential Bonferroni post-hoc; ★ P at least ≤ 0.05 vs floxed mice.

Food intake was measured in a small group of 2 month-old males (N = 7-11) from

tenth backcrossing after individualization over a 15-days period before resident-intruder behavioural tests. Average daily food intake was only marginally increased in Ast-IL-6 KO male respect floxed but not WT mice (Ast-IL-6 KO: 3.59 ± 0.07; Floxed: 3.24 ± 0.17; WT: 3.50 ± 0.11; $P = 0.075$) in a Generalized Linear model. Unfortunately, data from individualized older mice was not assessed.

4.4 Behavioural tests

4.4.1 Hole-Board

The behavioral traits observed in the Hole-Board are shown in Figure 4.3 and statistical results are shown in Table 4.2.

Horizontal exploratory activity was measured by counting total squares crossed by animals and it is shown separated by external (squares adjacent to a wall) and internal ambulations. Total ambulation was clearly decreased in Ast-IL-6 KO mice compared to floxed mice (Ast-IL-6 KO: 75.95 ± 4.58 and 47.13 ± 3.14; floxed: 88.94 ± 3.70 and 69.47 ± 3.13; $P \leq 0.05$ and 0.001 for 5th and 10th backcrossing, respectively). The separate analysis of the external and internal activities revealed a consistent decrease in external activity of Ast-IL-6 KO animals in all conditions, regardless of sex, age and backcrossing. Internal deambulations, more related to anxiety, were also decreased in Ast-IL-6 KO animals respect to floxed mice, especially in animals from the 10th backcrossing group. No differences due to sex effects was seen in 5th backcrossing animals but in the 10th backcrossing group external deambulations were increased in females and both internal and external ambulations were increased in old-adults compared to young mice.

In contrast to horizontal activity, vertical exploratory activity (rearings) was only significantly decreased in Ast-IL-6 KO males from 10th backcrossing. Sex did not have a clear-cut effect, as females from 5th backcrossing showed less rearings compared to males but in 10th backcrossing the inverse situation was seen.

The exploratory activity was further analyzed by looking at the number of head-dips and the time head-dipping. A clear sex-, age- and backcrossing-dependent effect of astrocyte IL-6 deficiency was observed, since in 5th backcrossing group head-dipping behavior was significantly altered in male mice only in 10th backcrossing group and the opposite was observed in young female mice. There were some differences between males and females in the overall head-dipping behavior in 5th backcrossing group (floxed females presented increased numbers compared to floxed males but Ast-IL-6 KO presented same levels in both genders); but again this does not seem a consistent sex difference as it was not observed in animals from 10th backcrossing. The latter did show differences due to age, as young animals performed increased numbers and time of head-dipping. However, this age-related differences in the number and time of head-dippings occurred exclusively in floxed mice, specifically in females, as showed after decomposing genotype and age; and genotype, age and sex interactions.

Other important parameters to assess emotionality are the number of defecations and those of squares not explored. Ast-IL-6 KO showed, in general, a significant increase in defecations compared to floxed mice, especially at younger ages in 10th backcrossing.

	Ast-IL-6 5th backcrossing			Ast-IL-6 10th backcrossing			
	Genotype (G)	Sex (s)	Gs	Genotype (G)	Sex (s)	Age (a)	Interactions
Hole-Board							
Deambul.	*	-	-	***	*	***	Gs = *
External	*	-	-	***	*	***	-
Internal	-	-	*	***	-	*	-
Rearings	-	*	-	-	*	-	Gs = *
HDn	-	*	**	*	-	**	Gsa = **
HDt	**	***	*	-	-	***	Gsa = *
Def.	-	*	-	***	*	**	Ga = **
Unexpl.	*	-	*	***	-	*	Gs = *
Plus-Maze							
CEn	**	-	-	***	-	-	-
TO	-	-	*	-	-	-	Ga = ***
TCe	*	-	-	***	-	-	Gsa = **
Def.	-	*	-	***	-	-	-
Rearings	-	-	*	*	-	***	sa = *
HDprot	*	-	-	***	-	-	Gsa = *
HDopen	*	-	**	-	-	-	Ga = ***

Table 4.2: Summary of significances obtained in Hole-board and Plus-maze behavioral tests.

Gs, Gsa, Ga and sa denote interaction between genotype and sex; genotype, sex and age; genotype and age; or sex and age, respectively. Deambul. stands for total deambulation, HDn and HDt stand for number and time of head dipping, Def for defecations, Unexpl. for number of non-visited squares, CEn for number of close arm entries, TO for time spent in open arms, TCe for time spent in central square, HDprot for number of protected head-dips and HDopen for number of unprotected head-dips. *, ** and *** *P* at least ≤ 0.05, ≤ 0.01 and ≤ 0.001 respectively.

Defecation was increased in young mice compared to 8-month animals but only in Ast-IL-6 KO, as shown after the decomposition of genotype and age interaction. Ast-IL-6 KO mice, mostly males, showed a significant increase in the number of unexplored squares, which were also affected by age, leaving young mice more unexplored squares compared to adults.

4.4.2 Elevated Plus-Maze

The behavioral traits observed in the EPM are shown in Figure 4.4 and statistical results are shown in Table 4.2.

In accordance with the hole-board results, the general locomotor activity assessed as number of entries into the closed arms [650] (Figure 4.4) and total number of squares crossed (Supplementary Figure A.11) was significantly decreased in the Ast-IL-6 KO mice compared to floxed mice regardless of sex, age and backcrossing number.

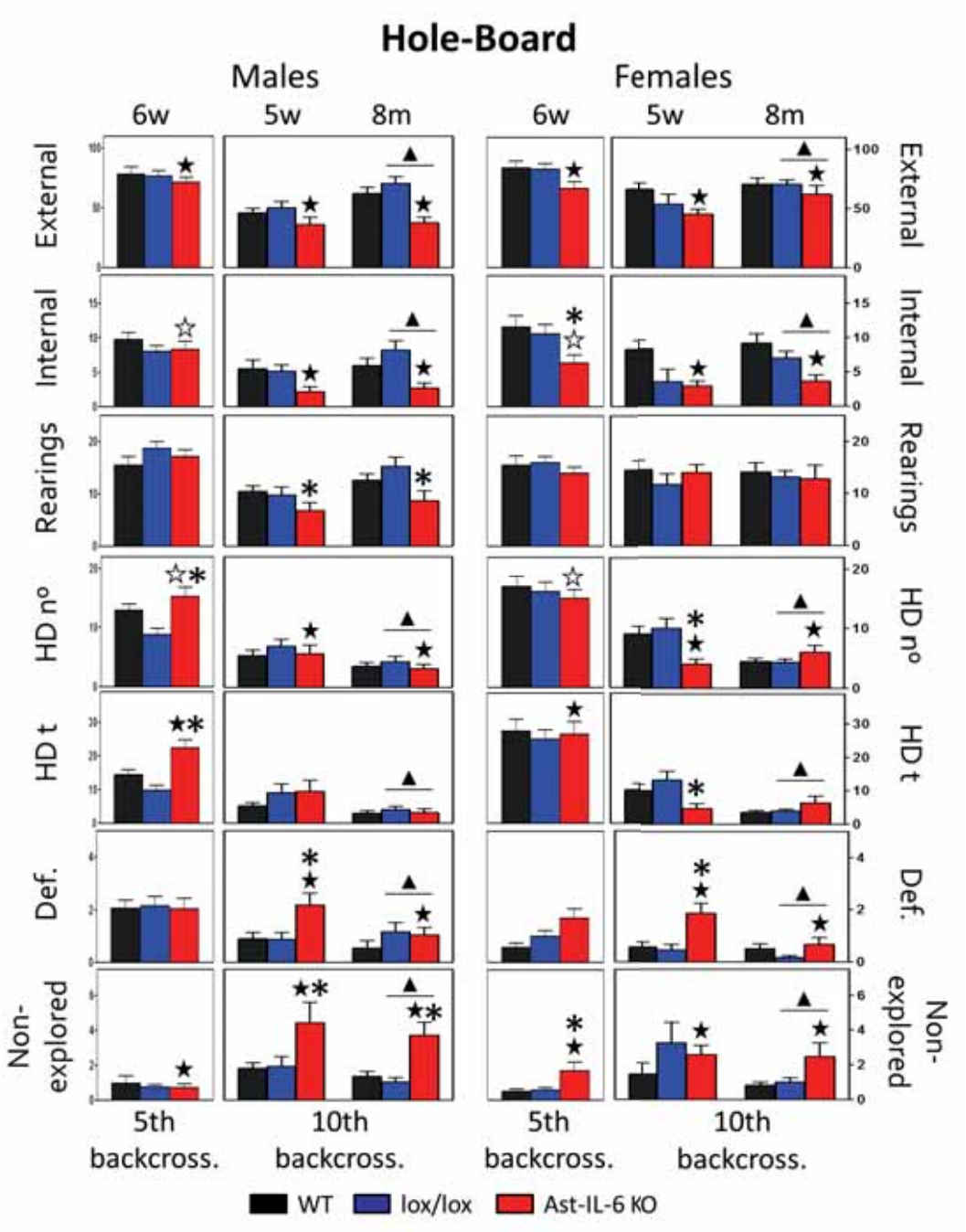


Figure 4.3: Behavioral traits analyzed in the Hole-board apparatus. Ambulation (External and internal, as number of external or internal squares crossed), vertical activity (number of rearings), exploratory behavior (number of head-dips (HD nº) and time (seconds) spent head-dipping (HD t)), defecations (Def.) and number of non-explored squares are shown for Ast-IL-6 KO, floxed and WT mice for both 5th (6 week-old) and 10th backcrossings (5 week-old and 8 month-old). (N = 11–37). ★ and ☆genotype effect P at least ≤ 0.05 or ≤ 0.1 vs floxed mice, ▲ P at least ≤ 0.05 vs young mice and ✻ P at least ≤ 0.05 vs floxed mice after genotype interaction decomposition.

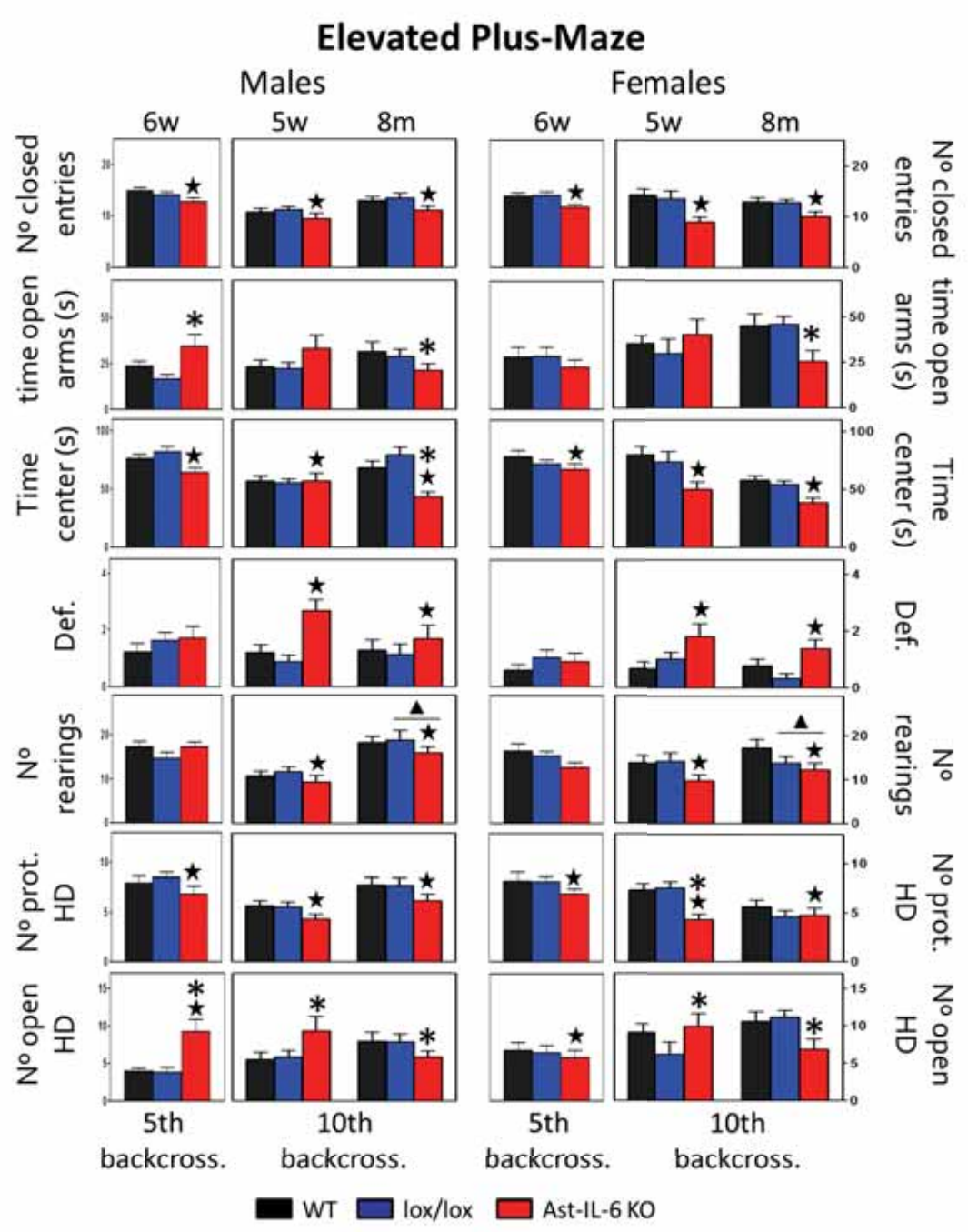


Figure 4.4: Behavioral traits analyzed in the Plus-maze apparatus. Number of entries to closed arms, time spent in open arms, time spent in the central square, defecations, number of rearings and number of both protected and unprotected (open) head-dips are shown for Ast-IL-6 KO, floxed and WT mice for both 5th (6 week-old) and 10th backcrossings (5 week-old and 8 month-old). (N = 11–37). ★ and ▲ P at least ≤ 0.05 vs floxed or young mice respectively. ✻ P at least ≤ 0.05 vs floxed mice after genotype interaction decomposition.

The anxiety level was assessed by measuring the number of entries and time spent into the open arms and calculating the percentage they represent respect total entries or total time spent in all arms of the EPM [650], as well as the time spent in the central square of the plus-maze apparatus. Due to the redundancy of these data only time spent in open arms and time spent in the center are plotted in Figure 4.4, the percentage of time and entries in open arms as well as time spent in close arms are presented in Supplementary materials (Figure A.11 and Table A.1). There is a strong interaction between age and genotype in time and percentage of time spent and entries in the open arms, tending to be increased in young Ast-IL-6 KO mice (significant for Ast-IL-6 males from 5th backcrossing group) but significantly decreased only in 8 month-old mice compared to floxed mice; showing a clear age effect as 8 months-old mice presented an opposite phenotype as young (5-6 week-old) mice regardless their backcrossing group. In relation with time spent in the central square, it was decreased in Ast-IL-6 KO mice respect to floxed animals independent of sex, age and backcrossing. However, no differences in latency time of the first escape from central square were found between genotypes (see Figure A.11).

Although the EPM is normally used for testing anxiety, other less conventional traits can be assessed, such as rearings and head-dips (total, protected and unprotected) [651], which can be compared with the results observed in the hole-board. Overall, the results of the EPM were quite consistent with those of the hole-board despite the different nature of the two apparatus. Thus, the number of rearings, which in hole-board was only decreased in Ast-IL-6 KO males, in EPM was decreased in both genders and ages in Ast-IL-6 KO mice from 10th backcrossing group, parallelling deambulation results. However, only in EPM test we observed an age effect in rearing behaviour, being increased in 8-months males in relation with 6 week-old ones. Regarding HD behavior, the total number was decreased only in 8 month-old Ast-IL-6 KO mice respect to floxed animals (see Figure A.11), but when differentiating them into protected and unprotected head-dips, we can see that Ast-IL-6 KO mice showed a decrease of protected head-dips regardless of age, sex or backcrossing group. Oppositely, young Ast-IL-6 KO mice show an increase of the unprotected ones (only males in the 5th backcrossing but both genders in the 10th backcrossing), whereas old Ast-IL-6 KO mice show a decrease respect to floxed controls, showing again a clear age effect. This is suggestive that young Ast-IL-6 KO mice are more prone to exploration in dangerous situations than floxed males, while old/adults Ast-IL-6 KO are less willing to do it.

Finally, defecations were strongly increased in both genders and ages of tenth backcrossing Ast-IL-6 KO mice compared to their respective floxed mice. In relation to that trait, we are not able to truly compare 5th with 10th backcrossing groups as, due to methodological differences, 5th backcrossing animals were weighed just before EPM and thus defecating in the other "new" environment likely altering our results.

4.4.3 Tail Suspension

Time mice spent in motion (agitation time) while being suspended by their tail throughout the experimental condition is shown in Figure 4.5. There are no significant effects of genotype or sex but a significant effect of time ($P \leq 0.001$) showing that all animals stayed

in motion significantly more time in the first 2-min block than in the other two, as usually described for this test. We also found a significant interaction between age and time of the test ($P \leq 0.001$) showing that during first 2 minutes 8 month-old animals agitated more than young animals ($P \leq 0.001$) while in the middle and last 2 minutes blocks this situation was reversed ($P \leq 0.01$ and 0.001, respectively), being young animals the ones who stayed in motion more time.

Moreover, there was also a significant interaction between genotype and time ($P \leq 0.05$) and a nearly significant interaction between genotype, sex, age and time ($P = 0.052$). Decomposition of these interactions with Sequential Bonferroni did not show any significant differences between Ast-IL-6 KO and floxed due to the severity of this post-hoc test. However, it is noteworth to point that with LSD post-hoc test a small significant increase in agitation was found in 8 month-old Ast-IL-6 KO male respect to floxed controls in the first and second blocks of 2-minutes test ($P \leq 0.05$).

Finally, we applied a GEE test for each condition (young males, adult males, young females and adult females) showing a significant effect of genotype in adult males ($P \leq 0.05$), being Ast-IL-6 KO males more time in agitation compared to floxed controls (4.5).

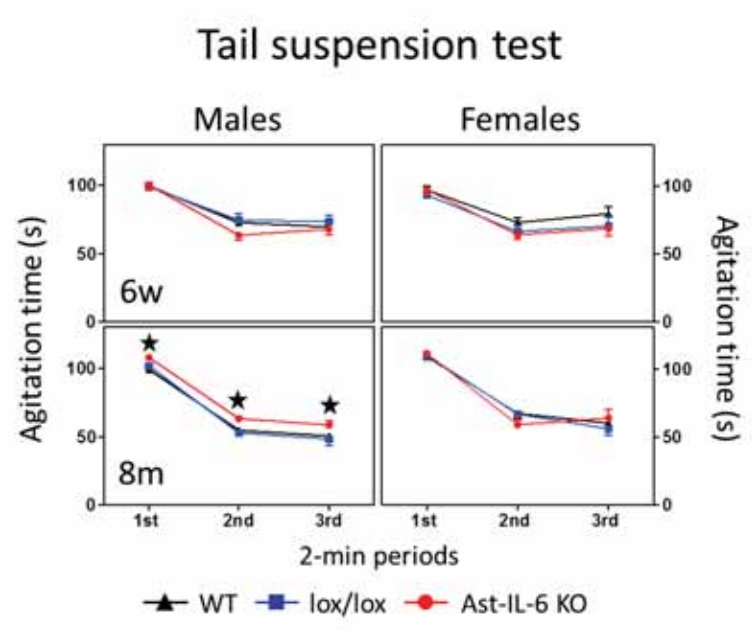


Figure 4.5: Time spent in motion during tail suspension test. Total duration of the test is shown in 3 blocks of two minutes and agitation time (in seconds) is presented (N = 11–32). ★ P at least ≤ 0.05 vs floxed mice.

4.4.4 Morris water maze

The behavioral traits observed in the Morris water maze are shown in figures 4.6 - 4.10; and several statistical results are shown in Table 4.3. Young (7 week-old, N= 11-23 for each

	Ast-IL-6 10th backcrossing				
	Genotype (G)	Sex (s)	Age (a)	Day (d)	Interactions
Cued learning					
Time plat.	***	-	-	n/a	Ga = ***
Spatial learning					
Time plat.	**	-	***	***	-
Probe 1h	*	-	***	n/a	-
Probe 24h	-	-	***	n/a	-
Reversal learning					
Time plat.	*	-	***	***	Gad = *
Probe new 1h	-	-	-	n/a	Gs = *
Probe new 24h	*	-	-	n/a	-
Probe old 1h	*	-	-	n/a	-
Probe old 24h	**	-	-	n/a	-

Table 4.3: Summary of obtained significances in Morris water maze test.

Ga, Gad and Gs denote interaction between genotype and age; genotype, age and day or genotype and sex, respectively. Time plat. stand for time needed to reach the platform and, in reversal learning, probe new refers to percent of time spent in the quadrant that allocated the platform during reversal learning, whereas old refers to the quadrant that allocated it during spatial learning. *, ** and *** P at least ≤ 0.05, ≤ 0.01 and ≤ 0.001. n/a not applicable.

sex) and old-adults (8 month-old, N= 11-21 for each sex) from 10th backcrossing group have been studied.

As stated in Section 3.3.4, the first test protocol that we performed in MWM was Cued learning, testing the animals for their ability to learn to swim to a cued goal. Animals were given four trials; a mean value was calculated and is presented in Figure 4.6. Ast-IL-6 KO mice showed a significant impairment in cued learning needing more time to find the platform. Also, a significant interaction between sex and age was found, showing that only in females, 8 month-old animals performed significantly worse than young ones. Moreover, 7 Ast-IL-6 KO mice out of 69, 4 floxed mice out of 89 and 0 WT out of 74 were incapable of climbing the platform in any trial. However, these non-performers animals were not excluded from the test because in the following test protocols, all of them were able to find and climb the submerged platform. We did try to avoid poor eyesight by excluding animals with visible alterations of the eyes (one eye permanently closed, cataract or excessive rheum), we also discarded locomotor deficits altering swimming and poor eyesight by checking mean swimming speed in a subset of animals (N = 10-14 per genotype and sex in young animals) with SMART v2.5.21 (Panlab S.L.U) with no differences between genotypes (Ast-IL-6 KO: 23.81 $\pm$ 0.88; Floxed: 23.80 $\pm$ 0.68; $P = 0.841$) but a sex effect, with a significant increase in swimming speed in males respect to females (males: 24.72 $\pm$ 0.65, females: 22.84 $\pm$ 0.77 cm/s; $P \leq 0.05$).

In first trials many animals did not want to swim away from the wall or quickly jumped back into the water once they climbed the platform, but this was clearly improved at the

end of cued learning test. Animals performing floating behavior at some point one or more test-days were equally distributed among genotypes (20% in Ast-IL-6 KO mice and 18% in Floxed mice) and, as they also presented periods of swimming, they were not excluded from the experiment.

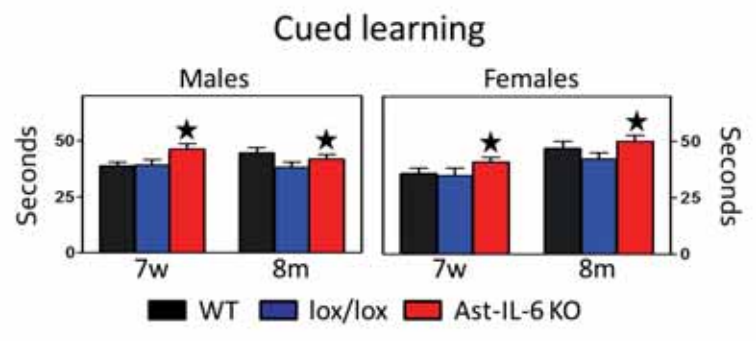


Figure 4.6: Time to climb to the visible platform in MWT. Tenth backcrossing animals are showed separated by sex and age (7 weeks and 8 month) (N = 11–23). ★ P at least ≤ 0.05 vs floxed mice.

The second protocol was to test spatial learning acquisition. Animals were given four trials a day during four days and the mean for each trial is showed in Figure 4.7, where we can observe that animals are learning the spatial location of the platform as the time to reach it is significantly diminished daily. Although they were all learning, Ast-IL-6 KO mice showed a significant increase in the amount of time needed to find the platform respect to floxed mice. Moreover, an important age effect is seen showing that 8 month-old mice spent significant more time to reach the platform compared to 7 week-old ones. There is a marginally significant interaction between sex and day ($P = 0.073$) that together with the visible absence of genotype effect in males made us analysed both genders separately applying a GEE for each sex, confirming that only in females there was a significant effect of genotype ($P \leq 0.01$), although both genders still present significant age and day effects ($P \leq 0.01$).

To assess reference memory at the end of those 4 days learning, a probe trial (a 30 seconds trial without platform) was carried out 1 hour after last trial and 24 hours later, in order to differentiate between short- and long-term memory. Percentage of time spent in the quadrant which previously allocated the platform was presented in Figure 4.8; these results show a clear deficit in spatial memory in older animals, which stayed in the 25% borderline limit of chance, in relation with younger animals. Regarding genotype differences, Ast-IL-6 KO mice showed impaired memory only with probe trial carried out 1 hour after the last training session but not with that 24 hours later.

Immediately after the second probe trial, animals performed two days of reversal learning with four trials a day, with the platform located in the opposite quadrant to that in initial spatial learning acquisition. Mean results for learning days are shown (4.9). Mice did learn the new location as they needed significant less time to find the platform in the second day. Again, we see important differences between younger and older animals, being learning impaired in the latter, and Ast-IL-6 KO mice also showed a significant poorer

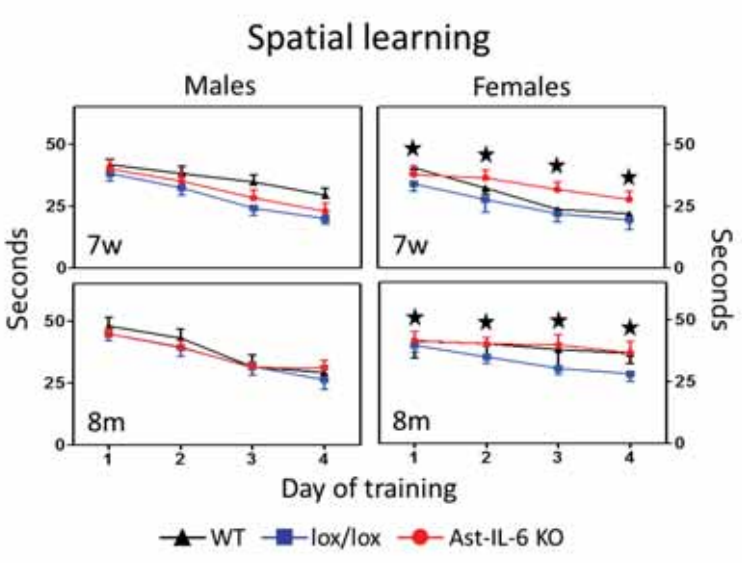


Figure 4.7: Mean time needed to reach the submerged platform. (N = 11–23). Data analyzed separately by sex. ★ *P* at least ≤ 0.05 vs floxed mice.

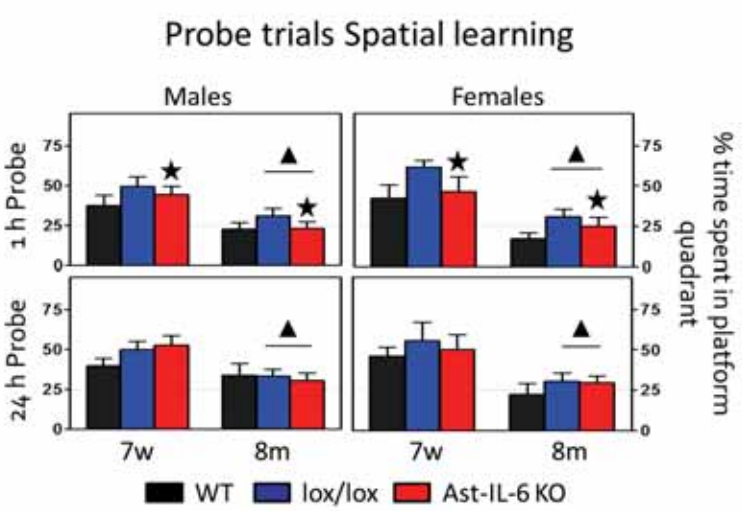


Figure 4.8: Percentage of time spent in the platform quadrant during Probe trials. (N = 11–23). In the top data is shown from the probe trial given 1 hour after last trial and, at the bottom, data from probe trial made 24 hours later. ★ and ▲ *P* at least ≤ 0.05 vs floxed mice and young animals, respectively.

performance than floxed controls. However, the decomposition of the significant interaction between genotype, age and day found, shown that learning deficits in Ast-IL-6 KO mice were present only in 8 month-old animals in the second day of training.

Once reversal learning was finished animals were also given two probe trial 1 and 24 hours later the last trial. In those probe trials it was measured not only the percentage of

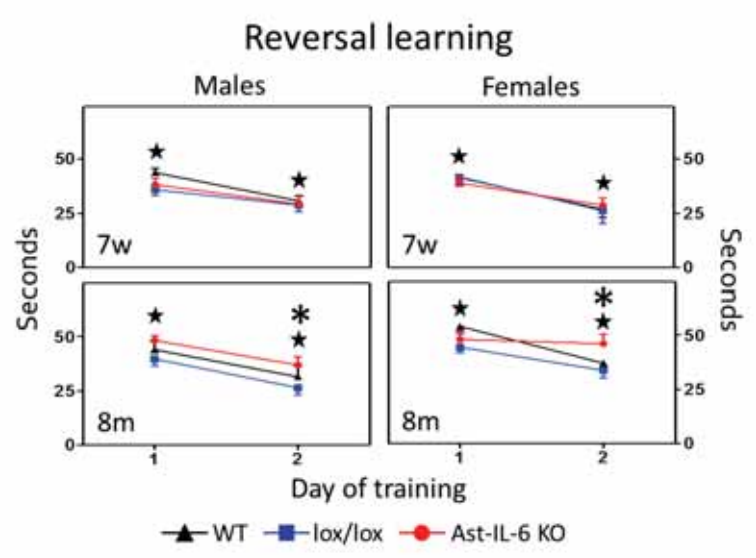


Figure 4.9: Mean time needed to reach the submerged platform in a new location. (N = 11–23). ★ at least $P \leq 0.05$ vs floxed mice. ✻ P at least ≤ 0.05 vs floxed mice after genotype interaction decomposition.

time spent in the quadrant with the newer location of the platform (the opposite from the spatial acquisition) but also the percentage of time spent in the old platform quadrant (the one that allocated the platform during spatial acquisition). Results, seen in Figure 4.10, show that animals barely pass borderline 25% chance line, not remembering the new location of the platform, in that case with any age effect. Also, Ast-IL-6 KO mice stayed in the second probe trial (24 h later) even less time in this quadrant respect to floxed mice. However, when analysing time spent in the quadrant with the old location of the platform we see it is still largely revisited and, specialy Ast-IL-6 KO mice, spent an increased amount of time searching for the old location platform in comparison with floxed animals.

As a general comment, in MWM we have not obtained significant differences between genders in any variable.

4.4.5 Dominance tube

Young (8 week-old) animals from tenth backcrossing group have been studied in the dominance tube test and results, presented as percentage of victories over all duels, are shown separately by sex in Figure 4.11. Although there was an overall significant increase in the percent of victories of Ast-IL-6 KO versus floxed mice (KO: 73.39 ±6.57; Floxed: 30.53 ±6.87, $P \leq 0.001$) there was a strong interaction between genotype and sex ($P \leq 0.01$) since percent of victories in Ast-IL-6 KO is only significant increased in females.

Percent of victories of Ast-IL-6 KO and floxed mice when they were faced with WT mice was not affected by neither genotype nor sex (percent of victories over WT: KO: 53.84 ± 10.18; Floxed: 50.09 ± 10.18, $P = 0.794$ and 0.210 for genotype and sex, respectively; N =

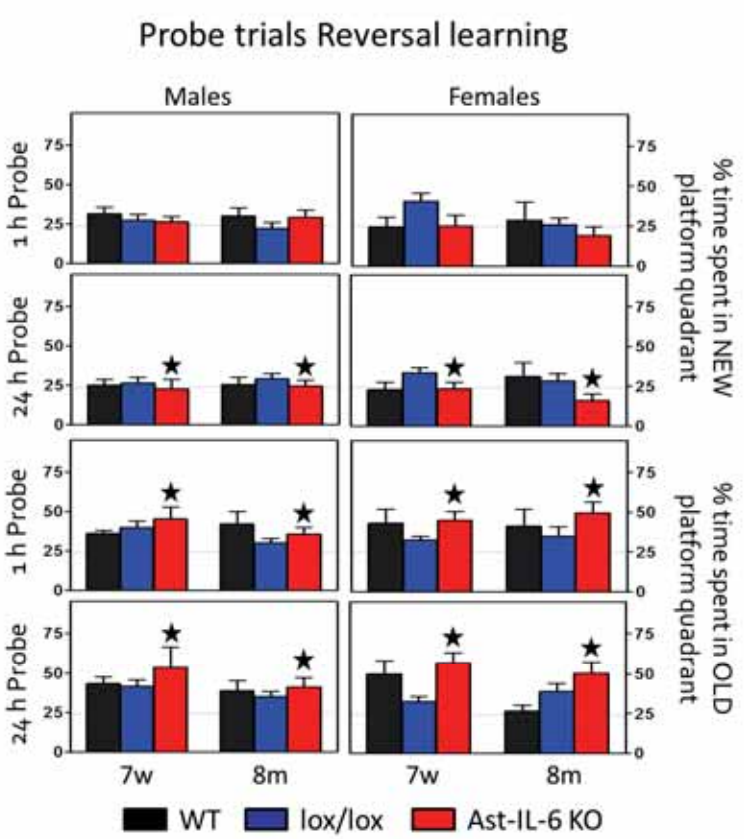


Figure 4.10: Percentage of time spent in both new and older platform quadrant during reversal probe trials. (N = 11–23). In the first two panels it is shown the percent of time spent in the quadrant that allocated the platform during reversal learning (the new location) for 1 and 24 hours probe trials. In the two bottom panels it is presented the percent of time spent in the quadrant that allocated the platform during spatial learning (the old location) for 1 and 24 hours probe trials. ★ P at least ≤ 0.05 vs floxed mice.

7-13).

4.4.6 Resident Intruder

The behavioral traits observed in the resident-intruder test are shown in Figure 4.12 and statistical results analyzed with GzLM and sequential Bonferroni (separately for resident and intruder condition) for genotype and age, as main factors, are presented in Table 4.4. Young (10-11 week-old; N = 7-11) and adult/old males (9 month-old; N = 8-10) from 10th backcrossing group have been studied.

Regarding aggressive behaviour, in the latency to first attack there was a significant interaction between genotype and age when Ast-IL-6 mice acted as intruders, since it tended to be decreased in adult Ast-IL-6 KO but increased in young KOs respect to floxed controls. However, the interaction decomposition was only significant with LSD post-hoc test (and marginally significant with sequential Bonferroni test, $P = 0.061$) in young ani-

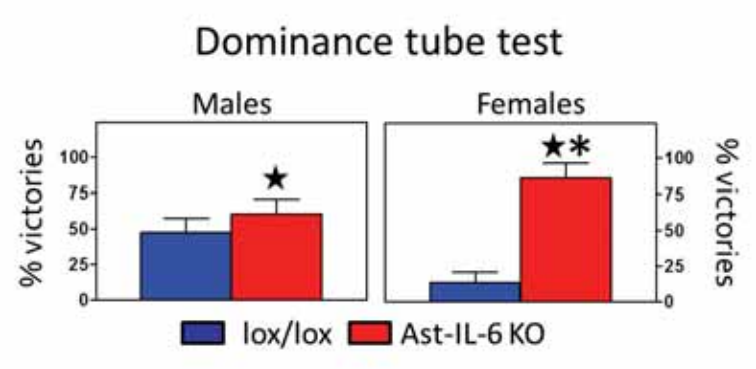


Figure 4.11: Percentage of victories in the dominance tube test. Data showing results of Ast-IL-6 KO and floxed encounters in the tube test. (N = 11-16). ★ P at least ≤ 0.05 vs floxed mice. ✻ P at least ≤ 0.05 vs floxed mice after genotype interaction decomposition.

mals. Consistent with that is the data from the number of attacks, which also showed a significant interaction between genotype and age in intruders, due to the fact that adult Ast-IL-6 KO fighted more than floxed mice while young KOs fighted less. However, the interaction decomposition was again only significant in young mice with LSD post-hoc test (and marginally significant with sequential Bonferroni test, $P = 0.066$). Other agressive behaviour such as active and passive chases, tail rattles, crouched postures and upright postures were analysed and presented together, showing no differences between animals when acting as a resident but, acting as intruders, 9 month-old mice showed a decreased of this aggressive behaviour and a significant interaction between genotype and age was found, as it was increased in adult Ast-IL-6 KO and decreased in young Ast-IL-6 KO in comparison with floxed animals, althougth the interaction decompositin was not significant.

When analysing affiliative behaviour (nose/body/ano-genital sniffs, crawl unders and allogrooming) there were no differences regarding age or genotype found. However, crawl-under behaviour was marginally significant for an interaction between genotype and age ($P = 0.089$) and marginally decreased in adults respect to young mice ($P = 0.071$) when acting as residents.

Floxed and WT mice presented an overall similar phenotype in this test. We did find important differences regarding condition (resident or intruder), although being analysed separately, if compared we found a significant increase in aggressive behaviour and a significant decrease in affiliative behaviour in intruder mice compared to residents.

4.5 Body temperature

Body temperature measured between 03:00 to 06:00 PM, in 7 month-old basal mice in the afternoon did not show differences due to genotype, sex or interaction between them when it was analysed by a GzLM with genotype and sex as main factors (WT: 36.13 ±0.11 ºC ; FLoxed = 36.29 ±0.9 ºC; Ast-IL-6 KO = 36.32 ±0.15 ºC).

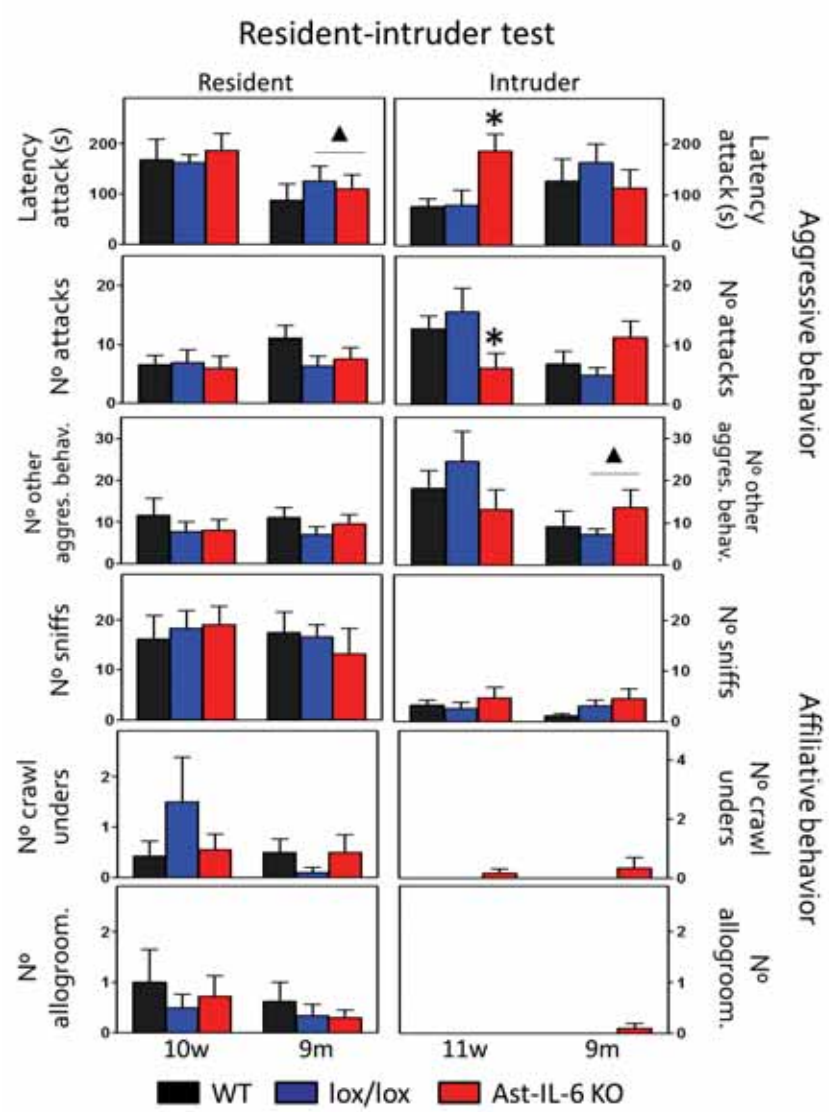


Figure 4.12: Behavioral traits analyzed in the Resident-intruder test. Latency to the first attack (seconds), number of attacks, number of aggressive behaviour other than attacks (active and passive chases, tail rattles, crouched and upright postures) and number of affiliative behaviours separated in sniffs (nose, body and anogenital), crawl unders and allogroomings, are shown for 10-11 week-old and 9 month-old mice (N = 7-11). ▲ P at least ≤ 0.05 vs young mice. ✻ P at least ≤ 0.07 vs floxed mice after genotype interaction decomposition.

Regarding the response to exposure to cold environment (8±1 ºC), a significant decrease of body temperature ($P \leq 0.001$) was observed 4 and 6 hours after cold exposure regardless of genotype, with GEE statistical analysis. Body weight was also significantly decreased at the end of the cold exposure ($P \leq 0.001$) without differences between genotypes (Figure 4.13).

Regarding body temperature measurements after 1 mg/kg LPS or saline injection, a

	Ast-IL-6 10th backcrossing					
	Resident			Intruder		
	Genotype (G)	Age (a)	Ga	Genotype (G)	Age (a)	Ga
Latency attack	-	*	-	-	-	*
Nº attacks	-	-	-	-	-	**
Nº aggres. behav	-	-	-	-	*	*
Nº sniffs	-	-	-	-	-	-
Nº crawl-unders	-	-	-	-	-	-
Nº allogroomings	-	-	-	-	-	-

Table 4.4: Summary of obtained significances in Resident-intruder test.

Ga denotes interaction between genotype and age. Nº aggres. behav. stands for the number of agressive behaviour other than attacks (Nº active and passive chases, tail rattles, crouched posture and upright posture). * and ** P at least ≤ 0.05 and ≤ 0.01.

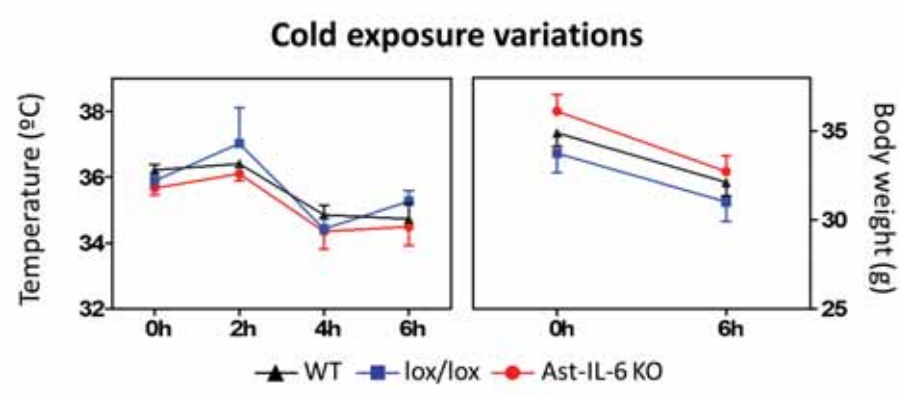


Figure 4.13: Body temperature and weight during cold exposure. Nine month-old male mice (N = 8-10) were individually placed in a cold room (8±1 ºC) at 9:00 AM with no access to water or food during 6 hours. Temperature was measured every two hours and body weight was assessed at the beginning and at the end of the experiment.

GEE for repeated measures was performed with genotype, time, treatment (LPS or saline) and sex as main factors. The first three of them were significant ($P \leq 0.01$); as were the interaction between genotype, sex and treatment ($P \leq 0.01$) and the interaction between genotype, treatment and time ($P \leq 0.01$). Data was then separated by treatment (Figure 4.14). Animals injected in the afternoon with saline showed a slight increase in body temperature ($P \leq 0.05$) without any difference between genotype or sex, while animals injected with LPS (in the morning) did show a significant effect of genotype ($P \leq 0.001$, showing Ast-IL-6 KO a decreased body temperature compared to floxed), time ($P \leq 0.001$, showing injected animals a decreased body temperature) and interactions between genotype and sex ($P \leq 0.01$) and genotype and time ($P \leq 0.01$). The interactions decomposition with sequential Bonferroni test showed in the first one that only Ast-IL-6 KO males have

a decreased body temperature compared to floxed mice and, in the second one, that only Ast-IL-6 KO mice before the injection have a decreased body temperature compared to floxed mice (Figure 4.14).

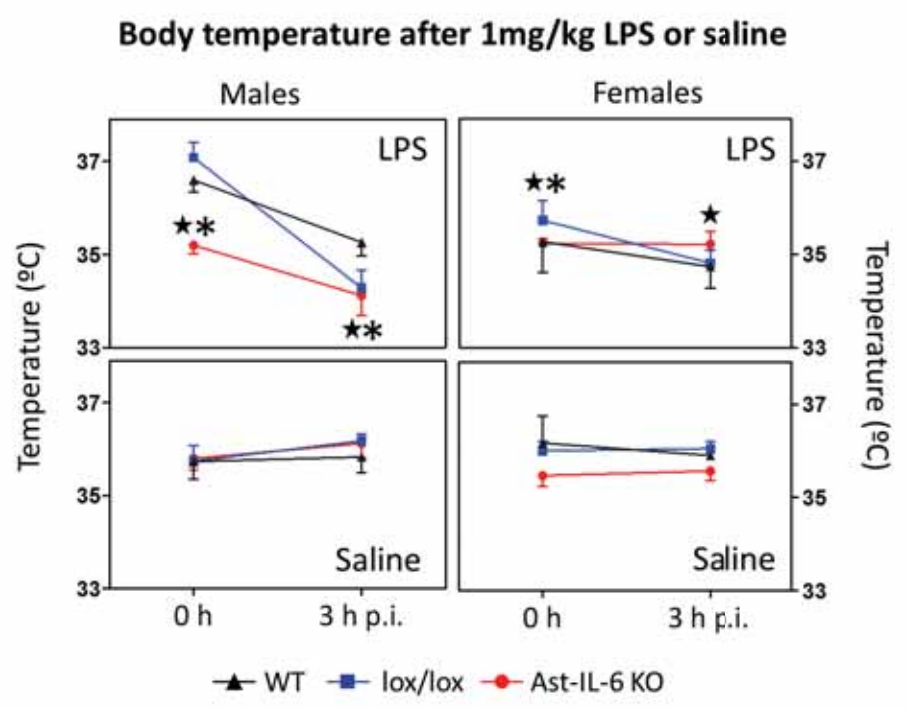


Figure 4.14: Body temperature variation after either 1 mg/kg of LPS or saline injection. Nine month-old mice (N = 5-8 in Ast-IL-6 KO and floxed mice and N = 3 in WT mice) were injected ip with either LPS (top) or saline (bottom) and body temperature was measured just before the injection (0 h) and 3-hours later (3 h p.i.). ★ P at least ≤ 0.05 vs floxed mice and ✻ P at least ≤ 0.05 vs floxed mice after genotype interaction decomposition.

In order to study hyperthermia responses in our animals, as LPS-dervided fever in total IL-6 KO mice is blunted [386], a lower dosage (10 μg/kg) of LPS was injected in the morning and temperature was measured 1, 2 and 3 hours later in floxed and Ast-IL-6 KO mice (N = 3-8 per genotype and sex). Results are presented separately by sex in Figure 4.15. A GEE with genotype, sex and time showed a significant effect of both genotype (P ≤ 0.01) and time (P = 0.01) but no sex effect or interaction, revealing that LPS did induce hyperthermia but this response was impaired in Ast-IL-6 KO animals.

Finally, circadian rhythm was analyzed in those same animals (one week before the hyperthermia study) in order to explain variability seen between morning and afternoon temperature data (Figure 4.16). Statistical analysis with GEE model for genotype, sex and time did not show a significant effect for genotype or sex but revealed a significant effect of time (P ≤ 0.001) and interaction between genotype, sex and time (P ≤ 0.05). Body temperature was increased during dark hours in all animals but Ast-IL-6 KO males tended to present lower temperature values respect to floxed controls.

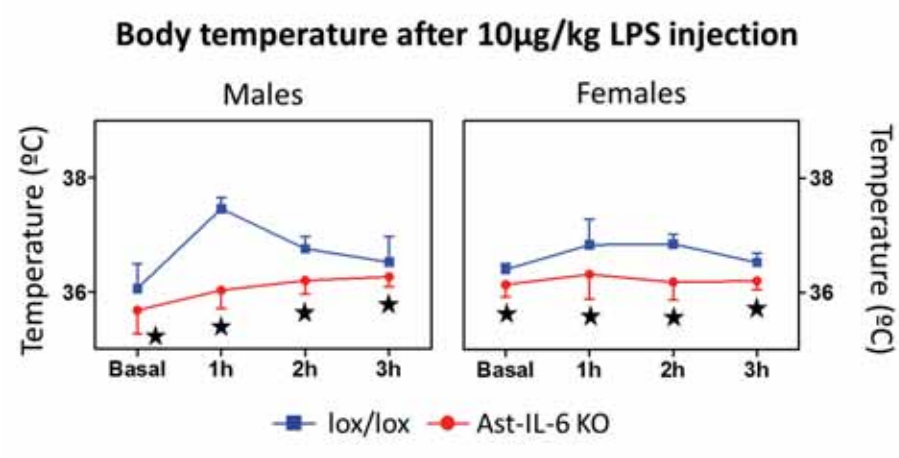


Figure 4.15: Body temperature variation after 10 μg/kg of LPS. Four to six month-old animals (N = 3-8) were injected ip with LPS and body temperature was recorded just before the injection (Basal) and 1, 2 and 3 hours later. ★ P at least ≤ 0.05 vs floxed mice.

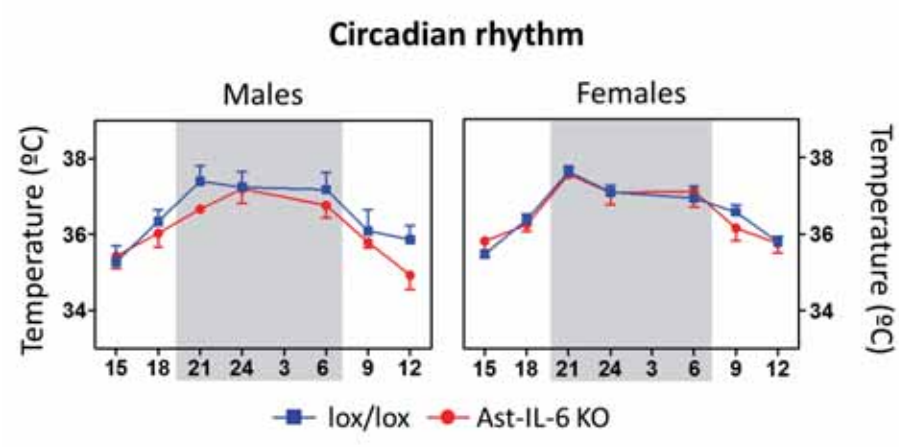


Figure 4.16: Circadian rhythm analysis. In 4-6 month-old animals (N = 3-8), their temperature in basal conditions was recorded every 3-hours in a 24-hour period (except for the measure at 3 AM). Grey denotes dark period (8 PM to 8 AM).

4.6 CNS histochemistry and immunohistochemistry

Regarding CNS histochemistry and immunohistochemistry, gliosis, evaluated by GFAP (astrocytes) and lectin (microglia), and IL-6Rα stainings of encephalon of 7 week-old mice from the 5th backcrossing were performed (Figure 4.17). For the seek of clarity only data from Ast-IL-6 KO and floxed mice is presented and analysed, although complete data with WT and heterozygous mice can be found in the Supplementary data (Figure A.13). All data is presented separately by sex (Figure 4.17).

The number of microglia was significantly increased in Ast-IL6 KO mice compared

to floxed mice in both sexes in the cerebellum having a significant effect of genotype. In cortex there was also a significant effect of genotype but also a significant interaction between genotype and sex ($P \leq 0.001$), also found in Hippocampus, revealing that microglia numbers were significant increased only in males in both hippocampus and cortex. Regarding astrogliosis, assessed as percentage of stained area, it was decreased throughout the CNS in Ast-IL6 KO mice; statistical significance of genotype effect was obtained in cerebellum and cortex and nearly significant in hippocampus ($P = 0.054$). Ast-IL6 KO mice did not show major significant differences regarding IL-6Rα staining respect to floxed mice in most cases, but a significant interaction between genotype and sex was found in cerebellum ($P \leq 0.05$) in which Ast-IL-6 KO female showed a significant decrease in staining. Also a nearly significant effect of genotype ($P = 0.053$) was seen in cortex, showing a tendency to be decreased in Ast-IL-6 KO mice cortex (Figure 4.17).

4.7 EAE

4.7.1 Symptomatology

Figure 4.18 shows the clinical score and the body weight changes observed following MOG_{35-55} immunization in data pooled from three different experiments upon 20 dpi in 5th backcrossing group, together with data from 10th backcrossing group animals. Clinical score and the body weight of the three independent experiments in the 5th backcrossing are shown in supplementary Figures A.14 and A.15.

As we can see in Table 4.5, average incidence of the disease was around 93% in 5th backcrossing and 100% in 10th backcrossing group. Mortality rate was low and without significant differences due to genotype. All genotypes showed the prototypical ascending paralysis course with concomitant body weight loss but Ast-IL6 KO from 5th backcrossing showed a significant delay in the disease onset respect floxed controls, which was not observed in 10th backcrossing experiment.

Regarding clinical score, a GEE for genotype, sex and time was performed, obtaining in both 5th and 10th backcrossing group a significant interaction between genotype, sex and time ($P \leq 0.001$ and 0.01, respectively). Data was separated by sex and only days from 10 to 19th post-induction were considered, GEE test indicated a significant effect of time in both sexes and backcrossing groups ($P \leq 0.001$) and a significant decrease in clinical score of Ast-IL-6 KO females compared to floxed mice in both 5th ($P \leq 0.01$) and 10th backcrossing females ($P \leq 0.05$). However, the latter, also showed interaction between genotype and time, when decomposed with sequential bonferroni there was a significant difference only at day 17, but if decomposed with the less strict LSD post-hoc, differences between genotypes were found from day 15 onwards. Ast-IL-6 KO females from 10th backcrossing also showed a decreased peak score and cummulative score respect to floxed controls (Table 4.5). Moreover, both Ast-IL-6 KO males and females from 10th backcrossing showed an increased grade of remission respect to floxed animals.

Regarding body weight, a GEE made from days 0 to 19 dpi showing no significant effect of genotype but significant effects of sex ($P \leq 0.001$) and time ($P \leq 0.001$) in both 5th and 10th backcrossing groups, and significant interactions between genotype, sex and

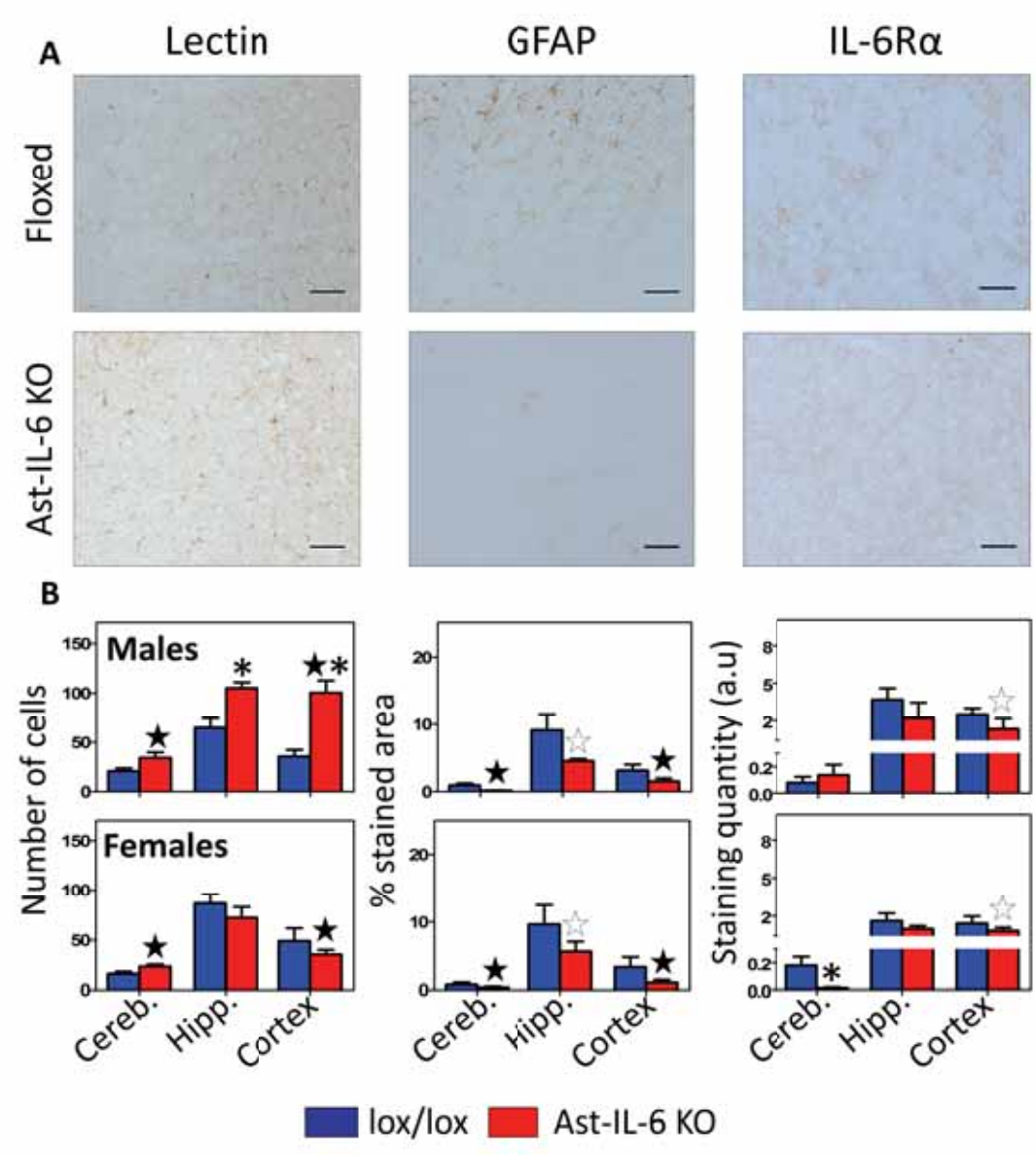


Figure 4.17: Lectin, GFAP and IL-6Rα stainings and quantifications in Ast-IL-6 KO and floxed mice. A. shows representative cortex stainings from Ast-IL-6 KO males (bottom) versus floxed controls (top). Magnification 100X (Bar 50μm). B. GFAP and IL-6Rα staining quantifications in cerebellum (Cereb.), hippocampus (Hipp.) and cortex are shown; lectin-positive cells were counted instead since blood vessels are also stained. N = 4–9. ★and ☆P at least <0.05 or <0.06, respectively, vs floxed mice. ✻ P at least <0.05 vs floxed mice after decomposition of significant interactions between genotype and sex.

time ($P \leq 0.05$) in the 5th backcrossing and between genotype and day and between sex and day (both $P \leq 0.001$) in the 10th backcrossing. In 5th backcrossing group, either by

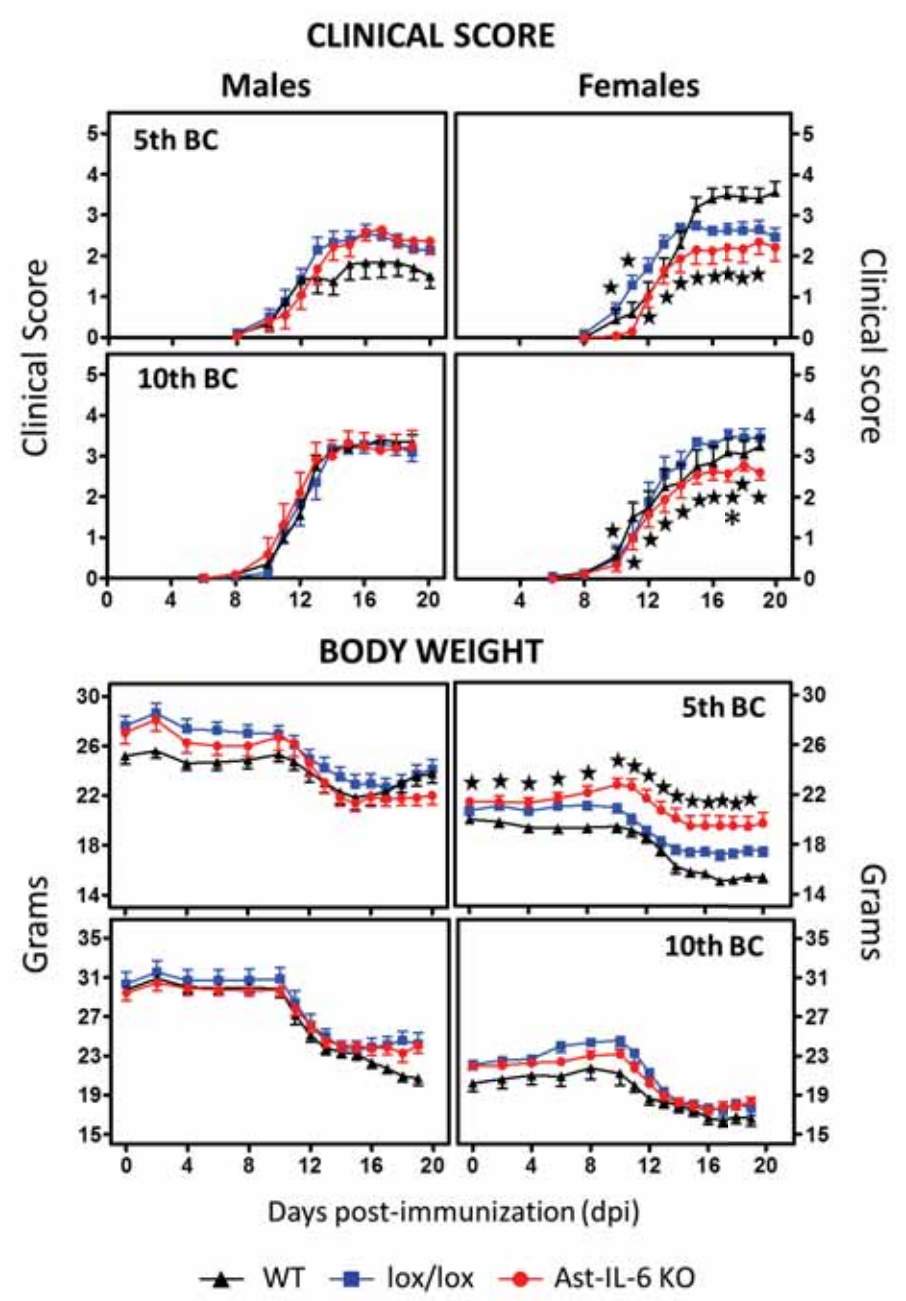


Figure 4.18: Clinical score and body weight changes following EAE induction. Data from 5th (N = 11-28) and 10th (N = 9-19) backcrossing groups are presented. GEE test for genotype and time is used in each sex from 10 to 19 dpi in clinical score and at all days in body weight analysis. ★ P at least ≤ 0.05 vs floxed mice. ✻ P at least ≤ 0.05 vs floxed mice after genotype interaction decomposition.

decomposing another significant interaction (genotype and sex, $P \leq 0.05$) or by perform-

ing a GEE only in females showing a significant effect of genotype ($P \leq 0.01$), we observed that Ast-IL-6 KO females presented an increased body weight respect to floxed animals. No significant differences between genotypes were seen in males or in either males or females from 10th backcrossing group.

4.7.2 HC and IHC of spinal cord and encephalon in 5th backcrossing group

4.7.2.1 Reduced cellular infiltrates and demyelination in spinal cord of EAE-induced Ast-IL-6 KO females

Cellular infiltrates in the longitudinal lumbar-cervical spinal cord of non-induced and EAE-induced animals at 20, 22 and 46 dpi were assessed. Total number of infiltrates in white matter was counted in a cresyl violet/ luxol fast blue staining and in a CD3 IHC counterstained with hematoxylin. A very similar number of infiltrates were obtained in both stainings and the mean of both countings was analyzed with a Generalized linear model using genotype and dpi (non-induced or 0 dpi, 20-22 and 46 dpi, in females) as main factors for floxed and Ast-IL-6 KO animals, separately by sex.

There was a significant dpi effect in females ($P \leq 0.001$) as infiltrates were significantly increased at both 20-22 and 46 dpi compared to non-induced controls and at 20-22 dpi compared to 46 dpi. We also found a significant effect of genotype ($P \leq 0.01$) and a marginally significant interaction between genotype and dpi ($P = 0.085$) in females, due to a significant decrease in the number of infiltrates in Ast-IL6 KO females respect to floxed controls in EAE-induced mice (Figure 4.19). Also, area occupied by T-lymphocytes was measured in five random infiltrates on each animal, in order to assess the size of the infiltrates, without significant differences between genotypes.

Total numbers of CD3 infiltrates were also assessed in brain without differences between genotypes (see Figure A.16). However, regarding lymphocytes occupied area in cerebellum, Ast-IL6 KO females presented a tendency to be decreased respect floxed mice ($P = 0.057$, considering controls, 20 dpi and 46 dpi together). Non-induced brains also presented lymphocytes, although to a lower extent, and if control brains were analyzed separately, they did show a significant decrease in Ast-IL-6 KO females respect to floxed controls ($P \leq 0.05$) (see Figure A.16).

Finally, demyelination in spinal cord white matter was assessed measuring the percentage of stained area in luxol fast blue stainings (see Figure 3.16). Dpi had a significant effect, being EAE-induced animals clearly demyelinated compared to controls. When data was split by group, it was observed that Ast-IL-6 KO females presented a significantly lower demyelination respect floxed animals at 20 dpi ($P \leq 0.05$) (Figure 4.19).

4.7.2.2 Slightly reduced gliosis and vasogenesis in spinal cord and brain of EAE-induced Ast-IL-6 KO females

Gliosis was evaluated by GFAP (astrocytes) and lectin (microglia) staining of both encephalon and spinal cord of EAE-induced animals.

Regarding astrogliosis, a distinct morphology between astrocytes from grey and white matter was clearly noted although the morphology of astrocytes did not appear to change

5th backcrossing group:

GENOTYPE				DAY 20			
Females	**Incidence**	**Mortality**	**Day of onset**	**Time of Peak Score**	**Peak score**	**Cumulative score**	**Grade of remission**
WT	16/16	2/16	12.75 ± 0.42	17.75 ± 0.58	3.69 ± 0.16	26.15 ± 2.32	0.46 ± 0.12
Floxed	28/28	0/28	11.57 ± 0.29	14.80 ± 0.49	3.45 ± 0.16	24.16 ± 1.32	0.91 ± 0.11
Heterozygous	12/13	0/13	12.16 ± 0.32	16.37 ± 0.87	3.12 ± 0.21	22.12 ± 1.96	0.66 ± 0.17
KO	21/23	3/23	13.38 ± 0.59 ★	14.30 ± 0.53	2.80 ± 0.23	19.40 ± 2.55	0.64 ± 0.13
Males	**Incidence**	**Mortality**	**Day of onset**	**Time of Peak Score**	**Peak score**	**Cumulative score**	**Grade of remission**
WT	10/12	0/12	11.70 ± 0.63	14.90 ± 1.06	2.90 ± 0.25	18.70 ± 2.73	1.15 ± 0.18
Floxed	17/18	0/18	11.65 ± 0.49	13.76 ± 0.62	3.14 ± 0.12	22.61 ± 1.34	0.88 ± 0.13
Heterozygous	4/4	0/4	13.00 ± 1.25	15.00 ± 1.35	2.62 ± 0.31	17.12 ± 5.02	0.25 ± 0.25
KO	11/11	0/11	12.27 ± 0.52 ★	15.18 ± 0.80	3.18 ± 0.18	20.32 ± 1.64	0.82 ± 0.26

10th backcrossing group:

GENOTYPE				DAY 19			
Females	**Incidence**	**Mortality**	**Day of onset**	**Time of Peak Score**	**Peak score**	**Cumulative score**	**Grade of remission**
WT	10/10	0/10	12.80 ± 0.92	16.10 ± 0.72	3.50 ± 0.20	22.80 ± 3.23	0.25 ± 0.15
Floxed	9/9	1/9	12.00 ± 0.56	14.66 ± 0.81	3.66 ± 0.17	25.61 ± 2.17	0.22 ± 0.12
KO	19/19	0/19	12.31 ± 0.49	14.31 ± 0.52	3.23 ± 0.06	20.02 ± 1.51	0.63 ± 0.17 ★
Males	**Incidence**	**Mortality**	**Day of onset**	**Time of Peak Score**	**Peak score**	**Cumulative score**	**Grade of remission**
WT	10/10	0/10	12.00 ± 0.51	15.20 ± 0.69	3.70 ± 0.13	24.85 ± 1.34	0.35 ± 0.16
Floxed	11/11	2/11	11.81 ± 0.35	13.72 ± 0.42	3.31 ± 0.19	24.63 ± 2.30	0.22 ± 0.07
KO	11/11	2/11	11.81 ± 0.42	14.00 ± 0.71	3.68 ± 0.26	25.68 ± 3.49	0.45 ± 0.19 ★

Table 4.5: EAE features in induced animals. Animals from both 5th and 10th (euthanized 19 dpi) backcrossing groups are shown separated by sex. For each animal we determined the time to disease onset (clinical score or ataxia ≥ 1), time to peak disease, peak-score, cumulative score (sum of all scores from disease onset to day 19 or 20), outcome (final score at day 19 or 20, not shown) and grade of remission (difference between peak score and outcome). Results are mean ± SEM. For Statistical analysis, a GzLM for genotype and sex was performed for each backcrossing group. ★ P at least ≤ 0.05 vs floxed mice. Peak score in 10th backcrossing group present a significant interaction between genotype and sex ($P \leq 0.05$) that was not significant after decomposing.

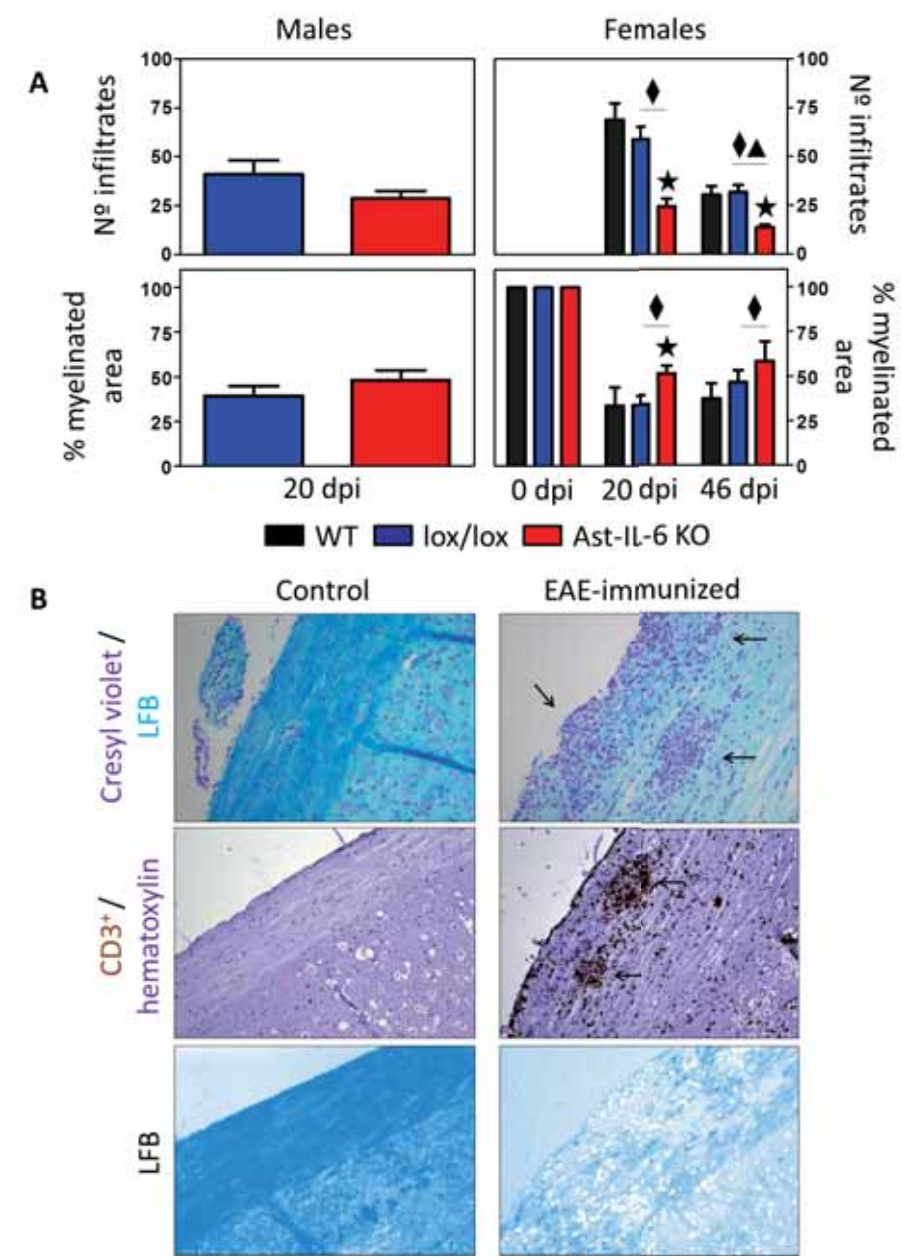


Figure 4.19: Cellular infiltrates and demyelination. A. Assessment of the total number of cellular infiltrates and demyelination in the longitudinal lumbar-cervical spinal cord white matter from non-induced animals (0 dpi, N= 3 all groups) and EAE-induced animals at 20 dpi (KO N = 8-14; floxed N = 8-20; WT = 5) and 46 dpi (N = 5-8). Results are mean ± SEM. A GzLM for each sex was performed. ★, ♦ and ▲ P at least ≤ 0.05 vs floxed mice, control group or 20 dpi group, respectively. B. Representative sections showing infiltrates (arrows) in spinal cord stained with cresyl violet (top) and CD3 (middle) in non-immunized (control) and immunized mice; demyelination shown by LFB following color deconvolution (bottom). All images at magnification 100X.

between genotypes (Figure 4.20). Area occupied by astrocytes was measured in both grey and white matter of spinal cord. Data from Ast-IL-6 KO and floxed females was analysed with GzLM with sequential Bonferroni correction with genotype and dpi (20-22 or 46 dpi) as main factors. A significant group effect was found in grey matter ($P \leq 0.01$), being astrogliosis increased at 20 dpi compared to 46 dpi. When data was separated by time, a significant decreased astrogliosis was seen in grey matter of Ast-IL-6 KO females at 46 dpi compared to floxed mice ($P \leq 0.01$), as well as a marginally significant decrease in white matter astrogliosis of Ast-IL-6 KO females at 20 dpi ($P = 0.066$). Ast-IL-6 KO males showed a marginally significant decrease in astrogliosis only in white matter ($P = 0.084$) (Figure 4.20).

Regarding astrogliosis in encephalon (cerebellum, cortex and hippocampus studied), when compared induced and non-induced females, we found a significant effect of dpi in all three areas studies ($P \leq 0.01$) due to a staining increase seen at 20 dpi respect to 46 dpi and control mice. For the sake of simplicity we followed the same scheme as before and a GzLM for genotype was performed for each dpi. Ast-IL-6 KO females presented a significant increase in astrogliosis in the hippocampus at 20 dpi ($P \leq 0.05$) but a significant decrease at 46 dpi ($P \leq 0.05$) respect to floxed mice, as well as a marginally significant decrease in cortex at 20 dpi ($P = 0.069$) compared to controls. No changes between genotypes were seen in males, which were only studied at 20 dpi (Figure 4.21).

In respect to microgliosis in spinal cord, quantification of the occupied area did not show significant differences between genotypes but, as quantification is not able to separate stained microglia from vessels, we counted microglia numbers, again with no significant differences between genotypes, neither in grey nor in white matter (Figure 4.20). Areas with increased microgliosis or macrophage infiltrates were counted along the spinal cord and they were significantly reduced in Ast-IL-6 KO females respect floxed controls without any significant differences in time ($P \leq 0.01$) and were also marginally decreased in Ast-IL-6 KO males compared to floxed mice ($P = 0.051$). However, there were no differences found between genotypes regarding stained intensity in the areas with infiltration (see Figure A.17).

Regarding microgliosis in encephalon, only females were analyzed and we found a significant effect of dpi in both cerebellum and hippocampus ($P \leq 0.001$ and 0.01, respectively), having increased numbers of microglia at 46 dpi compared to 20 dpi. Also, a significant interaction between genotype and dpi was found in hippocampus ($P \leq 0.05$), as microgliosis tended to be decreased in Ast-IL-6 KO at 20 dpi and increased at 46 dpi compared to floxed controls, although there were no significant results after decomposing the interaction. In cortex there was no differences (Figure 4.21).

Finally, vasogenesis was analyzed by counting lectin-stained vessels in both spinal cord and encephalon of EAE-induced mice. Vasogenesis in spinal cord white matter of Ast-IL-6 KO was significantly reduced respect floxed mice ($P \leq 0.001$) (Figure 4.20). However, in spinal cord grey matter there was only a marginally significant decrease in Ast-IL-6 KO vasogenesis ($P = 0.078$) but there was a significant effect of dpi, being increased at 46 respect to 20 dpi (Figure 4.20). Regarding encephalon, there was a significant effect of genotype ($P \leq 0.05$) in cerebral cortex, and a marginally significant effect in hippocampus ($P = 0.085$), showing Ast-IL-6 KO females a reduced number of vessels compared to

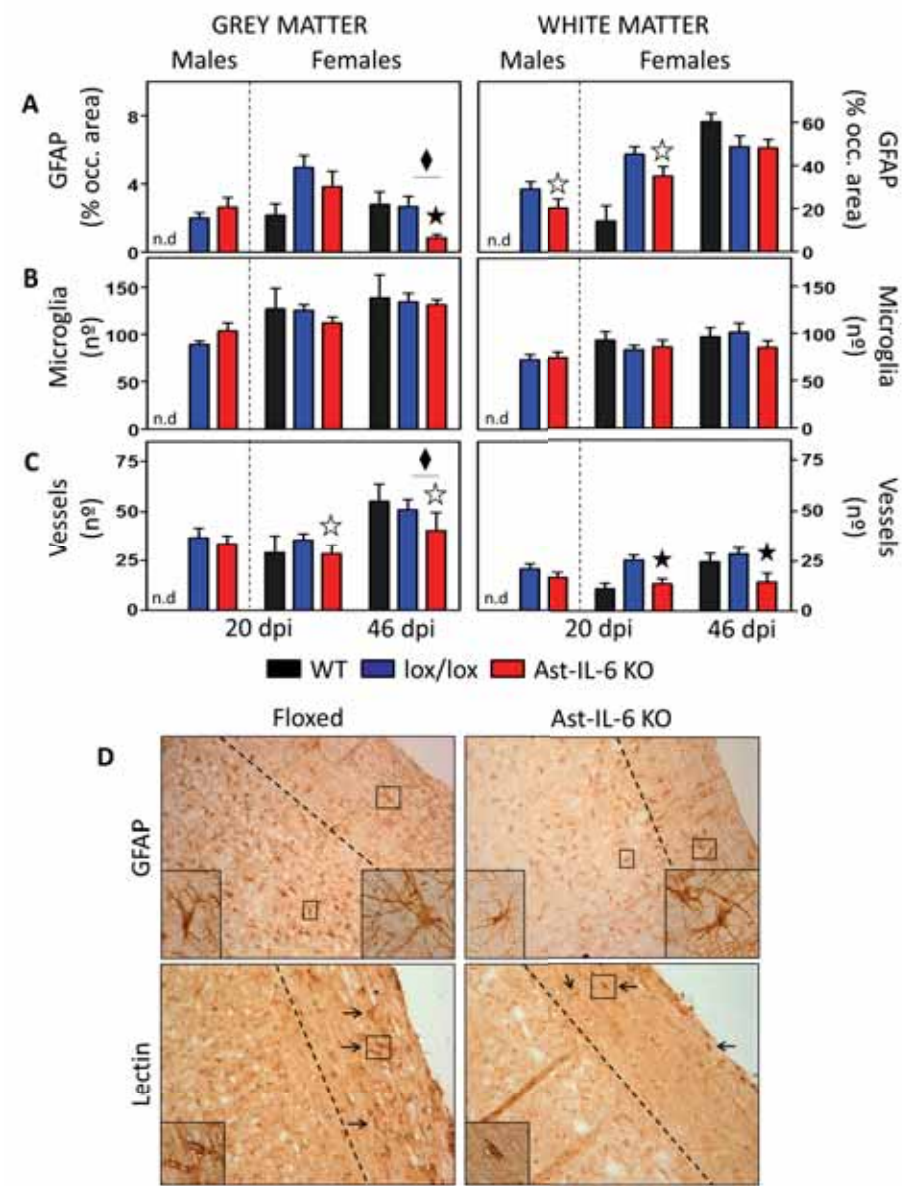


Figure 4.20: Gliosis and vasogenesis in spinal cord of EAE-induced animals. A-C. Results from GFAP and lectin stainings in the spinal cord (grey and white matter) of EAE-induced animals at 20-22 dpi (Ast-IL-6 KO N= 11-14; floxed N= 17-20; WT= 4) and 46 dpi (N= 5-8). GFAP overall immunostaining (A) and the number of lectin-positive microglia (B) and vessels (C) were analyzed. A GzLM was performed for each sex in Ast-IL-6 KO and floxed mice. ★ and ⧫ P at least ≤ 0.05 vs floxed mice or 20 dpi group, respectively. ☆$P \leq 0.1$ vs floxed mice. (n.d = not done). D. Representative GFAP at 100x (top) and lectin at 150x (bottom) stainings of 46 and 20 dpi animals, respectively. Arrows indicate vessels. The discontinuous line separates grey matter (left) from white matter (right). All inserts are at 400X, in the top they show astrocytary morphology in both grey and white matter and at the bottom they show vessels.

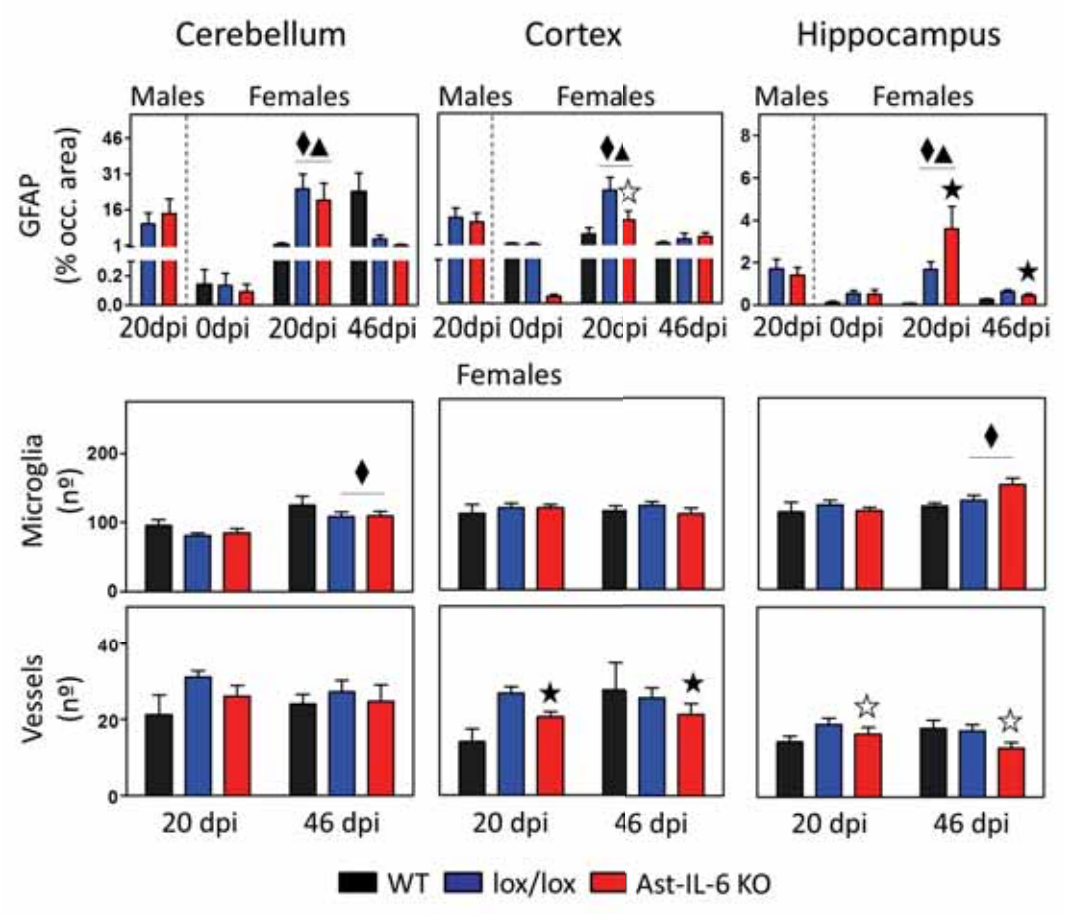


Figure 4.21: Gliosis and vasogenesis in encephalon of EAE-induced and non-induced animals. A. Results from the GFAP staining in different brain areas (Cerebellum, cerebral cortex and hippocampus) of non-induced (N = 4) and EAE-induced females at 20 (Ast-IL6 KO N= 14; Floxed N= 20; WT= 4) and 46 dpi (N= 5-8); as well as EAE-induced males at 20 dpi (N= 11-18). B. Results from tomato lectin staining at 20 and 46 dpi. We measured the number of microglia and vessels in different brain areas. ★, ♦ and ▲ P at least ≤ 0.05 vs floxed mice, 46 dpi group or control group, respectively. ☆$P \leq 0.1$ vs floxed mice.

controls (Figure 4.21).

4.7.3 IL-6 levels

IL-6 concentration in blood serum at the moment of sacrifice was measured with an ELISA kit (see Section 3.12) in 5th backcrossing animals from the first EAE experiment (N = 3-10 per genotype and sex) with no significant differences between genotypes (Data not shown). However, a significant increase in IL-6 levels in females was found compared to males (females: 51.65 ± 9.10; males: 27.47 ± 15.16 (pg/ml); $P \leq 0.05$) paralleling score results found between males and females (see Figure A.14).

4.7.4 Splenocytes nitrite production

Splenocytes from EAE females from the 5th backcrossing (experiments 2 and 3) were cultured and stimulated with either MOG or LPS at 10 μg/ml during 72 or 24 hours, respectively. Controls without any stimulation were also included. MOG- and LPS-stimulated splenocytes showed a significant increase in nitrite production compared to non-stimulated controls ($P \leq 0.001$ and 0.05, respectively). However, no significant differences between genotypes were found, but there was a non-significant trend in floxed and Ast-IL-6 KO animals to have lower nitrite production than WT animals (Figure 4.22).

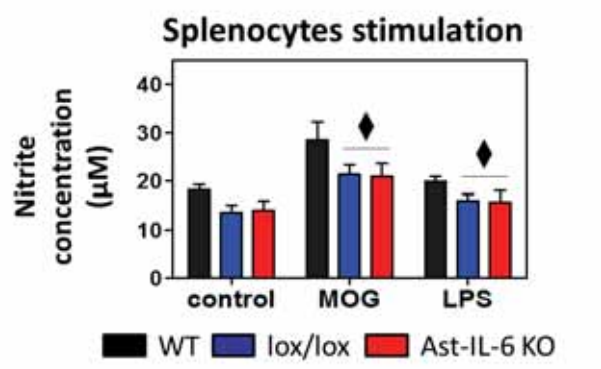


Figure 4.22: Nitrite production by splenocytes from EAE animals stimulated with 10μg/ml of either MOG or LPS. Culture supernatants were collected and, accumulated nitrite, a stable product of NO production, was measured using Griess reagent. (N = 13-16, WT = 7). Results are mean ± SEM. ♦ P at least ≤ 0.05 vs controls.

4.8 Cryolesion

4.8.1 Mortality rate and injury size

In 5th backcrossing group animals, mortality due to this procedure was similar in all genotypes (7.5%). However, in 10th backcrossing group animals mortality was higher and different between genotypes, being around 9% in floxed animals and 19% in Ast-IL-6 KO animals. Also, if Ast-IL-6 KO males and females are analyzed separatedly, we observe a 26% mortality rate in males while in females it is only 9% (data not shown).

Regarding total injury volume, which was calculated in 3 and 10 dpl floxed and Ast-IL-6 KO males and females, we again found important differences between 5th and 10th backcrossing animals (Figure 4.23).

In 5th backcrossing animals, a significant effect of genotype, sex, day of lesion ($P \leq 0.001$ in all cases) and interactions between genotype and day of lesion ($P \leq 0.05$) and a nearly significant interaction between genotype and sex ($P = 0.059$) were found. Ast-IL-6 KO mice and females showed a reduced injury volume compared to floxed animals and males, respectively. Also, injury volume was clearly decreased at 10 dpl versus 3 dpl in both genotypes. After decomposing the interaction between genotype and day of lesion,

we observe a significant decrease in lesion volume in Ast-IL-6 KO at 3dpl and a significant interaction between genotype and sex at 10 dpl ($P \leq 0.01$), because the injury volume at this day is decreased only in males ($P \leq 0.001$).

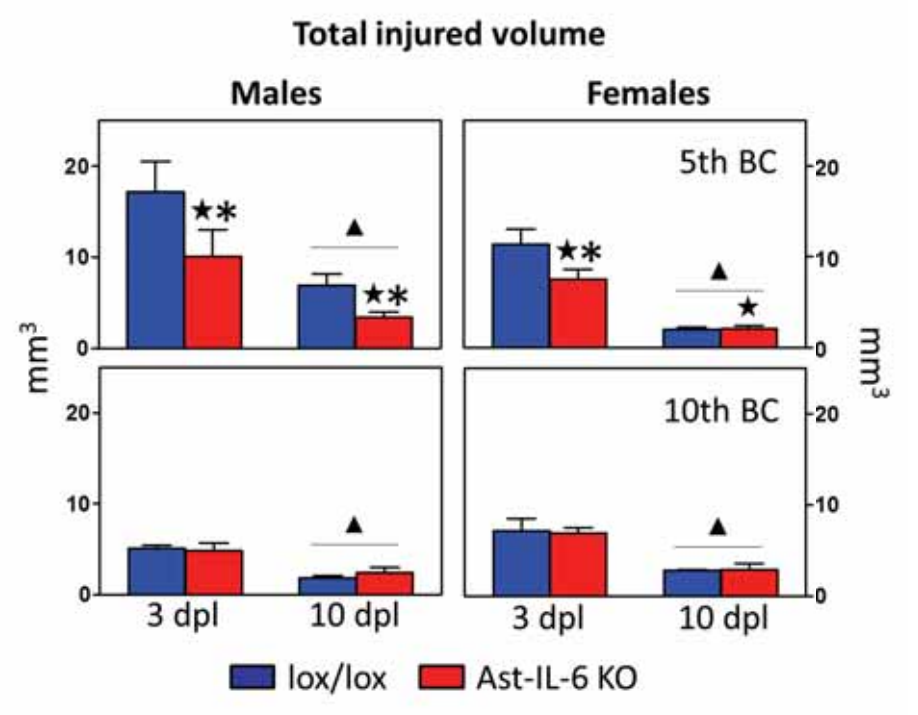


Figure 4.23: Total injury volume in Ast-IL-6 KO and floxed mice after cryolesion procedure. Injured volume is shown in 5th backcrossing (5th BC) (N = 3-8) and in 10th backcrossing (10th BC) group (N = 2-9). Results are mean ± SEM. ★ and ▲ P at least ≤ 0.05 vs floxed mice or 3 dpl group, respectively. ✻ $P \leq 0.05$ vs floxed mice after genotype interaction decomposition.

However, in 10th backcrossing animals genotype differences were not confirmed, with no significant differences between genotypes. Although total injury volume in 10th backcrossing animals was smaller than in the previous experiment, differences between sacrifice day are maintained, as injury volume is bigger at 3dpl compared to 10 dpl ($P \leq 0.001$). However, sex differences have been reversed, showing females an increased injury volume compared to males ($P = 0.001$).

4.8.2 HC and IHC of encephalon from 5th backcrossing group

4.8.2.1 Lesioned Ast-IL-6 KO presented no changes in astrogliosis but an altered gender-dependent microgliosis.

Astrogliosis in the border of the injured area presented a significant effect of gender ($P \leq 0.001$, being increased in females compared to males), day ($P \leq 0.001$) and interaction between them ($P \leq 0.05$). There were significant differences between controls and lesioned

animals and between 3 dpl and 10 dpl in all genotypes, being astrogliosis significantly increased at 3 dpl respect controls and at 10 dpl respect both 3 dpl and controls. However, there were no significant differences between genotypes (Figure 4.24). Hypertrophic reactive astrocytes appeared around the injured area with shorter and thicker processes; at 3 dpl they mostly showed isomorphic astrogliosis, mainly without overlapping neighboring astrocytes. At 10 dpl anisomorphic astrogliosis was more common, showing astrocytes hypertrophy of both the cell body and processes and overlapping neighboring astrocytes, as well as forming the glial scar, a strongly compacted limiting glial margin. In order to study reactive astrocytes morphology and intensity, the reaction with DAB was stopped when reactive cells were properly stained, which was sooner than the time needed to stain non-reactive astrocytes, that caused controls to appear without stained astrocytes. However, non-reactive astrocytes were properly studied before (see Section 4.6).

In order to assess putative differences in unlesioned tissue, astrogliosis was also evaluated in the hippocampus and the most distant area of the cerebral cortex (Figure 4.24), in both cases without any visible effect of the lack of astrocytary IL-6, although hippocampus did show a significant effect of day, being astrogliosis increased after injury (data not shown).

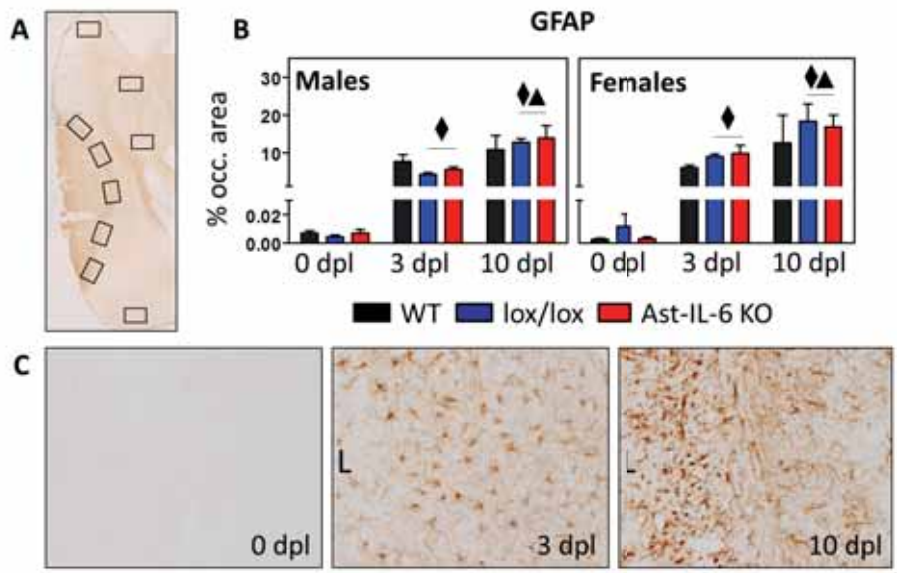


Figure 4.24: Astrogliosis assessment in Ast-IL-6 animals after cryolesion procedure. A. General view of a cryolesioned mouse encephalon. Black rectangles indicates where the images were taken to analyze GFAP staining (five images in the border of the injured area, two in the hippocampus and two in a remote part of the cortex). B. Quantification of the IHC for GFAP in the border of the injured area with a GzLM with sequential Bonferroni adjustment for genotype, sex and day of sacrifice in Ast-IL-6 KO and floxed mice. Results are mean ± SEM. N = 3-8 per genotype and sex. ♦ and ▲ P at least ≤ 0.05 vs control group or 3 dpl group, respectively). C. Representative images at 100X showing the typical process of astrogliosis after an injury in WT male mice. L stands for the lesioned side.

Microgliosis, measured by percentage of lectin staining in the border of the lesion and analysed with a GzLM with sequential Bonferroni for Ast-IL-6 KO and floxed mice, showed a significant effect of sex ($P \leq 0.01$), day of lesion ($P \leq 0.001$), interaction between them ($P \leq 0.001$) and interaction between genotype and day ($P \leq 0.05$). Thus indicating that lesioned animals presented an increased microgliosis in the border of the lesion compared to the same area in non-lesioned animals. Also, gender differences pointed out that females presented increased microgliosis compared to males and the significant interaction between genotype and day of sacrifice revealed that Ast-IL-6 KO tended to have increased microgliosis at 3 dpl and decreased microgliosis at 10 dpl respect to floxed mice. However, after decomposing this interaction we only find a marginally significant increase of microgliosis in Ast-IL-6 KO males at 3 dpl compared to floxed controls ($P = 0.063$) (Figure 4.25). Microgliosis staining was also evaluated in the hippocampus and the most distant area of the cerebral cortex, as stated for astrogliosis, without any significant effects (data not shown).

In order to discriminate between microglia and vessels (both stained with tomato lectin HC) both of them were counted in the border of the lesion and microglia was classified as basal (or ramified), reactive (hypertrophied cell with less and shorter processes) and fully activated (or round) (Figure 4.25). Statistical analysis of the results showed a significant effect of day of sacrifice in all microglia countings ($P \leq 0.001$ in all cases); a significant effect of sex in basal ($P \leq 0.01$) and reactive microglia countings ($P \leq 0.05$), being increased in females compared to males; and a significant interaction between genotype, sex and day of sacrifice in both basal and fully activated microglia ($P \leq 0.05$ and 0.01, respectively), as well as a significant genotype effect in fully activated microglia ($P \leq 0.05$), being decreased in Ast-IL-6 KO mice compared to floxed controls. Thus showing after decomposing interactions, a significant decrease in basal microglia countings from non-lesioned Ast-IL-6 KO females compared to floxed mice and a significant increase in round microglia countings from Ast-IL-6 KO males at 3 dpl (Figure 4.25).

Regarding vessels number, a significant effect of genotype, day of lesion, sex and interactions between genotype and sex and between sex and day were seen ($P \leq 0.001$), showing a decreased number of vessels in Ast-IL-6 KO compared to floxed controls and in lesioned animals compared to non-lesioned, presumably because of the reactivity of the tissue. Also, females presented an increased number of vessels respect to males. Finally, after decomposing the interaction between genotype and sex, we observe that only Ast-IL-6 KO females have a significantly decreased number of vessels compared to floxed mice (Figure 4.25).

4.8.2.2 Lesioned Ast-IL-6 KO presented an increased lymphocytary infiltration in the injured area respect to floxed mice.

Regarding lymphocytary infiltration in the brain, CD3 positive cells were counted at the border of the lesion at 3 and 10 dpl and in the equivalent area in controls (0 dpl) in Ast-IL-6 KO, floxed and WT mice, showing a significant effect of genotype ($P \leq 0.01$; being increased in Ast-IL-6 KO mice compared to floxed animals), day of lesion ($P \leq 0.001$; being increased at 10 dpl compared to both controls and 3 dpl) and interaction between geno-

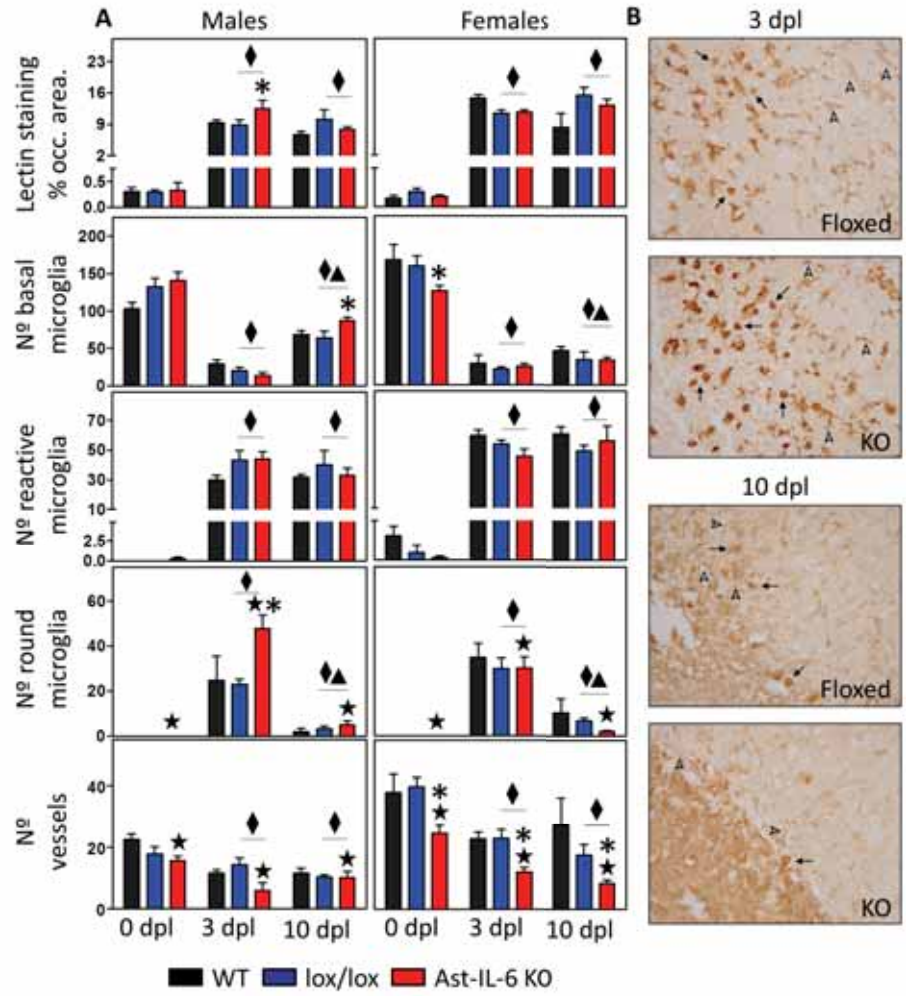


Figure 4.25: Microgliosis and number of vessels assessment in 5th backcrossing Ast-IL-6 animals after cryolesion procedure. A. Results from tomato lectin staining (N = 3-8 for gender and day post-lesion (dpl)). Percentage of stained occupied area, as well as the number of resting, reactive and fully activated microglia and vessels, are showed in the border of the lesion, at 3 or 10 dpl and in the equivalent place in non-injured mice (0 dpl). Results are mean ± SEM. A GzLM for Ast-IL-6 KO and floxed animals was performed with sequential Bonferroni adjustment. ★, ♦ and ▲ P at least ≤ 0.05 vs floxed mice, control group or 3 dpl group, respectively. ✻ $P \leq 0.05$ vs floxed mice after genotype interaction decomposition. B. Representative images at 200X of lesioned mice at 3 or 10 dpl. Arrows indicate fully activated, round microglia. Arrowheads show vessels.

type and day ($P \leq 0.01$), showing that CD3 increase in Ast-IL-6 KO compared to floxed mice was found in lesioned animals.

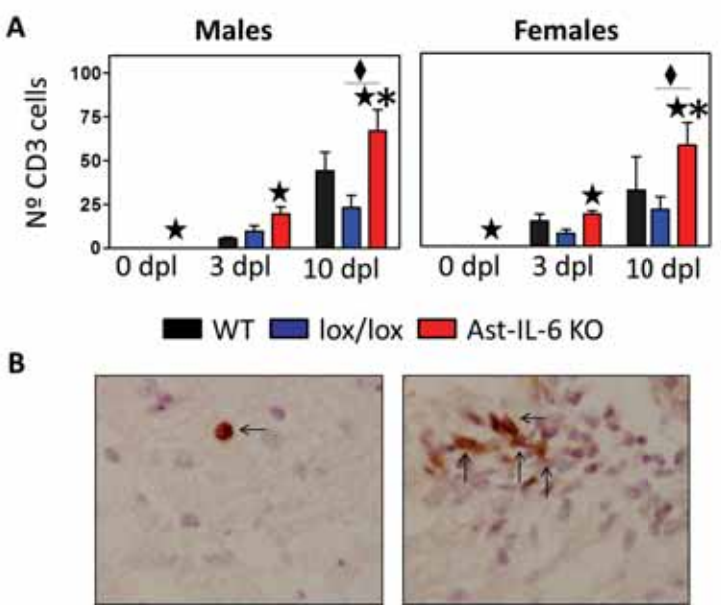


Figure 4.26: Lymphocytary infiltration in 5th backcrossing Ast-IL-6 animals after cryolesion procedure. A. Quantification of the IHC for CD3 in the brain separated by gender. Lymphocytes were counted in the border of the injured area at 3 and 10 dpl and in the equivalent area in controls at 0 dpl (N = 4-8; controls = 3-5). Results are mean ± SEM. A GzLM for Ast-IL-6 KO and floxed animals is performed with sequential Bonferroni adjustment. ★ and ♦ P at least ≤ 0.05 vs floxed mice or both control and 3 dpl group, respectively. B. Example images from CD3 IHC counterstained with hematoxylin, at 400x, of 10 dpl cryoinjured mice. Arrows mark lymphocyte CD3-positive cells.

4.9 LPS response

Ast-IL-6 males, from 5th backcrossing group (N = 5-9) were injected LPS at 1 mg/kg ip and killed 1 hour later. Both Ast-IL-6 KO and floxed mice showed significantly lower IL-6 levels in blood serum compared to WT animals ($P \leq 0.001$) with no significant differences between genotypes, although Ast-IL-6 KO animals presented a non-significant tendency ($P = 0.16$) (Figure 4.27).

Moreover, in these animals IL-6 gene expression was measured in nervous and non-nervous tissues such as cerebellum, liver and spleen. Again, floxed animals presented an important phenotype showing both Ast-IL-6 KO and floxed mice a significant decrease of IL-6 gene expression in cerebellum, liver and spleen (the last one only in Ast-IL-6 KO) compared to WT mice.

4.10 Astrocyte-enriched cell cultures

Cultures from cerebellar astrocytes did not reach confluence in any of the experiments performed. On the other hand, astrocytes from cortex worked well reaching confluence and being stimulated by LPS and INFγ (see Section 3.11.2.

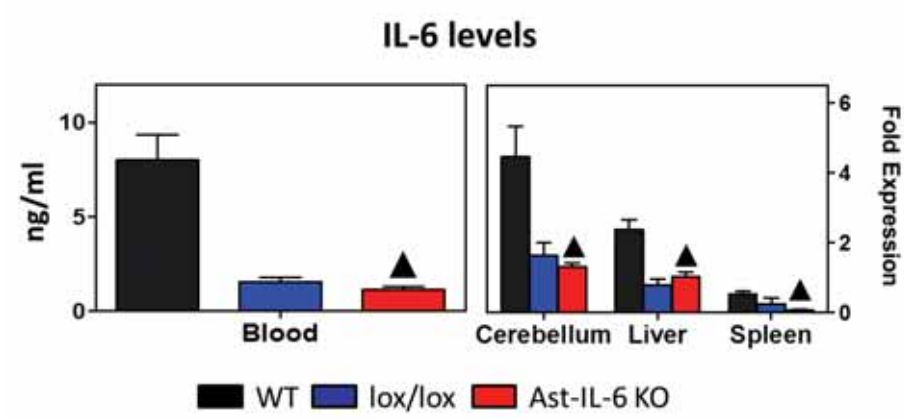


Figure 4.27: IL-6 levels in blood and tissues after 1 mg/kg LPS injection (N = 5-9). IL-6 concentration in blood serum measured with an ELISA kit (left). Fold expression of IL-6 gene in cerebellum, liver and spleen tissue measured wit rt-PCR (right). GzLM test for WT, floxed and KO mice with genotype as main factor and sequential Bonferroni adjustment was performed. ▲ P at least ≤ 0.05 vs WT mice.

IHC for GFAP did not reveal any significant differences in size or morphology between Ast-IL-6 KO, floxed or WT astrocytes. HC for lectin showed a strong presence of microglia in the cultures, possibly explaining the following ELISA results with cells supernatant (4.28).

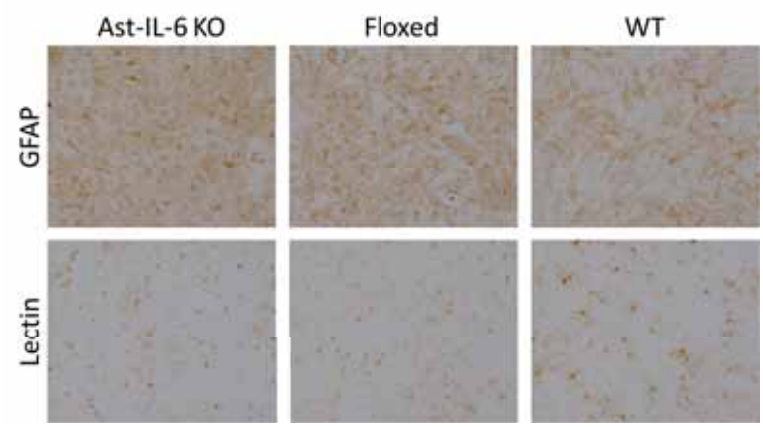


Figure 4.28: Stimulated astrocyte-enriched cell culture stainings. Representative images from IHC for GFAP (top) and HC for tomato lectin (bottom) in Ast-IL-6 KO, floxed and WT cultures after 24 hours stimulation with 10 ng/ml of LPS and 10 ng/ml of INFγ.

Neither control nor 2-hour-stimulated cells produced enough amount of IL-6 to be detectable for the ELISA kit. However, 24-hours stimulated cells of all three genotypes produced large amounts of IL-6 that required proper dilution for IL-6 analysis. A significant decrease in IL-6 levels was observed in culture media of Ast-IL-6 KO compared to both floxed and WT mice ($P \leq 0.001$ in both cases) (Figure 4.29), likely due to an impaired

astrocyte-derived IL-6 production. Floxed cells also presented significantly lower IL-6 production than in WT mice ($P \leq 0.01$).

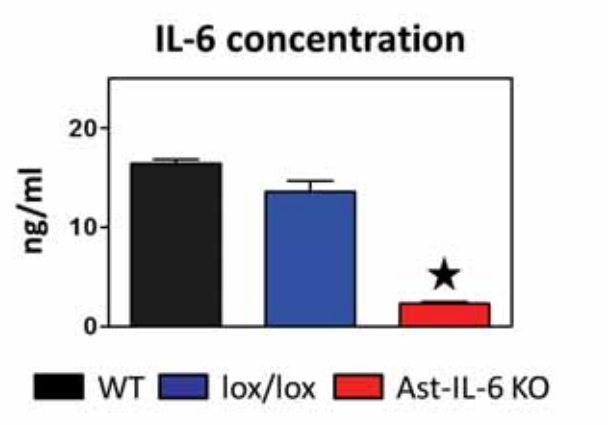


Figure 4.29: IL-6 production in astrocyte-enriched cultures. Data from three replicate wells of cells from 8 Ast-IL-6 KO, floxed and WT mice after 24 hours stimulation with 10 ng/ml of LPS and INFγ. GzLM test for WT, floxed and KO mice with genotype as main factor and sequential Bonferroni adjustment was performed. Results are mean ± SEM. ★ P at least ≤ 0.05 vs floxed mice.

CHAPTER 5

CHARACTERIZATION OF ASTROCYTIC IL-6 RECEPTOR DEFICIENCY

5.1 Frequencies at weaning

A total of 372 Ast-IL-6R mice were produced (Table 5.1). The distribution of sexes was normal, but the distribution of genotypes did not follow a Mendelian ratio, and as it happened in Ast IL-6 KO, Ast IL-6R KO mice were also clearly underrepresented at weaning ($P \leq 0.01$). Genotype of the dead mice between birth and weaning was not assessed in these animals but very few deaths were noticed. After weaning and up to four months of age, few deaths were observed, normally caused by teeth malocclusion or injuries due to fights between males.

	Ast-IL-6 Receptor		
	Observed N	Expected N	Residual
Genotype			
WT	104	91.3	12.8
Floxed	105	91.3	13.8
Heterozygous	94	91.3	2.8
KO	62	91.3	-29.3
Total	365		
Sex			
Females	179	186	-7
Males	193	186	7
Total	372		

Table 5.1: Genotype and sex frequencies at weaning in Ast-IL-Receptor animals.

At weaning Ast IL-6R KO mice were underrepresented ($\chi(3, N = 365) = 13.312$, $P = 0.004$), demonstrating a prosurvival role of astrocyte response to IL-6. Sex distribution was as expected ($\chi(1, N = 372) = 0.527$, $P = 0.468$).

5.2 Body weight

Regarding body weight, it appears that IL-6 receptor deficiency in astrocytes does not affect body weight in either males or females, being Ast-IL-6R KO animals indistinguishable from controls (Figure 5.1).

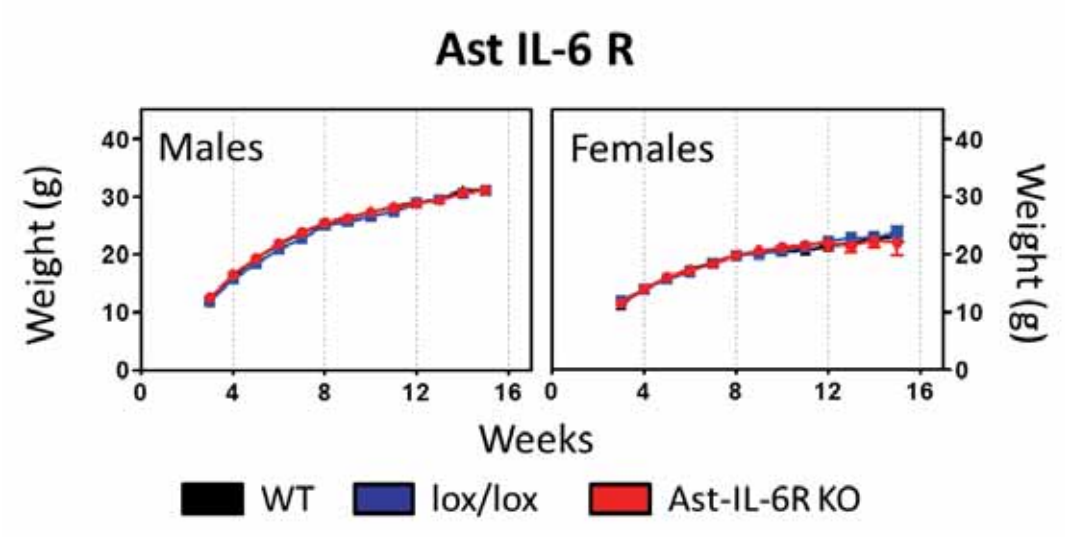


Figure 5.1: Body weight growth was not affected by astrocyte IL-6R deficiency. Mice were weighed weekly until 15 week-old (N = 21–48 at 3 weeks and N = 3–15 at 15 weeks). The GEE statistical analysis for floxed and Ast-IL-6R KO mice revealed that sex and time had a significant effect ($P \leq 0.001$, increasing weight with age and weighting males more than females), whereas there was no significant effect of genotype and any interaction between factors was significant.

5.3 Behavioural tests

5.3.1 Hole-Board

The behavioral traits observed in the Hole-Board are shown in Figure 5.2. Three month-old males and females (N = 19-30 per group) have been studied.

Total ambulation was clearly decreased in Ast-IL-6R KO mice compared to floxed mice ($P \leq 0.001$). The analysis of the separate external and internal activities revealed a consistent decrease in external activity in both sexes of Ast-IL-6R KO animals ($P \leq 0.001$). Internal deambulations, which are more related with anxiety, were also decreased in Ast-IL-6R KO animals compared to floxed controls. No differences due to sex effects were seen. Also, number of non-explored squares was significantly increased in Ast-IL-6R KO mice compared to floxed animals ($P \leq 0.05$) presumably due to the lack of motivation to explore or an increased anxiety to enter the internal squares.

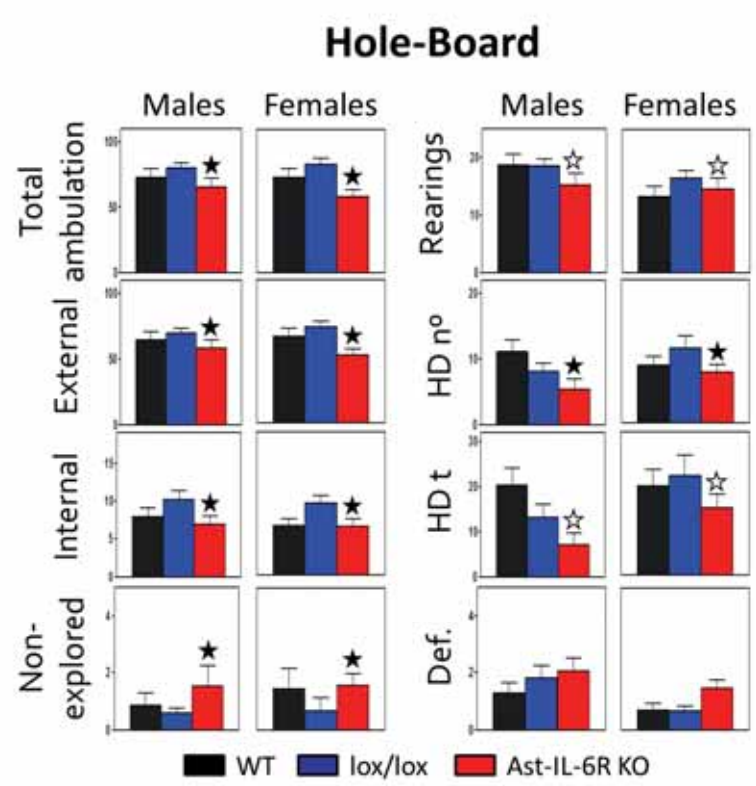


Figure 5.2: Behavioral traits analyzed in the Hole-board apparatus in Ast-IL-6 Receptor animals. Ambulation (total, external or internal squares crossed), non-visited squares, vertical activity (number of rearings), and exploratory behavior (number of head-dips (HD nº) and time (seconds) spent head-dipping (HD t)) are shown for Ast-IL-6R KO, floxed and WT mice (N = 19–30). ★ and ☆ P at least ≤ 0.05 or ≤ 0.1 vs floxed mice.

In contrast to horizontal activity, vertical exploratory activity (rearings) was only marginally significant decreased in Ast-IL-6R KO animals ($P = 0.091$). When analysing head-dipping behaviour, we observed a decreased exploratory activity in Ast-IL-6R KO animals, seen by a significant decrease in the number of head-dips and a marginally significant decrease in the time head-dipping ($P = 0.057$) in Ast-IL-6R KO animals respect to controls. Moreover, there were important differences between males and females in the overall head-dipping behavior, presenting females and increase in both number and time spent head-dipping compared to males ($P = 0.06$ and < 0.05, respectively).

Defecation was not affected by genotype, but by gender, showing males an increased defecation rate compared to females ($P \leq 0.05$).

In general, floxed mice presented either a similar phenotype than WT or an intermediate one between Ast-IL-6R KO and WT animals, although for example in internal deambulations, floxed animals showed a marked phenotype.

5.3.2 Elevated Plus-Maze

The behavioral traits observed in the EPM test for Ast-IL-6 Receptor animals are shown in Figure 5.3 and A.12.

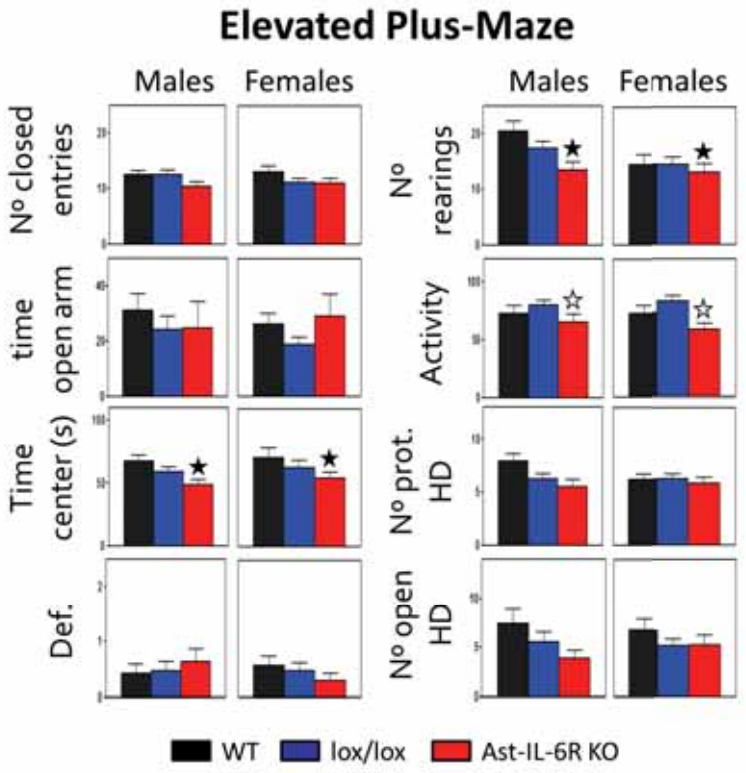


Figure 5.3: Behavioral traits analyzed in the EPM apparatus for Ast-IL-6R animals. Number of entries to closed arms, time spent in open arms, time spent in the central square (time center), defecations (Def.), number of rearings, activity (total number of squares crossed) and number of both protected and unprotected (open) head-dipps are shown for 3 month-old Ast-IL-6R KO, floxed and WT mice. N = 19–30. The results for floxed and KO mice were analyzed with GzLM for genotype and gender. ★ and ☆at least ≤ 0.05 and ≤ 0.1 vs floxed mice.

The anxiety level was assessed by measuring the number of entries and time spent into the open arms and calculating the percentage they represent respect to total entries or total time spent in all arms of the EPM [650], as well as the time spent in the central square of the plus-maze apparatus. There were no significant differences due to genotype effect except for time spent in the central square, which was significantly decreased in Ast-IL-6R KO compared to floxed controls ($P \leq 0.05$). However, there was no difference found in latency time of the first escape from center square between genotypes (see Figure A.12).

Less conventional traits for testing anxiety, as rearings and head-dips (total, protected and unprotected) [651], were assessed. The number of rearings, as in hole-board, was decreased in Ast-IL-6R KO compared to floxed mice ($P \leq 0.05$). However, regarding the

head-dipping behavior, both the total number and protected and open head-dips showed a non-significant trend to be decreased specially while being compared to WT mice. Finally, defecations were again not affected by genotype.

In contrast to the hole-board results, the general locomotor activity assessed as number of entries into the closed arms [650] did not show alterations due to genotype effect. However, total number of squares crossed tended to be decreased in Ast-IL-6R KO mice compared to floxed mice ($P = 0.06$). No differences between sexes were observed.

Any of the behavioral traits analysed in plus-maze test for Ast-IL-6 Receptor group showed a significant sex effect or interaction between genotype and sex, in contrast to Ast-IL-6 group in which this was common. As in HB results, generally floxed mice presented either a similar phenotype than WT or an intermediate one between Ast-IL-6R KO and WT animals.

5.3.3 Tail Suspension

Time mice spent in motion (agitation time) while being suspended by their tail is shown in Figure 5.4.

Three months-old animals from both genders (N = 4-13 per group) have been studied. We have used Generalized estimating equations (GEE) in Ast-IL-6R and floxed animals for genotype, gender and time. There was no significant effect of genotype but a significant effect of gender ($P \leq 0.05$) and time ($P \leq 0.001$) showing that females remained more time in agitation than males and that all animals stayed in motion significantly less time in the second and third 2-min block than in the first one.

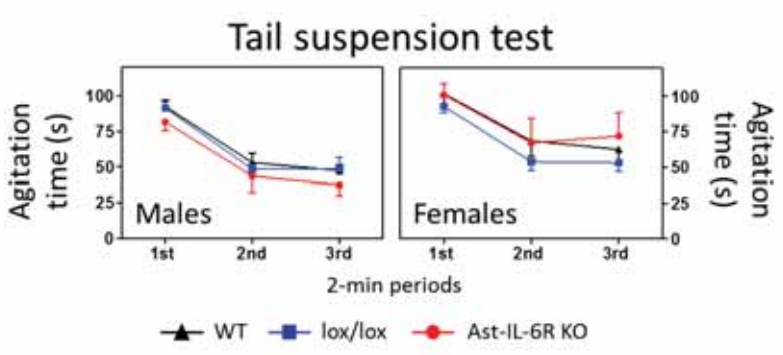


Figure 5.4: Time spent in motion during tail suspension test in Ast-IL-6R animals. Total duration of the test is shown in 3 blocks of two minutes and agitation time (in seconds) is presented (N = 4–13).

5.3.4 Morris water maze

Three month-old males and females Ast-IL-6R KO, floxed and WT mice have been studied are results are presented in figures 5.5 and 5.6.

The first test protocol performed in MWM was Cued learning, which tests animals for their ability to learn to swim to a cued goal. Animals were given four trials and the mean is presented in Figure 5.5.

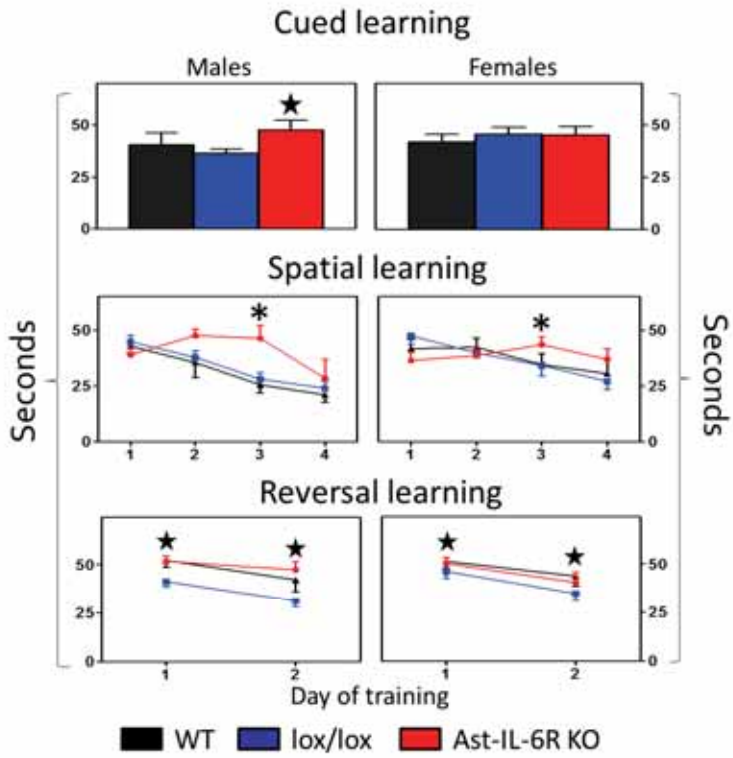


Figure 5.5: Time to reach the platform in cued, spatial and reversal learning in MWM. Three month-old animals from Ast-IL-6 Receptor group have been studied (N = 6–20). Ast-IL-6R KO and floxed mice were analyzed with GzLM for genotype and gender in cued learning and with GEE for genotype, gender and day for spatial and reversal learning. All corrected with Sequential Bonferroni adjustment for multiple comparisons. ★ P at least ≤ 0.05 vs floxed mice. ✻ ≤ 0.05 vs floxed mice after genotype interaction decomposition.

As in Ast-IL-6 experiment, in first trials many animals did not want to swim away from the wall or jumped back into the water after climbing the platform, but this was clearly improved at the end of cued learning last trial. There was no significant effect of genotype or sex but a marginally significant interaction between genotype and sex ($P = 0.083$) was present, due to a tendency to be increased only present in Ast-IL-6R KO males. If a GzLM was performed for each sex, the increased time to find the platform in Ast-IL-6R KO males was significant ($P \leq 0.05$).

All animals were capable in at least one trial to reach and climb the visible platform. Animals performing floating behavior one or more test-days were equally distributed among genotypes (data not shown) and they were not excluded from the experiment because they also presented periods of swimming.

The second protocol tested spatial learning acquisition. Animals were given four tri-

als a day during four days and the mean for each day is shown in Figure 5.5, where we can observe that the last day (4th) animals had learnt the spatial location of the platform as the time to reach it was significantly faster compared to the first three days (day effect: $P \leq 0.001$). There was no significant effect of genotype or sex but a significant interaction between genotype and day was found ($P \leq 0.001$), showing that Ast-IL-6R KO mice learned slower as they needed significantly more time to find the platform at day 3 ($P \leq 0.05$).

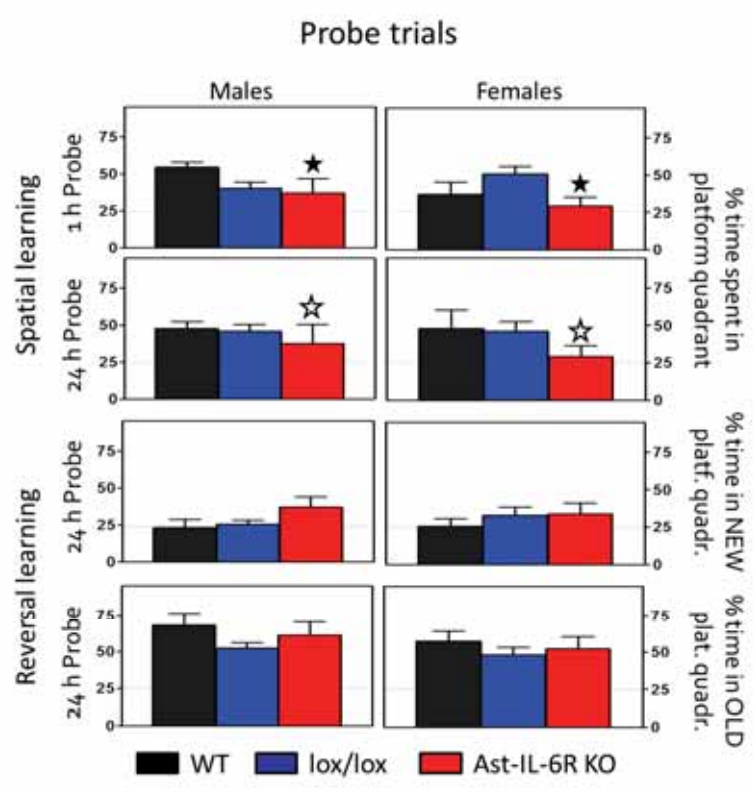


Figure 5.6: Percentage of time spent in the platform quadrant during Probe trials in Ast-IL-6R animals. Three month-old Ast-IL-6R KO, floxed and WT mice have been studied (N = 6–20). In the top two panels it is shown probe trials for spatial learning (1 and 24 hours after last trial). In the bottom two panels it is shown the percent of time spent in the quadrant that allocated the platform during reversal learning (the new location) and that in the quadrant that allocated the platform during spatial learning (the old location) for 24 hours probe trials for reversal learning. Ast-IL-6R KO and floxed mice were analysed with GzLM for genotype and gender. ★ and ☆P at least ≤ 0.05 and ≤ 0.1 vs floxed mice, respectively.

To assess reference memory at the end of those 4 days learning, a probe trial (a 30 seconds trial without platform) was given 1 hour after last trial and 24 hours later to differentiate between shorter and longer memory. Percentage of time spent in the quadrant which previously allocated the platform are presented in Figure 5.6. Results show that animals had learnt the location of the platform, staying more than the 25% of the time in the correct quadrant. Nevertheless, Ast-IL-6R KO showed significant impaired memory

1 hour after the last training session ($P \leq 0.05$) and a marginally significant impairment 24 hours later ($P = 0.094$), thus supporting worse learning in Ast-IL-6 KO. There were no significant effect of sex or interaction.

Immediately after the second probe trial, animals performed two days of reversal learning with four trials a day, with the platform located in the opposite quadrant than that in spatial learning acquisition. Mean results for each day are shown in Figure 5.5. Mice do learn the new location as they needed significant less time to find the platform in the second day (day effect: $P \leq 0.001$). Ast-IL-6R KO mice again performed worse this task since they showed a significant increase in the time needed to find the new location of the platform compared to floxed controls, without differences between sexes.

Once reversal learning was finished animals were given a probe trial 24 hours after the last trial, where it was measured not only the percent of time spent in the quadrant with the newer location of the platform (the opposite from the spatial acquisition) but also the percent of time spent in the old platform quadrant (the one that allocated the platform during spatial acquisition). Results, presented in Figure 5.6, show that animals barely did better than the 25% chance time, not remembering the new location of the platform, with no significant differences between genotypes or sexes. Surprisingly, when analysing time spent in the quadrant of the old location of the platform we see it is still largely revisited, even more than in spatial probe trials, with no significant differences between genotypes or sexes.

Generally, in all MWM tests, floxed and WT mice showed a similar phenotype.

5.3.5 Resident Intruder

The behavioral traits observed in the resident-intruder test are shown in Figure 5.7 and statistical results analyzed with GzLM for genotype in each condition (resident or intruder) and comparing both conditions are presented in Table 5.2.

Regarding aggressive behaviour, we have analysed latency to first attack, in which there was a significant increased latency in resident Ast-IL-6R KO compared to floxed mice, and the opposite non-significant tendency in intruders. Consistent with this data are the number of attacks, which were marginally significant decreased in resident Ast-IL-6R KO ($P = 0.098$). Freezing time was also analysed without any genotype differences (data not shown). The summation of other aggressive behaviours different from the number of attacks, such as active and passive chases, upright and crouched postures and tail rattles showed no differences between genotypes. However, when analysing tail rattles alone, Ast-IL-6R KO intruders showed a marginally significant increase compared to floxed mice (Ast-IL-6R KO: 3.6 ± 1.6; Floxed: 1.25 ± 0.61; $P = 0.081$) being increased in Ast-IL-6R KO intruders. When comparing both conditions together, we observed that these agressive behaviours were performed in greater number when animal acted as intruder, likely in an attempt to escape from the territorial defensive behaviour of the other male ($P \leq 0.01$).

Regarding affiliative behaviour, there were no differences in genotype when analysing separately each condition. However, when analysing resident and intruders together, we observed that both crawl-under and allogrooming behaviours were significantly increased in Ast-IL-6R KO compared to floxed mice ($P \leq 0.05$). Affiliative behavior was also

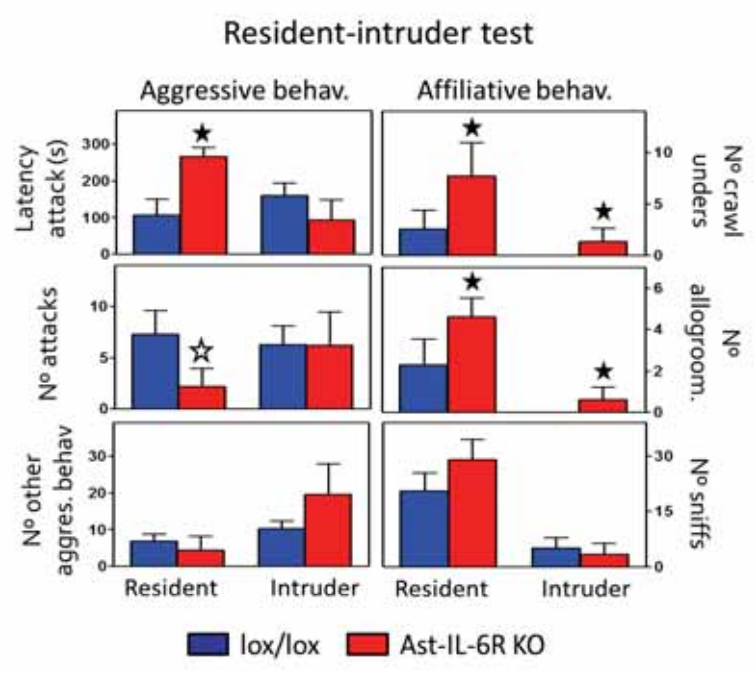


Figure 5.7: Behavioral traits analyzed in the Resident-intruder test for Ast-IL-6R mice. Latency to the first attack (seconds), number of attacks and number of aggressive behaviour other than attacks (activee and passive chases, tail rattles, crouched postures and upright postures) are shown as aggressive behaviour. Meanwhile, number of crawl-under behaviour, number of allogrooming behaviour and number of sniffings (as the summation of nose, body and ano-genital sniffs) are shown as affiliative behaviours in four month-old Ast-IL-6R KO and floxed male mice. N = 5-8. ★ and ♦ at least ≤ 0.05 vs floxed or resident mice, respectively.

significantly increased in residents respect to intruders (Crawl-unders and allogroomin: $P \leq 0.001$ and sniffs: $P \leq 0.01$).

5.4 CNS immunohistochemistry

Gliosis was evaluated by GFAP (astrocytes) and lectin (microglia) staining of encephalon of 7 week-old mice. There were no significant differences in gliosis between Ast-IL6R KO mice and floxed mice in any of the brain areas studied (Figure 5.8). Regarding IL-6Rα staining, Ast-IL6R KO presented significantly less staining compared to floxed mice in both cerebellum and cortex (Figure 5.8).

5.5 EAE

5.5.1 Symptomatology

We have carried one EAE experiment in Ast-IL-6R KO, floxed, WT and heterozygous mice. The latter genotype is not shown for the sake of simplicity. Figure 5.9 shows the clinical

	Ast-IL-6R				
	Resident	Intruder		Together	
	Genotype	Genotype	Genotype	Condition	Interaction
Latency attack	**	-	-	-	**
Freezing time	-	-	-	-	-
Nº attacks	-	-	-	-	-
Nº aggres. behav	-	-	-	**	-
Nº crawl-unders	-	-	*	**	-
Nº allogroomings	-	-	*	***	-
Nº sniffs	-	-	-	***	-

Table 5.2: Summary of obtained significances in Resident-intruder test for Ast-IL-6R animals.

By condition is meant acting as a resident or as a intruder. Nº aggres. behav. stands for the number of agressive behaviour other than attacks (Nº of active and passive chases, tail rattles and crouched and upright postures). *, ** and *** P at least ≤ 0.05, ≤ 0.01 and ≤ 0.001.

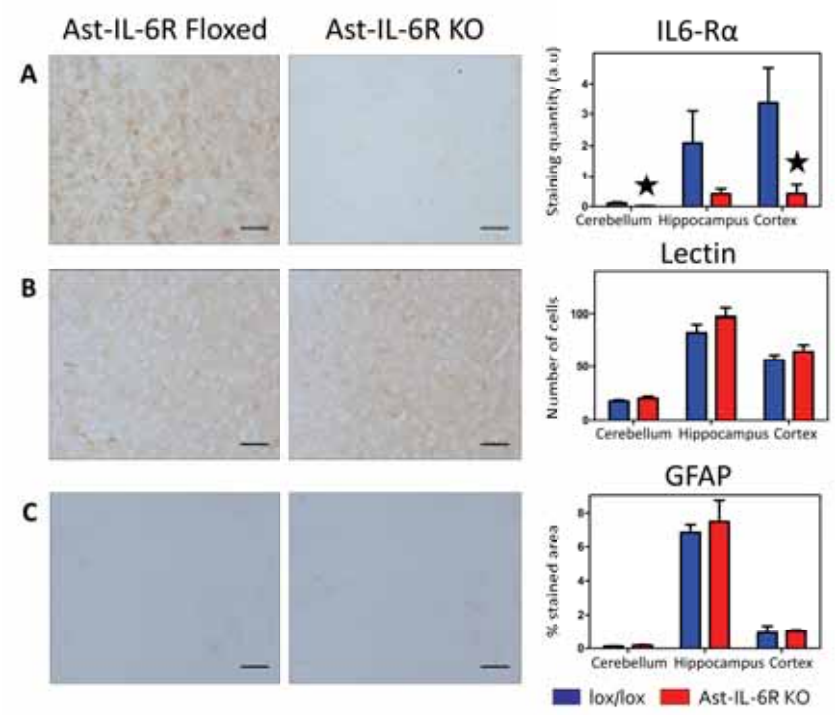


Figure 5.8: IL-6Rα, Lectin and GFAP stainings and quantifications in Ast-IL-6R KO and floxed mice. A-C. Representative cortex stainings from Ast-IL-6R KO and floxed controls (left) and staining quantifications in cerebellum (Cereb.), hippocampus (Hipp.) and cortex (right). A. IL-6Rα staining and quantification. B. Lectin staining and lectin-positive cells countings instead of quantification, since blood vessels were also stained. C. GFAP staining and quantification. Magnification 100X (Bar 50μm). N = 4–9. ★ P at least ≤ 0.05 vs floxed mice.

score and the body weight changes observed following MOG_{35-55} immunization (day 0) until 22 days post-immunization (dpi).

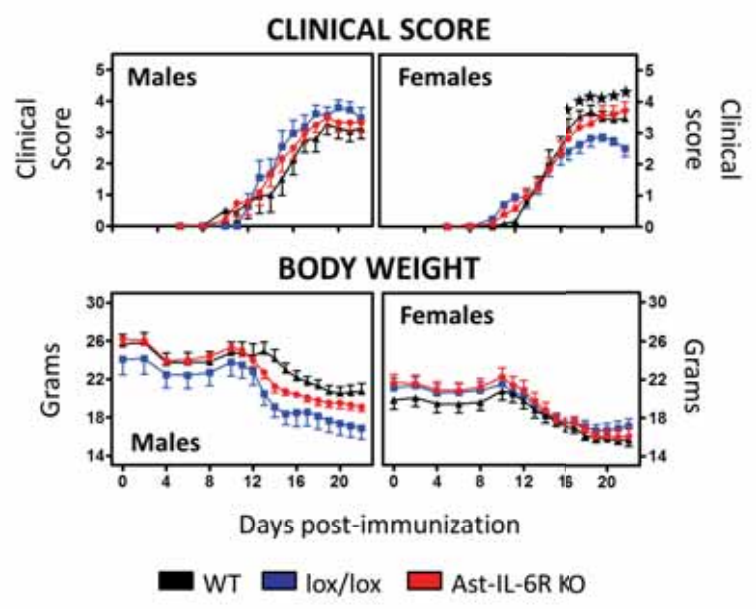


Figure 5.9: Clinical score and body weight changes following EAE induction. Data from 3 month-old Ast-IL-6R KO, floxed and WT mice (N = 12-14 for genotype and gender) is presented. GEE statistical model for genotype, gender and day as main factors with sequential bonferroni adjustment, is used from day 17 to 22 post-induction, when differences arises between genotypes. ★ at least ≤ 0.05 vs floxed mice.

As we can see in Table 5.3, incidence of the disease was 100% in this experiment. Mortality rate was around 16% without significant differences due to genotype effect. All genotypes showed the prototypical ascending paralysis course with body weight loss without any delaying due to genotype effect. However, we observed an impaired remission of the disease at day 22 in Ast-IL-6R KO mice from both sexes compared to floxed animals, as well as an increase of the peak score in Ast-IL-6R KO females. Moreover, outcome score presented a significant interaction between genotype and gender ($P \leq 0.5$) showing an increased outcome score in KO females compared to floxed ($P \leq 0.01$) in accordance with grade of remission data.

Regarding clinical score, a GEE for genotype, gender and day, with sequential Bonferroni adjustments for multiple comparisons is made from day 8 to 22 after induction, obtaining a significant effect of day and a marginally significant interaction between genotype and day ($P = 0.000$ and 0.079, respectively). However, when data only from day 17 to 22 is considered, there is a significant interaction between genotype and sex ($P \leq 0.05$) since a GEE in females showed a significant increase in the clinical score of Ast-IL-6R KO compared to floxed controls ($P \leq 0.01$), while the opposite tendency was observed in males.

Regarding body weight, a GEE made from days 0 to 22 after induction showed a significant interaction of genotype, gender and day ($P \leq 0.05$) due to a significant loss of

Ast-IL-6 Receptor				DAY 22			
Females	**Incidence**	**Mortality**	**Day of onset**	**Time of Peak Score**	**Peak score**	**Cumulative score**	**Grade of remission**
WT	12/12	2/12	14.25 ± 0.49	17.50 ± 0.39	3.79 ± 0.18	27.12 ± 1.94	0.33 ± 0.16
Floxed	12/12	0/12	13.66 ± 0.80	16.50 ± 0.89	3.33 ± 0.15	24.29 ± 2.30	0.83 ± 0.21
KO	14/14	3/14	13.64 ± 0.59	18.21 ± 0.64	3.96 ± 0.24 ☆	27.25 ± 1.88	0.25 ± 0.09 ★
Males	**Incidence**	**Mortality**	**Day of onset**	**Time of Peak Score**	**Peak score**	**Cumulative score**	**Grade of remission**
WT	12/12	2/12	14.91 ± 0.67	17.41 ± 0.74	3.41 ± 0.25	24.62 ± 4.56	0.29 ± 0.11
Floxed	12/12	3/12	14 ± 0.45	16.75 ± 0.95	3.91 ± 0.23	30.54 ± 3.52	0.45 ± 0.19
KO	13/13	3/13	13.23 ± 0.56	16.23 ± 0.83	3.65 ± 0.24	28.30 ± 3.44	0.34 ± 0.11 ★

Table 5.3: EAE features in Ast-IL-6R induced animals. Animals, killed 22 dpi, are shown separated by gender and genotype. For each animal we determined the time to disease onset (clinical score or ataxia ≥ 1), time to peak disease, peak-score, cumulative score (sum of all scores from disease onset to day 22), outcome (final score at day 22, fig 5.9) and grade of remission (difference between peak score and outcome). Results are mean ± SEM. For Statistical analysis, a GzLM for genotype and gender was performed with sequential Bonferroni adjustments. ★ or ☆ at least ≤ 0.05 or ≤ 0.01 vs floxed mice, respectively.

body weight after EAE induction in all animals and a non-significant trend in KO males to have a decreased body weight loss respect to floxed animals. When analysing data only from days 13 to 22 after induction this trend became marginally significant for males (P = 0.098).

5.6 Cryolesion

5.6.1 Mortality rate and injury size

Mortality due to this procedure was extremely elevated in this animals (around 50%) compared to Ast-IL-6 group, which forced us to perform the cryolesion procedure in two separate experiments in order to achieve enough animals to be studied. Although this experiment must be replicated in the future with another backcrossing strategy, we nevertheless show here results obtained until now. In the first experiment all animals were killed at 3 dpl and in a second experiment months later all animals were killed at 10 dpl. In this second experiment mortality was again very high, being around 50% in WT mice, 46.6% in floxed mice and 59% in Ast-IL-6R KO mice. When separated by gender, we observed that floxed and WT females presented an slightly lower mortality rate respect to males (40% in WT females compared to 60% in WT males and 42.8% in floxed females compared to 50% in floxed males) but in Ast-IL-6R KO mice we saw the opposite situ-

ation, with KO males showing a mortality rate of 37.5% while females presented nearly 78% of mortality (The number of animals under cryolesion procedure were N = 8-11 for floxed and KO mice and N = 5-7 for WT, all per gender and day).

Regarding total injury volume, which was calculated in the same way as in Ast-IL-6 animals, in 3 and 10 dpl WT, floxed and Ast-IL-6R KO mice, we did not find a significant genotype effect but we found a significant effect of gender ($P \leq 0.05$), day of sacrifice ($P \leq 0.01$) and interaction between genotype and day ($P \leq 0.05$) and between gender and day ($P \leq 0.05$) when Ast-IL-6R KO and floxed mice were analysed with a GzLM with sequential Bonferroni adjustments. Thus showing that females presented a decreased injured volume compared to males and that injured volume at 10 dpl was bigger than those at 3 dpl. After decomposing the interaction between genotype and day we found no differences at 10 dpl but a marginally significant decrease in Ast-IL-6R KO injured volume at 3 dpl compared to floxed animals ($P = 0.066$). However, is important tot point out that these are not reliable comparisons as 3 and 10 dpl sacrifices were performed at different days and both showed extremely high mortality rates.

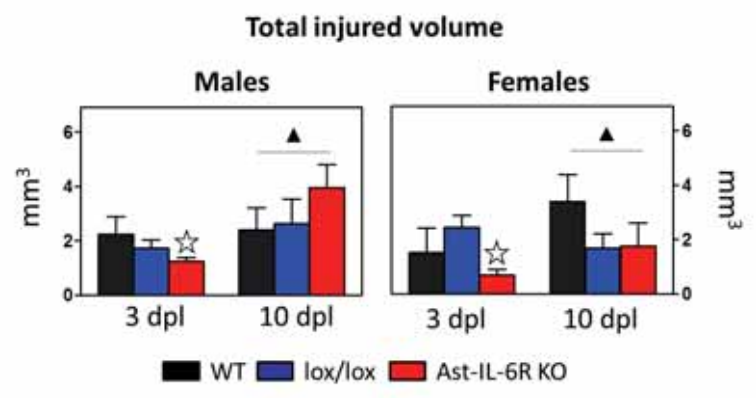


Figure 5.10: Total injured volume in Ast-IL-6R KO, floxed and WT mice after cryolesion procedure. Injured volume is shown at 3 dpl (N = 4-8) and 10 dpl (N = 2-5). Results are mean ± SEM. ▲ at least ≤ 0.05 vs 3 dpl group, and ☆at least ≤ 0.1 vs floxed mice.

5.6.2 IL-6 levels in blood

Blood from lesioned animals was collected at 3 dpl and IL-6 levels were analysed in serum. Data was analysed with a GzLM for genotype and gender as main factors with sequential Bonferroni adjustment. A significant effect of genotype ($P \leq 0.05$) was found, indicating that IL-6R KO presented an increased IL-6 concentration in blood 3 days after traumatic brain injury (see Figure 5.11). There were no significant effect of sex or any interaction between genotype and sex.

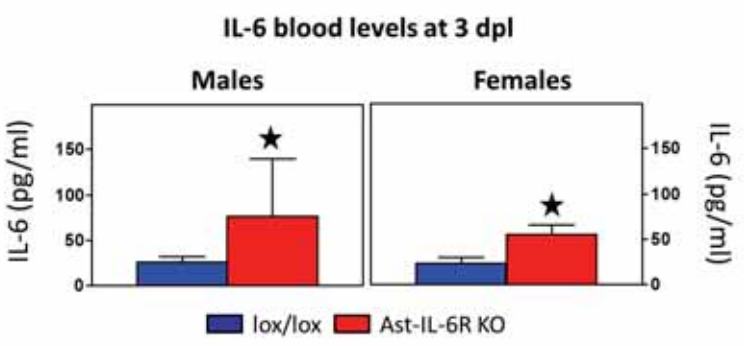


Figure 5.11: IL-6 blood levels at 3 dpl. IL-6 concentration was measured in blood serum with an ELISA kit at 3 dpl (N = 4-8 per group). Data was analysed with GzLM for genotype and gender with sequential Bonferroni adjustment for multiple comparisons. Results are mean ± SEM. (★ at least ≤ 0.05 vs floxed mice).

CHAPTER 6

CHARACTERIZATION OF TRANS-SIGNALING IN MEDIATING THE BIOLOGICAL ACTIONS OF IL-6

6.1 The severity of gliosis is diminished in GFAP-IL6/sgp130 mice

As the development of astrogliosis and microgliosis is particularly pronounced in the cerebellum of the GFAP-IL6 mice [252] the degree of gliosis in the cerebellum of the GFAP-IL6 versus GFAP-IL6/sgp130 mice by GFAP (astrocyte marker) IHC and lectin HC (microglial marker) was examined.

Regarding cerebellar astrogliosis, a GzLM for genotype and age revealed a significant effect of genotype ($P \leq 0.001$), age ($P \leq 0.05$, increasing with age) and interaction between genotype and age ($P \leq 0.001$); therefore data was split by age and sequential Bonferroni post-hoc analyses were carried out at each age. Cerebellar astrogliosis was increased prominently in GFAP-IL6 mice, while there was an age-dependent inverse opposing effect of soluble sgp130 protein: a complete reversal of the GFAP-IL6 phenotype was observed in GFAP-IL6-sgp130 mice at 1.5 months of age while no significant differences were seen at 9 month-old mice (Figure 6.1). Regarding astrogliosis in cortex, statistical analysis revealed a significant effect of genotype ($P \leq 0.001$) and age ($P \leq 0.001$), sequential Bonferroni post-hoc analyses were carried out at each age, showing that GFAP staining was increased in both GFAP-IL6 and GFAP-IL6/sgp130 mice compared to sgp130 and WT controls, but GFAP-IL6/sgp130 mice also presented decreased astrogliosis respect to GFAP-IL-6 mice, before 9 month-old (Figure 6.1).

Regarding microgliosis (Figure 6.2), a GzLM for genotype and age revealed a significant effect of genotype ($P \leq 0.001$), age ($P \leq 0.001$, increasing with age) and interaction between genotype and age ($P \leq 0.001$) showing that the number of lectin-positive cells with activated morphology and increased size was dramatically increased in cerebellum of GFAP-IL6 mice at all ages studied; these increases were significantly reduced in GFAP-IL6-sgp130 mice, althougth they were still increased compared to WT and sgp130 mice. No significant difference in the number of activated microglial cells was found in WT versus GF-sgp130 mice. On the other hand, the number of lectin-positive cells with resting

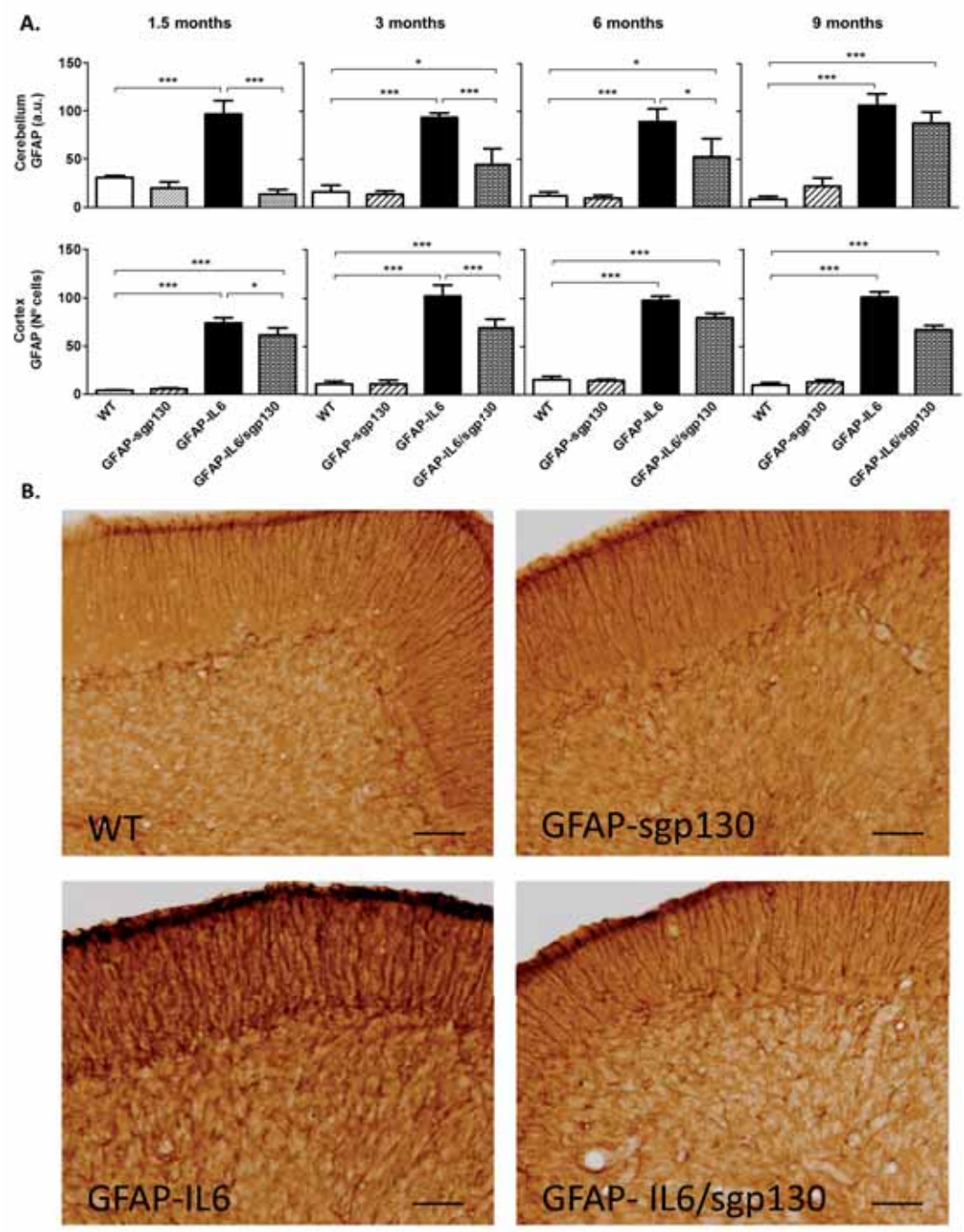


Figure 6.1: Astrogliosis assessment in GFAP-IL6, GFAP-IL6/sgp130, GFAP-sgp130 and WT mice. A. Staining intensity of GFAP IHC data at different ages. N = 3-4. Results are mean ± SEM. For Statistical analysis, a GzLM for genotype and gender was performed with sequential Bonferroni post-hoc adjustment for multiple comparisons. *, ** and *** at least ≤ 0.05, 0.01 and 0.001 between the selected genotypes. B. Representative images at 200X showing GFAP IHC in cerebellum from 1.5 month-old animals. Bar: 50 μm.

morphology showed an age-dependent decrease in the GFAP-IL6 mice that was reversed in GFAP-IL6-sgp130 mice. In contrast to cerebellum, minor changes in lectin staining were observed in cortex and hippocampus (data not shown).

6.2 Reduced vascular alterations and blood-brain barrier leakage in GFAP-IL6-spgp130 mice

Vasculopathy with BBB leakage and angiogenesis principally affecting the cerebellum has also been described previously in the GFAP-IL6 mice [252]. The number of blood vessels was initially quantified in lectin-stained brain sections in mice from 1.5 to 9 month-old and showed that there was a significant increase in the cerebellum of GFAP-IL6 but not GFAP-IL6/sgp130 mice (Figure 6.3). This increase was not seen in cortex (Data not shown). Consistent with the change in the number of blood vessels, angiogenesis as evaluated with endothelial cell BrdU labeling was increased significantly in cerebellum of GFAP-IL6 mice but not in GFAP-IL6/sgp130 mice when compared with the control genotypes (Figure 6.3). These findings indicated that there was a marked decrease in overall vascular disturbance and in angiogenesis in the cerebellum of the GFAP-IL6/sgp130 mice compared with those of GFAP-IL6 mice.

In parallel, Dr Campbell's group examined the overall morphology of the blood vessels in the brain of young animals using laminin IHC. Compared with WT and sgp130 control littermate, in cerebellum from GFAP-IL6 mice there was increased laminin deposition and associated marked dilation of the blood vessels [633]. These vascular changes observed in the GFAP-IL6 cerebellum were reduced noticeably in cerebellum from the GFAP-IL6/sgp130 animals. Also, to assess the degree of BBB leakage, Western blot analysis for murine IgG on lysates of cerebellum prepared from mice that had been extensively perfused with PBS was performed. The results showed that there was a significant increase in IgG present in lysates prepared from cerebellum of the GFAP-IL6 mice compared with WT and GFAP-sgp130 mice. Although significantly higher than in WT and GFAP-sgp130 mice, the level of IgG present in lysates of cerebellum from the GFAP-IL6/sgp130 mice was significantly lower than in the corresponding GFAP-IL6 mice [633].

6.3 Hippocampal neurogenesis is rescued in GFAP-IL6/sgp130 mice

It is known that hippocampal neurogenesis is markedly impaired in GFAP-IL6 mice [314]. In order to assess whether this impaired neurogenesis was influenced by IL-6 trans-signaling, doublecortin (DCX) and DCX/BrdU positive neurons were examined by IHC in the dentate gyrus of the hippocampus (Figure 6.4). A GzLM for genotype and age revealed a significant effect of genotype ($P \leq 0.001$, being decreased in GFAP-IL-6 compared to controls), age ($P \leq 0.001$, decreasing with age) and a nearly significant interaction between genotype and age ($P = 0.057$ and 0.055) in both DCX- and DCX/BrdU- positive cell counts; therefore data was split by age and sequential Bonferroni post-hoc analyses were carried

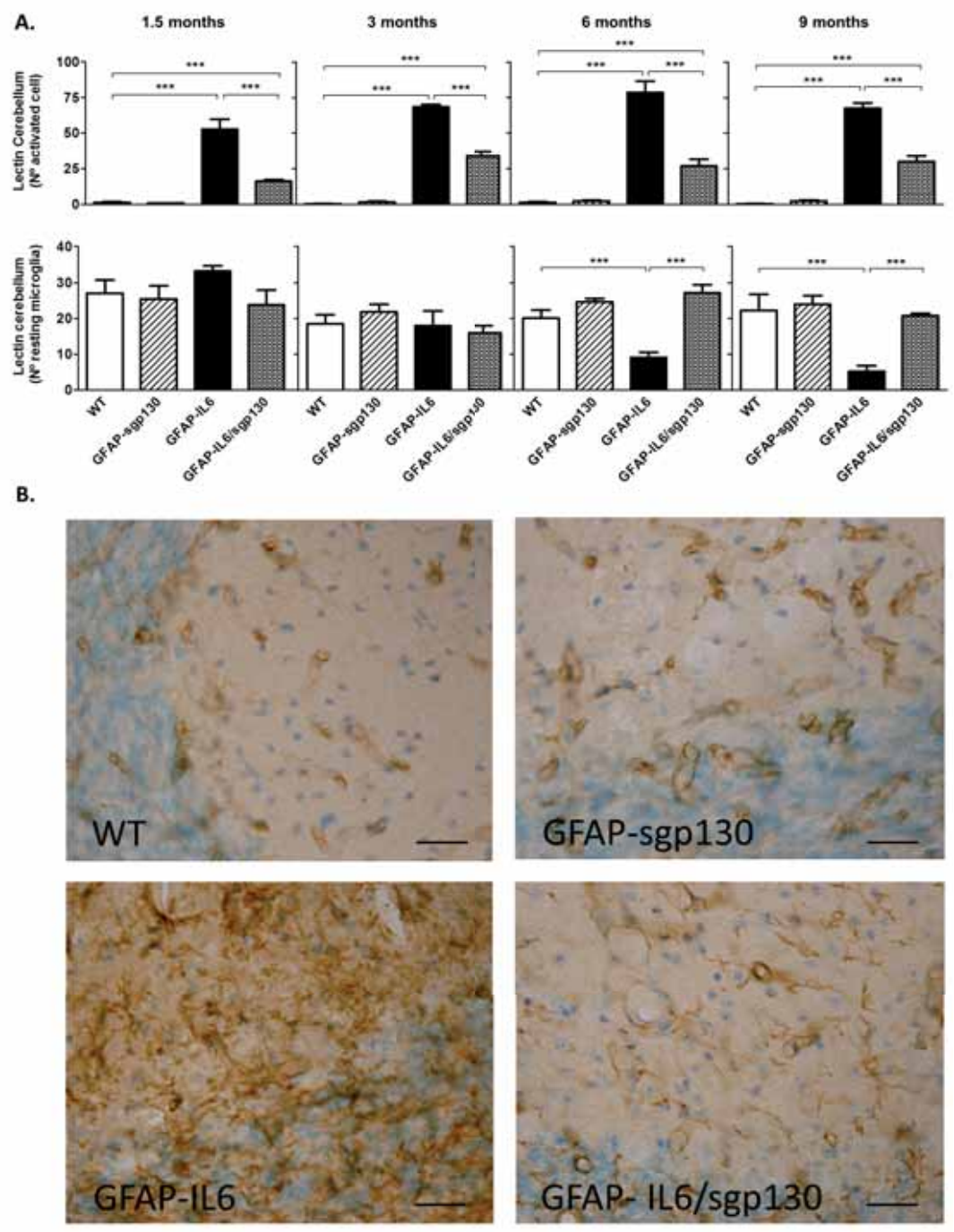


Figure 6.2: Microgliosis assessment in GFAP-IL6, GFAP-IL6/sgp130, GFAP-sgp130 and WT mice. A. Number of activated and basal microglia cells at different ages. N = 3-4. Results are mean ± SEM. *** at least ≤ 0.001 between the selected genotypes. B. Representative images at 400X showing lectin staining counterstained with Nissl in cerebellum from 3 month-old animals. Bar: 25 μm.

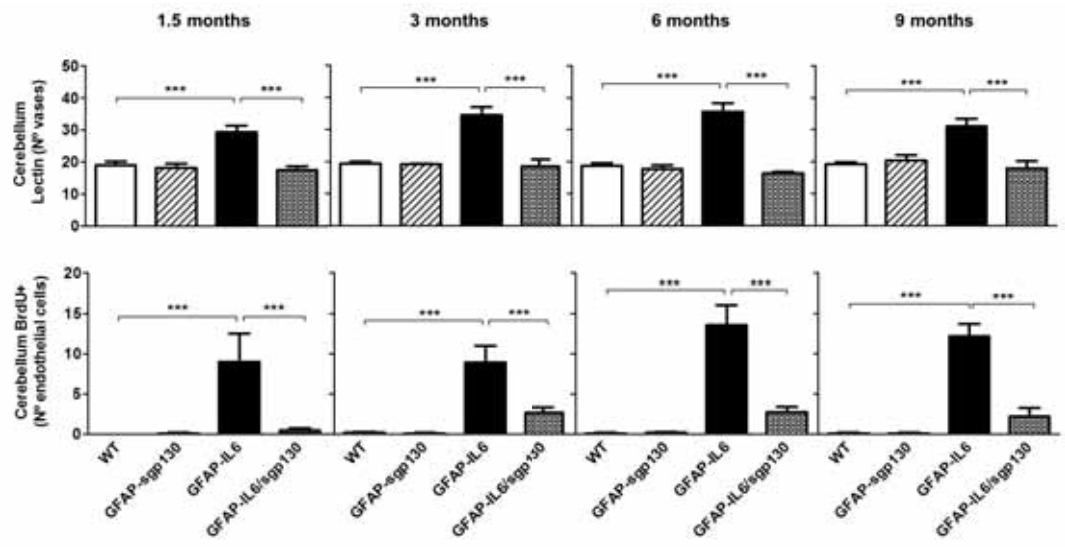


Figure 6.3: Blood vessels and BrdU+ endothelial cells assessment in cerebellum of GFAP-IL6, GFAP-IL6/sgp130, GFAP-sgp130 and WT mice. Number of blood vessels (top) and BrdU+ endothelial cells (bottom) in cerebellum from different age mice of the four genotypes of study. N = 3-4. Results are mean ± SEM. *** at least ≤ 0.001 between the selected genotypes.

out at each age. In accordance with the previous report [314], compared with controls, the number of DCX-positive cells was significantly lower in GFAP-IL6 mice ($P \leq 0.001$) at all ages. However, a significant age-dependent rescuing effect was observed in GFAP-IL6/sgp130 mice in which the number of DCX-positive cells were similar to WT at 1.5 months of age but had diminished significantly by 3 months of age.

Regarding gliogenesis in dentate gyrus, we counted BrdU positive and DCX negative glial cells and a non-significant trend to be increased in GFAP-IL6 mice was observed in 1.5 month-old mice (data not shown).

6.4 Reduced neurodegeneration in GFAP-IL6-spgp130 mice

Progressive neurodegeneration develops in the cerebellum of the GFAP-IL6 mice [252]. Therefore, we evaluated whether the blockade of IL-6 trans-signaling influences this process. A GzLM for genotype and age revealed a significant effect of genotype ($P \leq 0.001$) and a significant interaction between genotype and age ($P \leq 0.01$). Sequential Bonferroni post-hoc analyses were carried out at each age, showing evidence of neurodegeneration, as reflected by a significant loss of Nissl staining in cerebellum, in GFAP-IL6 mice compared with all other genotypes (Figure 6.5). This neurodegeneration in GFAP-IL-6 mice was already present at 1.5 months of age and was accentuated with age, showing gross disruption of the architecture of the molecular and granule cell layers and a loss of cellularity in the cerebellum. In contrast to the GFAP-IL6 mice, the cerebellum from GFAP-IL6/sgp130 mice, from all ages, exhibited similar Nissl staining area as the WT control animals and both the architecture of the molecular and granule cell layers, as well as the degree of cellularity, appeared to be largely normal in these mice (Figure 6.5).

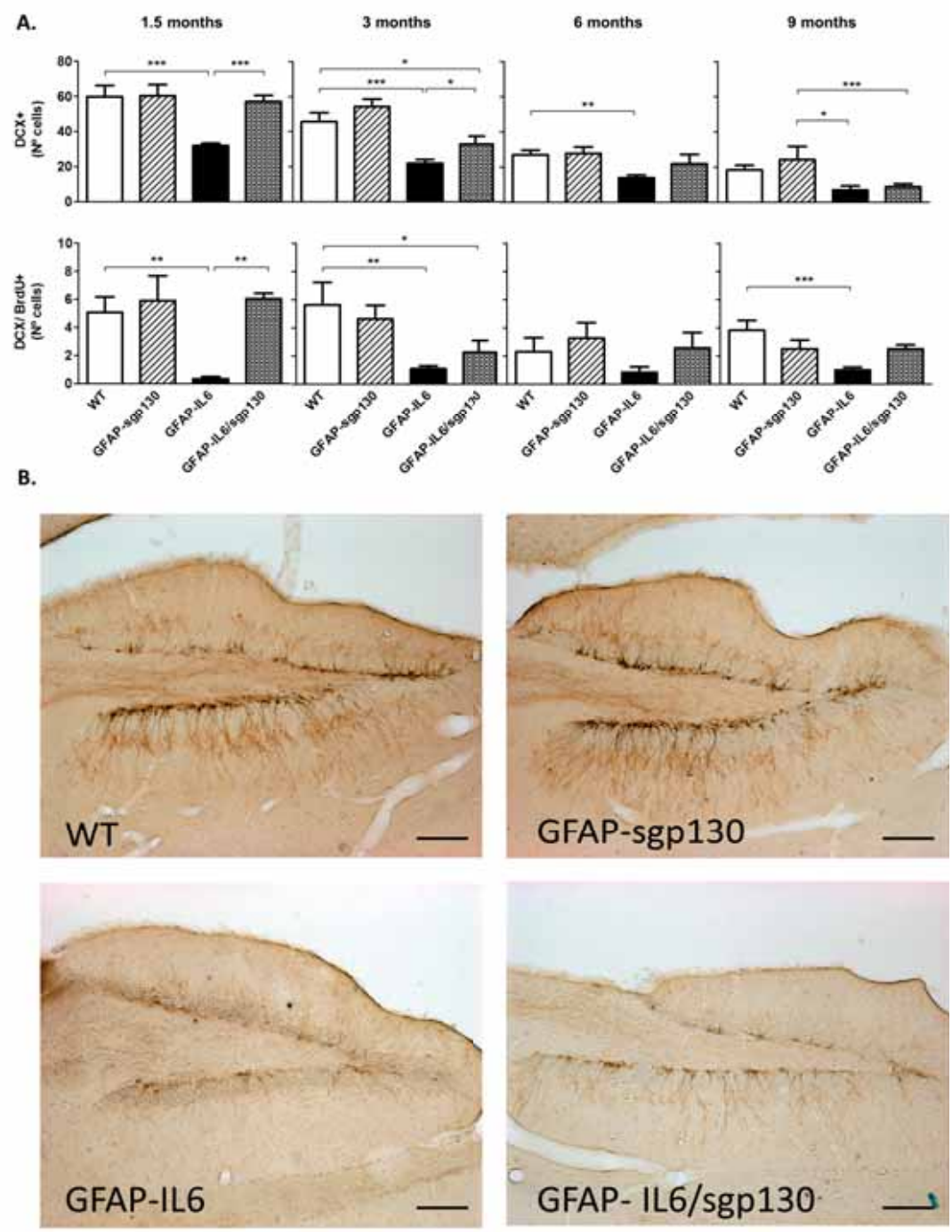


Figure 6.4: Neurogenesis assessment in dentate gyrus of GFAP-IL6, GFAP-IL6/sgp130, GFAP-sgp130 and WT mice. A. Number of DCX+ (top) or DCX/BrdU+ (bottom) cells in dentate gyrus at different ages. N = 3-4 animals. At least 3 different hippocampus sections per animal were counted. Results are mean ± SEM. *, ** and *** at least ≤ 0.05, 0.01 and 0.001 between the selected genotypes. B. Representative images showing DCX staining in dentate gyrus from 3 month-old animals. Bar: 100 μm.

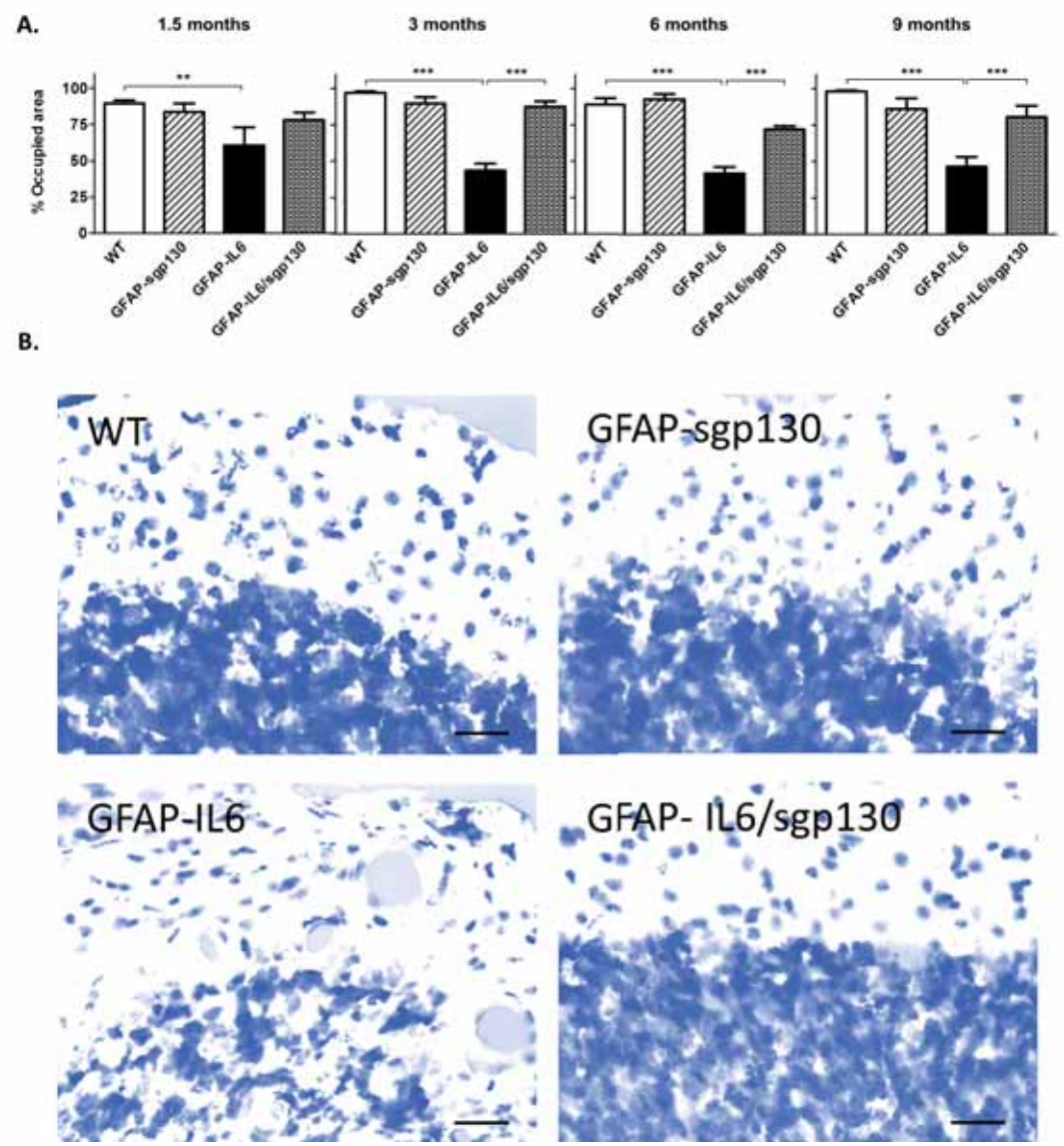


Figure 6.5: Neurodegeneration assessment in cerebellum of GFAP-IL6, GFAP-IL6/sgp130, GFAP-sgp130 and WT mice. A. Percentage of Nissl staining area in cerebellum at different ages. N = 3-4 animals. Results are mean ± SEM. *, ** and *** at least ≤ 0.05, 0.01 and 0.001 between the selected genotypes. B. Representative images showing Nissl staining in cerebellum from 3 month-old animals. Bar: 25 μm.

CHAPTER 6

DISCUSSION

7.1 Validation of astrocyte-derived IL-6 and IL-6R KO mice

Once the floxed mice for IL-6 were generated we could demonstrate CNS-specific recombination of the IL-6 gene, then several approaches to detect CNS-specific decreases of IL-6 expression were made. Detection of IL-6 expression by in situ hybridization was tried, but transcript levels were too low in basal mice as to be detected by this approach; also, attempts to measure tissue IL-6 by a commercial ELISA or by IHC (in either paraffin or free-floating sections using a commercial antibody) failed. Stimulated cortical astrocyte cell cultures, although with a big presence of microglia, were performed and showed a significant decrease in IL-6 production in Ast-IL-6 KO animals compared to controls. Moreover, we finally set up RT-qPCR, which did identify a decrease of IL-6 mRNA levels in basal conditions (animals not subjected to any manipulation other than normal handling) in cerebellum from young animals. The cerebellum was chosen because in transgenic mice using the GFAP promoter to drive cytokine expression it is normally found that this brain area has the highest transgenic expression [252, 652]. To assess any putative compensatory changes in other cytokines we measured TNFα and IL-1β in the cerebellum and spleen of floxed and Ast-IL6 KO mice, and certainly there is an inverse tendency for both cytokines to be elevated in the latter mice, which is significant for TNFα in the spleen. This increase is consistent with the often observed inhibitory effect of IL-6 on the expression of these two cytokines, although this is a very complex subject. Overall, the differences were small, but the relatively small effect of deleting IL-6 in astrocytes is to be expected considering the fact that the cytokine is expressed in other cells as well. Regardless the small reduction observed in Ast-IL6 KO mice of IL-6 expression in bulk CNS tissue, the fact is that very clear effects were observed in a number of physiological and behavioural traits that will be explained in the following sections.

Knocking-out IL-6Rα in astrocytes had a dramatic effect in the overall receptor staining throughout the CNS. Without doing co-staining with specific cellular markers, we cannot conclude the type of cells being stained. Previous studies suggested that in the mouse hypothalamus IL-6Rα staining is abundant in neuronal fibers and cell bodies and that little co-staining with astrocyte and microglia markers is achieved [357, 359]. Yet,

Ast-IL6R KO mice showed a dramatic reduction in IL-6Rα staining; from our experiments we cannot conclude whether this is related to knocking out the gene in neuronal and astrocyte precursors, or to the fact that the soluble IL-6R is going to be detected (at least that bound to membrane gp130) by IHC; also, by affecting astrocyte physiology because of lack of normal response to IL-6, that might decrease the neuronal expression of the IL-6R indirectly.

7.2 Prosurvival role

Our results showed that the early (intrauterine life) survival is influenced by the astrocyte production of and response to IL-6. This cytokine was found to be expressed as early as the eight-cell stage embryo [653], but two different total IL-6 deficient mouse lines showed normal mendelian patterns [654, 269]. This was also the case for total IL-6Ra deficient mice, also born in the expected Mendelian ratio [631]. Thus, when all sources of IL-6 are shut off survival is not affected, but if only the astrocyte system is eliminated then a prosurvival role of IL-6 in early stages emerges.

As mortality between birth and weaning was equally distributed among genotypes, we conclude that in principle intrauterine rather than newborn mortality is the more likely cause for this genotype effect.

Interestingly, regarding the prosurvival role of astrocyte-derived IL-6 receptor, our results are in line with previous studies that found extremely altered Mendelian ratios and highly elevated embrionary and perinatal deaths (progressively dying between 12.5 days postcoitum and term) in gp130-deficient mice [655, 656, 657]. The fact that inactivating the gp130 receptor avoids signaling from nearly all members of the IL-6 cytokine family, including not only IL-6, but also IL-11, IL-27, LIF, OSM, CNTF and so on; and also that this receptor is blocked in all cells, explains why its effects are so dramatic. Necropsies of gp130 KO mice indicated, depending on the genetic background used, heart and hematopoietic abnormalities [655] as well as histological bone changes among others [656]. It is worth to mention that ligand-binding of gp130 receptor results in the coordinated and simultaneous activation of the STAT1/3 and SHP2-Ras-ERK pathways, which negatively control each other (see section 1.1.2); but it seems that only STAT3 signal is required for embryonic development, as only mice deficient for STAT3 pathway showed altered mendelian ratios [657, 658, 659]. Ernst et al. [659] showed in a very elegant way that the altered birth ratios were due to a defective maternal embryo implantation, as female deficient for gp130/STAT pathway mated with males of any genotype showed no external signs of pregnancy, while blastocysts of all genotypes recovered from these KO females developed to term when surgically transferred to wt recipient mice [659].

Interestingly, this same maternal defect of embryo implantation had been previously described in mice deficient for LIF [660, 661] and for the IL-11 receptor α subunit (IL-11Rα KO), the later due to defective uterine response because of incompetent decidua formation [662, 663]. Also, mice deficient for the low-affinity leukemia inhibitory factor receptor (LIFRβ) exhibited perinatal death, disrupted placental architecture, profound mineralized bone loss, increased glycogen levels in liver and a reduction in the number

of spinal cord and brainstem astrocytes [664]. These results show once more the partial functional redundancy between IL-6 family members given specific premises and the complexity of this cell-type-specific responses to pleiotropic-acting growth factors and cytokines and STAT1/3 or the SHP2/ERK cascade, together accounting for the majority of intracellular gp130 signalling (reviewed in [665]). We have been able to identify embryonic death in two new models of IL-6 cytokine family, Ast-IL-6 KO and Ast-IL-6R KO, but it is now mandatory to study if the altered mendelian ratios are due, totally or partially, to a defective maternal embryo implantation and assess any disturbance in the reciprocal negative regulation between the SHP2/ERK and STAT1/3 pathways, as well as any putative compensatory cytokine levels and receptor expression.

Finally, from weaning to adulthood no differences in survival were observed between genotypes in our animals. Also, our Ast-IL-6 KO and Ast-IL-6R KO appeared normal without any major visible anatomical, morphological or physiological abnormalities rather than the ones discussed in the following section (7.3).

7.3 Role of astrocyte-derived IL-6 and IL-6R in physiological conditions

7.3.1 Body weight, food intake and energy expenditure

One major physiological effect observed in Ast-IL-6 KO mice was a significantly increased body weight gain in males from 8 week-old onwards in the fifth backcrossing group. These results are consistent with previous studies with total IL-6 KO mice, which demonstrated that body weight increased at mature ages but not in young animals [345], and with those showing that either chronic i.c.v. administration of IL-6 [348] or CNS-specific transgenic expression of IL-6 decreases body weight [352]. When the experiment was refined with 10th backcrossing animals differences between genotypes were weaker and appeared later (24 week-old onwards), paralleling the evolution of total IL-6 KO mice described by Fald et al. that describes young IL-6 KO mice leaner than wt controls whereas 7 month-old adult IL-6 KO mice developed obesity [366]. The reason for this remains unknown, but low body weight in young individuals during certain circumstances can be associated with obesity later in life [666]. Regarding the variation in our different backcrossing groups, it can be possible that genetic background is the responsible for the delay, but we cannot discard that changes in the environment and psychological stress caused by behavioural tests and new environment in the 5th backcrossing group had in some way accentuated and accelerated body weight changes due to for example different activity levels to cope with these new situations. In fact, it is described that IL-6 KO mice present a blunted increase in energy expenditure after psychological stress [362].

Despite all, our results indicate that, at least in males, IL-6 release by astrocytes has an inhibitory role controlling body weight; until 35 week-old. Whether or not this will occur in females at even later ages seems difficult but remains to be established. On the other hand, the absence of mIL-6R signaling in astrocytes did not affect body weight gain, clearly indicating that cells other than astrocytes are likely relevant in the central effects of

IL-6 on body weight, although we cannot rule out a role of soluble IL-6R. However, there are important controversies in the field since other studies do not find such an increase of body weight in total IL-6 KO mice, or even find a lower body weight when fed a high fat diet [349, 90]. The reasons for these discrepancies are unknown, but as peripheraly and central IL-6 could have different roles in body weight, as shown for example by the fact that males with IL-6 transsignaling pathway blocked in the periphery [415] showed decreased body weight compared to controls, it is therefore difficult to predict in total IL-6 KO the importance of each component in the final output. Incidentally, in our lab total IL-6 KO mice do get obesity, more clearly in males than in females (unpublished data).

So far the published studies suggest that IL-6 exert these inhibitory effects on body weight mainly through increased energy expenditure rather than controlling food intake [366, 345, 362]. Also supported by the fact that rats exposed chronically to high levels of IL-6 either by icv injections [348] or adenovirus-associated viral vector injection [353] and both GFAP-IL6 [352] and a different transgenic mouse line with neuronal expression of IL-6 [667], all showed no effects on food intake despite lower body weight gain. However, this conclusion should be taken with caution as, in some cases, animals were not individualised and, in other cases, results were expressed in food intake per gram of body weight; which, as transgenic GFAP-IL-6 or IL-6 icv injected animals had a decreased body weight, it caused that there were no overall differences in food intake. But, when results where reported without body weight normalization they did show a significant decrease in food intake [348, 667].

Therefore, the role of food intake in the increased body weight should not be totally discarded. Regarding Ast-IL-6 KO, in an experiment with individualised males, absolute food intake presented a tendency to be increased in KO animals compared to floxed controls, in line with the above-mentioned data, [58] and that showing that total IL-6 KO mice had increased absolute food intake [345], specially at older ages, which could partly explain this increased body weight gain and has to be studied in both genders and at older ages.

Respect to energy expenditure, existing data demonstrates that IL-6 injected icv increases oxygen consumption, carbon dioxide production and thermogenesis [347, 353, 345] and that total IL-6 KO presented a blunted increase in energy expenditure during new-cage stress and cold ambient paradigm [362]. However, we have been unable to test our animals for oxygen consumption or carbon dioxide production and, in relation to thermogenesis, we did not find robust effects although we did find a tendency in Ast-IL-6 KO males to have a decreased body temperature respect controls at certain conditions (room temperature) and times (morning), in accordance with total IL-6 KO mice showing lower temperature levels at thermoneutrality and in basal conditions compared to WT [362].

Also, the clear and consistent decrease in the activity levels of the Ast-IL-6 KO mice (see section 7.3.3.1) could be critical for the increased body weight of these mice. Yet, it is equally obvious that the activity is reduced in both genders while weight is only affected in males, thus indicating that other factors are contributing. However, data from our laboratory do show that when Ast-IL-6 KO animals are under a hight-fat diet both males and females have increased obesity respect floxed controls (unpublished data).

To be able to have this inhibitory effect on body weight, IL-6 appears to influence the expression of critical hypothalamic factors which control food intake and energy expenditure [357, 359, 668]. Some of these factors are being analysed in our group with promising results to verify if they are altered in our models.

Finally, it should also be taken into account that, although Ast-IL-6 KO mice parallel total IL-6 KO, other CNS-cells, different than astrocytes, and also peripheral IL-6 could still exert lipolytic and fat-burning effects when released from the periphery and reach CNS [336, 669].

7.3.2 Body temperature effect of astrocyte-secreted IL-6 in adult males

Body temperature is regulated by cytokines. IL-6 is an endogenous pyrogen [670, 347, 385] that when administered icv rapidly elevates the body temperature and reduces locomotor activity [58]; IL-6 delivered directly into the rat hypothalamus increases thermogenesis [353].

IL-6 KO mice in basal conditions have lower body temperature than WT mice at room temperature (only marginally significant), at thermoneutrality (30 ºC) and especially during cold exposure (4 ºC), having a decreased capacity for cold-induced thermogenesis [362]. We could not test our animals at thermoneutrality (30 ºC) but at room temperature we observed a significant decrease of the body temperature of Ast-IL-6 KO male mice when measured in the morning, in line with total IL-6 KO mice. Although literature described that both IL-6 KO and GFAP-IL-6 mice have impaired cold-induced thermogenesis [362, 352], this effect was not seen in our Ast-IL-6 KO male mice.

It seems likely that the decrease in body temperature in total IL-6 KO mice is due to the decreased energy expenditure mentioned in the previous section (see 7.3.1), as the fact that those mice could be losing more heat to the environment than WT mice seems less plausible, as they also present differences at thermoneutrality, when heat loss to the environment should be minimal [362]. The fact that in our Ast-IL-6 KO mice there is tendency to present a compensatory elevation of TNFα in the brain, which is an antipyretic factor [671], could contribute to the decreased temperature of our animals.

Daily rhythm of temperature changes at thermoneutrality is described as not affected in IL-6 KO mice respect WT [386] but, baseline measurements of body temperature at both thermoneutrality and at room temperature in IL-6 KO had been studied calculating the average data of a 8-hours period [362], not being able to discriminate the exact timing of body temperatures differences in IL-6 KO mice. Regarding gender differences, it is again difficult to compare with data existing for IL-6 KO mice, as the majority of studies are only performed in males. But as in body weight, differences seems to exist only in males.

Moreover, the fever induced by different types of inflammatory stimuli, specially LPS, is blunted in IL-6 KO mice [386, 672, 671, 673]. In rats, systemic administration of LPS can lead to monophasic fever, multiphasic fever, or hypothermia depending on the dose, the route of administration, and the ambient temperature [674]. We tested two different dosage of LPS injection in Ast-IL-6 KO mice.

LPS injected at 1 mg/kg produced a significant hypothermia only in males without differences due to genotype. This result is in agreement with other data showing that elevated doses of LPS around 1 mg/kg or 2.5 mg/kg induced hypothermia in WT mice [675, 672]. Data from Morrow et al. also showed a significant hypothermia in IL-6 deficient mice at a dose of 100 μg per animal [673], which, if considering that average mice weigh 25 grams it would give us a 2.5 mg/kg of LPS. In that case, they found a hyperthermia in WT animals when we also found hypothermia in WT males, may be it is due to their LPS injected dose, more than double ours. Gender differences are difficult to compare with bibliography as most of the time experiments are only performed in males or the gender is not specified. Regarding differences between genotypes, our results indicates that LPS response at hight dosage is much more powerful in floxed mice than in Ast-IL-6 KO animals, showing a role for astrocyte-derived IL-6 in LPS-derived hypothermia.

LPS injected at 10 μg/kg induced hyperthermia in our floxed controls but this response was impaired in Ast-IL-6 KO animals, both males and females. These results are in line with total IL-6 KO that failed to evoke fever response at 50 μg/kg of LPS and, if correctly replicated with more animals, would indicate that not only central IL-6 is the responsible of LPS-induced fever but that astrocyte-derived IL-6 has the major role in it.

7.3.3 Behaviour

To assess the phenotype of our conditional KO mice regarding normal behavioral traits, we tested them in battery for the Hole-board, Elevated plus-maze, Tail suspension, Morris water maze, Dominance tube (only Ast-IL-6 group) and Resident-intruder paradigms in order from least to most stressful tests.

7.3.3.1 Emotional reactivity

Remarkably, in both tests assessing locomotor activity (HB and PM), the finding that the Ast-IL-6 KO mice were less active than floxed mice was very consistent (judged by measuring the total number of squares crossed and, in EPM, also by the number of enters in closed arms). This decrease in ambulation was found in both genders, at all ages studied and in both backcrossing groups. It is also supported by a a decrease in the number of rearings (which also has a very important activity component [676]) in Ast-IL-6 KO animals. Interestingly, these results are in agreement with IL-6 KO data that showed a decrease in ambulation [439], indicating that both astrocyte-derived IL-6 and astrocyte response to IL-6 (therefore central IL-6) have a major role in baseline ambulation of the IL-6 KO mice. As already discussed in the previous section, this decreased activity could easily contribute to the increased body weigh from both IL-6 and Ast-IL-6 KO mice. To this matter, it would be valuable to know if groups which reported no decrease in locomotor activity of IL-6 KO males where able to find an increased in body weight, unfortunalely they did not report it. Finally, since Ast-IL-6R KO mice showed a decreased locomotor activity but normal body weight, there should be a different mechanism to prevent them from gaining weight.

Ambulation reflects spontaneous locomotor activity but also exploration and anxiety, both behavioral traits difficult to differentiate in the open field test [677] and for which HB and EPM are probably best suited. The HB was originally devised by Boissier and Simon [636] who claimed that it allows to separate locomotion from exploration (measured by head-dippings), a discrimination improved by the reduction from 16 to 4 holes, which avoided correlations between ambulation and head-dipping behavior [678]. Exploratory behaviour is a more complicated topic to analyse as it showed not only gender-specific differences but also differences between both backcrossings groups (5th and 10th). While young Ast-IL-6 KO males from 5th backcrossing group showed an increase in both number and time of head-dipps compared to controls in HB test, this effect disappeared in both young and adults IL-6 KO males from 10th backcrossing, while Ast-IL-6 KO females from 10th backcross showed a decrease in head-dipping at young ages while an increase at older ages, showing once more the importance of gender, age and of course the backcrossing group in mice [439].

When adding the information extracted from EPM test, the so-called unprotected head-dipping behavior in the EPM was again higher in young Ast-IL-6 KO males from 5th backcrossing group but also in young mice (both males and females) from 10th backcross, always compared to floxed controls. Interestingly, in old/adult mice (again in both genders) the opposite situation is observed, showing Ast-IL-6 KO mice less unprotected head-dipps.

When these behavioral traits were analyzed in Ast-IL-6R KO mice, it was obvious that the phenotype was essentially the same than that of Ast-IL-6 KO mice regarding ambulation and rearings (a decrease in Ast-IL-6 KO animals from both genders compared to floxed mice), and the opposite regarding exploratory behavior, being reduced in young Ast-IL-6R KO animals. Thus, these results strongly suggest that IL-6 released by astrocytes in normal, basal conditions increases the locomotor activity in mice, and that the response of astrocytes to IL-6 via mIL-6R is contributing to such a behavioral trait. Presumably, the IL-6 released by astrocytes will function by subsequent autocrine/paracrine actions on astrocytes themselves, and the phenotype of Ast-IL-6R KO mice is more prominent because of other cellular sources of IL-6 besides astrocytes are relevant in this behavior. Regarding the opposing phenotype of Ast-IL-6 KO and Ast-IL-6R KO mice at younger age, the results can only be reconciled if CNS cells other than astrocytes are being affected by IL-6 too. Our group is now studying this behaviour in neuronal IL-6 deficient mice to gain insight into the role of neurons in this regard.

These results are only partly consistent with those obtained in total IL-6 KO mice, which also showed reduced levels of ambulation but, in contrast, their head-dipping behavior was also decreased [439], not only in old animals as in our case. The HB also allows to get some knowledge of the anxiety of the mice, for instance by comparing if the putative changes observed in the squares crossed differ between the external squares (those close to the walls of the apparatus) and internal squares (those of the center). If the animal avoids the internal squares, that may be considered as an anxiety trait [676, 638]. In our study, Ast-IL-6 KO and Ast-IL-6R KO mice showed reduced ambulation in internal squares compared to their respective floxed mice, which do support a role of astrocyte release of and response to IL-6 regarding the control of anxiety. Which in Ast-IL-6 KO an-

imals is further supported by an increased defecation rate and more unexplored squares than floxed controls, which altogether indicate increased anxiety.

To further analyze this possibility, we used one of the most commonly used tests, the elevated Plus-Maze [679], first described in 1985 by Pellow and collegues [637], which relays on the preference of rodents for closed spaces instead of open (and elevated) ones. In the EPM, old Ast-IL-6 KO males and females spent more time in open arms than floxed mice, supporting an increased anxiety level and the HB results on internal squares activity only at this age, as young mice presented the opposite tendency, suggesting a decreased anxiety in young Ast-IL-6 KO. Thus, supporting unprotected head-dipps data, which is elevated in young Ast-IL-6 KO (which seems to indicate a lower fear to explore in unprotected areas such as open arms [680]) and decreased in old Ast-IL-6 KO respect to comprols. However, defecation rates, as in HB test, are increased in Ast-IL-6 KO mice from both ages.

Again, these results partially agree with those obtained in male total IL-6 KO mice, which show clear increases in anxiety in the EPM at 8-9 week-old [439, 440]. No differences were observed in most of the behavior displayed in the EPM between Ast-IL-6R KO mice and their corresponding floxed controls (except the ones regarding to ambulations and rearings, which were clearly decreased in Ast-IL-6R KO), which strongly suggests that it is unlikely that the response of astrocytes to IL-6 via mIL-6R is relevant in the control of anxiety. In contrast, when examining the time spent in the central square, which is a measure of decision making [639], we noticed that both Ast-IL-6 KO and Ast-IL-6R KO mice spent a shorter time. We think that is also in agreement with the fact that Ast-IL-6 KO animals spent significantly more time in each entry to both open and close arms than controls, likely to mean that once they enter to an arm they are more predisposed to explore it.

7.3.3.2 Despair behaviour

After observing that IL-6 deficiency seemed to render mice more emotional, our group predicted that it might in turn increase the vulnerability of these animals to stress-related pathology [439]. Further experiments with IL-6 deficient mice confirmed IL-6 role in stress-related pathology but in the opposite way, showing IL-6 KO mice reduced despair behavior in both the forced swim test and the tail suspension test, enhanced hedonic behavior and a strong resistance to develop learned helplessness following intense stress [442, 454]. This apparent contradiction seems not such thing, as increased anxiety as a consequence of stress appears to be independent from depressive-like behaviour to some authors [457, 458]. However, others suggest anxiety-depression association [681, 682, 683], showing the last study a complex relationship in which rats with low levels of anxiety exhibited an inverse correlation between anxiety and depression (the tendency observed in our animals), while rats with high anxiety showed a direct correlation between anxiety and depression.

Results from our Ast-IL-6 KO showed a modest increase in old Ast-IL-6 KO males agitation time compared to controls. Interestingly, these results are in line with all published data about total IL-6 KO (to the author knowledge) even those in disagreement between

them, as works which found a reduced despair behaviour in IL-6 deficient mice were performed only in adult males (between 3-6 month-old) [442, 454] but that the one showing no differences between IL-6 KO and WT was in fact performed in young IL-6 KO at 6 weeks of age, besides of being in a different genetic background [443]. Altogether, those results suggest that age is a crucial factor in mediating IL-6 role in depression-like behaviours, as it is for example in mediating antidepressant activity with the forced swimming test [684]. Regarding gender-related differences or age effect in another genetic background, we are for the time being, unable to know if those genotype-related differences are also present in females or young IL-6 KO mice in C57BL/6 background. Ast-IL-6R KO mice did not show any significant differences between genotypes.

It is noteworthy that, of the two experiments demonstrating reduced despair behaviour in IL-6 KO mice, one is performed during the light phase of the cycle [442] and the other during the dark one [454], demonstrating that IL-6 deletion had the same effect in both cases yet IL-6 is secreted in a circadian rhythm and it is correlated with the sleep-wake cycle. Also, IL-6 KO mice are resistant to induction of depression-like behavior by light deprivation in the constant darkness paradigm by NF-κB pathway [685].

Increasing amounts of data suggest that inflammatory responses have an important role in the pathophysiology of depression based upon a large series of reports of elevated immune parameters in depressed individuals and in animal models (greatly reviewed in [686, 687, 688]). To sum up, proinflammatory cytokines, including IL-6, have been found to access and interact virtually every pathophysiologic domain that characterize depression (like neurotransmitter metabolism, neuroendocrine function, synaptic plasticity and behavior) being increased, together with acute phase proteins, chemokines and cellular adhesion molecules, in depressed patients and model animals; being IL-6 one of the most reliable peripheral biomarkers in depression. Sustained elevation of IL-6 in the CNS is a key contributor of both depressive-like phenotypes and resistance to antidepressant medications, while blocking of classical and trans-signaling pathways prevented the IL-6-induced increases in immobility [689]. Also, intrahippocampal IL-6 treatment leads to increased depression-like behavior in the forced swim test in rats [690] and long-term exposure to this proinflammatory cytokine, as well as to chronic stress (a precipitating factor in depresion), impair hippocampal neurogenesis in mice [314, 691]; which also has a role in the action of pharmacological and non-pharmacological interventions for depression in animals [692]. Psychological stress (like TST performance) activates the immune response both peripherally and in the brain [688] and increases NF-κB activity in healthy individuals while leads to an exacerbated stress response in depressive patients [693]. NF-κB pathway is critical in mediating depressive behavior and its required for the antineurogenic and anhedonic effects of repeated stress, being enhanced in mouse hippocampus following an acute stress procedure [694] and it seems that NF-κB effects on depression depend on IL-6 presence, at least in depression-like behavior in the constant darkness paradigm [685]. It is logical to expect that in Ast-IL-6 KO animals the resistance to depression-like behavior is much more weak compared to total IL-6 KO, as in this case both neurons and microglia, among other cells, would be secreting IL-6 in response to psychological stress. Not having any genotype-related differences in Ast-IL-6R KO implies that astrocyte-derived response via membrane receptor to IL-6 is not necessary for IL-6-

derived depression-like behaviour, as neurons, microglia or astrocytes via trans-signaling are being stimulated in a feed-back loop.

Despite the weak results obtained with Ast-IL-6 KO mice, a more profound study with other test to assess depression-like behaviour or hedonic alterations are required. It would also be desirable to re-check despair behaviour with forced swim test (Porsolt) instead of TST, as it is more suitable for C57BL/6 mice due to their propension to climb their tails [695]. However, in our battery design, FST was not an option in order to avoid learning interferences with the following behavioral tes, Morris water maze.

7.3.3.3 Learning and memory

Results from MWM test indicate an impaired learning in both spatial and reversal short memory acquisition of Ast-IL-6 KO mice, measured by the time animals need to find the submerged platform, in all genders and ages studied. This results are in agreement with those from Baier et al. who found impaired hippocampus-dependent and -independent learning in total IL-6 KO mice [442]. However, there is a strong controversy with spatial learning in IL-6 KO as other authors described no change [405] or even an improved spatial learning in IL-6 KO at both young and adult ages [445] but, it should be commented that in that study it could be criticized the fact that the radial maze measures spatial learning only when transparent walls are used [451], which was not the situation. Interestingly, exogenous administration of IL-6 led to suppressed long-term-potentiation (LTP; the underlying cellular/molecular mechanism sub-serving memory consolidation [696]) in hippocampus [697, 698] and impaired spatial learning in rats [447]. Also, rodents with cerebral overexpression of IL-6 had reduced hippocampal LTP, reduced hippocampal neurogenesis, and impaired avoidance learning [698, 450, 446]. Altogether, these observations that elevated central IL-6 levels are pernicious for learning and memory, led to the hypothesis that IL-6 deficient mice should show improved memory. This hypothesis do not seem true, although the fact that IL-6 has a role in learning and memory is out of doubt.

There are several reasons which could explain the slightly unexpected impaired learning performance in both IL-6 KO and Ast-IL-6 KO mice (see figure 7.1), as for example the reduced locomotor [439] and exploratory activity [439, 440, 442], the increased levels of anxiety [439, 440], the increased susceptibility to stress [442], a impairment of object recognition memory [442, 452], as well as impaired cued learning (the ability to learn to swim to a marked goal), discussed below.

Cued learning was assessed in Ast-IL-6 animals, which indicated again an impairment of Ast-IL-6 KO mice at all ages and genders compared to floxed controls. In the first trials many animals did not want to swim away from the wall or jumped back into the water after climbing the platform, possibly due to the lack of recognition of the platform as the unique escape route. Cued learning is basically a control procedure as if subjects are impaired in cued learning, there is the possibility about whether a spatial deficit is present, as it requires the same basic abilities (correct eyesight, swimming ability), basic strategies like swimming away from the wall or climbing on the platform, and the same motivation as in the spatial version [641]. Although we discarded motor swimming

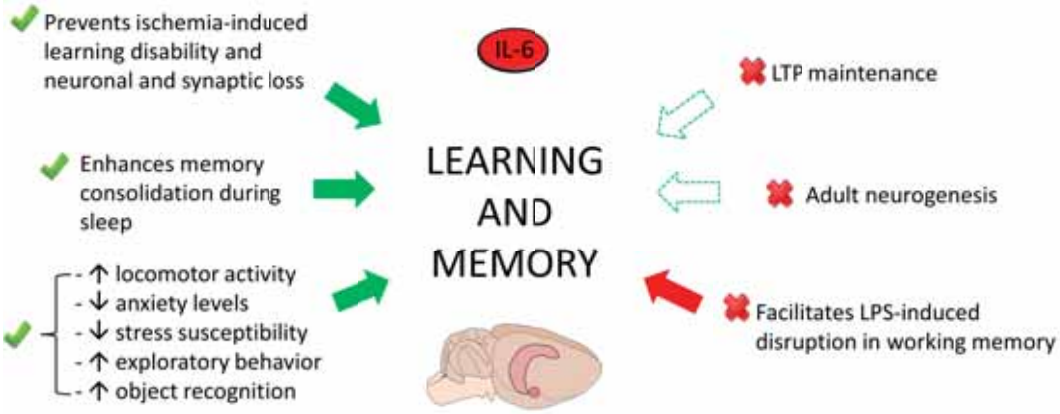


Figure 7.1: Schematic illustration of IL-6 involvement in learning and memory. Among detrimental actions, IL-6 blocks the maintenance of LTP [449] and impairs adult neurogenesis [314, 315], thus influencing the consolidation of learning and memory. It also facilitates LPS-induced disruption in working memory [405]. On the other side, it prevents ischemia-induced learning disability and neuronal and synaptic loss due to its trophic effect on ischemic hippocampal neurons [489]. It is also believed to enhance memory consolidation during sleep [699] and, IL-6 administration increases locomotion, rearings and ambulatory exploration [700] while IL-6 deficits in transgenic mice have shown alterations in locomotion, exploration, anxiety, object's recognition and stress susceptibility [439, 440, 442, 452], which can affect the learning and memory processes in many ways.

deficits, as there were no swimming speed differences between genotypes, and poor eyesight by excluding from the study animals with detectable eyes alteration by visual examination we cannot discard non-visible eye affectation by IL-6 (as already described in IL-6 KO [701]) or any other alteration causing that spatial deficits in Ast-IL-6 KO mice are totally or partially due to their deficits to learn to use distal cues.

Interestingly, only Ast-IL-6R KO males showed cued learning deficits but both sexes presented impaired spatial learning acquisition, showed by both an increased time to reach the platform compared to controls and, during probe trials, by a decreased percentage of time spent in the platform quadrant, specially when short-time memory (1 hour after the last training session) was tested, deficits in KO animals for reference memory (24 hours after the last training session) were only marginally significant. Ast-IL-6R KO mice also performed worse in reversal learning, needing more time to find the platform. There were no differences in time spent in the platform quadrant during probe trial, possibly due to the fact that all genotypes have been unable to properly learn the new location of the platform as the time spent in the quadrant which allocated it is around 25%, the normal by chance. As in Ast-IL-6 KO mice, decreased locomotion and exploratory behaviour could be partly responsible for this bad performance, although the role of astrocyte response to IL-6 in learning and memory should be further studied.

Finally, results from probe trials of Ast-IL-6 animals, have been deliberately left last to be able to discuss them widely. Although there were no differences due to gender, an important age effect was seen, staying old/adult mice less time in the platform quadrant

compared to young. Probe trials 1 hour after the last spatial learning training showed, as happened during spatial learning acquisition, a decreased percentage of time spent in platform quadrant of the Ast-IL-6 KO mice from both genders and ages respect to controls, as a measure of worse learning and decreased short-term memory. Curiously, no differences due to genotype were found 24-h after the last training session, as Ast-IL-6 KO animals maintain the percentage of time spent in the platform quadrant while other genotypes decreased it, pointing that Ast-IL-6 KO mice could be showing better memory consolidation. This hypothesis was further supported when data from probe trial from reversal learning were analysed.

In both 1 and 24 hours after last reversal training session all genotypes spent around 25% of the time in the platform quadrant, indicating that the new location was not properly learnt, but, even in that case, Ast-IL-6 KO mice showed a worse performance (paralleling reversal acquisition data) specially 24 hours later the last trial session. However, we also wanted to analyse the percentage of time spent in the old platform quadrant (the one that allocated the platform during spatial acquisition trials) in order to assess if these animals were capable of extinguish their initial learning of the platform position and learn the new goal position. All genotypes spent more than 25% of the time in this old platform quadrant, demonstrating that they were revisiting it, as, even after multiple trials, mice do not completely abandon their initial learning strategy and begin trials by starting to move towards the original platform position, in opposite to rats, which rapidly switch their search strategies to the new goal position [641]. In fact, Ast-IL-6R mice spent more time in the original platform quadrant 3 days-after the last trial than on the immediate probe trial, indicating that learning curve could be delayed. Whether or not this persistence to revisit the first location and the lack of cognitive flexibility is the cause of the slow reversal learning remains to be elucidated.

Interestingly, despite all genotypes revisit the original platform position, Ast-IL-6 KO mice stayed significantly more time in it compared to floxed controls at both ages and genders. In fact, they stayed in the original platform quadrant the same amount of time as three days before, during the first spatial probe trial, possibly showing a stronger consolidated memory of what they learnt during spatial acquisition. These results are in accordance with those from Balschun et al. showing that blockage of endogenous IL-6 resulted in a reinforcement of LTP and an improvement of retention when tested 24 h later under the same conditions [449]. Thus, suggesting an inhibitory role of IL-6 in the mechanism controlling the consolidation of long-term synaptic plasticity and hippocampus-dependent spatial learning. As in the CNS IL-6 is principally synthesized by astrocytes, it is reasonable that lacking only astrocyte-derived IL-6, it effect in memory consolidation is still visible and strong as it happens in Ast-IL-6 KO mice. It would be interesting to assess memory consolidation in total IL-6 KO mice, however, as far as I know the only published data in MWM with IL-6 KO mice is with working memory (short-time memory) [405] or with a similar protocol than us but, unfortunately, without assessing the percentage of time animals stayed in the original platform quadrant during reversal probe trial [442]. However other authors suggested greater spatial memory consolidation in IL-6 deficient mice with another behavioral test [702].

Moreover, recent studies suggest an important role of adult neurogenesis in mem-

ory consolidation and some types of hippocampal-dependent learning and being IL-6 an important regulation of neurogenesis, specially by transsignaling pathway (see 7.5), it is mandatory to study hippocampal neurogenesis in our Ast-IL-6 KO mice.

7.3.3.4 Social behaviour

Data from dominance tube test performed in young Ast-IL-6 mice shown an overall significant increase in the percent of victories of Ast-IL-6 KO versus floxed mice, significant only in females. Total IL-6 deficient males from the same age but in a different genetic background (129/SV/EV strain) also demonstrated increased dominant score specially after repeated encounters [444]. In that study dominance was not assessed in females and, in our case, we did not assess it again after resident-intruder sessions. Dominance status in adult/old animals, as well as in Ast-IL-6R mice, remains to be assessed in the near future.

Regarding resident-intruder test, our individualised Ast-IL-6 and Ast-IL-6R animals were first tested as residents with a CD1 male and, a week later, as intruders with a different CD1 male. Those CD1 males were highly aggressive and had previous fight experience (always with one week of rest between different tests); also, they were not matched for body weight with our experiment mice, being much bigger compared to our young Ast-IL-6 animals (weighting an average of 20 gr more). It is possible that CD1 extreme aggressiveness and increased size dissuaded intruder Ast-IL-6 mice to fight for the CD1 territory, as they significantly try to escape from CD1 chases and avoid any affiliative behaviour, in opposite as when they acted as residents.

Only results from our 9 month-old Ast-IL-6 KO animals are in line with those of total IL-6 KO presented by Alleva et al. [444], presenting a more aggressive behavior demonstrated by decreased latency to attack, when acting both as resident and as intruder; an incresed number of attacks when being intruder and an increased number of other aggressive behavior different than attacks, such as tail rattles, upright posture, etc. However, young Ast-IL-6 KO mice (10-11 week-old) more similar to the 7 week-old IL-6 KO mice from Alleva's study, showed the opposite tendency, with what seemed a decreased aggressive behavior due to the tendency to present an increased latency to the first attack, a reduced number of attacks and other aggressive behaviour. Nevertheless, it should be noted that young IL-6 KO in Alleva's study did not show significant decreased latency of attack and increased number of attacks in the first encounter with the intruder male (as in our case) but in the subsequent ones, and besides, the different genetic background could be influencing. High aggressiveness is correlated with low cerebrospinal fluid concentrations of the 5-HT (Serotonin) metabolite [703] and by pharmacologically increasing 5-HT levels aggressive behavior is reduced [704, 705]. As IL-6 administration induced profound elevations of serotonin activity in the hippocampus and prefrontal cortex [700] it is reasonable to think that it's putative deficit its potentiating aggressiveness in old Ast-IL-6 KO mice by decreasing serotoninergic system, which should be studied in our animals. However, as steroid hormones, such as androgens, affect aggressiveness [706] and IL-6 is also interrelated with the HPG axis (see 1.3.3.5) it could also be contributing to the final effects observed. Regarding affiliative behavior, neither IL-6 KO nor Ast-IL-6 KO mice

differed significantly from their controls.

Interestingly, Ast-IL-6R KO presented significantly more affiliative behaviour (such as crawl unders, allogrooming, sniffs...) compared to floxed controls both when acting as residents and as intruders, although when they were intruders the number of affiliative beehaviour was significantly lower compared to the other condition, possibly due to the highly aggressiveness of CD1 mice when defending their territory. Regarding to aggressive behaviour, Ast-IL-6R males when acting as residents they exhibited an increased latency to attack and a tendency to have lesser number of attacks, indicating that they were less motivated to aggressively defend their territory, those differences were not seen when they acted like intruders, possibly because for the intruder it test another type of aggression different from the territorial one, as it could be either defensive or fear-induced aggression [707]. Curiously Ast-IL-6R mice presented a similar phenotype regarding affiliative behavior of that shown by mice overexpressing IL-6 under neuronal promoter (NSE-hIL-6) [444] with the addition of decreased aggressive behavior that was not seen in those NSE-hIL-6 mice. Whether these changes are specifically due to lack of astrocyte response to circulating IL-6 via classic signaling or whether compensatory mechanisms derived from a putative increase in central IL-6 levels due to a decreased regulatory feedback on astrocyte-derived IL-6 or any change due to astrocyte dysfunction or other cytokines levels alterations remains to be elucidated.

7.3.3.5 Mechanisms of action

Exact mechanisms by which IL-6 could alter emotionality and behavior are not completely known. It has been described in microarray analyses that IL-6 affects the expression of hundreds of genes in the mouse cortex [473, 474]. There is also indication that synaptic proteins of the hippocampus may be affected by this cytokine [708]. Furthermore, IL-6 is involved in the control of the Hypothalamic–Pituitary-Adrenal (HPA) axis and its connections with the hippocampus and the amigdala, which are important brain centers in the stress response and stress-related behavioral alterations [709, 710]. Also, regarding with aggressiveness behaviour, IL-6 has a putative role inducing glucocorticoids in some scenarios [711] and, since some glucocorticoids can downregulate a subtype of serotonin receptor [712] and thus serotonin [713], Il-6 can have a great impact in intermale aggression [706].

A very recent report showed that IL-6 could decrease the inhibition/excitation ratio in the cortex, being thus a potential contributing mechanism to stress-induced alterations present in several neurological and psychiatric conditions [714]. Also, we have carried out the behavioral tests with lights on, and it will be interesting to do it with lights off considering that IL-6 might affect sleeping patterns/circadian rhythm which could, in turn, affect behavior [715].

Interestingly, we also observed altered gliosis in Ast-IL6 KO mice. The number of microglia lectin-positive cells was increased in males throughout the CNS; in females this was seen only in the cerebellum. Such an increase in microglial cells is in principle consistent with the observed tendency for TNFα and IL-1β to be upregulated in the Ast-IL6 KO mice, since microglia is a source of these cytokines and IL-6 has been shown to in-

hibit their expression in other scenarios [271, 716, 717]. GFAP staining, in contrast, was decreased in Ast-IL6 KO mice, suggesting that a reduction in astrocyte physiological functions might be in place. The interplay between microglia, astrocytes and neurons, including IL-6 production and effects, is well-known, and could have a role in the phenotype of our mice as well. For instance, inhibition of IL-6 trans-signalling in the brain facilitates recovery from LPS induced sickness behavior [718]. Somewhat surprisingly, no differences were observed in the Ast-IL6R KO mice regarding gliosis, which points out once more to the importance of sIL-6R and trans-signaling pathway in IL-6-induced astrogliosis [719], but further experiments will be needed to test that possibility.

It is mandatory in order to correctly understand memory consolidation, to assess adult neurogenesis in hippocampus of both Ast-IL-6 KO and Ast-IL-6R KO mice in a similar way as already done for GFAP/sgp130 animals (see 6.3. Also, levels of central IL-6, TNFα and IL-1β in Ast-IL-6R KO mice would give us valuable clues of how lacking astrocyte-derived IL-6 membrane receptor is affecting locomotion, learning and aggressive behavior.

In summary, the results demonstrate a complex role of IL-6 on mice behaviour. By using mice deficient specifically in the production of IL-6 by astrocytes, as well as mice with the deletion of the membrane IL-6 receptor of astrocytes (which can still respond to circulating IL-6-IL-6R), we demonstrate the importance of this cell type in complex behaviors as measured in the well-known paradigms used.

7.4 Role of astrocyte-derived IL-6 and IL-6R in pathological conditions

7.4.1 EAE

IL-6 is implicated in the pathogenesis of autoimmune disorders such as MS in humans [720, 721]. A critical role of IL-6 in the animal model of MS, EAE, is obvious as total IL-6 KO mice are resistant to EAE [531, 530, 529, 532] and, also, neutralization of IL-6 with antibodies leads to a reduced disease [525], by yet not well defined mechanisms. However, other studies have demonstrated that the transgenic expression of IL-6 in the CNS by viral systems also reduces EAE [526], and that the systemic administration of IL-6 also reduces the clinical symptoms in a viral model of EAE [528]. Taking all data together, it is obvious that IL-6 can potentiate but also inhibit EAE, reflecting the complexity of its actions, whether they are peripheral or central and the type of cell producing the cytokine or responding to it, among others.

Since the production of this cytokine during the course of EAE arises from diverse cellular sources both in the periphery and in the CNS, the specific contribution of each source of IL-6 to the development of the disease needs to be established. Our group have previously demonstrated the type of EAE shown by mice expressing IL-6 only in the brain but not in the periphery (GFAP-IL6-IL-6 KO mice), which did not show resistance to classical symptomatology of MOG_{35-55} immunization and developed a type of EAE that was similar to that shown by GFAP-IL6 mice [555]. Thus, showing that the CNS local milieu

may have dramatic effects and it is able to induce EAE in the absence of peripheral IL-6, although it has always being considered a disease mostly induced peripherally. Also, in experiments of adoptive transference, EAE is less severe in IL-6KO mice than in wild-type mice, which suggests that IL-6 also has to mediate locally the disease in the CNS [530]. The next question to be answered was what happens when it is the brain-produced IL-6 the one that is missing. Due to the major role of astrocyte-derived IL-6 in neuroinflammation [252] and being astrocytes the most abundant cell in the CNS, astrocyte-derived IL-6 was an excellent candidate to be deleted.

Once we successfully achieved and phenotyped at basal conditions conditional Knockout animal for astrocyte-derived IL-6 and IL-6 membrane receptor (Ast-IL-6 KO and Ast-IL-6R KO), as previously explained in this thesis, they were phenotyped in an MOG_{35-55}-induced EAE model. Results indicate that lack of astrocyte-derived IL-6 is unable to prevent EAE induction and pathology. However, in a clear sexual-dimorphism, we found a decreased intensity in the symptomatology of the Ast-IL-6 KO females seen by a decresead clinical score from day 10 onward and by decreased body weight loss, again in Ast-IL-6 KO females, but only in 5th backcrossing group. These results were further supported by a slightly delay in the onset disease and by increased grade of remission at 19-20 dpi in Ast-IL-6 KO mice, both genders together, in 5th and 10th backcrossing group, respectively. Also, females from 10th backcross showed a tendency to have a decrease peak score and cummulative score compared to controls. Differences between backcrossing could be due to the genetic background or also due to the different age of the animals (2 versus 3 month-old), which could empathize only some of the differences while hiding others. All these results together showed a weak, but consistent, role of astrocyte-derived IL-6 in EAE pathology, reinforcing the general idea exposed by our group that central IL-6 must be taken into account in EAE pathology [552, 555], which should be further studied, specially central cytokines levels and cellular population, by means of rt-PCR and flow cytometry at different times post-induction, as already performed in GFAP-IL6-IL-6 KO mice.

Regarding the lack of astrocyte-derived IL-6 membrane receptor, we can appreciate an slightly increased symptomatology, specially in females, understood as increased clinical score from 17 dpi onwards in Ast-IL-6R KO females and a decreased grade of remision at 22 dpi in both genders of Ast-IL-6R KO, always compared to floxed controls. These results are in agreement with previous studies showing that immunized mice lacking cell surface expression of astrocyte-derived gp130 (GFAP-Cre gp130 KO) not only succumbed to chronic EAE, but also developed clinically a significantly more severe EAE and with worse recover rate than control mice, mainly because of increased astrocyte apoptosis, increased numbers of CD4 t cells in the CNS and increased demyelination [722].

Increased severity is much more obvious in this gp130 KO than in our Ast-IL-6 KO receptor, it is probably because of lacking gp130 receptor impairs signal transducing of nearly all IL-6 cytokines family, not only IL-6, being therefore more difficult to compensate one for each other. We still have to assess cytokines levels in CNS in our model but, interestingly, these GFAP-Cre gp130 KO showed strongly upregulated expression of IL-6, LIF, OSM, and IL-27 in spinal cord at 14 dpi, whereas at 25 dpi IL-6, LIF, OSM, and IL-27 levels declined [722]. As GFAP-Cre also affects a subpopulation of neurons,

this group decided to delete gp130 in neurons to assess whether astrocytes or neurons were responsible for aggravation of EAE in GFAP-Cre gp130 KO mice, showing that only the absence of gp130 on astrocytes, but not on neurons is the responsible for the increased susceptibility phenotype [722]. They further demonstrated that diminished activation of the gp130-SHP2/Ras/ERK pathway reproduced all pathological features observed in GFAP-Cre gp130 KO mice, including astrocyte loss, lack of astrogliosis, a significantly more severe clinical course, increased T-cell infiltration, and severe demyelination; while mice with intact gp130-SHP2/Ras/ERK signaling but impaired STAT activation in astrocytes developed a similar clinical course compared to floxed controls and decreased compared to GFAP-Cre gp130 KO and GFAP-Cre gp130-SHP2/Ras/ERK KO, due to an astrocyte-dependent reduction of autoimmune t cells in the CNS [722]. It would be interesting to assess SHP2/Ras/ERK pathway activation in our model animals.

Although symptomatology differences between our knock-out animals and controls were not very robust, histological and immunohistological changes were more consistent and with greater differences in Ast-IL-6 KO mice from 5th backcrossing group. Thus, Ast-IL-6 KO females presented a reduced inflammatory state in spinal cord, as revealed by a decrease in T-lymphocytes infiltrates; there were also signs of decreased gliosis, and a reduced demyelination and a decreased angiogenesis.

Autoreactive T-cells can result in inflammatory demyelination of the CNS and knowing that the frequency of Tregs in MS patients is unchanged from controls [723] (although their function is impaired) could explain the decreased demyelination seen only in Ast-IL-6 KO females, the only which presented a decreased in T-lymphocytes infiltration. IL-6 has also a major role in Th17 cell differentiation from naive CD4+ T-cells (reviewed in [538]); particularly in the EAE model [539, 527]. Moreover, showing the importance of infiltrating cells in EAE pathology, EAE-resistant IL-6 KO mice demonstrated a deficiency in Th17 cells infiltrated in the CNS [83]. When responsiveness to IL-6 is eliminated only in T helper cells there is resistance to EAE, as IL-21 pathway is intact but not active in the absence of IL-6 [550]. Th17 cells produce IL-17 (among other cytokines) which enhances IL-6 production by astrocytes, which in turn induces differentiation of Th17 cells in a positive feedback loop between IL-17 and IL-6 via activation of NF-κB and STAT-3 [223, 540] that would not be present in our Ast-IL-6 KO animals. However, as Ast-IL-6 KO animals are not resistant to EAE induction and finally reached the same score as WT, we can venture to say that astrocyte-derived loop is not necessary for the development of the disease as it is probable that neuronal, endothelial and microglial IL-6 allow this positive feed-back between IL-17 and IL-6. In order to check all these hypothesis, a detailed study of the exact lymphocytic population present in the infiltrates is needed and planned to do in the near future in the 10th backcrossing group as well as in Ast-IL-6R mice.

7.4.2 Traumatic brain injury

IL-6 is also a critical molecule during a CNS injury with a very complex role due to its plurifunctionality and outcome's duality. Thus, it is mandatory the study of IL-6 context-dependent role during the inflammatory response after a CNS injury and the mechanism

of action that will ultimately determine either a beneficial or a detrimental role of this cytokine to the final output.

IL-6 is upregulated in several animal models of brain injury [466, 467, 465] and shows a myriad of actions as suggested by studies in GFAP-IL-6 and IL-6 KO mice. The transcriptomic analysis of IL-6KO mice versus WT mice [473] and that of GFAP-IL-6 mice [474] in a model of brain cortex cryoinjury revealed that IL-6 modulates the expression of many genes involved in inflammation, apoptosis, oxidative stress and synaptic activity among others. GFAP-IL-6 mice showed more rapid healing and recovery after traumatic brain injury [469, 468], while IL-6 KO mice presented a slower rate of recovery and healing [468]. Clinically, IL-6 levels in CSF and brain parenchyma correlate with improved outcome after severe TBI in humans [477, 478] confirming IL-6 neuroprotective effects after TBI. However, there are conflicting data showing that icv administration of anti-IL-6 antibody did not influence the edema or the acute cognitive and neurological motor deficits after injury [475] and that IL-6 KO mice showed no changes after TBI in BBB integrity, neurological impairment and inflammatory cell recruitment [476]. The reasons for these discrepancies remain unclear.

Our results with mice, from 5th backcrossing group, lacking astrocyte-derived IL-6 indicate no changes in survival but a clear effect in healing (measured as injury size), in which Ast-IL-6 KO animals presented a faster reduction of the damaged area respect floxed mice. We also observe a clear sexual dimorphism in which females are more resistant to injury and have faster recovery rates, presumably because of higher antioxidant expression [724], being at 10 dpl nearly completely recovered in both genotypes preventing to see differences between them. However, a totally different picture emerges when analysing data from 10th backcrossing group. In that case, we observe a generalised increase in mortality compared to 5th backcrossing group; but shockingly, in Ast-IL-6 KO mice mortality rates were even higher, specially in males, reaching up to 19% mortality. These results, totally opposing the first idea given by the experiment with 5th backcrossing mice that astrocyte-derived IL-6 had detrimental role in brain injury resolution seems to indicate just the contrary, being in line with literature showing that IL-6 deficiency is detrimental in TBI [472]. However, results from total injured volume in 10th backcrossing animals showed no differences between genotypes and a deeply decreased injured volume compared to 5th backcrossing animals, maybe due to the fact that in this case, animals with increased injury size died. These strong and disturbing differences between both genetic backgrounds are difficult to understand and explain, mice age variation of only 1 month between both experiments make it unlikely to have major effects. Experimenters who performed the procedure, as well as the protocol, where the same in all cases. Being in partially or totally in C57BL/6 genetic background is also unlikely to have alone so strong effects. The only visible difference that we are aware of is a lesser quality of the dry ice pellets used in the second procedure, we had recently changed our manufacturer and the dry ice pellets were more fragile and opaque, indicating that it was old. Unfortunately, we continued with the experiment and it is possible that this bad quality of the pellet made ice crystals splintered into the nervous tissue aggravating the pathology, although unfortunately, it does not seem very realistic.

Regarding IL-6R animals, mortality due to this procedure was extremely elevated (around

50%) in both of the two separate experiments we performed. At least one of them was with bad quality dry ice pellets (as it was the same day we performed Ast-IL-6 mice from 10th backcrossing. However, a new backcrossing strategy will be performed before performing a new experiment in order to discard the IL-6 receptor deficiency as the cause for he elevated mortality. Ast-IL-6R KO mice showed an increased mortality rate compared with controls, especially because of females. In relation with injured size, females showed a decreased lesioned area compared to males and Ast-IL-6R KO mice showed a marginally significant decrease in injured volume compared to floxed controls which although it is a priori contradictory with the increased mortality rate in KOs, it could be in fact a result of this increased mortality, as only animals with the smallest lesions would be able to survive. In fact, Ast-IL-6 KO females, which showed the highest mortality, are the ones with the more accused decrease of the injured size. Reasons underlying this increased mortality in Ast-IL-6R KO females remains unknown. Our results also indicate an increased lesioned volume at 10 dpl compared to 3 dpl but, as both times were performed in two different experiments (as the hight mortality prevented to do together) it is not correct to compare them, as external conditions (such as dry ice pellet state) could be influenting.

Histological changes in Ast-IL-6 animals from 5th backcrossing group showed a normal increase in astrogliosis after the lesion without major differences between genotypes. Regarding microgliosis, a significant increase of activated microglia at the border of the injury in Ast-IL-6 KO males respect floxed controls was seen at 3 dpl; although, both sexes of Ast-IL-6 KO showed a faster recovery at 10 dpl of the normal resting phenotype, as males had an increased number of basal microglia and females a reduction of fully active cells respect to controls. Those results indicate that, not only gliosis is not impaired in Ast-IL-6 KO in contrast to total IL-6 KO mice [472, 471, 257], but, in the case of microgliosis, it is increased in our conditional KO mice in a gender-dependent way. Increased microgliosis and a competent astrogliosis might be beneficial for lesion recovering as seen in GFAP-IL-6 mice and in other models such as axonal lesion or Alzheimer disease, presumably by enabling cellular debris phagocytosis and growth factor production, reviewed in [725].

Lymphocytic infiltration is a key factor for the tissue remodelling and indicates the magnitude of the inflammatory response subsequent to the injury. IL-6 under inflammatory conditions facilitates leukocytes infiltration but, surprisingly, Ast-IL-6 KO mice presented an increase of lymphocytic infiltration at the border of the lesion respect to controls at both 3 dpl and, even more pronounced, at 10 dpl, again in contrast to total IL-6 KO mice, which presented decreased lymphocyte recruitment [258]. However, after CNS injury and BBB rupture there is a massive entry of leukocytes, proportionally to the severity of the lesion, which will produce IL-6 in our conditional KO mice as opposed to total IL-6 KO animals. Also, compensatory mechanisms due to the lack of astrocyte-derived IL-6 previously seen in normal conditions, such as increased microglia numbers in encephalon and a tendency to have increased TNF-α level [630] (which also stimulate lymphocytic infiltration) are likely to contribute to lymphocytic infiltration in our animals.

Finally, regarding the number of vessels, we observe a decrease in Ast-IL-6 KO mice from both genders at 3 dpl and in females at 10 dpl compared to floxed animals. We

also find this decrease in non-injured females, presumably due to lower IL-6 levels in the intact brain [630] exactly the opposite that happens with GFAP-IL-6 mice, showing extensive revascularization, both in basal condition [252] and after an injury [468]. Results are in agreement with published works showing that IL-6 promotes vasogenesis [726].

In conclusion, we have shown that lack of astrocyte-derived IL-6 is not sufficient to prevent EAE disease but it is able to slightly ameliorate clinical scoring and the inflammatory component. In contrast, astrocyte IL-6 deficiency caused a significantly better resolution of a traumatic brain injury. Those results highlight again the dual role of IL-6, which is highly contextual as revealed by the important differences compared to total IL-6 KO mice.

7.5 Role of trans-signaling in mediating IL-6 actions

The importance of classic versus trans-signaling in mediating IL-6 actions in the CNS is relatively unknown, although it was recently reported that icv injection of sgp130 (a trans-signaling inhibitor) enhanced the recovery of rats from LPS-induced receptor activation and production of IL-6 and sickness behavior, suggesting a role for trans-signaling in this process [718].

The use of sgp130 to block IL-6 trans-signaling specifically has proven in various biological settings elsewhere [727] and in transgenic mice that overproduce sgp130 in the periphery, trans-signaling but not classic IL-6 signaling is markedly blocked [728]. The aim of our study was to examine if IL-6 trans-signaling blockage was capable to prevent or ameliorate some of the consequences of the neuroinflammatory and neurodegenerative model GFAP-IL-6 mice at different ages. A transgenic mouse model with sgp130 production targeted to the CNS, under GFAP promotor, was intercrossed with GFAP-IL6 transgenic mice generating bigenic mice with coproduction of IL-6 and sgp130 in the brain, allowing to assess the role of trans-signaling in mediating IL-6 actions in the CNS. The validity of this model is illustrated somewhere else [633].

The strong astrogliosis seen in the cerebellum from GFAP-IL6 mice [252, 157] was prevented at the earliest age and significantly diminished up to 6 months-old in bigenic mice. Astrocyte reactivity was also increased in cerebral cortex of GFAP-IL6 mice and significantly decreased in bigenic mice at young ages. STAT3 signaling is thought to be involved in astroglial responsiveness being modulated by other gp130 family members [729, 316, 730, 731]. Also, mice with a conditional deletion of STAT3 in astrocytes have reduced astrocyte activation in response to injury [261]. Enhanced trans-signaling is alone capable to produce astrogliosis in the CNS, as showed by transgenic mice with systemic production of human IL6/sIL-6R but not IL-6 alone, exhibiting astrogliosis and neurologic signs [254]; but its blockage by sgp130 is translated into the prevention/reduction in astrogliosis only at young ages. Although the cause of this loss of effect with age is mainly unknown it should be examined whether or not there is insufficient sgp130 concentration to effectively block trans-signaling at older ages. Exogenous icv injections of sgp130 could be administered at adult age before discarding trans-signaling as the main pathway driving to astrogliosis.

Chronic IL-6 production in the CNS is also associated with robust microgliosis in the cerebellum of GFAP-IL6 mice, but we found that the extent of microgliosis was markedly decreased in the cerebellum of the bigenic mice regardless of age. Interestingly, it has been shown that the development of neurodegenerative changes and progressive learning deficit in GFAP-IL6 mice correlated with the degree of microgliosis [446], thus, a behavioral analysis of bigenic mice would give us extremely valuable information on the extend of neuroinflammation effect on cognitive impairment. The decreased microglial response to IL-6 in the presence of sgp130 could be caused by reduced trans-signaling or by other factors such as the decreased astrogliosis, vascular disease and neurodegeneration, but astrogliosis seems quite unlikely as it is only present at younger ages while the decreased microgliosis is found at all ages. As attenuation of microgliosis occurred despite evidence for STAT3 activation in these cells and, in rodent microglia, STAT3 activation is induced by IL-6 via classic signaling [264], other factors may be involved in the reduced microglial response besides reduced IL-6 trans-signaling. However, cultured microglia requires the presence of sIL-6R and thus trans-signaling mediation, to modulate certain cellular responses of these cells; despite being able to respond to IL-6 alone [264]. A differential response from the same cell to classic versus trans-signaling may have important implications for our IL-6 understanding, with differential responsiveness of specific genes to classic versus trans-signaling [633].

IL-6 production in the CNS highly affects the cerebrovascular endothelium as shown by several physical, molecular and functional changes observed in this tissue in the cerebellum of GFAP-IL6 mice [252, 267]. Despite the fact that human [732] and murine [726] cerebrovascular endothelial cells can respond to IL-6 by classic signaling, a significant reduction in vascular alterations in the cerebellum of GFAP-IL6/sgp130 mice at all ages studied was observed, shown by reduced vascular endothelial cell proliferation in the cerebellum. One outcome of chronic angiogenesis in the cerebellum of the GFAP-IL6 mice is the loss of BBB integrity and increased leakage of the blood vessels [267]. Consistent with our findings here, the significant reduction in vascular endothelial cell proliferation in the cerebellum of the GFAP-IL6/sgp130 mice was accompanied by a marked decrease in BBB leakage [633].

Several molecular and cellular changes in GFAP-IL6 mice, specially a marked neurogenesis reduction [314], contribute to progressive neurodegeneration with loss of neuronal integrity and function [252, 267]. As STAT3 activation pattern in GFAP-IL6 mice neurons indicates that these cells are relatively unresponsive to chronic IL-6 production [633] it is suggested that the neurodegeneration in these mice may be mediated by a neurotoxic environment generated by other factors. The reduction in the cerebellar neurodegeneration observed in the GFAP-IL6/sgp130 mice, regardless of age, may reflect the overall decrease in these pathogenic changes including reduced gliosis and BBB leakage. In accordance to this, previous studies showed there is a close correlation between the degree of microglial activation and progressive neurodegeneration and learning impairment in the GFAP-IL6 mice [446], which also makes very interesting to study bigenic mice in a learning parading such as Morris water maze.

Moreover, a role for inflammation and microglial reactivity has been associated to decreased neurogenesis [313, 315]. Interestingly, although there was a complete rescue

of neurogenesis in GFAP-IL6/sgp130 mice at 1.5 months of age this was not sustained at older ages examined, being neurogenesis very sensitive to IL-6-caused perturbations.

Finally, our findings indicate that trans-signaling is crucial for IL-6 responsiveness by selected cellular and molecular targets in the brain including astrocytes and Bergmann glia. However, despite this selectivity, trans-signaling blockage had broader benefits reducing many detrimental effects of IL-6 in the brain and may represent a useful therapeutic approach for the treatment of the numerous neuroinflammatory and neurodegenerative diseases with IL-6 implication. The use of GFAP-IL6/sgp130 mice in other neuroinflammation models such as EAE at different ages is promising [534] and would give us valuable information about the expectations of targeting trans-signaling to prevent and treat CNS diseases.

CHAPTER 8

CONCLUSIONS

Regarding astrocyte-derived IL-6 and astrocytic mIL-6R:

1. Tissue-specific IL-6 (Ast-IL-6 KO) and mIL-6R (Ast-IL-6R KO) knock-out mice have been obtained.

2. Lack of astrocyte-derived IL-6 and astrocytic IL-6R influenced the early survival, presumably due to intrauterine death.

3. Body weight was affected by astrocytic IL-6 deficiency in a gender-dependent manner, being increased in Ast-IL-6 KO males. Astrocytic IL-6R deficiency had no effects on body weight.

4. Astrocyte-derived IL-6 and astrocytic IL-6R deficiency influenced behaviour:

    - A decreased locomotor activity (horizontal and rearings) were observed in Ast-IL-6 KO and in Ast-IL-6R KO mice in both Hole-Board (HB) and Elevated Plus-Maze (EPM) tests.
    - A more anxious phenotype was seen in adult Ast-IL-6 KO mice in HB (internal deambulations and defecation) and in EPM (open arms, defecation and unprotected head-dips). This behaviour, in contrast, was reversed in some parameters (open arms and unprotected head-dips) in young animals. No clear phenotype was observed in IL-6R KO mice.
    - Ast-IL-6 KO and Ast-IL-6R KO mice showed reduced exploratory behaviour in HB test (head-dips and unexplored squares) and Ast-IL-6 KO also in EPM test (protected head-dips).
    - Lack of astrocyte-derived IL-6 moderately reduced despair behaviour in Tail Suspension Test in 8 month-old mice.
    - Astrocyte-derived IL-6 deficiency produced spatial learning impairments in Morris Water Maze but seemed to have a beneficial role in memory consolidation. Lack of astrocytic IL-6R also produced impaired learning and suggested a delayed learning curve.

- Regarding social dominance and aggressiveness, Ast-IL-6 KO females showed more dominant behaviours than floxed mice in the dominance tube test. 11 week-old Ast-IL-6 KO males acting as intruders were less aggressive than controls, while 9 month-old Ast-IL-6 KO males showed the opposite tendency. Ast-IL-6R KO males exhibited more affiliative behaviours and were less aggressive when acting as residents.

5. Basal Ast-IL6 KO mice showed increased microglia and decreased astroglia stainings in the CNS while Ast-IL-6R KO mice showed no significant differences.

6. Astrocytic IL-6 deficiency did not fully prevent EAE's prototypical ascending paralysis course but it significantly decreased its symptomatology in females. In agreement, reduced cellular infiltration, demyelination, and gliosis were seen in the spinal cord of EAE-induced Ast-IL-6 KO females. Ast-IL-6R KO mice showed the EAE prototypical course, but showed an impaired remission at 22 days post-induction (dpi), and females showed an increased clinical score after 17 dpi.

7. The preliminary results obtained suggest that astrocytic IL-6 deficiency showed a detrimental role in traumatic brain injury remission in 5th backcrossing group, which was not consolidated in 10th backcrossing animals. Injured Ast-IL-6 KO presented no changes in astrogliosis but an altered gender-dependent microgliosis and increased CD3 infiltration in the injured area respect to floxed mice.

Regarding IL-6 trans-signaling:

8. Blockage of IL-6 trans-signaling in the CNS reduced many of the detrimental effects that IL-6 have in GFAP-IL-6 neuroinflammation model; such as the severity of the gliosis, vascular alterations, impaired neurogenesis and neurodegeneration.

APPENDIX A

SUPPLEMENTARY DATA

A.1 Behavioral programs guide

In order to use our two behavioral programs, a Microsoft Excel© platform is needed. Once Excel is opened (see Figure A.1) we should initiate Visual Basic Editor by going to *Tools, Macro, Visual Basic Editor*.

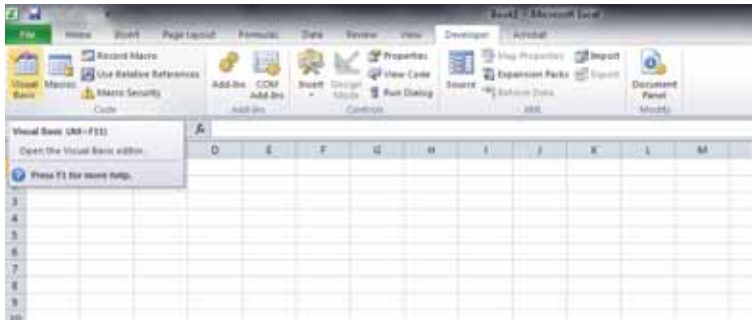


Figure A.1: Accessing Visual Basic for Applications environment.

On the top of Visual Basic Editor, in the *Insert* menu, select *Module* to open the *Module window* that will allow us to insert the program code (see Figure A.2).

In the *Module window*, the code we provide in the supplementary material of our published article by Quintana et al. [630] should be pasted (see Figure A.3).

Also, one form for each behavioral test should be created (HoleBoard and PlusMaze) in which we construct the desired interface for our programs, defining buttons and Text boxes. Below there is as an example of our form for Hole Board test (see Figure A.4).

To acquire data we just have to run our program side by side with the video of the recorded animal test and follow animals' route by pressing each square in which the animal enters and press Rearing button when this behavior is performed (see Figure A.5). After one animal's test, total defecation and head dipping values should be introduced in their respective test boxes, pressing *Save* and *New* to start another animal.

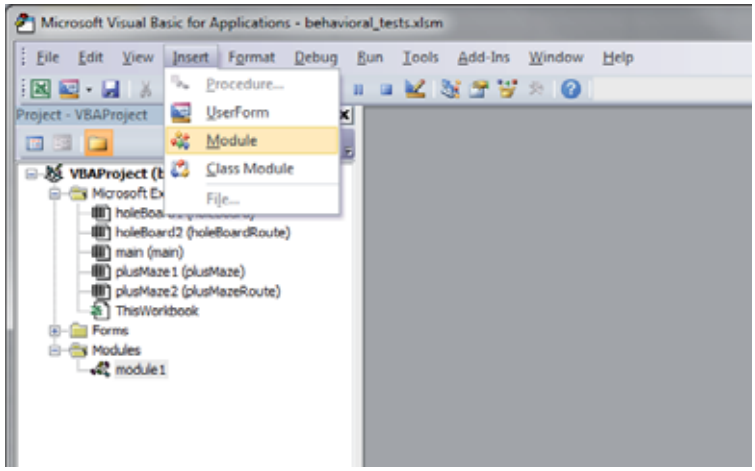


Figure A.2: Creating an empty module in Visual Basic environment.

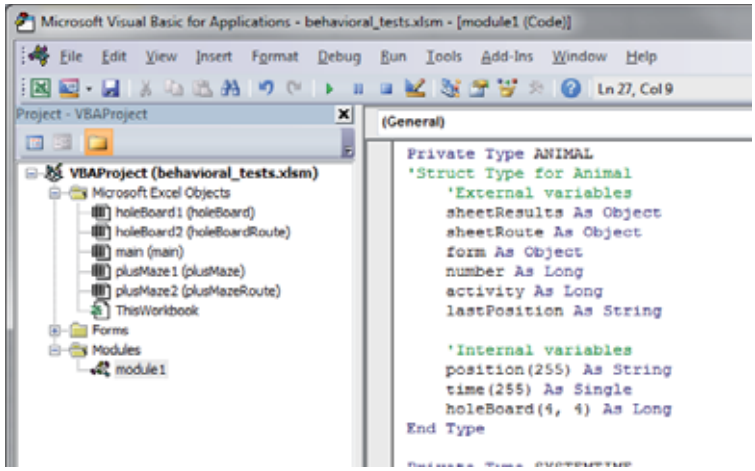


Figure A.3: Pasting the code in the empty module.

When we wish to stop we just have to press Close button and the form will disappear leaving an excel sheet with results: Activity, as total number of squares crossed, Internal and External as numbers of internal and external deambulations, respectively, and non-visited as the number of squares in which mice did not enter (see Figure A.6).

To use Plus Maze program we open Plus Maze userform side by side with animals' recorded test and follow mouse's route by clicking each button crossed as well as clicking the Head Dipping button (HD) and Rearing button when necessary (see Figure A.7).

In this case, when we finish, the software will provide the total number and time in which mouse is performing Head dippings, number of performed rearings, total activity as total number of squares crossed, time spent in each compartment (center, open arms and close arms) and the number of enterings in both open and close arms (see Figure A.8).

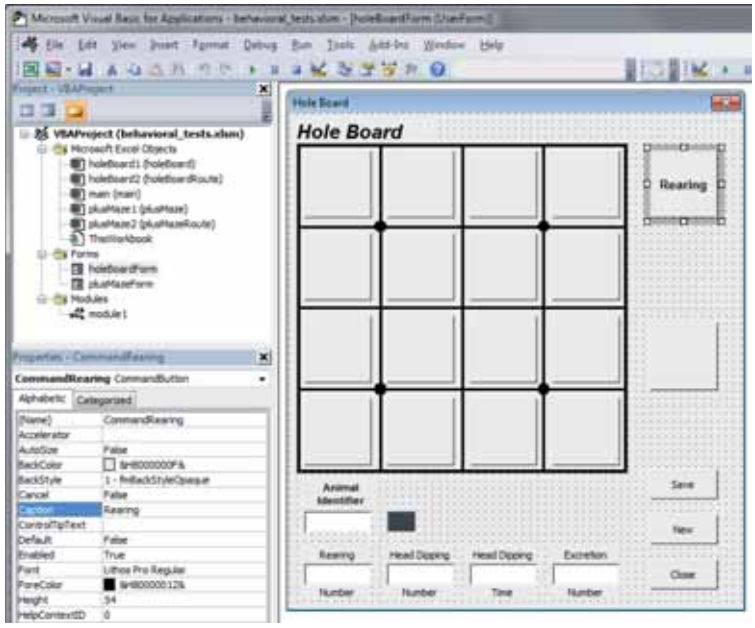


Figure A.4: Form example of our HoleBoard test.

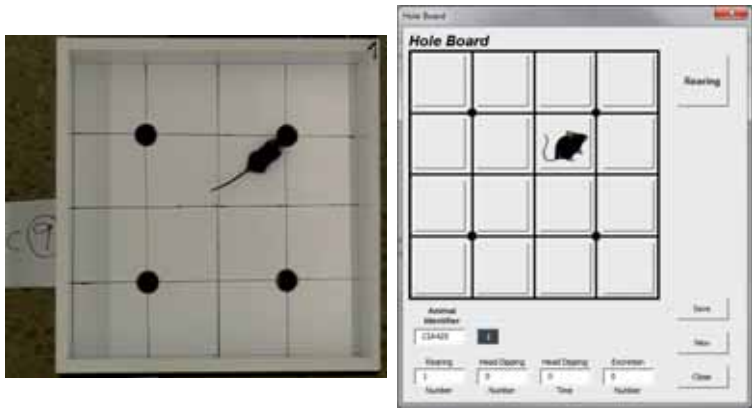


Figure A.5: Example of HB testing procedure. Photogram of a recorded test video (Left), side by side with the HB programme interface during the analysis (Right).

Once we have our results, we can observe that in both HoleBoard and PlusMaze it has been created another worksheet after the sheet containing our results, it is called *holeBoardRoute* and *plusMazeRoute*, respectively (see Figure A.9).

In this worksheet, the program stores the route that animal performed by saving each position of mice in the test apparatus, as well as the time, making possible to plot animal's route during the test, as the following example (see Figure A.10).

However, in the supplementary material of our published article by Quintana et al. [630], we also provide the working Excel file that we have used, in case anyone wanted to directly use or modify it.

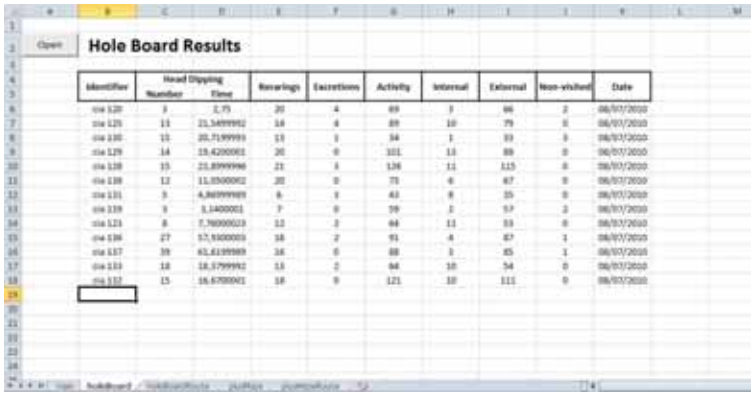


Figure A.6: Results as shown in Excel sheet.

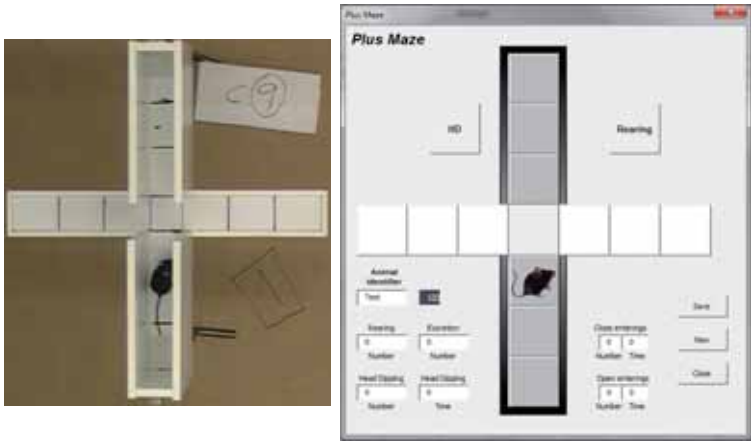


Figure A.7: Example of EPM testing procedure. Photogram of a recorded test video (Left), side by side with the EPM programme interface during the analysis (Right).

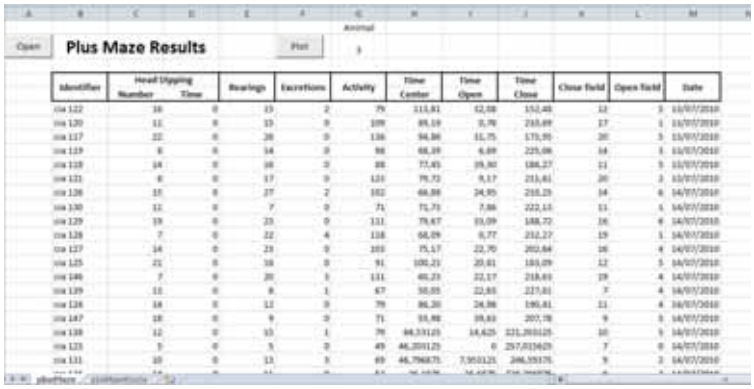


Figure A.8: Results for EPM as shown in Excel sheet.

Figure A.9: Example of the data saved for the EPM route.

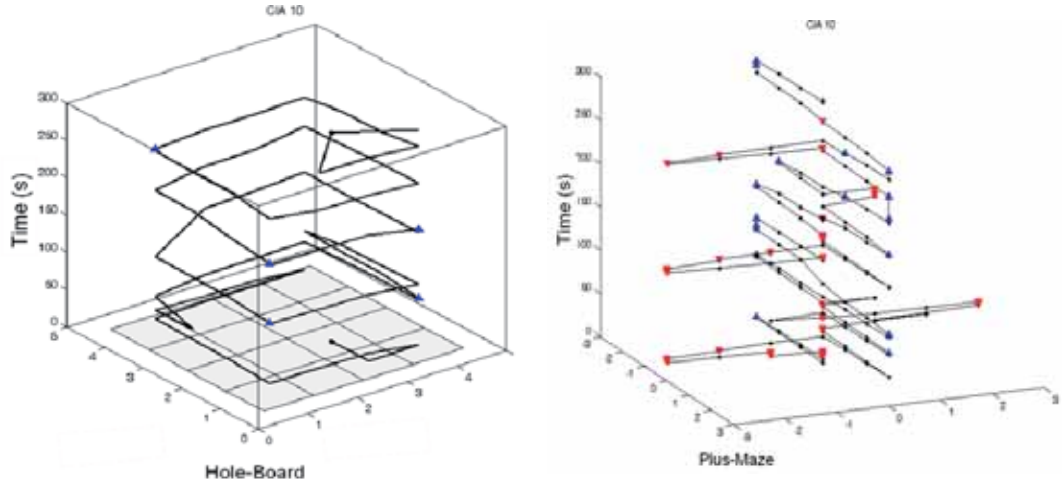


Figure A.10: Example of one mouse performance in both behavioral tests. Total duration of the test (5 min/ 300 seconds) is plotted in the vertical axis while in the horizontal axis the situation of each square is shown. As time passes, we observe movement patterns and mouse's behavior like rearings (blue marks) and head-dipps (red marks).

A.2 Additional results

A.3 Behavioral tests

A.3.1 Ast-IL-6 mice

Additional behavioural traits analysed in EPM are shown in Figure A.11 and statistical results are shown in Table A.1.

	Ast-IL-6 5th backcrossing			Ast-IL-6 10th backcrossing			
	Genotype (G)	Sex (s)	Gs	Genotype (G)	Sex (s)	Age (a)	Interactions
Plus-Maze							
Deambul.	**	-	*	***	-	-	-
%TO	-	-	*	-	-	-	Ga = ***
nºOE	-	-	-	-	*	-	Ga = **
%OE	-	-	-	-	-	-	Ga = ***
TC	-	-	-	**	-	-	Ga = ***
nºHD	-	-	*	-	-	-	Ga = **
latCen	-	-	-	-	-	-	-
meanTO	*	-	**	-	-	-	-
meanTC	*	-	-	***	-	-	-

Table A.1: Summary of significances obtained regarding additional parameters analyzed in EPM behavioral test.

Ga denotes interaction between genotype and age. Deambul. stands for total deambulation, %TO for percentage of time spent in open arms, nºOE and %OE for number, and percentage respect total arms entries, of open arms entries, TC for time in close arms, nºHD for total HD, latCen for latency to leave central square and mean TO or mean TC stands for mean time per entry in open and closed arms, respectively (s). *, ** and *** P at least ≤ 0.05, ≤ 0.01 and ≤ 0.001 respectively.

A.3.2 Ast-IL-6R mice

Additional behavioural traits analysed in EPM are shown in Figure A.12.

A.4 Brain IHC at physiological conditions

A.5 EAE

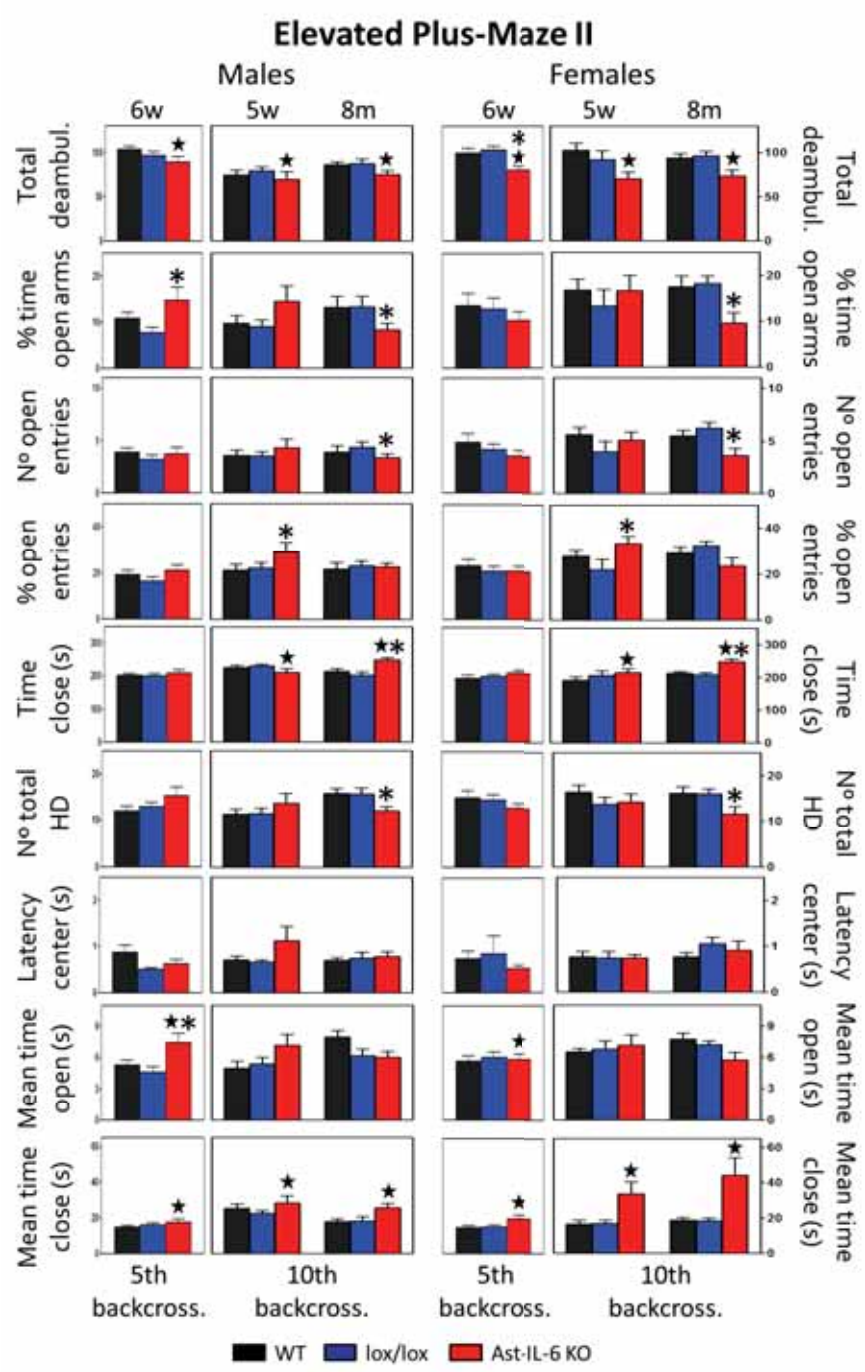


Figure A.11: Additional behavioural traits analyzed in the EPM apparatus. Total deambulation (Total deambul.), percentage of time spent in open arms, Nº and percentage of open arms entries, time spent in close arms (Time close), total number of HD and latency to leave the central square (Latency center) and mean time spent per each entry in either open and closed arms are shown for Ast-IL-6 KO, floxed and WT mice for both 5th (6 week old) and 10th backcrossings (5 week-old and 8 month-old). N = 11–37. ★ *P* at least ≤ 0.05 vs floxed mice. ✻ *P* at least ≤ 0.05 vs floxed mice after genotype interaction decomposition.

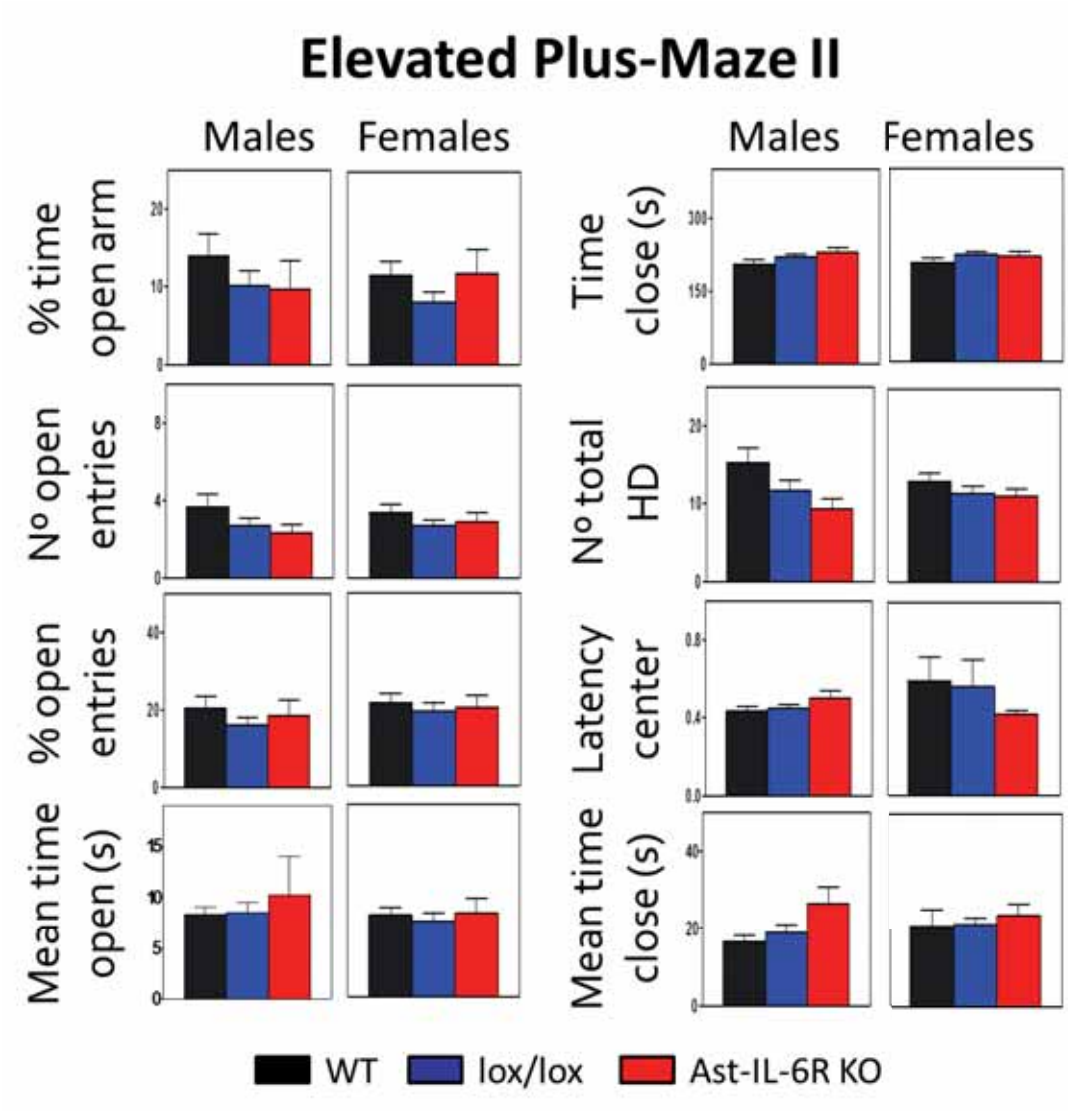


Figure A.12: Additional behavioural traits analysed in the EPM apparatus. Percentage of time spent in open arms, Nº and percentage of open arms entries, time spent in close arms (Time close), total number of HD, latency to leave the central square (Latency center) and mean time spent per each entry in either open and closed arms are shown for Ast-IL-6R KO, floxed and WT mice. N = 19–30.

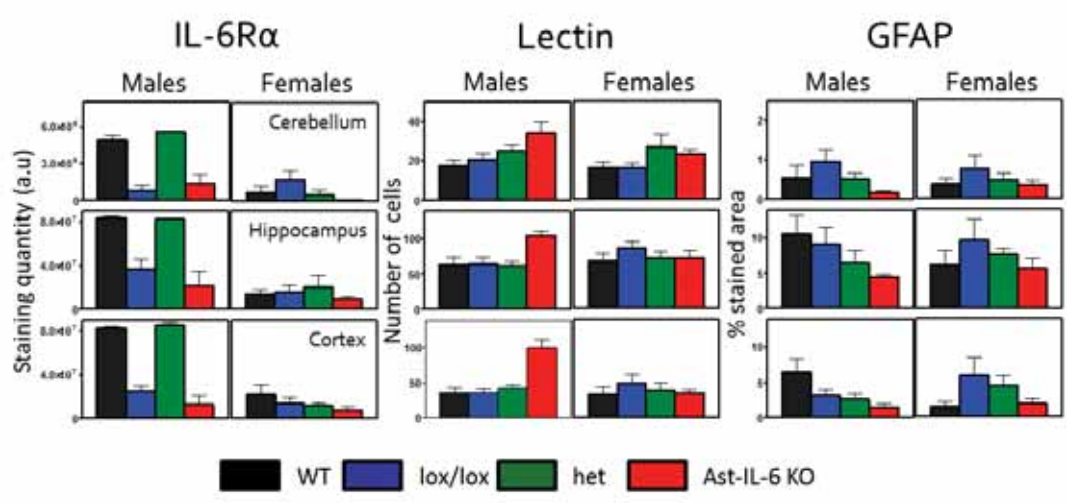


Figure A.13: IL-6Rα, Lectin and GFAP quantifications in encephalon of Ast-IL-6 KO, floxed, WT and heterozygous mice.

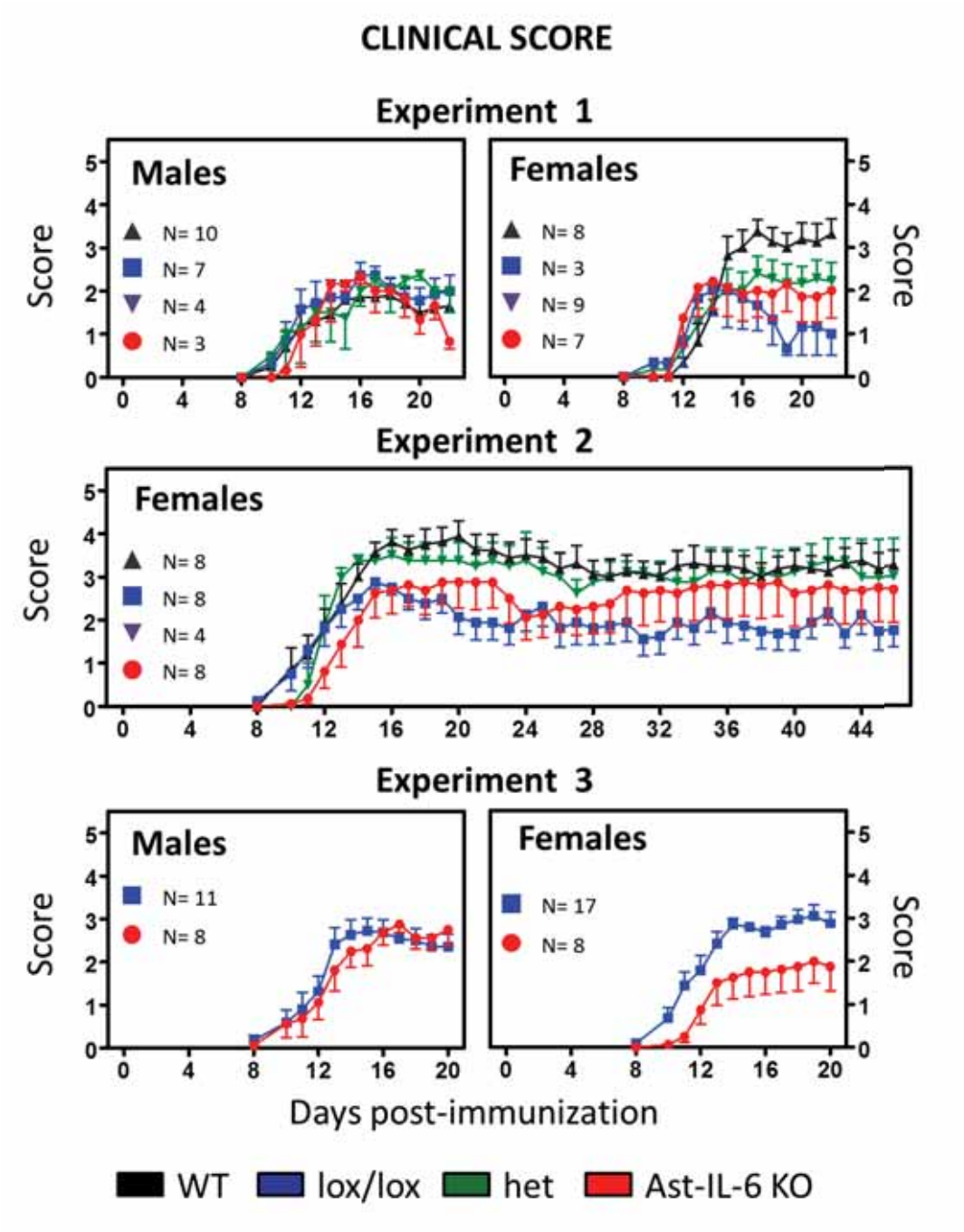


Figure A.14: Clinical scores of EAE induced animals from 5th backcrossing group. Three separate inductions were carried out with the duration and number of animals showed in the figure.

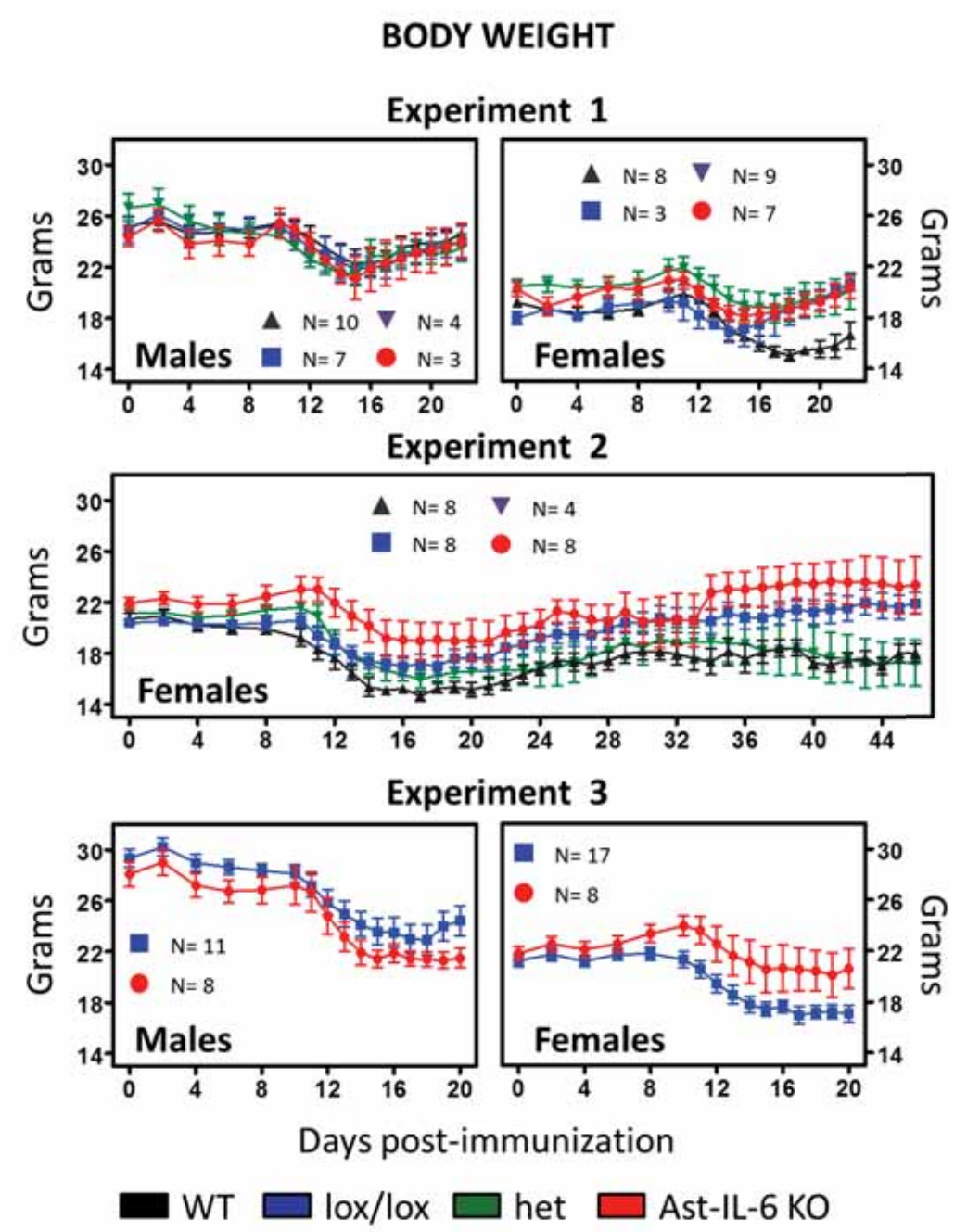


Figure A.15: Body weight of EAE induced animals from 5th backcrossing group. Three separate inductions were carried out with the duration and number of animals showed in the figure.

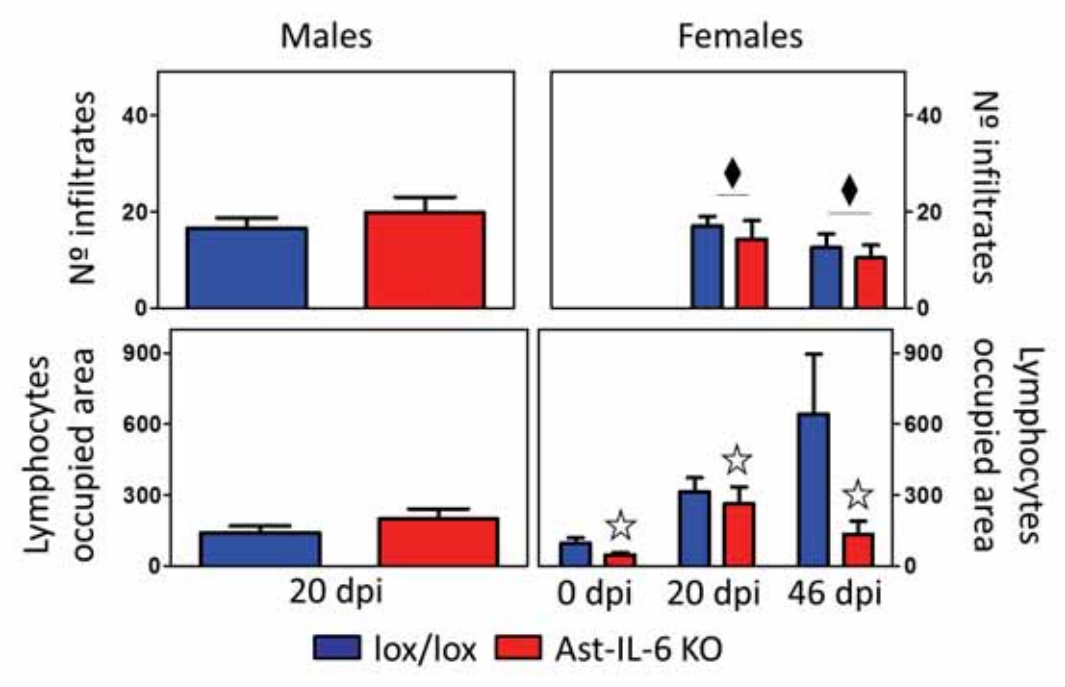


Figure A.16: Number of infiltrates and lymphocytes occupied area in brain. Assessment of the total number of CD3 infiltrates (top) and lymphocytes occupied area (bottom) in brain (0 dpi, N= 4) and EAE-induced animals at 20 and 46 dpi (N = 5-20). Results are mean ± SEM. A GzLM was performed for each sex. ☆P = 0.057 vs floxed mice. ♦ P at least ≤ 0.05 vs control group.

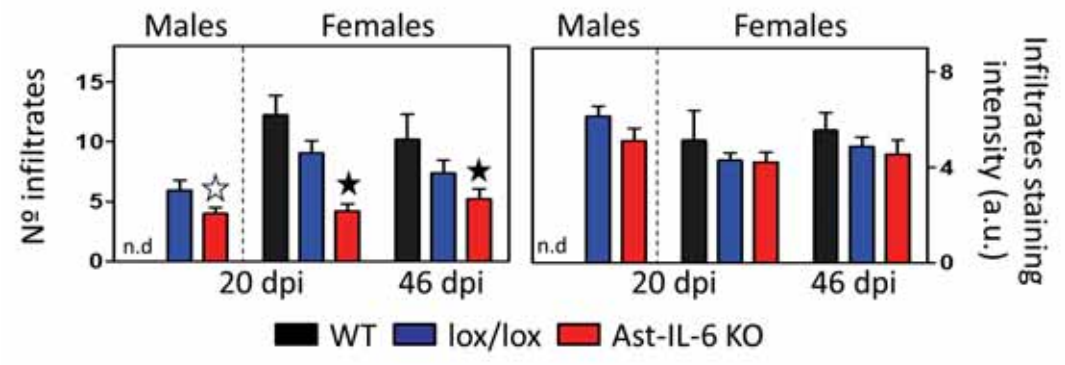


Figure A.17: Areas with increased microgliosis or macrophage infiltrates in EAE-induced animals. Both the number of infiltrates and their stained intensity were assessed in spinal cord of EAE-induced animals at 20 and 46 dpi (N = 5-20). Results are mean ± SEM. A GzLM was performed for each sex. ★ and ☆P at least ≤ 0.05 or ≤ 0.1 vs floxed mice, respectively.

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