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## Julien GENTY

Born on 20 October 1988 in Soyaux (France)

# CONSEQUENCES OF EARLY LIFE STRESS FOR PAIN PROCESSING AND COPING WITH STRESS IN LATER LIFE: BEHAVIOURAL AND BIOCHEMICAL STUDIES IN THE RAT

## Dissertation defence committee

Dr Fernand Anton, dissertation supervisor
*Professor, Université du Luxembourg*

Dr Andrea Ebersberger
*Senior Lecturer, Universität Jena*

Dr Paul Heuschling, Chairman
*Professor, Université du Luxembourg*

Dr Nadya Freund
*Associate Professor, Universität Bochum*

Dr Pascal Darbon
*Associate Professor, Université de Strasbourg*

*"Nothing exists in our intelligence that was not first in our senses"*

Democritus
460-370 BC

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To Lucie.

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## List of abbreviations

ACC: Anterior cingulate cortex

ACTH: Adrenocorticotropic hormone

AMPA: α-amino-3-hydroxy 5 methyl-4-isoxazeleproprionic acid

AmpB: Amphotericin b

ANO1: Anoctamine 1

ANS: Autonomic nervous system

AVP: Arginine Vasopressine

ASICs: Acid-sensitive ion channels

ATP: Adenosine triphosphate

BDNF: Brain derived neurotrophic factor

CAM-KII: Calcium/calmodulin-dependent kinase II

CCI: Chronic constriction injury

CFA: Complete Freund's adjuvant

CGRP: Calcitonin gene-related peptide

C-MCs: C-fibers mechano-chemicals

C-MHCs: C-fibers mechano-heat-chemicals

C-MHs: C-fibers mechano-heat

CNS: Central nervous system

CORT: Corticosterone

Cox-2: Cyclooxygenase

CREB: c-AMP response element-binding protein

CRH: Corticotropin releasing hormone

DAG: Diacylglycerol

DEG/ENaC: Degenerin/epithelial Na+ channel

DH: Dorsal horn

DRG: Dorsal root ganglia

EAAC: Excitatory amino acid

EAATs: Excitatory amino-acid transporters

ELS: Early life stress

EPSCs: Excitatory post-synaptic currents

ERK: Extracellular signal-regulated kinase

FSH: Follicle-stimulating hormone

GABA: γ-Aminobutyric acid

GAD: Glutamate decarboxylase

GC: Glucocorticoid

GDNF: Glial-cell derived neurotrophic factors

GLAST: Glutamate/aspartate transporter

GLT-1: Glutamate transporter-1

GnRH: Gonadotropin releasing hormone

GPCR: G-protein coupled receptor

GR: Glucocorticoid receptor

GRE: Glucocorticoid response element

HPA: Hypothalamo-pituitary adrenal

IASP: International association for the study of pain

IL-1β: Interleukin 1β

IL-6: Interleukin 6

InsP3: Inositol-(1, 4, 5)-trisphosphate

KCC2: Potassium chloride cotransporter 2

LBC: Ligand binding core

LH: Luteinizing hormone

LTP: Long term potentiation

MC2R: Melanocortin 2 receptor

mGluR: Metabotropic receptor

MR: Mineralocorticoid receptor

MS: Maternal separation

Nac: Nucleus accumbens

NGF: Nerve growth factor

NK-1: Neurokinin-1

NMDA: N-methyl-d-aspartate

NTS: Nucleus tractus solitaris

PAFs: Primary afferent fibers

PAG: Periaqueductal gray

PFC: Prefrontal cortex

PGE2: Prostaglandin E2

PKA: Protein kinase A

PKA: Protein kinase A

PLC: Phospholipase C

PND: Post-natal day

PNI: Peripheral nerve injury

PRL: Prolactin

PTSD: Post-traumatic stress disorder

PVN: Paraventricular nucleus

RVM: Ventral medulla

SC: Spinal cord

SCC: Side chain cleavage

SHRP: Stress hypo-responsive period

SNRP: Stress non-responsive period

SS: Social stress

TG: Trigeminal ganglia

 TNF-α: Tumor necrosis factor-α

TN-α: Tumor necrosis α

TRH: Tyrotropin-releasing hormone

TRP: Transient receptor potential

TRPA: Transient receptor potential subfamily A

TRPC: Transient receptor potential subfamily C

TRPM: Transient receptor potential subfamily M

TRPV: Transient receptor potential subfamily V

TSH: Thyroid stimulating hormone

VTA: Ventral tegmental area

WDR: Wide dynamic range neurons

WHO: World health organization

# Chapter I: Preface

Stress is commonly defined as the response to a non-specific situation presenting a psychological and/or physical challenge. In order to react in an appropriate manner to environmental threats the body will trigger a wide range of defence mechanisms. However cases where challenges are sustained and the individual does not have the ability to cope with the stress are nowadays believed to be a main factor for the onset and exacerbation of a broad range of disorders. Among these are psychiatric disorders such as depression but also pain affections. Pain is described by the International Association for the study of Pain (IASP) as an unpleasant sensory and emotional experience associated with actual or potential tissue damage. As for stress, this system aiming at preserving the body integrity can become defective and enhance pain sensitivity or foster the development of chronic pain. These two health problems categories each represent a considerable issue of public health. Indeed the World Health Organization (WHO) estimates that 27% of the European adult population had experienced at least one episode of mental disorder and the IASP reported that 19% of the pan-European population experienced chronic pain (Macfarlane, Pain 2016). Furthermore, chronic pain and stress-related disorders are greatly comorbid, having deleterious effects on the efficacy of treatments. Despite the raising awareness of clinical and pre-clinical research on their overlapping pathways, common mediators and interactions, the nature of the relationship between chronic pain conditions and stress-related disorders is not yet elucidated.

The studies I undertook during my Ph.D. aimed to understand how chronic stress, with an emphasis on early-life stress, is linked to altered nociceptive transmission and to modified chronic pain vulnerability. Early life stress (ELS) was of particular interest as this period of life is subjected to an intense neuronal plasticity of notably stress and pain systems. Furthermore it is increasingly accepted that early life factors are linked to the susceptibility to develop chronic pain conditions in adulthood. As pain is a multidimensional system, I had to restrict my studies to one of the relay stations for the transmission of pain. In the

context of chronic stress, most of the work was done on the brain circuits underlying the affective part of pain but little is known about the effect of chronic pain on spinal nociceptive processes. Since chronic stress is a broad phenomenon altering not only processing at the brain level, I focused my studies on spinal dorsal horn noxious transmission.

The first stage of my work was to assess the impact of ELS on neuropathic pain, a type of chronic pain arising from nerve lesions. In a second step, I sought to determine if the results obtained in this first study were specific to the type of pain (neuropathic) or also were valid for another type of chronic pain, e.g. chronic inflammatory pain. The third study aimed to determine if ELS would predispose to enhanced vulnerability to stress exposure later in life and to concomitant alterations of chronic pain. To finish, during the last year of my Ph.D. I investigated the possible mechanisms underlying the behavioural results using pharmacological manipulations and initiated a separate project devoted to in vivo electrophysiological characterization of the response behaviour of nociceptive spinal dorsal horn neurons.

In the following introduction, I will give an overview of the stress systems, focusing on the hypothalamo-pituitary adrenal (HPA) axis and early life stress. Then I will outline the different part of the pain system and concepts of central sensitization involved in chronic pain.

# Chapter II: Stress mechanisms

## 2.1. Introduction

Stress is a term used in order to define an emotionally and/or physically challenging situation. This possible challenge will be interpreted by the amygdala that will in turn signal the hypothalamus for a coordinated response. The perceived threat will hence induce a so-called "fight or flight" response of the organism by promoting a hallmark of physiological and behavioral adaptations primarily due to an activation of the sympathetic nervous system (SNS). In case where the stress is prolonged, an adaptation syndrome will be triggered involving in this case the endocrine system and the hypothalamo-pituitary adrenal axis.

In chapter II, I will briefly describe the role of sympathetic nervous system during stressful events and I will then focus on the HPA axis and its implication in chronic stress.

## 2.2. Sympathetic nervous system:

The sympathetic and the parasympathetic nervous systems are the two main functional subdivisions of the autonomous nervous system (ANS). They both regulate the function of a multitude of tissues. As they often have opposite effects, the activation of one will decrease the activity of the other on a given tissue. During stressful events, the sympathetic nervous system is predominantly activated resulting in the stimulation the adrenal medulla hence releasing adrenaline and noradrenaline into the blood stream. This leads to a whole body response comprising, among others, bronchial dilatation or increase of heart rate and contraction strength (for review McCorry 2007).

The ANS and its two subdivisions have often been regarded as structures functioning independently from central nervous, endocrine or immune system. It is however now clear that the ANS interacts and regulates various physiological systems (Kenney and Ganta 2014). Many studies revealed a

crucial role of the ANS in mediating nervous and immune systems' interactions (Elenkov et al., 2000; Helwig et al., 2007; Kang et al., 2008; 2009). The relay of information from the immune system to the CNS has be shown to be performed via two main pathways. The neural communication pathway involves mainly the vagal nerve that transmits peripheral immune signals to the brain (Maier et al., 1998). Non-neural mechanisms providing transmission of immune signals to the CNS are mainly related to the blood brain barrier (Banks and Erickson, 2010). Despite the different nature of the communication, both pathways largely depend on the actions of cytokines. These molecules are signaling proteins synthesized and release throughout the body by a wide variety of cells such as neurons, microglia, astrocytes and other immune cells (Galic et al., 2010). The sympathetic nervous system is of high biologic relevance during acute challenge. In physiological conditions, HPA and SNS work in concert to modulate the immune response (Niijma et al 1991; Borovikova et al., 2000). However an "uncoupling of SNS and HPA-axis" has been observed that during chronic pathological conditions and is thought to play an important role in the etiology of diseases (Straub et al., 2006).

The first autonomic response generally subsides within several minutes, the hypothalamus triggers a second and more sustained bodily response: the HPA axis activation. Despite the importance of the ANS, the HPA axis is known to be largely responsible for long term adaptations, psychiatric disorders and vulnerability/resilience phenomenon (for reviews McEwen 2007; Krishnan et Nestler, 2007; Lupien et al, 2009; Daskalakis et al, 2013; McEwen 2017).

## 2.3. Hypothalamo-pituitary adrenal axis

The HPA axis is a central neuro-endocrine system mediating not only the stress response but also involved in the regulation of the immune system's function, energy expenditure and storage rendering this structure an essential component for organisms' homeostasis (Ulrich-Lai and Herman, 2009). As its name

indicates, this system is composed by three major structures: the hypothalamus that integrates information and orchestrates the response, the pituitary gland, capable to secrete and/or release hormones in the blood stream controlling many endocrine functions and finally the cortical part of the adrenal glands, the final effector capable of synthetizing steroid hormones.

### 2.3.1. Description of the axis

#### 2.3.1.1. Hypothalamus

The hypothalamus is a complex structure located in the anterior part of the diencephalon regrouping eleven major nuclei that can be regrouped based on their location in the hypothalamus regions and zones. The nucleus composing the hypothalamus can be regrouped in three sub-regions. The anterior region includes the supra-optic and paraventricular nucleus. The median region encompassing the mediodorsal, medioventral and the arcuate nucleus. The posterior region is composed by the dorsal hypothalamic area and the pre- and supra-mammilar nuclei.

The periventricular zone is bordering the third ventricle. In this zone are located two important nuclei involved in neuroendocrine regulation: the arcuate and the paraventricular nucleus (PVN). The medial zone is adjoining to the periventricular zone and regroup several nuclei specialized in the autonomic and neuroendocrine systems. Finally, the lateral hypothalamus contains a large fibers pathway connecting the cerebral cortex and the spinal cord.

Table 1 summarizes the different nucleus, zones, regions of the hypothamlmaus and their functions.

*Table 1: Summary table of the repartition of the hypothalamic nuclei according to the zone and regions*

| Nucleus | Zone(s) | Region(s) | Functions |
|---|---|---|---|
| Paraventricular | Periventricular, Medial | Anterior,Tuberal | Fluid balance, milk let-down, parturition, autonomic & anterior pituitary control |
| Preoptic | Medial, Lateral | Anterior | Lateral anterior thermoregulation, sexual behavior |
| Anterior | Medial | Anterior | Lateral anterior thermoregulation, sexual behavior |
| Suprachiasmatic | Medial | Anterior | Biological rhythms |
| Supraoptic | Medial, Lateral | Anterior | Fluid balance, milk let-down, parturition |
| Dorsomedial | Medial | Tuberal | Emotion (rage) |
| Ventromedial | Medial | Tuberal | Appetite, body weight, insulin regulation |
| Arcuate | Periventricular, Medial | Tuberal | Control of anterior pituitary, feeding |
| Posterior | Medial | Posterior | Thermoregulation |
| Mammillary | Medial | Posterior | Emotion and short-term memory |
| Lateral Complex | Lateral | Tuberal | Appetite and body weight control |

The regulation of the hypothalamic nuclei function is complex due to a multitude of neural and non-neural interconnections between the brain and the periphery. Indeed the hypothalamus regulates emotions and learning through its connections to limbic circuit structures such as amygdala and hippocampus. It also regulates feeding and insulin release via visceral and somatosensory input and output. Most importantly for my Ph.D. thesis framework, periventricular neurons connect to the median eminence where it can release corticotropin-releasing hormone (CRH) and vasopressin (AVP) hormones into the hypothalamic-neurohypophyseal portal system (specialized region of the circulation consisting of two capillary beds directly connected by a set of blood vessels and linking these two structures) in order to control the hormone secretion of the anterior pituitary gland (Amett et al., 2016).

### 2.3.1.2. Pituitary gland

The pituitary gland is a neuroendocrine structure located in the sphenoid bone, at the skull basis and consisting in one anterior and one posterior lobe. The pituitary gland is linked to the brain by the infundibulum (or pituitary) stalk. The

posterior part of the pituitary gland is a site of storage and release of two neurohormones synthesized in hypothalamic nuclei, the vasopressin and oxytocin respectively controlling body water balance and parturition. Interestingly it was shown recently that oxytocin is also involved in social bonding, stress (Olff et al., 2013) and analgesia (Eliava et al., 2016)

Contrary to the posterior pituitary gland, its anterior counterpart is a pure endocrine gland. However, the release of posterior pituitary hormones is still under control of the hypothalamic parvocellular neurons secreting releasing or release-inhibiting hormones from the arcuate, periventricular and supraoptic nuclei. Each releasing hormone will then target a specific cell group specialized in synthetizing one of the six anterior pituitary hormone (fig.1):

- Lactotroph cells under the influence of prolactin releasing factors (activators) and dopamine (inhibitor) will control the liberation of prolactin (PRL)
- Growth hormone secretion is under the influence of somatostatin (inhibitor) and the growth hormone-releasing hormone.
- Gonatrophins secretion, regrouping the luteinizing hormone (LH) and the follicle-stimulating hormone (FSH), is controlled by the gonadotropin releasing hormone (GnRH)
- The tyrotropin-releasing hormone (TRH) controls the secretion of the thyroid stimulating hormone (TSH)
- Finally, CRH governs the release of adrenocorticotropic hormone (corticotropin) (ACTH) in the blood stream in order to stimulate glucocorticoid (GC) secretion.
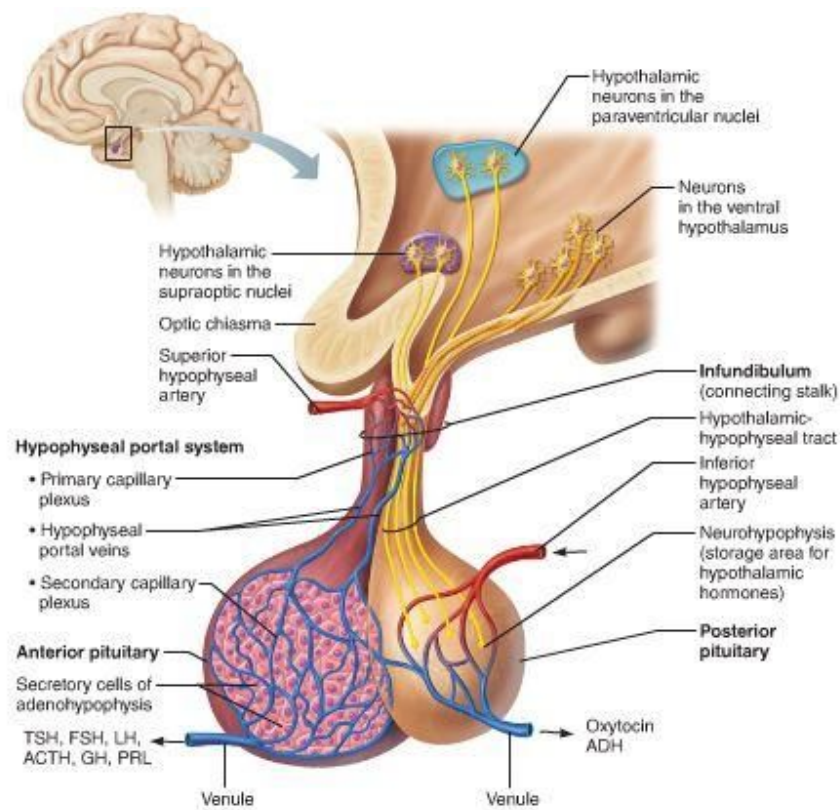
*Figure 1: Schematic representation of the Hypothalamo-pituitary system. From Boron and Boulpaep, Medical physiology, second edition.*

Upon release in the median eminence CRH enter the hypophyseal blood portal to reach the anterior pituitary. The corticotroph cells capable of synthetizing ACTH express the CRH receptor at their membrane surface. The latter is a G-coupled protein receptor (GPCR) that triggers the cAMP pathway. Upon binding of CRH on its receptors, intracellular concentration of cAMP is rising which in turn activates the protein kinase A (PKA). Hence, PKA stimulates L-type calcium channels leading to a general increase of intracellular concentration. This intracellular rise of calcium will lead to the exocytosis of ACTH in the blood stream. Interestingly, this also leads to the enhancement of proopiomelanocortin (POMC) gene expression. POMC is a large gene translated to several proteins such as ACTH and β-endorphin. β-endorphin is a neurotransmitter mainly found in the hypothalamus, pituitary gland and nucleus tractus solitaris (NTS), structures involved in stress and/or pain processing

(Mercer et al., 2013). β-endorphin has been proposed to have important ramifications for pain, stress and immune functions (Merenlender et al., 2009; Jessop, 1995). Indeed, the analgesic effect of CRH during acute stress (Lariviere and Melzack, 2000) is thought to be mediated by β-endorphin. Furthermore, peripheral inflammation and/or sustained stress have been shown to upregulate both CRH and β-endorphin in lymphocytes, thereby acting as anti-inflammatory and analgesic agent on the site of inflammation (Jessop et al., 1995).

### 2.3.1.3. Adrenal glands

The adrenal glands are located above the upper limit of each kidney in the retroperitoneal space. Each adrenal is composed of an inner medulla and an outer cortex (fig. 2).

The adrenal medulla is composed by neuroendocrine cells, the chromaffin that derive from the neural crest and migrate into the center of the adrenal gland. Chromaffins synthetize and secrete catecholamines, especially adrenaline and to a lesser extent noradrenaline. The secretion of these catecholamines is principally regulated by acetylcholine released by preganglionic sympathetic fibers of the splanchnic nerve. Once in the general circulation, adrenaline and noradrenaline will have global impact on the organism regulating systemic arterial blood pressure, bronchial and pupil dilatation, the blood flow of active muscles etc.

The outer part of the adrenal axis (adrenal cortex) is subdivided in three cellular layers. The external one, the glomerulosa layer is responsible for the synthesis of aldosterone, the main human mineralocorticoid. Its secretion is principally controlled by the angiotensin hormone in order to promote water retention by the kidneys. The two other layers, fasciculata (in the middle) and reticularis (near the medulla/cortex junction) contain specialized cells synthetizing the main glucocorticoid of the organism, cortisol, under the control of ACTH.

Androgenic steroids are also secreted by adrenal cortex but they are to a large extent a byproduct of the production of cortisol.
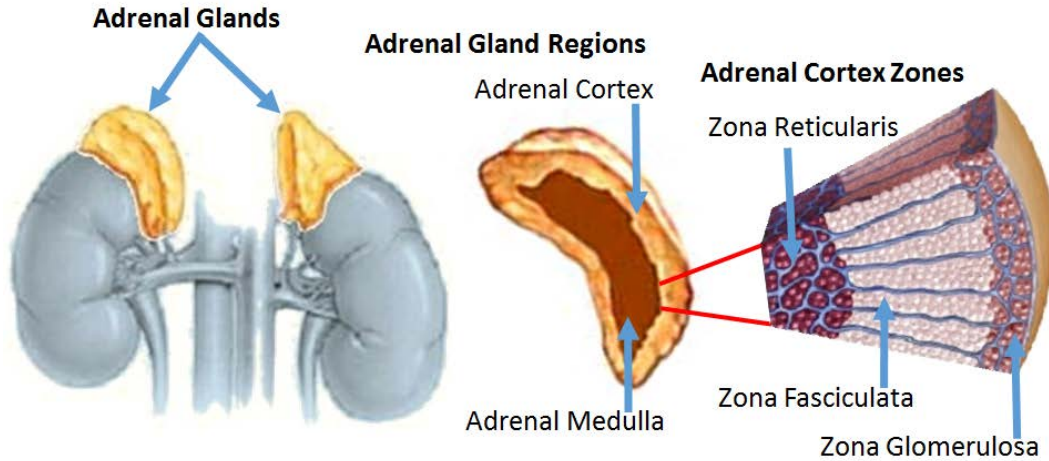


*Figure 2: Location and structure of the Adrenal glands (https://www.yourhormones.com/adrenal-glands/)*

At the cellular level, ACTH binds to the melanocrotin 2 receptor (MC2R) on every layer of the adrenal cortex. However, only the cells located in the fasciculata and reticularis layers contain the 17α-hydroxylase enzyme necessary in order to synthetize cortisol. MC2R is GPCR stimulating adenylate cyclase and hence triggers the cAMP pathway. The rapidly rising intracellular calcium stimulates the side chain cleavage (SCC) enzyme and enhances the conversion of cholesterol into pregnenolone, the rate-limiting step of cortisol synthesis. In the longer term, ACTH also increases the synthesis of several other proteins needed for cortisol synthesis.

### 2.3.2. Glucocorticoids & glucocorticoid receptors: final effector

The name GC was originally given because this steroid was recognized to elevate glucose plasma concentration. In Humans the main GC is cortisol but corticosterone (CORT) is its equivalent in rats. As for every steroid the two analogues derive from cholesterol and are therefore very similar, the only difference emerging from the lack of OH group in position 16. Cortisol is a hydrophilic compound and is therefore soluble in the plasma. However once it

reaches its target tissue, the steroid is still capable to cross the plasma membrane without requiring transporters. Cortisol can bind to the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR).

MR has a higher affinity for GCs and is nearly saturated during the diurnal through of GC and in physiological conditions. As GR has a lower affinity for GCs, it is mainly bound during diurnal peaks and following an exposure to stress. GR belong to the superfamily of nuclear receptors and can exist as homo- or heterodimers. They contains six domains, from A to F (Turner and Muller, 2005) (fig.3).

- The A/B domain, located at the N-terminal extremity, represents the transactivation domain 1 which is involved in the regulation of target gene transcription
- The C domain or DBD (for DNA binding domain) allows the receptor to bind to the DNA "zinc fingers" structures and also to form dimers.
- The D region, also called hinge, contains the nuclear localization signal
- The domain E is responsible for hormone binding and, as the C region, is involved in dimerization through a basic zipper region. Similarly to the A/B region, it also bears the transactivation domain 2.
- The F domain of the GR, localized at the C-terminus end seems to not have a known function yet.
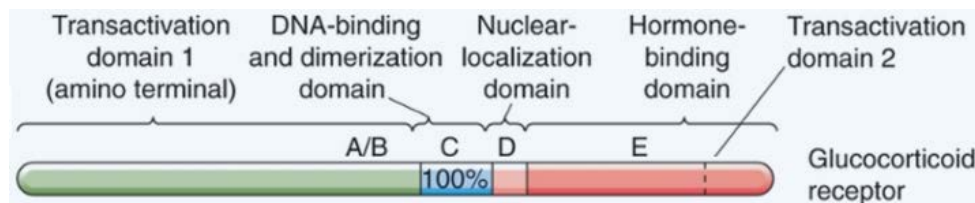


*Figure 3: Simplified representation of the GR domains. From Boron and Boulpaep, Medical physiology, second edition.*

GRs are mainly located in the cytoplasm bound to a heat shock protein HSP90. Upon binding with GCs, HSP90 and GR segregate and the new GC/GR complex translocate in the nucleus. A dimerization is however necessary in order for GR to be functional. Once in the nucleus the GR/GC complex will bind to a target gene's GRE (glucocorticoid response element) site where it can enhance or repress their expression. The majority of GC actions are through genomic regulation. This process is well-known in many somatic tissues such as:

- The liver where GC upregulates the expression of enzymes involved in the metabolism of amino acids
- In muscles, GCs promote the breakdown of protein hence supplying the liver with amino acids
- In fat tissue, a similar process as in the muscles happen and favor the degradation of fatty acids leading to higher plasmatic glucose concentration.

More interesting in the framework of my Ph.D., GCs are increasingly considered due to their action on the immune system and the regulation of behaviors (Tronche et al., 1999; Silverman and Sternberg, 2012).

Briefly, chronic inflammatory conditions such as asthma or rheumatoid arthritis involve the activation of many inflammatory and immune cells releasing multiple inflammatory proteins. The genomic upregulation of most of these inflammatory mediators requires the activation of the nuclear factor-κB (NF-κB) and the activator protein-1 (AP-1). The predominant effect of GCs is to interfere with this upregulation by reversing histone acetylation or bind to NF-κB (through non genomic effect) and to upregulate anti-inflammatory gene expression (Barnes, 2006).

GCs also act on the CNS through genomic and non-genomic mechanisms. The role GCs play in the regulation of mood and behavior seems to be biphasic. For example, adequate levels of GCs protect against traumatic stress-induced

synapse remodeling in the basolateral amygdala (BlA) (Rao et al., 2012) while chronically elevated levels induce dendrite elongation (Mitra and Sapolsky, 2008). Importantly, in the framework of the present work, it was demonstrated that spinal GRs contribute to the establishment of neuropathic hyperalgesia through genomic and non-genomic modulation of glutamatergic receptors and transporters (Wang et al., 2004, 2005, 2006).

### 2.3.3. Regulation of the HPA axis:

We saw above that cortisol is an essential hormone for the maintenance of homeostasis. As for most hormones, daily rhythm and environmental factors have the capacity to induce a surge. Therefore, it is essential that, after the loss of homeostasis, the organism brings back cortisol levels to its physiological range (allostasis). For this purpose, the HPA axis secretion is regulated by endocrine and neuronal higher CNS influences.

Endocrine regulation includes:

- In the corticotrophs of the anterior pituitary: cortisol binds to the cytoplasmic GR and inhibits the synthesis of both CRH receptor and ACTH via negative GRE sites (nGRE). Elevated plasmatic cortisol levels also reduces ACTH release.
- In the paraventricular cells of the hypothalamus: Through similar mechanisms as in the pituitary gland, cortisol inhibits the synthesis and the release of CRH.

Neuronal regulation includes:

- Catecholaminergic neurons of the nucleus of the solitary tract (NTS) receiving inputs from cranial nerve from thoracic and abdominal viscera and hence relaying sensory information to the PVN cells. NTS cells also receive projections form limbic structures regulating behavioral adaptations to stress such as prefrontal cortex (PFC) and amygdala.

- Hypothalamic GABAergic neurons of the dorsomedial nucleus and preoptic area are able to inhibit glucocorticoid surge after environmental stress (Roland and Sawchenko, 1993; Cullinan, 2000). Also hypothalamic neurons of the arcuate nucleus involved in allostasis have been shown to possess the capacity to increase CRF, ACTH and corticosterone levels (Smith and Vale, 2006).

- Limbic neurons also regulate the HPA axis activity and are thought to have a role in the etiology of psychiatric disorders. Indeed, structures such as the hippocampus and the prefrontal cortex have the capacity to inhibit hypothalamic PVN cells (Jacobson and Sapolsky, 1991; Herman et al., 2005). On the contrary amygdala projections have been shown to activate the HPA axis. However, similarly to the hippocampus and the PFC, the amygdala influence on the HPA axis seems to depend on several factors such as the duration and the nature of the stressor.
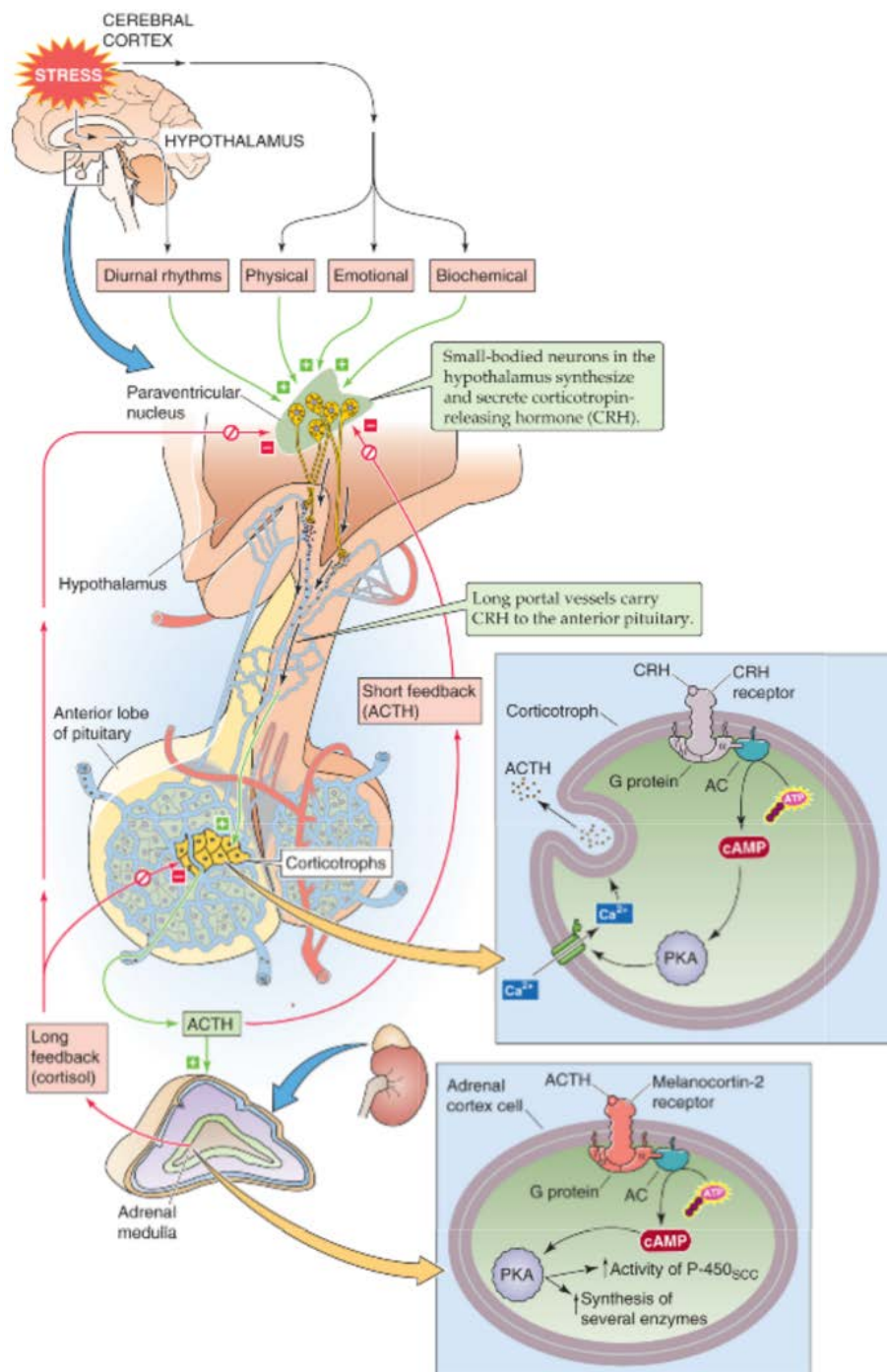
*Figure 4: Summary of the HPA axis functioning*

*From Boron and Boulpaep, Medical physiology, second edition.*

## 2.4. Chronic Stress

Stress response is essential for an organism in order to adapt to changes of environment and to maintain homeostasis. However, environmental challenge can be sustained and lead to a failure to the organism to further cope. Hence, this system originally optimized to promote survival is dysregulated. This failure of allostasis also called "allostatic load" (McEwen and Stellar, 1993) is thought to be at the root of many pathologies due to the general repercussions on neuroendocrine, autonomic, metabolic and immune systems. Despite significant advances these last decades, the mechanisms by which environmental factors can trigger maladaptive responses remain poorly understood. Indeed, the elucidation of these mechanisms is of utmost complexity since many factors are interacting such as the nature (psychological or physical, sustained or transient, mild or intense etc…) and the temporal aspects (prenatal, postnatal, adolescence, adulthood) of the stressor will determine how an organism responds depending on past experience, sex, genetics background etc...

The immune system is very sensitive to stress modulation. Indeed, although an acute stressor can trigger an immune response via catecholamines and glucocorticoids in order to fight infection (Dhabhar, 2009; Dhabhar et al., 2012), the sustained exposition to a stressor may result in immune suppression (McEwen, 2017). Nevertheless, it should be noted that immune suppression may not only be deleterious. Thus, it can be beneficial in cases of auto-immune disorders while deleterious during infection. Also, robust evidences show that activation of the immune system in circumstances such as peripheral inflammation can activate neuro-endocrine stress-related structures (Butts and Sternberg. 2008). Hence, neuroendocrine maladaptation such as dysfunction of the HPA axis observed during chronic stress-related pathological states have been shown to play a critical role in the physiopathology of diseases.

### 2.4.1. Early life stress

#### 2.4.1.1. Introduction

The maladaptation that living organisms undergo during chronic stress periods largely depends on neuroplastic changes. The HPA axis is a structure highly sensitive to reprogramming during the neonatal period. Therefore, given the importance of the HPA axis for the integration of the stress and the stress response, it seems logical that early adverse experiences alters behaviors and brain structures as seen in depression or stress related disorders. It was proposed recently by Heim and colleagues (2008), that maltreatments during childhood sensitize neurobiological systems involved in the stress response and enhance the risk to develop depression. For example, it was later shown that physical stressors such as harsh corporal punishment (Tomoda et al, 2009) or emotional (Van Harmelen et al, 2010) stressors occurring during childhood are linked with reduced frontal cortex volume in adults as well as with suffering from depression and/or anxiety disorders. Other structures important for stress response and adaptations have been implicated in early life stress and later depression such as the hippocampus, the amygdala and the HPA axis (for review McCrory et al, 2011). Human data on the HPA axis alterations are mixed. Indeed, increased cortisol, ACTH and CRH have been reported in maltreated children but mostly when associated with depression (Kaufman et al, 1991; 1997). However this was also seen in non-maltreated children displaying affective disorders (Tarullo and Gunnar 2006). In adults, a loss of adequate HPA axis regulatory negative feedback loop was reported in depressed men that underwent maltreatment during infancy (Heim et al., 2008). On the other hand, post-traumatic stress disorder (PTSD) in the context of childhood abuse has been associated with low cortisol levels (Meewisse et al. 2007). Therefore, despite the clear link between a dysregulation of the HPA axis and consequences of early life stress, it seems yet not clear what distinct pattern of adaptations there is. Despite much work ahead to do, fundamental research has already determined several mechanisms

by which early life stress affects emotional regulation and neuroendocrine functions. Indeed, excitotoxicity (Pickering et al., 2006), oxidative stress (Diehl et al., 2012) and inflammation (O'Mahony et al., 2009; Roque et al., 2015; Green et al., 2011) were implicated.

### 2.4.1.2. Interaction with the immune system

The first studies linking early life adversity to altered immune system were conducted in the 1980s (Coe et al., 1988; Coe and Laudenslager, 2007). It is now established that one of the major effect of early life stress is to cause an immediate as well as delayed and prolonged alterations of the immune response as seen in Human and rodents (Carpenter et al., 2010; Roque et al., 2015).

Apart from its well-known involvement in host defense against infections, the immune system also has a capital role for maintaining homeostasis and behaviors notably through its interaction with the CNS. For instance, sickness behavior is characterized by reduced food and water intake, exploration, social and sexual interaction. This state is coordinated by the immune and nervous system in order to promote host survival and arises from the activity of immunocompetent cells such as microglia or astrocytes and their interaction with the brain via cytokines (Bilbo and Schwarz, 2009). Furthermore, recent advances have established that on top of their capacity to modulate behavior during acute illness, cytokines and glial cells also have a role in the establishment and perpetuation of CNS alterations (Dantzer 2010)

Due to ethical considerations, few human studies have investigated the impact of early life adversity on immunological markers in infants. However, the short delay consequence of adverse life conditions during childhood seems to be a heightened immune system responsivity as shown by increased C reactive protein and interleukin-6 levels (Slopen et al., 2013; Miller et al., 2011). In humans, this enhanced immune system responsivity seems to be maintained in adults (Carpenter et al., 2010; Lacey et al., 2013; 2014; Tiejen et al., 2012). Even

though sensitized immune responses could represent an adaptive advantage (e.g. better response to infection or injury), many clinical studies provided evidence of the association with an amplified immune response in early life and later alteration of the CNS in relation with pathologies such as schizophrenia and Parkinson's disease (for reviews Ganguly and Brenhouse 2015).

### 2.4.1.3. Animal models and studies

The first reports suggesting that early life experiences are shaping individuals' future life can be traced back to the beginning of the 20th century as Freud describes the formation of behavioral or psychological symptoms (what he called neurosis) as a result of repressed impulses often built during infancy. It is only later in the 1950s that first experimental evidence started supporting the hypothesis that childhood experiences, and especially aversive ones, could have long lasting psychological and neuro-endocrine consequences (Weininger, 1954). Early experiments determined then that the HPA axis plays a major role in the potential repercussions of early life adversity. The development of primates and rodents' HPA axis present extensive similarities, hence rodents have been used as a model to understand how an alteration of the HPA axis can modulate brain functions and behaviors for life. Even though experimental models using rodents are very useful, the respective paradigms may not completely correspond to human conditions and results must interpreted with caution. Indeed, while humans are born with a functional HPA axis, its development is still going on in newborn rats. As a consequence, a stress during the first week of rodent's life does not mimic a stress during the same period in humans but rather match a stress during the third trimester of gestation (Lupien et al., 2009). In these animals, the HPA axis is not activated following mild stress during the first 2 weeks of life. First termed stress non-responsive period (SNRP) (Schapiro, 1962), this sensitive interval of time was later better defined by the pioneers Seymour Levine (Levine, 1967) and later by Robert M. Sapolsky

and Micheal J. Meaney (1986) as a stress hypo-responsiveness (SHRP) rather than a non-responsive period. SHRP ranges from postnatal days (PND) 1 to 10 in mice and 3 to 14 in rats and is characterized by lower baseline glucocorticoid plasma levels than normal and by attenuated corticosterone secretion following the exposition to a stressor (De Kloet et Daskalakis, 2012; Schmidt, 2010).

Several hypotheses have been advanced to explain why the HPA axis is hypo responsive at birth such as immature hypothalamic neuronal connections (De Kloet et al., 1988), an impairment of the CRF cascade (synthesis, release, transport) (Grino et al., 1989), or enhanced negative feedback (Walker et al., 1986). However it seems that the strongest inhibitory factor comes from the reduced response of the adrenals to ACTH (Rosenfeld, 1991). Despite remaining uncertainties, researcher have now established that maternal presence and care is a key regulator of HPA axis hypo-responsiveness during the first 2 weeks of rodent's life. Hence, the main models of early life stress in rodents are capitalizing on these conclusions and removing the pups from their dam has become a standard method. Several variations of maternal separation (MS) exist, mostly relying on the period at which the separation is done (number of days and PND), the length of these separations (from 2~3 hours to 24 hours) and whether the pups are separated all together or separately from the dam (deprivation). Furthermore, accumulating data show that small variations of protocols can greatly impact the outcome (good example of MS variations on BDNF levels see Daskalakis et al., 2015). Nevertheless, two maternal separation paradigms may be differentiated, a model using prolonged maternal separation, often at one specific day during the SHRP and a model using repeated shorter maternal separations done on several consecutive days during the SHRP.

Evidence shows that one long MS (also called maternal deprivation) during the SHRP is sufficient to activate the HPA axis. Consequently neonates display elevated basal and stress-induced CORT levels (Stanton et al., 1988) and reduce the amount of CRH binding site in the pituitary gland (Anisman et al., 1998).

Also the consequences of prolonged MS are not limited to the HPA axis since it increases the density of CRH receptors in many other brain areas such as the PFC, the amygdala, and the hippocampus. These effects will then lead to enhanced anxiety mostly through an enhancement of HPA axis activity (Roceri et al., 2002; Fenoglio et al., 2006; Schulkin et al., 1998, Daskalakis et al., 2015).

On the contrary, a single episode of short MS (up to 3 hours) does not lead to a direct HPA axis activation. Only a daily MS is capable to induce a sensitization of the HPA axis response and the resulting plasma CORT and ACTH elevation following exposition to the stressor (Lippman et al., 2007; Huot et al., 2002; Francis et al., 2002). Similarly to the protracted maternal separation paradigm, the short repeated MS-induced alterations extend beyond the HPA axis. Indeed, altered BDNF protein expression was noted in the cortex, amygdala, ventral tegmental area (VTA), hippocampus, nucleus accumbens (Nac) and striatum (Lippman et al., 2007). It has also been demonstrated that, following short MS, hippocampal astrocytic but not microglial density was decreased coupled with an enhanced microglial activity and increased IL-1β. In the hypothalamus, other pro-inflammatory cytokines, TN-α and IL-6, saw their levels increase (Roque et al., 2015).

Overall, despite common alterations in different brain areas, it is very difficult to draw a uniform consequence of early life stress. Indeed, the multiplicity of studies and different paradigms used in fundamental research indicates that every protocol should be very carefully compared with existing literature. Nevertheless, research indicates that MS-induced perinatal programming modulates immune and central nervous systems which can have life-long repercussions on anxious and noxious behaviors (Kalinichev et al., 2001; Weaver et al., 2007; Hennessy et al., 2011; Chocyk et al., 2011; Diehl et al., 2012; Rana et al., 2015). Therefore, in order to understand how early life stress experiences can predispose individuals to develop chronic pain disorders, further studies remain essential.

### 2.4.2. Adult social stress

#### 2.4.2.1. Introduction

In addition to the stressful events occurring during perinatal and peri-adolescence periods, those taking place at adulthood can also lead to enhanced probability to develop diseases. From an evolutionary perspective, civilizations have at least partly developed in order to provide social structures ensuring survival of the individual and hence also of the society. Hence, stable societies aim to reduce threats that could lead to potential harm or stress. In this context, set of rules maintaining social cohesion are established. They are often dynamic and require individuals to adapt to them. Consequently, some circumstances such as isolation, social inequalities (poverty), loss of a loved one, bullying or other forms of psychological or physical abuse but also environmental factors can be important sources of stress and have been associated with high levels of violence in human society (Gilbert, 2000; Bushman et al., 2005; Kennedy et Adolphs, 2012). On the other hand, stress-related illnesses (from social origins or not) are often associated with dysfunctional social behaviors (Rygula et al., 2005). Indeed, clinical studies have reported that PTSD or depression is often associated with reduced social motivation due to enhanced anxiety and group avoidance but also anger and violence when facing stressful situations (Nitesh et al., 2007; Baddeley et al., 2012).

#### 2.4.2.2. Animal models

Similarly to human beings that generally live in close proximity to conspecifics, rodents are living in groups and hence are subjected to stress from social nature (e.g. during the establishment of hierarchy). Early studies established that one of the greatest stress sources of social origin comes from either fighting for control and/or losing control (Barnett, 1958; 1964). The analogy to human social stressors led scientists to develop several experimental procedures aiming to mimic behavioral and biological alterations leading to enhanced vulnerability.

Hence, scientists have used two main experimental paradigms in order to evoke pronounced psycho-social stress: the chronic social defeat and the resident-intruder paradigm (Berton et al., 2006; Barik et al., 2013; for review Salttery and Cryan, 2014; Koolhaas et al., 2017). In order to create emotional stress the models use violent social interactions that are often due to the establishment of dominance hierarchy (Pryce and Fuchs, 2016). Classically these paradigms are based on the encounter of two foreign individuals (rats, mice or tree shrews) in the home cage of one of the two. The resulting fight will give rise to a winner and a looser (often the intruder). Biologically, both rodents are undergoing a strong stressful situation associated with HPA axis activation and the resulting plasma corticosterone elevation (Koolhaas et al., 2017). However, the outcome of the conflict is going to determine the subsequent impact. Indeed, the HPA axis recovery of a submissive individual will be much slower (Koolhaas et al., 2011). The weaker negative feedback originates from the uncontrollability of the situation (Swenson et Vogel, 1983) and is mediated in part by non-genomic actions of GC through MR and GR (De Kloet et al., 2008). The lack of control experienced by a submissive individual is often considered as aversive. Interestingly however, the control of a group can also be a stressor. Indeed, as social groups are a dynamic structure, the dominance can be challenged. Hence, a dominant male can experience difficulties to maintain its position and be challenged and these difficulties to maintain social hierarchy have been associated with stress pathologies (Fokkema et al., 1995; Sapolsky, 1995). Another determining factor for the severity of the stress response comes from the predictability of the stressor. This parameter was defined as "the rates at which environmental harshness varies over time and space" (Brumbach et al., 2009). It was shown experimentally that this parameter is a capital trait for modeling environmental conditions leading to psychiatric disorders (Nederhof and Schmidt, 2012).

As mentioned above, social groups are a dynamic structure but they are also persistent. However many models are relaying on episodic aggression, defeat, or intrusion. Even, when the exposure is repeated and hence chronic (chronic intermittent stress), it does not perfectly represent the conditions in human society.

In the framework of the present studies, we used a paradigm combining aspects of different chronic social stress models. The experimental design was introduced in Mormède's study (1990) and readapted by Schmidt and colleagues (2007). This paradigm is based on creating a continuous social instability for 6 weeks during which animals have to readapt to new congeners and establish social hierarchy twice per week. Therefore, rats are facing an unpredictable environment maintained over several days from which they cannot escape. This model has been shown to induce anxio-depressive state characterized by symptoms such as reduced sucrose preference and food intake (Herzog et al., 2009), reduced time and entries in plus-maze open arm (Schmidt et al., 2007) but also noxious sensitivity (Le Coz et al., 2017) and social deficits (Saavedra-Rodriguez and Feig., 2013). It was established that the consequences of such a stress may be very long lasting as they could be seen three generations after the stress exposure through epigenetic changes but also presumably through constitutive alterations of gene regulated by stress hormones (Saavedra-Rodriguez and Feig, 2013). Biological alterations of the HPA axis and limbic structures were also observed such as increased adrenal weight and sensitivity to ACTH, enhanced plasma corticosterone levels, decreased hippocampal GR expression and alter neuro-immune responses in amygdala and hippocampus (Schmidt et al., 2007; Nowacka et al., 2014).

Altogether these findings suggest that this model is adequate to study psychosocial stress while mimicking conditions observed in human (e.g. mobbing).

### 2.4.3. Concepts of vulnerability and resilience

Initially it has been hypothesized that adverse life conditions occurring during critical periods of development (perinatal period or adolescence) or at adulthood automatically lead to the development of enhanced vulnerability to diseases. A couple of assumptions were discussed in this context. The neurotoxicity hypothesis suggests that chronic stress and hence sustained exposure to GC weakens the ability of neurons to resist to new insults and enhances their vulnerability to toxic challenges (Sapolsky et al., 1986). Although not incompatible with neurotoxicity, the vulnerability hypothesis considers that neurobiological alterations seen in some diseases are not a consequence but rather a pre-existing risk factor originating from adverse early-life experiences (Gilbertson et al., 2002).

Even though these two hypotheses may not be completely dismissed, harsh environmental conditions especially when occurring in early life will not uniformly lead to a disadvantage (enhanced vulnerability) later on and the consequences of early life stress should be considered as a "double-edged sword" (Brunson et al., 2003). Indeed, experimental and clinical studies have shown that resilience to stress can be an outcome of early life stress (Macri et al., 2011). Resilience can be defined as the capacity to cope with challenges through neurobiological adaptations maintaining an efficient allostasis (Mitra and al., 2009; Karatoreos and McEwen, 2013). Experimental models have shown that the severity of stressors applied during early life is one of the determining factors for the appearance of resilience as opposed to vulnerability. Hence, the relationship between early life adverse conditions and the resilience in later life follow a U-shaped curve (Pfau and Russo, 2015). Therefore individuals exposed to a mild stress during infancy generally show a better outcome when facing stressful situations during adulthood whereas little or severe stresses may lead to enhanced vulnerability (Macri et al., 2011).

Others have also described resilience or vulnerability taking into consideration the accumulation of stress experiences. The two-hit (also sometimes called three-hit or multiple hit) or cumulative stress hypothesis is the classical view stating that early life adversity would predispose individuals to later pathologies due to a buildup of stress progressively increasing risks until individuals reach allostatic load (McEwen and Stellar, 1993; McEwen, 1998). However, a more evolutionary perspective assumes that adverse early experience may induce programming aimed at a better preparation for later and hence better coping with subsequent stressful challenges (fig. 5) (Nederhof and Schmidt, 2012; Daskalakis et al., 2013). However the match/mismatch notion is considered to be a context specific hypothesis which conjectures that adaptive attributes in a certain context can be detrimental in another one. In a similar manner genetic predispositions can also modulate vulnerability/resilience and should be taken into account and individual vulnerability/resilience should consider the interaction of genetic and environmental factors.
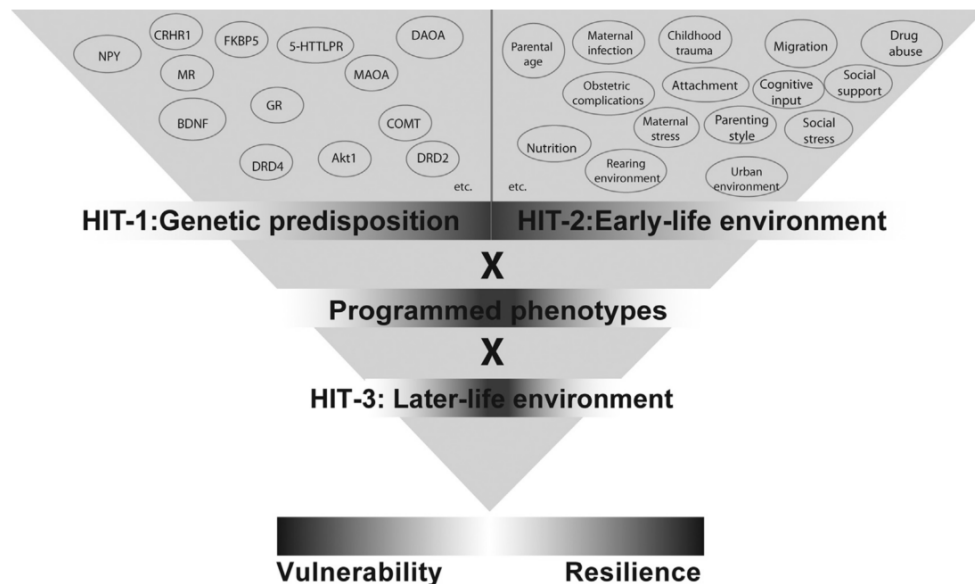


*Figure 5: Multiple hit concept of vulnerability and resilience. In this model the first hit is considered to be the genetic predispositions determining the biological stress substrate of individuals. The genetic heritage will then interacts with early environmental stimuli (hit-2) leading to a predetermined phenotype. This phenotype, when interacting with environmental challenges later in life will determine the vulnerability or the resilience of individuals to these challenges (Daskalakis et al., 2013).*

Vulnerability and resilience have been almost exclusively studied in the context of psychopathologies. However, many studies are showing that early life stress can predispose (or not) to a wide variety of other disorders such as cancer, neurological and immune disorders or chronic pain (Schuler and Auger, 2010; Hoeijmakers et al., 2017). Interestingly, central structures implicated in stress processing are often involved by the pain response and modifications within these structures were shown to impact the other systems' functioning (Felice et al., 2014) such that depression is often associated with chronic pain syndromes. Nevertheless, not many studies have investigated a possible enhanced resilience or vulnerability to such pathologies after stress experiences. Furthermore, stress is known to have a broader impact than solely on the brain and stress-related disorders, yet there is a paucity of studies investigating the transmission of noxious information from nociceptors to second order neurons at the spinal cord level (which carry the information to higher brain centers). Hence, in the present work, I sought to determine if and how early life and later stressful experiences could change pain sensitivity during chronic pain conditions and to gain insight about potentially involved alterations in spinal processing.

# Chapter III: Pain mechanisms

## 3.1. Introduction

Pain exerts a capital function of the central nervous systems as it gives essential information about potential or existing harmful stimulus, the ultimate goal being to avoid potential or further injury. Since these noxious stimuli (from noxia in Latin meaning damage) can be very diverse the body has developed a broad range of receptors, types of neurons and levels of integrations. Therefore, the sensation of pain involves a complex neuronal network. It is mediated in the periphery by primary afferent neurons named nociceptors, conducted through the spinal cord and processed within the brain. (Milan, 1999). This will be further detailed in chapter 3.2. An injury can lead to an enhancement of sensitivity to painful stimuli (Sandkühler, 2009). Hyperalgesia (exaggerated pain response to a noxious stimuli) and allodynia (the experience of a painful sensation with a normally innocuous stimuli) are common symptoms that can even be seen in response to small injuries such as a small cut. However, in some cases such as the phantom limb pain condition, these symptoms can persist long after the initial cause of pain has vanished. In this case, patients that underwent an amputation still have the feeling that the missing limb is attached and can experience itching, burning or aching in this area. This kind of spontaneous and or exaggerated pain can be seen in other conditions like neuropathy or chronic inflammation where pain has lost its alarm and protective aim and has become persistent and debilitating, constituting a disease of its own. Research has shown during the past decades that such pathologies are underlined by long lasting modifications of the different components of the pain network (Blackburn-Munro and Blackburn-Munro, 2001; 2003). However, due to the multidimensional organization of the pain system and the great comorbidity between painful and stress-related conditions, much work remains in order to understand and treat the different pain states.

## 3.2. Anatomy of the pain system

### 3.2.1. Nociceptors / primary afferent neurons
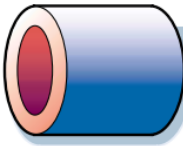
Classically, nociceptor is the name given to the sensory endings of high threshold primary somatosensory neurons which are specialized in the detection of noxious stimuli. Even though they are capable to encode noxious stimulus, some can also detect stimuli in the innocuous range. Alike other primary afferent sensory neurons, they are pseudo-unipolar. Their cell body is located in the dorsal root ganglia (DRG) or the trigeminal ganglion (TG) and projects one axonal branch to the periphery and the other one to the CNS where it makes a synapse with a second order neuron. In the periphery they are almost ubiquitous as they are found in the skin, ligaments, muscles, deep fascias and membranes, arterials and visceral tissues. The synapse connecting primary afferent and second order neurons will be detailed further. However, pseudo-unipolar neurons are of peculiar nature. Contrary to other neurons composed of dendritic receiving and axonal transmitting regions, the nociceptor can send and receive messages in both directions (Dubin and Patapoutian, 2010).

Nociceptors are classified according to their conduction velocity (which depends on the type of fibers they are associated with), sensitivity to noxious heat, cold or mechanical stimuli, their sensitivity and the repertoire of chemical transduction molecules they use. Nevertheless, it is important to bear in mind that most nociceptors are polymodal, they express different combinations of sensory sensors that allow them to detect different stimuli. Therefore their classification is rather complex and will be not detailed fully in this manuscript. As already mentioned the first classification differentiates nociceptors according to their conduction velocity. The acute and well localized "fast pain" is mediated primarily by medium-sized myelinated Aδ afferents while small diameter non-myelinated C fibers conduct a diffuse and slow "second pain". In addition to the environmental modality it answers to, a nociceptor's depolarization is also determined by the amplitude and duration of the stimulus. Hence,

electrophysiological studies have identified subclasses of Aδ and C-fibers (Basbaum et al., 2009; Julius and Basbaum, 2001). Briefly, the type I Aδ fibers respond to mechanical and chemical stimuli but not well to heat (only to very intense heat levels). On the contrary the type II Aδ nociceptors have higher mechanical but lower heat thresholds. The slowly conducting nociceptors are also subdivided between the C-fibers mechano-heat (C-MHs) (Perl, 2007), mechano-chemical (C-MCs), mechano-heat-chemicals (C-MHCs) sensitive (Dubin and Patapoutian, 2014). Another type of C-fibers exists, it is insensitive to heat and mechanical stimuli during physiological states but not after inflammation (Schmidt et al., 1995; Meyer et al., 1991).

Primary afferent axons

| | Thermal threshold |
|---|---|
| **Aα and Aβ fibres**<br>Myelinated<br>Large diameter<br>Proprioception, light touch | None |
| **Aδ Fibre**<br>Lightly myelinated<br>Medium diameter<br>Nociception<br>(mechanical, thermal, chemical) | ~ 53 °C Type I<br>~ 43 °C Type II |
| **C fibre**<br>Unmyelinated<br>Small diameter<br>Innocuous temperature, itch<br>Nociception<br>(mechanical, thermal, chemical) | ~ 43 °C |

*Figure 6: Summary of primary afferent fibers properties. Nociceptors classified upon their diameter. Large diameter myelinated A-α and A-β fibers are conducting innocuous stimuli. On the contrary myelinated A-δ and unmyelinated C-fibers are responsive to noxious stimulations. (Julius and Basbaum, 2001)*
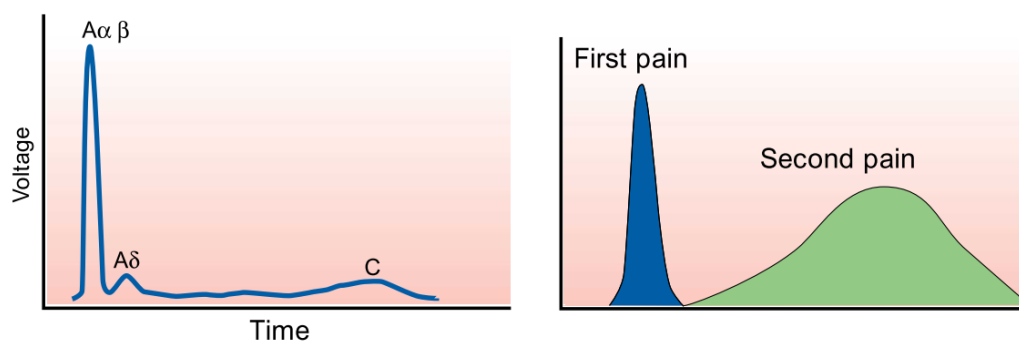
*Figure 7: Involvement of the different nociceptive fibers in the detection of pain. The fiber diameter directly determine the conduction velocity. Hence A-δ fibers will conduct the first pain while C-fibers will transmit the second pain (Julius and Basbaum, 2001).*

### 3.2.1.1. Detection of noxious heat

Capsaicin and vanilloid have the ability to produce a burning pain if applied on the skin. The comprehension of molecular mechanisms underlying innocuous and noxious heat detection started with the cloning of the capsaicin receptor and definition of the related vanilloid family receptors (Lumpkin and Caterina, 2007). The burning sensation produced by these compounds is principally due to their actions on polymodal C- and Aδ-fibers via the activation of the transient receptor potential cation channel subfamily V member 1 (TRPV1). This channel is one of the transient receptor potential (TRP) ion channel family comprising about 30 members. TRPV1 has been shown to be activated by temperatures ranging approximately from 43°C (up to 52 C°) which correlates with the human pain threshold. However studies show that TRPV1 is not solely responsible for the transduction of noxious heat. Indeed, TRPV2, expressed in Aδ-fibers type I has a threshold at around 52 °C and would be responsible for high threshold noxious heat (Lumpkin and Caterina, 2007). The most recently discussed channel participating in the detection and transduction of noxious heat is the Anoctamine 1 channel (ANO1). This non-TRP channel is activated by temperatures above 44°C.

### 3.2.1.2. Detection of noxious cold

The understanding of innocuous or noxious thermal discrimination is incomplete, especially concerning cold sensitivity. Pharmacological (using menthol or eucalyptol) and genetic studies have proposed several candidates from the TRP cation channel family to be involved in the transduction of cold stimuli. The TRP subfamily member 8 (TRPM8) was the first cold transducer identified (McKemy et al., 2002; Peier et al., 2002). Although the TPM8 channel is known for innocuous cold transduction (discriminate temperatures under 28°C) its implication in noxious cold sensitivity is still debated (Knowlton et al., 2011; Pogoezala et al., 2013). Recently, two other TRP channels were shown to be implicated in noxious cold transduction. TRP cation channel subfamily C member 5 (TRPC5) is expressed in DRG neurons (Zimmerman et al., 2011) but its role in noxious cold transduction still needs further enquiry. Nevertheless, TRP5C-/- mice did not present alteration of noxious cold sensation. TRP cation channel subfamily A member 1 (TRPA1) is also expressed in DRG and TG neurons and is thought to control the responsiveness to noxious cold (<5°C) (Gentry et al., 2010; Knolton et al., 2011). Primary sensory neurons contain a multitude of cation channels which are thought to play a capital role in innocuous and noxious cold transduction. Indeed, K+ channels (mainly TREK, TRAAK) and voltage gated Na+ channels (mostly Nav 1.7, Nav 1.8 and Nav 1.9) are thought to modulate the transducing properties of nociceptors (Lolignier et al., 2016).

### 3.2.1.3. Detection of noxious mechanical stimuli

Similarly to noxious temperatures, nociceptors have the capacity to detect noxious mechanical stimuli such as pressure or tissue deformation. However, contrary to heat, cold or chemical stimuli the identification of proteins involved in noxious and innocuous touch is challenging due to the difficulty to establish appropriate stimulation protocols that are valid in and especially ex vivo (Dubin

and Patapoutian, 2010). Nevertheless, few candidates are believed to transduce mechanical noxious stimulus. Members of the degenerin/epithelial Na+ channel (DEG/ENaC) family, the acid-sensitive ion channels (ASICs) 1 to 3 have been proposed to play a role in musculoskeletal and ischemic pain (Price et al., 2000; Basbaum et al., 2009). TRP channels are also candidates but their contribution remains unclear. Indeed, TRPV4 and TRPA1 channels have been proposed as modulators of mechanical transduction but results from genetically modified mice models are not always consistent (Petrus et al., 2007; Kwan et al., 2006). More recently, researchers have identified rapidly-adapting mechanically activated cation channels, Piezzo 1 and 2, expressed in DRG neurons and involved in nociceptive processes, however further research is needed (Coste et al., 2010; Kim et al., 2012).

### 3.2.2. Spinal synaptic transmission

#### 3.2.2.1. Introduction

Nociceptors will convert the noxious environmental stimuli into a train of action potentials. This signal will then be conducted and transmitted to a second order neuron at the spinal cord (SC) level. The dorsal horn (DH) of the SC is divided into 6 laminae based on neuron size and density (Rexed, 1952). Depending on the type of nociceptive fibers that conduct the information, the synapse between first and second order neuron will be located in a different layer of the DH. Indeed, Aδ fibers generally end up in the laminae I and V while the C-fibers finish in the superficial DH in the laminae I and II (Basbaum et al., 2009).
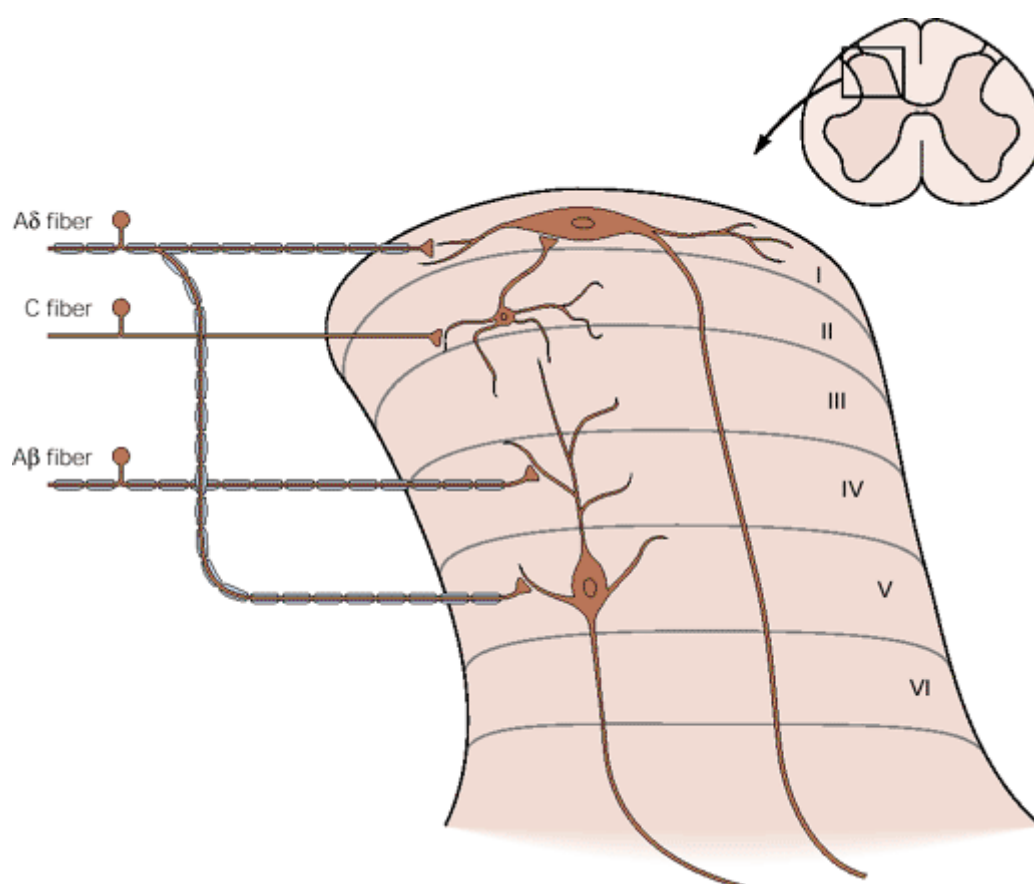
*Figure 8: Anatomical projections of nociceptive afferent fibers in the dorsal horn of the spinal cord (Kandel et al., 5th edition)*

Once C and Aδ fibers reach the DH, they will signal to a second order neuron via a glutamatergic synapse along with the co-release of peptides such as substance P and CGRP (Basbaum et al., 2009). Different types of second order neurons can be differentiated by their connections to primary afferents. The nociceptive-specific neuronal cells are primarily located in the superficial DH and therefore are connected to Aδ and C-fibers and transmit only painful stimuli. Neurons receiving inputs from Aβ-fibers are involved in proprioception. Finally, wide dynamic range (WDR) neurons are located in deeper laminae of the DH and receive inputs from Aβ, Aδ and C-fibers, hence these neurons can respond to a range of stimulations from innocuous to noxious stimuli (Collins and Ren, 1987). Additionally to these projection neurons, the DH contains glutamatergic and GABAergic interneurons that will either facilitate or inhibit

the transmission of nociceptive signals. Additionally, the non-neuronal microglial and astrocytic cells have raised increasing interest in the past 15 years. Indeed astroglial cells, are now known to be an important regulator of glutamatergic transmission (Ziak et al., 1998; Nie and Weng, 2009) and dysfunctional glial cell functions are associated with chronic pain (Romero-Sadoval et al., 2008; Nie and Weng, 2010; Beggs and Salter, 2010; Mika et al., 2013). Neurons in the spinal cord DH are also submitted to descending modulation from higher brain centers but this will be addressed later.

### 3.2.2.2. Glutamatergic system: ionotropic receptors

Glutamate is the main excitatory neurotransmitter of the CNS and is found in a very large majority of primary afferents regardless of their diameter and conduction speed. ATP had been proposed as a co-transmitter of noxious stimuli transmission however pharmacological studies failed to prove its role. Indeed, complete blockade of glutamatergic receptors extinguishes all excitatory postsynaptic currents in DH of lumbar SC (Li and Zhuo, 1998). Nevertheless, it was shown recently that ATP can act on non-neuronal cells of the DH (Beggs and Salter, 2010). Hence, glutamate remains the main excitatory amino acid in the SC and upon fixation to its post synaptic receptors it depolarizes second order neurons. Therefore glutamate is essential for nociceptive neurotransmission but also for synaptic plasticity and is involved in the establishment of chronic pain. Glutamate has four different subtypes of receptors located at pre, post and peri-synaptic sites: three are ionotropic receptors the α-amino-3-hydroxy 5 methyl-4-isoxazeloproprionic acid (AMPA) receptor, the N-methyl-d-aspartate (NMDA) receptor and the kainite receptor and one G-protein coupled receptor (GPCR) metabotropic receptor (mGluR). Most glutamatergic excitatory post-synaptic currents (EPSCs) are composed of a rapid AMPA/kainate current depolarizing the cell followed by an NMDA current component.

*AMPA receptor*

The AMPA receptor is a tetrameric structure composed of the assembly of one or more of the GLUA1-4 proteins. These subunits are derived from four distinct genes GluR1 to GluR4 coding for four different proteins composed of three transmembrane domains (Armstrong and Gouaux, 2000). The different combinations of GLUA proteins and post-transcriptional mechanisms of the four subunits results in a very large diversity of the AMPA receptors. Nevertheless, AMPA receptors exhibit a relatively fast opening and deactivation when compared to other glutamate receptors. Importantly, calcium permeable AMPA receptors have been shown to be of importance regarding synaptic strength in the DH (Bleakman et al., 2006). The permeability of AMPA receptors for calcium depends on their subunit composition. Most AMPA receptors are composed of at least one GLUA2 subunit, however some do not and are permeable to calcium. Therefore, the GLUA2-lacking AMPA receptors are believed to play an important role in long-term plasticity in the DH (Cull-Candy et al., 2004).
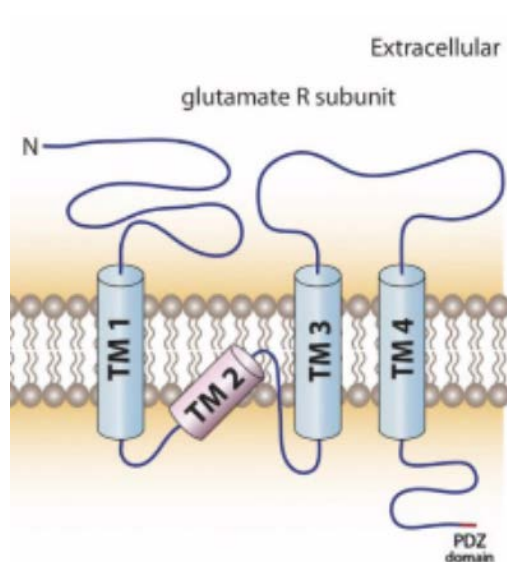


*Figure 9 : AMPA and NMDA receptors structure. The receptor is characterized by two regions S1 (extracellular region adjacent to TM1) and S2 (extracellular region between TM3 and TM4) corresponding to the ligand binding core (LBC). The intracellular domain plays an important role in the receptor's function as it is subjected to phosphorylation by various protein kinase such as CamKII, PKC or PKA.*

AMPA receptors have been found in the brain, spinal cord and in the periphery however I will focus on the spinal cord. AMPA receptors are present on pre- and postsynaptic sites.

Presynaptic AMPA sub-units are translated in the DRGs and transported to the synapse. There, they mediate nociceptor depolarization and glutamate release in the synaptic cleft (Bardoni, 2013). Interestingly immunolabelling studies showed that GluA2/3 subunits are mainly expressed in myelinated primary afferents (Lu et al., 2003) suggesting that most of presynaptic AMPA receptors are permeable to calcium and hence involved in synaptic plasticity. Nevertheless, about one fourth of GABAergic interneuron terminals are immune-reactive to GLUA2/4 antibody and two third of GLUA2/4 terminals are GABAergic and/or glycinergic (Lu et al., 2005; Engelman et al., 2006).

Postsynaptic AMPA receptors are responsible for physiological pain transmission and plasticity as most contain the GLUA2 subunits and are permeable to calcium. Recently accumulating evidence seems to support that AMPA receptor may have a role in chronic painful sensations (Wang et al., 2010)

*Kainate receptor*

Similarly to AMPA receptors, Kainate receptors are homo-or heterotrimeric. The kainate family comprises 5 genes coding for five proteins split into two subfamilies: the first one, composed of GluK1 to 3 subunits, presents a quite low affinity to glutamate and is capable to form a channel with members of this subfamily only. The second, formed by Gluk4-5, has a high affinity for glutamate but cannot form a channel solely with members of its own subfamily. Indeed, in order to form a functional channel, the kainate receptors necessitate at least one of the members of the low-glutamate affinity subfamily (Larsson, 2009). Kainate receptors have similar kinetics as the AMPA receptors except for a slower deactivation. These receptors are primarily present on the presynaptic terminals of C-fibers, but also on postsynaptic elements. However,

contrary to AMPA receptors they are believed to mediate the transmission of higher stimulation intensities due to their slower decay kinetics (Zhuo, 2017).

*NMDA receptor*

NMDA receptors are classically composed of 4 subunits, forming a homo or hetero-tetrameric channel. Seven genes are coding for the different channel subunits: $GLU_{N1}$, $GLU_{N2A}$, $GLU_{N2B}$, $GLU_{N2C}$, $GLU_{N2D}$, $GLU_{N3A}$, $GLU_{N3B}$ and the organization of the channel is similar to AMPA and Kainate receptors. However, as the $GLU_{N1}$ subunit bears the glycine co-agonist binding site and the $GLU_{N2}$ the agonist binding site for glutamate, each receptor is formed by 2 $GLU_{N1}$ in combination with two $GLU_{N2}$ and/or $GLU_{N3}$. The activation of this receptor requires the binding of glycine and glutamate but since glycine is naturally present in the extracellular space, most co-agonist sites are constitutively occupied. The binding of glutamate to the receptor is however not sufficient to open the channel. Indeed, under physiological conditions, at a resting neuronal potential (-80mV), the channel pore is blocked by a magnesium plug. It is only following a sustained neuronal depolarization that this $Mg^{2+}$ block is removed allowing a calcium influx. Once $Ca^{2+}$ is inside the cell it will active several signaling cascades that can promote plastic changes involved in long term potentiation.

In the spinal cord, the NMDA receptors subunits GLUN1, GLUN2A-D and GLUN2B have been detected both pre and post synaptically (Ma and Hargreaves, 2000; Boyce et al., 1999). When located on the presynaptic elements, NMDA receptors are involved in the modulation of neurotransmitter release of glutamatergic but also GABAergic neurons as the structural GLUN1 subunit has been detected on primary afferent fibers (PAFs) and GABAergic interneurons terminals in DH laminae I to IV (Lu and al., 2003; 2005). The postsynaptic repartition of the different NMDA receptors subunits is quite heterogeneous. While GLUN1 seems to be evenly expressed in the dorsal horn, GLUN2A seems to be localized rather superficially whereas GLUN2B is

distributed in laminae III-IV (Nagy et al., 2004). Postsynaptic NMDA receptors do not seem to be involved in normal physiological nociception. Indeed, it was demonstrated that removal of one of the main three NMDA receptor subunits; GLUN1, GLUN2A or GLUN2B using genetic models or pharmacological intervention does not alter basal nociception. Hence the role of these post-synaptic receptors would be restricted to long term potentiation seen in the framework of chronic pain states (South et al., 2003; Tan et al., 2005).

### 3.2.2.3. Glutamatergic system: metabotropic receptors

Metabotropic glutamate receptors (mGluRs) are a family of GPCR class C. This GPCR superfamily is classically composed of an N-terminal extracellular tail, a central core constituted of 7 transmembrane α helices linked by intra and extracellular loops (3 of each) and terminated by an intracellular C-term tail. However, contrary to other GPCR they form a dimer and possess a "two extracellular lobes" domain capable of binding natural ligands such as glutamate and (Pin et al., 2004). mGluR have another particularity compared to other C class GPCR, an extracellular disulfide bridge links the two subunits. This influences the functionality of the receptor as two glutamate molecules will be necessary to activate fully the receptor (Kniazeff et al., 2004). There are 8 mGluRs divided in three subfamilies based on their sequence homology, the intracellular cascade they trigger and their pharmacological profile.

Group I mGluRs comprise mGluR 1 and mGluR 5 and are mostly found on post-synaptic elements. mGluRs 1 and 5 are coupled with the Gq/11 family of G proteins. Upon activation of these receptors, the activation of phospholipase C (PLC) β1 will lead to the production of inositol-(1, 4, 5)-trisphosphate (InsP3) and diacylglycerol (DAG). InsP3 will act to release calcium from intracellular stores while DAG actives PKC. The liberation of calcium and the subsequent channel phosphorylation due to the activation of group I mGluRs have been shown to be a critical component of central sensitization in the context of

chronic pain notably by potentiating NMDA and AMPA responses and increasing the excitability of second order neurons (Jones and Headley, 1995; Walker et al., 2001a, b; Karim et al., 2001; Hu et al., 2006). Importantly, group I mGluRs have been detected in glial cells in several CNS regions such as hippocampus, the cortex but also the spinal cord (Danbolt, 2001). It has been suggested that glial mGluRs participate also in the establishment of chronic pain due to their capacity to release glutamate, modulate its transporters' activity and evoke synaptic responses from neighboring neurons but to my knowledge their role has not been investigated extensively in the spinal cord (Pocock and Kettenmann, 2007).

Group II (mGlu2 and 3) and group III (mGlu4, 6, 7, 8) sub-families are both negatively coupled with adenylate cyclase via a Gi/o-protein (Goudet et al., 2009). Both subtypes are mostly expressed in the presynaptic elements of sensory PAFs (Carlton et al., 2001) and serve as auto-receptors that regulate synaptic transmission and presynaptic neuron excitability in a negative way. Group II mGluR activation has been demonstrated to have analgesic effect as demonstrated by pharmacological activation (Yang and Gereau, 2003; Li and Neugebauer, 2006) and their expression is sensitive to painful experiences (Dolan et al., 2003). However, activation of the group III mGluRs does not seem to affect nociceptive threshold in healthy rats but to inhibit hyperalgesia associated with chronic pain (Neugebauer, 2000; Goudet, 2008). Some Group II and III mGluRs have been found on glial cells such as mGlu3 and mGlu4 where they are thought to have a neuroprotective role (D'Antoni et al., 2008).

### 3.2.2.4. Glutamatergic system: transporters

An important factor determining the transmission of the nociceptive signal to higher brain center is related to the concentration of glutamate within the synaptic cleft. The more glutamate in the cleft, the stronger the signal will be. Furthermore, an excessive extracellular glutamate concentration and hence an

excessive receptor activation are known to lead to excitotoxicity and neuronal death. Therefore, it is important that this amino acid is recaptured. Spinal glutamatergic synaptic clearance is predominantly provided by the excitatory amino-acid transporters (EAATs). They all derive from the same SLC1 gene-family of transporters and comprise 5 subtypes, however most of the glutamate uptake is secured by the glutamate/aspartate transporter (GLAST or EAAT1), the glutamate transporter-1 (GLT-1 or EAAT2) and the excitatory amino acid carrier (EAAC 1 or EAAT3). The two other glutamate receptors, EAAT4 and EAAT5, are primarily found in cerebellar Purkinje cells and retina respectively (Dehnes et al., 1998; Arriza et al., 1997). GLT-1 and GLAST have been shown to be expressed almost exclusively in glial cells next to the synapses while EAAC1 is found in neurons (Danbolt et al., 2016). Despite their ubiquitous distribution, studies suggest that GLT-1 is the major glutamate transporter, accounting for at least 80% of glutamate re-uptake (Danbolt, 2001). Glutamate transporters, and by extension glial cells, have been shown to greatly influence pain transmission, particularly during pathological states (Ni and Weng, 2010; Mika and al., 2013).

### 3.2.3. Higher brain centers and descending modulation

#### 3.2.3.1 Ascending pathways and supra-spinal centers

After the transmission of the noxious signal to the spinal cord, second order neurons will form bundles sending their projections to several brain structures. The main projections originating from the superficial DH are targeting the thalamus as it acts as the principal relay for sensory inputs.

Traditionally projections have been described to comprise two main pathways, medial and lateral. The medial system includes, spino-reticular, spino-parabrachiohypothalamic, spino-mesencephalic, spino-parabrachio-amygdaloid, paleospino-thalamic and spino-hypothalamic tracts while the lateral system comprises spino-cervical tract and neospino-thalamic tract. These

two parallel pathways act in a complementary manner. On one hand, the lateral system sends input to the primary and secondary somatosensory cortexes (S1 and S2) in order to process the sensory quality (stinging, burning, aching), the location and the duration of noxious signals. On the other hand, the medial pathway extend to the forebrain (anterior cingulate and prefrontal cortexes) in combination with limbic structures (amygdala, ventral tegmentum area and nucleus accumbens) and is mainly involved in the emotional and motivational responses to pain (Almeida et al., 2004). It is only after reaching the higher brain centers, allowing perception and emotional evaluation that noxious stimuli lead to the subjective experience of pain, until then the processing of noxious information is referred to as nociception.

Despite distinct path and projections the structures involved in the different aspects of pain are interconnected with each other and therefore nociceptive processing is not limited to the areas receiving direct projections. However, some structures are clearly identified and believed to play a role in distinct aspect of pain sensation. As the focus of my Ph. D. was the spinal cord, I will not provide too many details on the different supra-spinal targets.

### 3.2.3.2 Descending modulation

We mentioned earlier that the transmission of the nociceptive message in the DH is modulated by PAFs (through auto-receptors) as well as excitatory and inhibitory interneurons. Other regulation mechanisms allowing a very fine tuning of the nociceptive information are provided by descending inhibitory or facilitatory fibers originating from supraspinal brain centers. The best characterized descending modulatory pathway is the periaqueductal gray-rostral (PAG) ventral medulla (RVM) pathway. The PAG is a midbrain structure interconnected with the RVM and receiving inputs for cortical areas and limbic regions. The RVM also receive inputs from other supraspinal structures such as the thalamus and the locus coeruleus. These two structures have been shown to

influence spinal nociceptive transmission, however it is believe that the RVM is the final relay of descending modulatory neurons (Ossipov et al., 2014). The RVM is mainly the source of serotoninergic descending projections but GABAergic/glycinergic projections have also been described (Kato et al 2006). Depending on the receptor subtype activation, serotoninergic projections to the dorsal horn can either induce antinociceptive (via activation of 5-HT1A, 5-HT1B, 5-HT1D or 5-HT7 receptors) or pronociceptive effects (via activation of 5-HT2A or 5-HT3 receptors) (Suzuki et al., 2004; Dogrul et al., 2009; Bannister and Dickenson, 2016). Other monoaminergic projections are known to modulate spinal transmission. Noradrenergic fibers mainly arising from the locus coeruleus promote inhibition of the spinal cord activity via an activation of the $\alpha 1$, $\alpha 2C$ and $\beta$-adrenoreceptors present on GABAergic interneurons, hence enhancing inhibition of the noxious signal transmission (BABA et al., 2000). Lastly, the inhibitory D2-like dopamine receptors are predominantly expressed in the spinal cord compared to the D1-like ones (Bouthenet et al., 1987). Through this GPCR, dopamine promotes presynaptic inhibition of glutamate release and hence has antinociceptive actions (Roca-Lapirot et al., 2011). Altogether the complex actions of descending modulatory neurons allow cognitive factors and emotional states to influence the perception of pain (Bushnell and al., 2013). Indeed, negative expectation can counteract analgesics effects (Bingel et al., 2011) while distraction or positive anticipation can produce placebo analgesia (Benedetti et al., 2005; van der Meulen et al., 2017).

## 3.3. Chronification of pain: peripheral and central sensitization

### 3.3.1. Concept of peripheral sensitization

Peripheral sensitization mostly occurs during inflammation but can also be present in the framework of nerve damage. As a result, the threshold of activation by noxious stimuli (for heat, cold, pressure and chemicals) can be reduced and the general excitability of primary afferents fibers is increased.

Inflammatory mediators and growth factors are greatly involved in the induction and maintenance of peripheral sensitization. For instance, the nerve growth factor (NGF) has been shown to induce a prolonged thermal and mechanical hyperalgesia. Both effects components were thought not to be mediated by the same mechanisms. Indeed, NGF-thermal hyperalgesia is mediated by mast cell degranulation (Lewin et al., 1994). NGF, in combination with other growth factors such as GDNF (glial-cell derived neurotrophic factors) and BDNF (brain derived neurotrophic factor) can activate intracellular cascades activating kinases which can alter the expression and or phosphorylate channels such as TRPV1 but also voltage-gated sodium, calcium and potassium channels (Mantyh et al., 2011; von Hehn et al., 2012). Altogether, the release of growth factors and cytokines can change the electrical properties of injured and neighboring non-injured neurons leading to abnormal inputs from PAFs and painful sensations (Wu et al., 2002).

### 3.3.2. Concept of central sensitization

As stated above, acute pain has an important physiological role in preserving individuals from further damage or injury. However, in some circumstances such as neuronal injury and inflammation, pain can turn out to be a disease on its own. In this case, patients are not warned any longer from a potential danger by noxious stimuli, pain just becomes debilitating. Chronic pain states can arise from various causes and are subjected to huge differences in individual susceptibility. Chronic neuropathic pain originates from a nerve damage or a disease affecting the neurosensory system which can generate pacemakers-like potentials from nociceptors and altered processing within pathways involved in pain perception (Woolf and Mannion, 1999; Woolf, 2004). Chronic inflammatory diseases are mechanistically different since they may be accompanied by a sustained peripheral inputs originating from the affected tissues in the periphery and leading to central sensitization. Despite the very diverse etiologies of chronic pains syndromes several commonalities exist. Of

particular interest is the structural and functional remodeling with the DH of the spinal cord. For instance, recent studies are suggesting that comparable increases of synaptic spine density can be observed in paw inflammation and neuropathic pain models (Tan et al., 2012; Lu et al., 2015). These structural alterations were linked to a more general phenomenon termed central sensitization. The definition of central sensitization has been given by the IASP as an "increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input". This definition is however quite vague since the term "nociceptive neurons" a very heterogeneous neuronal population comprises. For instance, GABAergic and glycinergic neurons of the DH are involved in nociception but an increase of their activity leads to a reduced noxious sensitivity. On the other hand, an enhancement of PAFs activity generally leads to higher noxious sensitivity. Nevertheless, it is generally accepted that the term "central sensitization" implies an enhanced responsiveness to noxious stimuli associated with the subjective phenomena of hyperalgesia (a state of increased sensitivity to painful stimuli) and/or allodynia (a state during which normally non-noxious stimulation are eliciting pain sensations) and/or spontaneous pain (Sandkühler, 2009). In the spinal cord, central sensitization is characterized by a reduction of the thresholds eliciting a response, a widening of the nociceptive neuron's receptive fields (Cook et al., 1987), an enhanced synaptic transmission efficacy (Woolf and King, 1990) and the acquired capacity of Aβ fibers to transmit noxious stimuli (Woolf and King, 1990; Simone et al., 1989).

### 3.3.2.1. Mechanisms underlying spinal sensitization

Several distinct but not exclusive causes are believed to underlie the establishment of altered noxious transmission seen during chronic pain states.

*Long term potentiation (LTP):*

Originally discovered in the hippocampus (Bliss and Lomo, 1973) and considered to constitute a substrate for mechanisms underlying simple forms of learning, LTP was shown later at the level of the spinal cord (Woolf, 1983; Wall and Woolf 1984). Although hippocampal and spinal LTP share common mediators and mechanisms they are not identical in any case (Ji and al., 2003). Spinal LTP results from brief high-frequency (around 100 Hz) repeated trains of electrical nociceptor primary afferent stimulation and is claimed to constitute a potential mechanism involved in early-onset central sensitization that may involve homosynaptic as well as heterosynaptic effects. Briefly, homosynaptic plasticity refers to enhanced synaptic transmission between the stimulated neurons and their postsynaptic partners whereas heterosynaptic plasticity corresponds to the additionally altered processing of neighboring synapses, e.g via the co-release of neuropeptides. The reinforcement of synaptic strength occurring via repetitive noxious inputs to the spinal cord thus results in facilitation of noxious transmission (Woolf and Wall, 1986) characterized by a reduction of threshold of the nociceptive second order neurons, increased responsiveness and a widening of nociceptive receptive fields (Cook et al., 1987). A commonly used model selectively studying spinal homosynaptic plasticity is the so-called "wind up" paradigm that relies on trains of impulses typically applied at a lower frequency (around 1 Hz).

In the case of heterosynaptic plasticity the repeated stimulation of C-PAFs is capable to elicit a response of low threshold Aβ-PAFs (Ji et al., 2003). The recruitment of Aβ-PAFs has been proposed to be long lasting and involved in the pathogenesis of allodynia during chronic pain as Aβ-fibers have been shown to sprout into DH laminae I and II (Woolf and al., 1992). Furthermore, substance P, a neuropeptide involved in nociceptive processing, has been detected in Aβ primary afferents in models of inflammatory and neuropathic pain (Neumann et al., 1996). The long lasting facilitation will lead to activation of ligand gated

channels, especially the glutamatergic receptors. It will lead in turn to the activation of multiple kinases (PKA, PKC, and ERK) pathways and the phosphorylation of ion channels, subsequently increasing the synaptic efficacy and channel opening time. Furthermore, the opening of NMDA receptors will allow a massive calcium influx and autophosphorylation of the calcium/calmodulin-dependent kinase II (CAM-KII). The classical and more comprehensive concept of central sensitization underlying the enhancement and long-term maintenance of clinical pain states is however related to the co-release of neuropeptides together with glutamate under conditions of pronounced and ongoing nociceptive input. Slowly depolarizing neuropeptides like substance P may under these conditions act post synaptically via activation of metabotropic receptors like the neurokinin-1 (NK-1) receptor. This will lead to the subsequent activation of the PLC pathway, in turn releasing calcium from intracellular stores and activating PKC (Drdla and Sandkühler, 2008). Altogether, this will have several consequences such as maintaining NMDA (Garry and al., 2003) receptors in an open state and increasing AMPA receptors single channel conductance (Sheng and Kim, 2002). Finally, the described maintenance depends on CREB mediated pathways (Nguyen and Kandel, 1996) triggered by NMDA and mGluR but also by β-adrenoceptors and dopaminergic receptors partly underlying the effects of descending modulation (Sweatt, 2001; for review Ossipov and al., 2014).

*Disinhibition of GABAergic/glycinergic interneurons:*

Spinal inhibitory interneurons are an important regulator of DH nociceptive transmission, hence death or loss of these cells function can result in enhanced pain sensitivity. Most of the studies have investigated GABAergic interneurons, however there is a great overlap between GABA and glycine inhibitory interneurons (Prescott, 2015). The following studies on the loss of inhibitory interneurons as a maladaptive plasticity mechanism have focused on GABAergic interneurons which does not necessarily rule out similar effects for

glycinergic neurons (Moore et al., 2002; Polgar et al., 2003; 2008). Nevertheless other evidence points towards an alteration of these cells during chronic pain states. Indeed, other studies have shown diminished GABA and Glutamate decarboxylase (GAD) immunoreactivity (Ibuki et al., 1997; Eaton et al., 1998; Moore et al., 2002), reduced GABA release and presence at the synapse (Stiller et al., 1996; Schoffnegger et al., 2006). Despite those indications of a dysfunction of the GABAergic system in the DH, it was discovered later that these neurons remain functional (Schoffnegger et al., 2006). It is believed that the reason of this disinhibition may be related to the dysregulation of the potassium chloride cotransporter 2 (KCC2) (Coull et al., 2005). Indeed, this transporter is undergoing a downregulation following nerve injury and this seems to be sufficient to alter intracellular chloride levels and cause a disruption of anion gradient subsequently leading to a reduced GABAergic inhibitory tone (Beggs and Salter, 2010).

### 3.3.3. Importance of glial cells

Originally believed to have a role for the structural support for the CNS, glial cells have been largely investigated since and their role proven to be much broader. Glial cells represent 70% of the totality brain and spinal cord cells and are divided in two major types: astrocytes and microglia.

Microglia are macrophage-like resident cells of the central nervous system. During physiological conditions, microglial cells have a ramified shape but can adopt an amoeboid form upon activation. In physiological conditions microglial have a capital role since they regulate neuronal environment through their interaction with neuronal cells allowing them to provide a structural support, act as a nutrient stock and supplier but also to destroy and remove damaged or dead neurons. Upon insults these cells undergo rapid morphological and functional changes associated with genomic regulation and the release of a multitude of factors. These actions have been shown to be a critical factor for the

development of neuropathic and inflammatory pain (Lin and al., 2007; Raghavendra et al., 2003; 2004; Tanga et al, 2004; Romero-Sandoval et al., 2008).

Astrocytes are the most abundant macroglial cells of the CNS such that one astrocyte can enwrap tens of thousands of synapses. Originally believed to be the "nerve glue" (Virchow 1859), they actually express a multitude of channels and transporters at their cell surface allowing them to regulate ionic homeostasis of surrounding neurons. Indeed, astrocytes are believed to participate actively in synapse regulation. The "tripartite synapse" theory is based on the fact that astrocytes can sense neuronal activity, respond by intracellular calcium elevation and regulate synaptic strength (Arraque et al., 1999). Comparably to microglia, activated astrocytes undergo morphological and functional alterations during chronic pain conditions which are believed to underlie neuropathy and inflammation (König et al., 2014; Yan and al., 2014; Romero-Sandoval et al., 2008).

Microglial and astrocytic activation are believed to have a capital role in the establishment of central sensitization of the DH during chronic pain conditions. Despite sharing the common ability to release growth factors and pro-inflammatory cytokines, both glial cells subtypes seem to have complementary rather than overlapping roles (for an extensive review Ji and al., 2013). Indeed microglia have been claimed to play a greater role in the initiation of chronic pain conditions while astrocytes seem to be more involved in the maintenance of abnormal pain sensations (Raghavendra and DeLeo, 2003). Nevertheless, both cell types have overlapping roles in the establishment of DH sensitization and both are involved in the different stages of pain hypersensitivity (Ledeboer et al., 2005; McMahon et al., 2005).

Microglia does e.g. participate in the disinhibition of DH neurons. Indeed, the ATP released by primary afferents stimulates the P2X4 receptors expressed at the microglial cells' surface. Activation of P2X4 will in turn trigger the de novo

synthesis and release of BDNF. BDNF will subsequently reduce KCC2 expression of laminae I DH neurons leading to an intracellular chloride ion increase and impairing GABAergic inhibition (Coull et al., 2003; Tsuda et al., 2003; Trang et al., 2009; Tsuda et al., 2013).

Astrocytes have a tremendous impact on synaptic clearance. Both GLT-1 and GLAST are expressed on astrocytes cell membrane and they act as the major actor of glutamatergic clearance as they can recapture more than 90% of synaptic glutamate (Danbolt, 2001). Hence, the sustained downregulation of these transporters is believed to account for a great deal in abnormal pain sensations during inflammatory or neuropathic pain (Sung et al., 2003; Maeda et al., 2008).

On top of this, glial cells can both synthetize and release a number of pro-inflammatory cytokines, growth factors and chemokines. The tumor necrosis factor-α (TNF-α), the interleukin-1β and the interleukin-6 (IL-1β and IL-6) are the most studied glial mediators. They have been shown to be upregulated after peripheral neuropathy and inflammation (DeLeo and Yezierski, 2001; Watkins et al., 2001; McMahon et al., 2005). Furthermore, altogether they participate in the development and maintenance of abnormal pain sensation by modulating excitatory and inhibitory synaptic transmission. For instance, TNF- α, which is primarily produced by microglia, can alter the excitability of the CNS by reducing the expression of GLT-1 and GLAST (Sitcheran et al., 2005). Additionally, TNF-α possesses the ability to activate p38 MAPK consequently modifying AMPA receptor composition via phosphorylation thus increasing calcium AMPA permeability (Beattie and al., 2002). IL-1β has been found in glia and neurons and have the capacity to increase cell excitability through its actions on sodium, calcium and potassium channels (Schäfers and Sorkin, 2008) leading to an increase of amplitude and frequency of spontaneous excitatory postsynaptic currents (EPSCs). Furthermore, IL-1β and IL-6 were shown to act presynaptically leading to a reduction of the frequency of inhibitory

postsynaptic currents (Kawasaki et al., 2008). In addition to these actions on transporters pro-inflammatory cytokines can also elicit neuronal LTP by inducing the phosphorylation of transcription factors such as the CREB and promote the expression of pro-nociceptive genes (Ji et al., 2013).
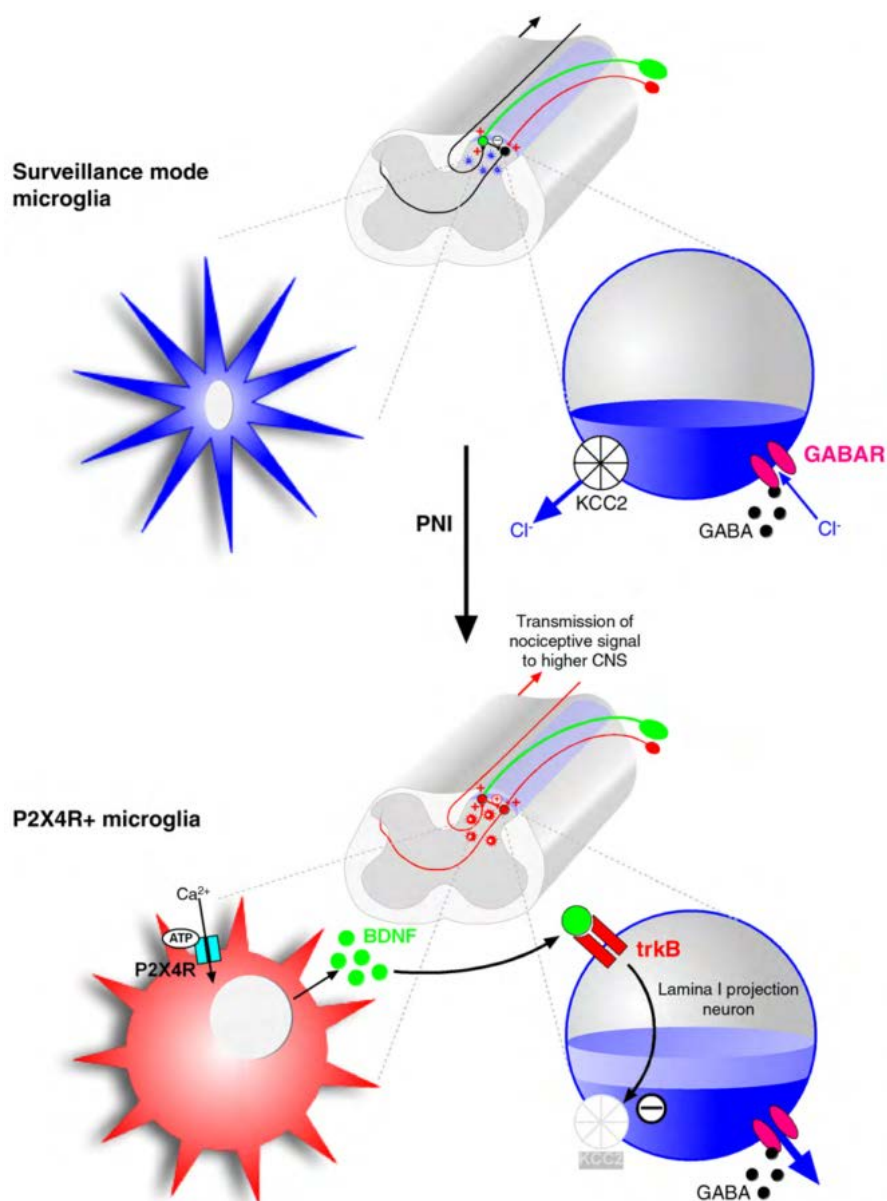


*Figure 10: Involvement of microglial cells in disinhibition. The top half part of the graph represents the physiological state, where microglia is resting and GABAergic neurons display normal functioning. Following peripheral nerve injury (PNI) microglia takes up its amoeboid form and release BDNF. BDNF will in turn act to downregulate neuronal KCC2. The extrusion of chloride will hence be slowed down and chloride intracellular concentration will rise leading to the impairment of GABAergic neurons to exert their inhibitory effect (*Beggs and Salter, 2010).

### 3.3.3. Neuropathic pain

#### 3.3.2.1. Introduction

The facilitation of pain transmission following injury or inflammation of peripheral tissues is a necessary set of adaptive changes evolutionarily set up in order to avoid further damage. This leads to the generation of enhanced pain sensitivity, as a defense mechanism allowing proper healing. In contrast, neuropathic pain occurring following nerve damage that can arise from multiple sources (diabetes, nerve injury, cancer, multiple sclerosis etc.), can lead to enduring changes in pain sensitivity. In addition to the prolonged and amplified pain sensations, concerned patients may have abnormal painful sensations triggered by innocuous stimuli (allodynia) and spontaneous pain. As these symptoms are underlined by sustained neuronal changes, neuropathic pain is nowadays regarded as autonomous nervous disease (von Hehn et al., 2012).

Neuropathic pain is quite common and can be found in the 7 to 8% of the general population (IASP report). Furthermore, this prevalence could be underestimating the actual number of patients suffering from neuropathic pain since it is often associated with another pathology. For instance, 37% of German patients suffering from low back pain have neuropathic pain. This statistical value represents 14% of female and 11% males Germans. In UK about 25% of patients with diabetes develop neuropathy, this could be a major health issue in the future since the prevalence of diabetes has been increasing over the years. Lastly, the American Institute of Medicine estimates that approximately 18% of people suffer from neuropathic pain (Toth et al., 2009). This debilitating disease greatly impairs the quality of life of patients and has a high impact on the economy of health systems notably because it is still poorly treated. Several genetic polymorphisms have found to confer vulnerability/resilience to neuropathic pain (Binder et al., 2011). Furthermore, neuropathic pain presents many comorbidities with other syndromes or pathologies such as poor sleep,

depression and anxiety. Another difficulty of finding adequate treatment resides in the fact that the disease may have peripheral and/or central origins.

### 3.3.2.2. Animal models of neuropathic pain

The first models of neuropathic pain started to emerge in the 1970s responding to the need to establish human-like etiologies and symptoms of chronic neuropathy. The oldest model is the axotomy model (Wall et al., 1974) consisting in a complete transection of the sciatic nerve in the mid-thigh region. The surgery leads to the development of a neuroma (regenerative fibers sprouting anarchically) at the proximal stump of the nerve (for review Jaggi et al., 2011). However this model is not commonly used anymore since complete nerve section is rare in the clinical setting and the symptoms observed in rodents such as autotomy (self-mutilation of the denervated limb) are ethically hardly acceptable. Newer models have hence emerged in the 1980s ~ 1990s. One of the most if not the most commonly used is the chronic constriction injury models (CCI) established by Bennett and Xie (1988). It consists of inducing a peripheral mononeuropathy (generally in rats) by placing 3 or 4 loose chromic gut ligatures (spaced of about 1 mm) around the sciatic nerve. The tightness of the ligature is a critical factor for the development of CCI-induced neuropathy since the epineural circulation should not be interrupted. Locally, the constriction induces an intraneural oedema, ischemia and Wallerian degeneration thereby sensitizing both A and C-PAFs and in turn initiating central sensitization (Gabay and Tal, 2004). CCI leads to a range of behavioral alteration such as thermal and mechanical hyperalgesia and spontaneous pain characterized by excessive licking, limping and avoidance to put weight on the paw ipsilateral to injury (Wang et al., 2005; 2006; Barrot, 2012; Le coz et al., 2017). These changes have been shown to develop rapidly (within less than a week) following CCI-surgery and to be sustained for up to 7 weeks (De vry et al., 2004). Hence, the CCI model is considered to correspond well to causalgia or complex regional pain syndrome observed in neuropathic patient (Sandkühler, 2009).
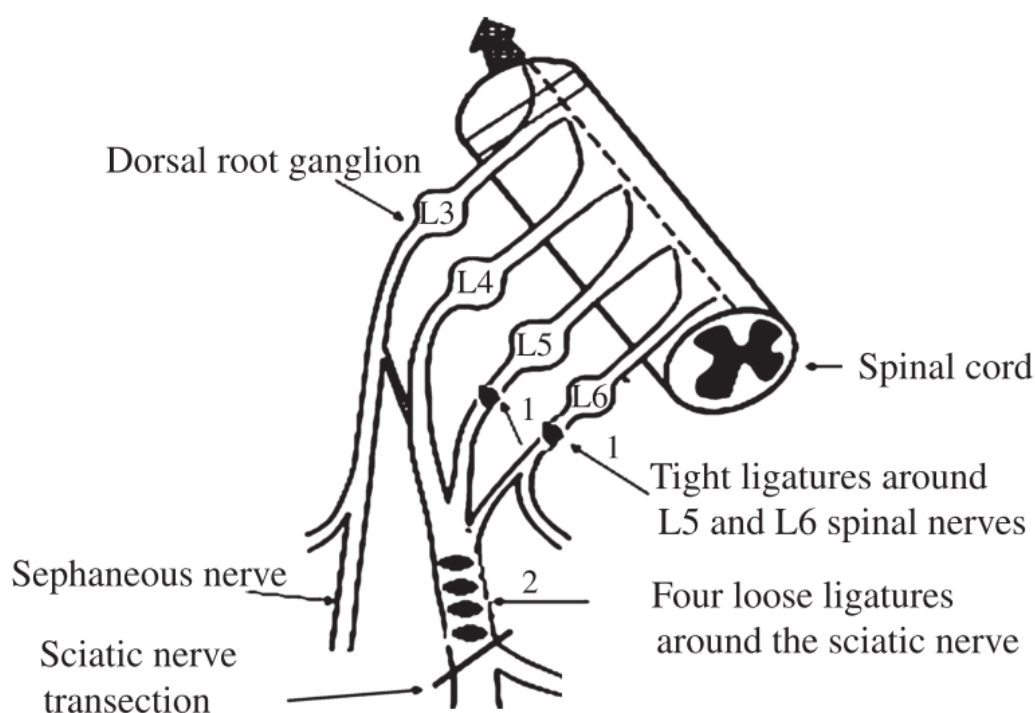
*Figure 11: Schematic representation of neuropathic pain models (**Jaggi et al., 2011**)*

### 3.3.4. Inflammatory pain

### 3.3.3.1. Introduction

Inflammation is a reaction of living tissues to an aggression. This reaction includes the activation of immune system cells and the release of a multitude of mediators such as pro-inflammatory cytokines (TNF-α, IL-1β, IL-6). In the same way as peripheral neuropathy, inflammation has the capacity to sensitize nociceptors, facilitate pain transmission and if it is sustained lead to chronic inflammatory pain. The peripheral sensitization of nociceptors has been for a long time attributed solely to the pro-inflammatory mediators found in the inflammatory "soup". However, even though these substances account for a great part of the enhanced excitability of nociceptors, other mediators such as growth factors have the capacity to sensitize PAFs. Although the chronification of inflammatory and neuropathic pain states share a great deal of overlapping mechanisms, a few differences have been identified (Latremoliere and Woolf,

2009). Indeed, it seems that inflammation-mediated pain hypersensitivity involves a Cox-2 (cyclooxygenase)/PGE2 (Prostaglandine E2) pathway which in turn potentiates AMPA and NMDA receptors and reduces glycinergic inhibition in the DH (Harvey et al., 2004).

### 3.3.3.2. Animal models of inflammatory pain

The great majority of inflammatory pain models rely on the activation of the immune system, which leads to a sensitization of the nociceptive system, following injection of a substance. The different models vary depending on the site of injection (often one of the knee, ankle or paw) and the injected agent such as formalin, carrageenan or complete Freund's adjuvant (CFA). Formalin is generally used as a short-term model of inflammation and allows to differentiate the direct activation of nociceptors (acute phase) from the inflammatory response (late phase) (Hunskaar and Hole, 1987). On the contrary, carrageenan and CFA have more antigenic potential and their injection induces thermal and mechanical hyperalgesia and swelling for at least several days (Barrot, 2012). These models are thought to mimic closely the time course of post-operative and long lasting pain (Ke Ren and Dubner, 1999). In the framework of the present work I injected CFA into one of the rat hind footpads. CFA is a heat-killed Mycobacterium tuberculosum that is suspended in oil. We preferred this to to carrageenan because the hyperalgesia onset time (2-6h) and duration of the inflammation (2-3 weeks) was closer to the time scale used during neuropathic pain studies. Furthermore, Tanga and colleagues (2004) have shown that CFA-induced paw inflammation produced a pattern of glial activation and proinflammatory cytokine expression that was comparable to the one observed under neuropathic conditions.

# Chapter IV: Objectives

Data have accumulated for several decades about the link between chronic stress-related diseases such as major depressive disorders and pain states. Indeed, alterations of pain thresholds, pain tolerance and alterations of the efficacy of analgesics and inversely, individuals suffering from chronic pain-related alterations in stress processing have been reported in clinical and laboratory settings (Sarlis et al., 1992; Aghajani et al., 2012; Green et al., 2011; Nishinaka et al., 2016; Alvarez et al., 2013; Uhelski et al., 2010; Kalinichev et al., 2001; Lentjes et al., 1997). As discussed in chapter 2, chronic stress often leads to alterations of the HPA axis. Furthermore, clinical and experimental data have demonstrated that many major peripheral and central pain mediators (such as cytokines, monoamines and hormones) are also implicated in the modulation of the HPA axis (Blackburn-Munro and Blackburn-Munro, 2001). Nevertheless, the relationship between stress-related disorders and chronic pain is still debated. Indeed, several hypotheses have been proposed: 1) both are independent diseases despite sharing common substrates and mechanisms, 2) stress-related disorders are the main pathology and chronic pain is a resulting consequence, 3) conversely some investigators consider that chronic pain induces diseases such as depression and 4) prior stress experiences predispose patients to develop psychiatric disorders once they are undergoing chronic pain.

Our laboratory has investigated the impact of HPA axis modulation on painful behaviors and on alterations of spinal processing associated with peripheral inflammation (Juif et al., 2016; 2014; 2011) and neuropathic pain (Le coz et al., 2017; 2014a; 2014b). These studies have used various approaches to investigate the role of HPA axis activity such as pharmacological manipulation (i.p. injection of dexamethasone or RU486), the use of different rat strains known for displaying hypo and hypercorticolism (respectively Lewis and Fisher rats) and administration of chronic stress (restraint stress or chronic social stress).

While these publications and others have added to the understanding and identification of molecular mediators that are common to stress- and nociception

processing such as cytokines, growth factors and monoamines, the respective interactions remain unclear. This is especially true regarding possible long term alterations at the spinal cord level induced by early life stress.

Early life stress has been shown to be a factor of vulnerability or resilience regarding several psychiatric disorders. Despite the tight association and overlap between stress- and pain-related systems very little is known about the potential influence of early life stress on pain sensitivity with respect to chronic pain conditions in later life. Furthermore, even though early life stress has been identified as a factor predisposing to chronic pain conditions, the large majority of the studies on potentially underlying mechanisms have been investigating supra-spinal but not spinal levels of processing.

This thesis sought to investigate if and how early life stress affects pain sensitivity and the associated spinal processing in Sprague Dawley rats. This research was done in several steps:

- The first study investigated the impact of early life stress (3h MS protocol) on pain sensitivity at adult age in the framework of physiological and neuropathic conditions (CCI surgery).
- The second study aimed to determine if early life stress had comparable consequences for (CFA-induced) inflammatory pain and spinal biomarkers as for neuropathy-related pain.
- In the third study, a more complex protocol was set up. Based on the multiple hits hypothesis, I aimed to determine if a second stress insult at adult age in animals previously exposed to early life stress would lead to an allostatic load potentially enhancing vulnerability to chronic pain.

The data collected during these experiments allowed me to write the 3 manuscripts composing this thesis. Furthermore, I participated to a study initiated prior my arrival on the effect of chronic social stress on neuropathic pain. This article is given in the supplement.

Two additional projects were started during the final years of my Ph. D. One aimed to determine the role of microglia in the alterations identified in the first study. Indeed, some of the data collected could suggest that reduced activity and/or number of microglial cells could underlie the reduced pain sensitivity during the onset of chronic pain. The hypothesis was that MS could lead to an early activation of enduring adaptive modifications of immune system. These alterations might thus have positive repercussions during the onset of neuropathic pain. Assuming that the respective microglial cells could be hypo-functional, I intended to pharmacologically enhance their activation. So far preliminary results have been obtained and are displayed in chapter 8.

The final project aimed to characterize the electrophysiological response behavior of spinal nociceptive neurons following early life stress. The use of in vivo electrophysiology allows the determination of the response behavior of nociceptive dorsal horn neurons in naïve and MS rats. Furthermore, this approach provides a possibility to investigate early life stress-related differences in central sensitization as well as to characterize the influence of potentially altered descending pain modulation pathways. A description of this project can also be found in chapter 8.

# Chapter V: Impact of early life stress on neuropathic pain

## 5.1. Abstract

Early life stress (ELS) leads to a permanent reprogramming of biochemical stress response cascades that may also be relevant for the processing of chronic pain states such as neuropathy. Despite clinical evidence, little is known about ELS-related vulnerability for neuropathic pain and the possibly underlying etiology.

In the framework of experimental studies aimed at investigating the respective relationships we used the established ELS model of maternal separation (MS). Rat dams and neonates were separated for 3 h/day from post-natal day 2 to 12. At adulthood, noxious mechanical and thermal thresholds were assessed before and during induction of neuropathic pain by chronic constriction injury (CCI). The potential involvement of spinal glutamatergic transmission, glial cells, pro-inflammatory cytokines and growth factors was studied by using qPCR.

MS per se did not modify pain thresholds. But, when exposed to neuropathic pain, MS rats exhibited a marked reduction of thermal sensitivity and a delayed development of mechanical allodynia/hyperalgesia when compared to control animals. Also, MS did not alter glucocorticoid receptor mRNA levels, but prevented the CCI-induced down-regulation of NR1 and NR2 sub-units of the NMDA receptor and of the glutamate transporter EAAT3 as observed at 21 days post-surgery. Additionally, CCI-provoked up-regulation of glial cell markers was either prevented (GFAP for astrocytes) or dampened (Iba1 for microglia) by MS. Pro-inflammatory cytokine mRNA expression was either not affected (IL-6) or reduced (IL-1β) by MS shortly after CCI. The growth factors GDNF and NGF were only slightly downregulated 4 days after CCI in the MS-treated animals. The changes in glutamatergic signaling, astroglial and cytokine activation as well as neurotrophin expression could, to some extent, explain these changes in pain behavior. Taken together, the results obtained in the described experimental conditions support the mismatch theory of chronic stress

where an early life stress, rather than predisposing individuals to certain pathologies, renders them resilient.

**Maternal separation stress leads to resilience against neuropathic pain in adulthood**

Julien Genty*, Milène Tetsi Nomigni, Fernand Anton, Ulrike Hanesch


Laboratory of Neurophysiology,

Institute for Health and Behavior,

University of Luxembourg,

162a, avenue de la Faïencerie,

L-1511 Luxembourg,

Luxembourg


* Corresponding author:

Julien Genty

162a, avenue de la Faïencerie,

L-1511 Luxembourg,

Luxembourg

Phone:+352 466644 6340

julien.genty@uni.lu

## 5.2. Introduction

The nervous and the immune system are involved in the stress response as well as in the processing of pain (Krishnan and Nestler, 2008; Schwaller and Fitzgerald, 2014). Stress-related structural or functional modifications within these systems are hence likely to impact pain sensitivity (Sandkühler, 2009). Early life is a critical period for the normal development of individuals. Preclinical (Schmidt, 2010) and clinical (Heim et al., 2010) studies have demonstrated that early life stress can have a major impact on neuronal circuits and immune system development, possibly leading to enhanced vulnerability to physio- and psychopathological states at adulthood. A well-established way of modelling early life stress in rodents is to expose new-born pups to maternal separation (MS) (Levine, 2001). MS has been shown to induce abnormal development of immune and nervous systems (Roque et al., 2015). Among these perturbations is a long term modification of the central neuronal circuitry involved in relaying noxious stimuli and in controlling pain sensitivity during normal and pathological states (Chung et al., 2007; Uhelski and Fuchs, 2010; Weaver et al., 2007). Neuropathic pain is a chronic pain state that generally occurs following nerve damage. As a consequence, significant peripheral and central remodeling leads to enhanced pain sensitivity (hyperalgesia), induction of pain by a normally non painful stimulus (allodynia) and to spontaneous pain (for review see: von Hehn et al., 2012). Despite significant advances in basic and clinical research, this condition remains difficult to treat since our understanding of the underlying pathophysiological mechanisms is still insufficient (von Hehn et al., 2012).

Although spinal synaptic transmission of noxious stimuli constitutes the first relay of this network, it has scarcely been studied in the context of stress. Among the molecular mediators involved in nociceptive transmission and in the establishment of neuropathic pain, several key players such as the glucocorticoid receptor (GR) (Ladd et al., 2004; Wang et al., 2005), glutamatergic receptors

and transporters (Toya et al., 2014), cytokines (Alvarez et al., 2013) and neurotrophins (Faure et al., 2007) are modulated by MS. Thus, it seems plausible that MS could have an impact on neuropathic pain vulnerability at least in a subset of individuals. In addition changes of neuro-immune and/or neuroendocrine systems induced by chronic stress could facilitate or hamper the development of chronic pain independently of classical stress processing pathways. To our knowledge, no study has investigated the effect of MS on pain thresholds and potential spinal molecular mediators involved in the change of nociceptive transmission under conditions of neuropathy. In the present work we used behavioral (mechanical and thermal thresholds) and biochemical (mRNA expression of spinal markers) approaches to assess the impact of MS on the onset and the maintenance of neuropathic pain. In order to induce this pain condition, we decided to use the well-established chronic constriction injury (CCI) model initially described by Bennett and Xie (1988).

## 5.3. Material and Methods

### 5.3.1. Animals

Female (nulliparous) and male Sprague Dawley rats were purchased from Harlan Laboratories (Netherland). They were then reared in our facility to provide the offspring used for the experimental studies. All animals were housed under standardized conditions: temperature controlled room (21-23°C), relative humidity $60 \pm 10\%$, 12 h light/dark cycle, food and water provided ad libitum. Rats were only briefly handled twice per week during the cage changes. Except for the maternal separation procedure in the respective groups, pups were left undisturbed until weaning at post-natal day 21. Experiments started when the animals reached the age of 8 weeks.

Animals were divided in 4 groups (see Fig. 1) depending on the stress and pain conditions they were exposed to: controls CON (no MS, no CCI; n=10),

CON+CCI (no MS but CCI; n=15), MS (MS but no CCI; n=15), and MS+CCI (MS and CCI; n=14).

All animal experiments were carried out in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and met the ARRIVE guidelines. The animal procedures were approved by the Animal Experimentation Ethics Committee (AEEC) of the University of Luxembourg (Project ID 15-SPM-01-UH) and the "Ministère de l'Agriculture, de la Viticulture et de la Protection des consommateurs".

### 5.3.2. Maternal separation

Maternal separation was carried out on at least four different litters. The day pups were first seen was marked as P0. From P2 to P12 pups were separated from the dam, placed on a heated pad at 33 °C (+/- 2°C) and left undisturbed for 3 hours/day. At the end of each separation period pups were returned to their home cage. No other manipulation was done than indicated.

### 5.3.3. Chronic constriction injury (CCI) surgery

At two months of age, after baseline behavioral testing, rats underwent the CCI surgery. They were deeply anaesthetized with isoflurane (4.5% for induction, 2.0-2.2% for maintenance) during the entire procedure using an anesthesia apparatus (Univentor 400, Zejtun, Malta). The right sciatic nerve was exposed in the mid-thigh and three natural chromic gut 4-0 (Stoelting Europe, Dublin, Ireland) loose ligatures were placed around the nerve at a distance of 1 mm. The muscle layer was closed with 4-0 silk sutures and the skin layer with surgical skin staples.

In case animals presented signs of autotomy in the course of the experiments, they were immediately removed and sacrificed in order to minimize their

suffering. For this reason 2 CON+CCI and 3 MS+CCI animals were sacrificed before the end of the neuropathy protocol and their results were discarded.

### 5.3.4. Behavioral tests

Noxious mechanical and thermal thresholds were assessed in 54 male rats using the von Frey monofilament test and the cold plate test respectively. All behavioral tests were done in the morning between 8:00 and 12:00 a.m. During each session, animals were moved to the experimentation room at least 1 hour before the start of the experiments to allow them to habituate to the environment. Rats underwent the von Frey monofilament test, followed by the cold plate test. Baseline thresholds were assessed on three consecutive days prior to the CCI surgery. To assess the impact of maternal separation on neuropathic pain, the pain sensitivity was tested at days 4, 7, 10, 14, and 21 after the CCI surgery.

### 5.3.4.1. Von Frey monofilament test

To evaluate mechanical pain thresholds, animals were placed on a metal wire mesh floor, covered by a Plexiglas chamber (19.5x19.5x14 cm) and given at least 15 minutes to acclimate, until exploratory activity ceased. Filaments (OptiHair, MarstockNervTest, Germany) were applied perpendicularly on the mid-plantar region of the hind paw and pressure was gradually increased until the deflection point of the filament. Pain thresholds were determined with the ascending and descending method of limits with forces ranging from 8 to 256 mN. The threshold force was defined as the first filament evoking at least a 40% response rate (two withdrawals out of five consecutive applications). Both hind paws were tested three times in an alternative order and the mean results were defined as the respective thresholds. Results were then normalized for each animal by computing the ratio of ipsi- to contralateral side expressed in percent. Finally, data for each group were averaged.

### 5.3.4.2. Cold plate test

This test was used to assess stress- and pain-related changes in thermal thresholds. To avoid sensitization it was performed at least 30 minutes after the von Frey test. In order to establish a baseline, rats were placed on the cold plate set at a temperature of 5°C for maximally three minutes (cut-off time). In each session, this procedure was repeated three times with a ten minute interval. Lifting or licking the paw as well as jumping are commonly considered as behavioral indicators of pain. We selected the number of paw lifts as behavioral parameter for the assessment of thermal thresholds since CCI-induced neuropathy produced pronounced limping and reduced the animal capacity to jump. The mean values of the three cold plate tests were defined as the score of a session.

### 5.3.5. Reverse transcription and Real-Time qPCR

Rats were rapidly killed by decapitation under deep anesthesia (Isoflurane 4.5%). Levels L4/L5 of the spinal cord were harvested and divided into ipsilateral-contralateral sides. Total RNA was extracted by the acid guanidium–thiocyanate–phenol–chloroform method using TRIzol® reagent (Life Technologies, Halle, Belgium). The aqueous phase containing RNA was collected and precipitated with isopropanol. The RNA pellet was washed with 70% ethanol, air dried, dissolved in Rnase free water (VWR, Leuven, Belgium) and stored at -80°C until further analysis.

RNA quality was assessed with the Experion system or the Experion Automated Electrophoresis Station (Bio-Rad Laboratories, Nazareth, Belgium) using StdSens chips (Bio-Rad). The RNA quality indicator was between 7 and 9. RNA concentration was measured using the Nanodrop 2000 spectrophotometer quantification system (Isogen Life Sciences, Netherlands). Reverse transcription was performed with the Improm-II reverse transcription kit (Promega, Leiden, Netherlands) to convert 500 ng of total RNA into cDNA with

0.5 µg/ml of Oligo dT15 primer by using a C1000 Touch thermocycler. qPCR experiments were carried out on a CFX 96 real time system (Bio-Rad, Nazareth, Belgium) with 12.5 ng of cDNA in a final volume of 20 µl using PerfeCTa® SYBR® Green SuperMix (VWR, Leuven, Belgium) containing 2X reaction buffer with optimized concentrations of MgCl2, dNTPs, AccuStart Taq Polymerase, SYBR Green I dye, stabilizers as well as forward and reverse primers at 2 µM. Primers were designed using Beacon Designer™ software, tested for sequence specificity using the Basic Local Alignment Search Tool at NCBI and validated on spinal cord (for list of primers used in the study, see Table 1). The following protocol was used: polymerase activation at 95°C for 3 min, 40 cycles of denaturation at 95°C for 10 s and annealing at 61°C for 30 s. Finally, the melting curves were recorded between 65°C and 95°C in 0.5°C intervals. Each qPCR experiment was run in triplicate and no-template controls were added as negative controls. Threshold cycle values (Cq) were used to compute the amount of target gene mRNA in relation to the reference gene mRNA (actin, beta). $\Delta$Cq represents the difference between the number of cycles that were necessary to detect the PCR products of the target genes and that of the reference gene. $\Delta\Delta$Cq indicates the difference between the $\Delta$Cq of the experimental groups (CON+CCI, MS and MS+CCI) and the $\Delta$Cq of the control animals (CON) animals.

The data were expressed as 2-$\Delta\Delta$Cq and the mean of the right injured side was computed for each group.

*Table 2: Summary of the primers used*

| Name gene | Accession | Sequence | Amplicon size (bp) |
|---|---|---|---|
| Actin β | NM_031144.3 | F: 5' GCT GAG AGG GAA ATC GTG CGT GAC 3'<br>R: 5' GGA GGA AGA GGA TGC GGC AGT GG 3' | 96 |
| GR NR3C1 | NM_012576.2 | F: 5' TGG AAA CCT GCT CTG CTT TG 3'<br>R: 5' GAG GAG ACA AAC AGC ATG TG 3' | 102 |
| NR1 GRIN1 | NM_001270608.1 | F: 5' GGT TGC GTG GGC AAC ACC AA 3'<br>R: 5' CCG TCC GCA TAC TTA GAA GA 3' | 80 |
| NR2a GRIN2a | NM_012573.3 | F: 5' CAG ATA ACA ATA AGA ACC ACA AG 3'<br>R: 5' AAC ATC GCT ACA GTC CTT 3' | 83 |
| NR2b GRIN2b | NM_012574.1 | F: 5' AGG AAC CAG GCT ACA TCA AAA A 3'<br>R: 5' TAG TGA TCC CAC TGC CAT GTA G 3' | 197 |
| EAAT2 Slc1a2 | NM_017215.2 | F: 5' ATG CTC CTC ATT CTC ACA G 3'<br>R: 5' CTA CAT TGA CCG AAG TTC TC 3' | 103 |
| EAAT3 Slc1a1 | NM_013032.3 | F: 5' TCA TAG TCG TGC GGA AGA AC 3'<br>R: 5' AGC GGA ATG TAA CTG GAA GG 3' | 111 |
| GFAP | NM_017009.2 | F: 5' TAC AGG AAA TTG CTG GAG GG 3'<br>R: 5' GAC ACA GAT TTG GTG TCC AG 3' | 104 |
| Iba1 AIF1 | NM_017196.3 | F: 5' AAT GAT GCT GGG CAA GAG AT 3'<br>R: 5' ACC TCC AAT TAG GGC AAC TC 3' | 129 |
| IL-1β | NM_031512.2 | F: 5' AGA GTG TGG ATC CCA AAC AA 3'<br>R: 5' GGA ACT GTG CAG ACT CAA AC 3' | 105 |
| IL-6 | NM_012589.2 | F: 5' CCA GAG TCA TTC AGA GCA ATA C 3'<br>R: 5' CTT CTC CAT TAG GAG AGC AT 3' | 116 |
| GDNF | NM_019139.1 | F: 5' GTG TTG CTC CAC ACC GCG TCT 3'<br>R: 5' GGT CTT CGG CGG GCG CTT C 3' | 73 |
| NGF | NM_001277055.1 | F: 5' CAC GGA CAT CAA GGG CAA GGA 3'<br>R: 5' GCT CGG CAC TTG GTC TCA AA 3' | 96 |

### 5.3.6. Statistical analysis

Data are presented as mean ± SEM. Homogeneity of variance was tested using Levene's test or Shapiro-Wilk test. Statistical analysis for the von Frey and the cold plate experiments was carried out using a two-way (time x group) repeated measures analysis of variance (ANOVA) followed by a Tukey's multiple comparison post hoc test to check for differences between groups.

For all gene expression experiments, the ipsilateral spinal cord of CON (no CCI) served as control and the relative expression level was set to 1. The expression levels of the treatment groups CON+CCI 4d, CON+CCI 21d, MS, MS+CCI 4d and MS+CCI 21d were expressed as fold of CON. For statistical analysis, these relative expression levels (fold) were compared using a two-way (stress x CCI) ANOVA with Tukey's multiple comparison post hoc test.

A summary of the statistical analysis is given in Table 2.
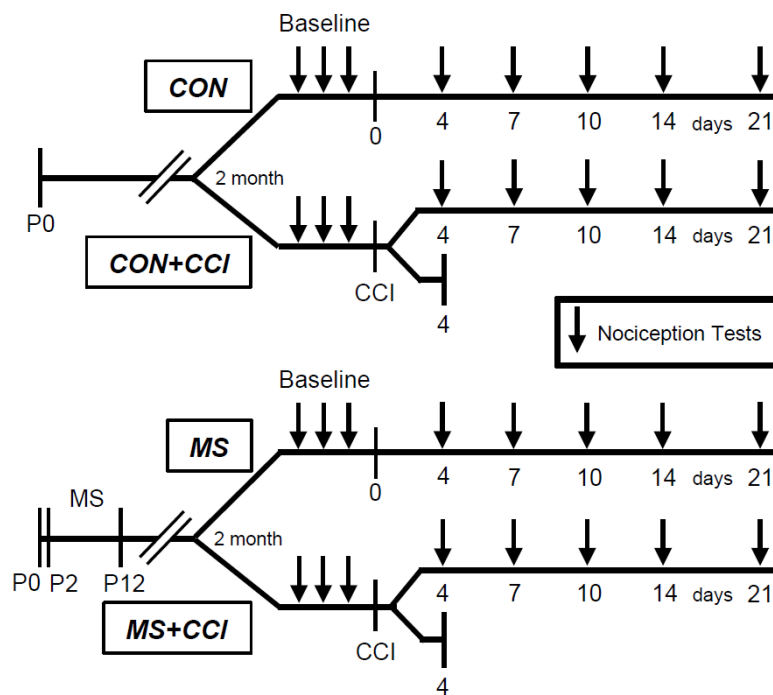


Figure 12: Experimental timeline. After birth (P0), rats were separated into 2 experimental groups. The first one was left undisturbed while the second underwent the maternal separation (MS) procedure from postnatal day 2 (P2) to P12. At two months of age, the animals in each of the two groups were either assigned to a non-operated group (CON resp. MS) or to a group that underwent chronic constriction injury (CON+CCI resp.

*MS+CCI). All four groups were then tested for noxious mechanical and cold sensitivity three days in a row to assess baseline values. At day 0, CCI was performed in the respective groups (CON+CCI and MS+CCI) and further behavioral tests were performed in all of the animals on days 4, 7, 10, 14 and 21. After the last testing, animals were sacrificed and the L4-L5 segments of the spinal cord were removed for qPCR analysis of biochemical markers. Two additional sets of animals in the CON+CCI and MS+CCI groups were included to study changes in the expression of biochemical markers early after CCI surgery. They did not undergo behavioral tests and were sacrificed 4 days post-CCI.*

## 5.4. Results

### 5.4.1. Effect of MS and CCI surgery on pain-related behavior

#### 5.4.1.1 Mechanical pain sensitivity

Baseline mechanical thresholds were assessed in all four groups before performing CCI-surgery in the respective groups at d0 (Fig. 13). No significant differences could be found between groups (CON: $103.27 \pm 5.65$; CON+CCI: $94.68 \pm 4.20$; MS: $99.29 \pm 5.30$; MS+CCI: $96.61 \pm 4.00$), indicating that MS per se had no effect on mechanical pain threshold. This was true for the entire period of testing in which the MS group did not present any significant differences as compared to CON (d4: CON $101.94 \pm 5.08$, MS $101.18 \pm 5.46$; d7: CON $104.44 \pm 6.34$, MS $108.46 \pm 4.91$; d10: CON $100.15 \pm 6.61$, MS $96.23 \pm 3.83$; d14: CON $94.48 \pm 6.04$, MS $99.96 \pm 2.66$; d21: CON $105.41 \pm 5.98$, MS $111.22 \pm 8.32$).

As expected, CCI-surgery resulted in a decrease of mechanical thresholds in CON+CCI and MS+CCI groups. In CON+CCI the threshold constantly dropped until d14 and then started to recover on d21 (d4: $71.12 \pm 10.22$; d7: $46.94 \pm 7.83$; d10: $41.85 \pm 5.82$; d14: $24.32 \pm 2.05$; d21: $42.14 \pm 6.47$). When compared to CON this decrease turned out to be significantly different at d4 ($p<0.01$) and highly significant from d7 on ($p<0.001$). The reduction of mechanical thresholds observed in MS+CCI animals did not display a parallel time course. Pain thresholds slowly but constantly decreased until the end of the experiment at d21 without showing recovery (d4: $93.98 \pm 9.16$; d7: $77.18 \pm 5.44$; d10: $78.21$

± 7.50; d14: 59.51 ± 9.95; d21: 43.86 ± 7.04). When comparing to the group only exposed to MS, significant differences were found for all time points (d4: p<0.05, d7: p<0.01, d14: p<0.001, d21: p<0.001).

Animals with a history of maternal separation (MS+CCI) exhibited a clearly higher mechanical threshold after induction of neuropathic pain than rats that were not subjected to this early life stress (CON+CCI). Shortly after CCI surgery at d4, a significant difference (p<0.05) between the two groups could already be seen. From d7 to d14, the difference between groups was pronounced (d7: p<0.01; d10 and d14: p<0.001). At d21 the mechanical thresholds of CON+CCI and MS+CCI groups became comparable again.

Taken together, these results indicate that MS protracts the appearance of mechanical hyperalgesia associated with neuropathy.
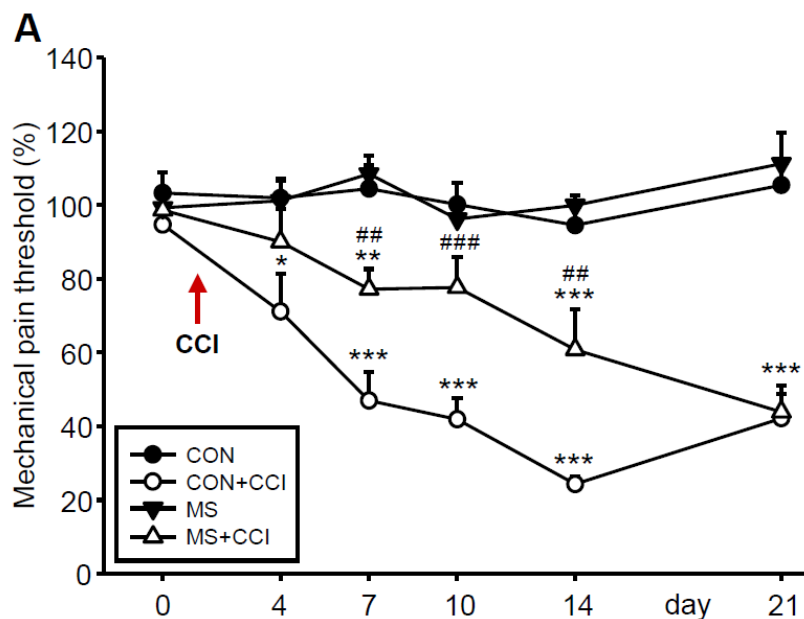


*Figure 13: Maternal separation stress reduces CCI-induced mechanical and cold hypersensitivity. Mechanical pain thresholds were measured by the Von Frey test before and during the 21 days of neuropathy and expressed as percent ratio of ipsi- to contralateral side. Before induction of CCI, all four groups presented similar mechanical thresholds. The control (CON, black circles) and the maternally separated group (MS, black triangles) were not subjected to CCI; their mechanical threshold was unchanged in the course of the experiment. In the control group that underwent CCI surgery (CON+CCI, white circles) the mechanical pain threshold decreased until day 14 and started to recover at day 21. The maternally separated group that was*

*subjected to CCI (MS+CCI, white triangles) reacted with a steady decrease of pain thresholds until the end of the experiment. MS+CCI animals were less sensitive than the CON+CCI group for up to 14 days.*

### 5.4.1.2. Thermal pain sensitivity

As described for the assessment of baseline mechanical thresholds, the thermal sensitivity was measured in the four experimental groups prior to CCI on d0 (Fig. 14). No significant differences in the number of paw lifts were observed between the groups (CON: $0.03 \pm 0.02$, CON+CCI: 0.00, MS: 0.00, MS+CCI: 0.00). The thermal sensitivity of CON and MS animals remained constantly low throughout the experiment; the two groups did not display any significant differences.

CCI surgery in the CON+CCI and MS+CCI groups resulted in a decrease of nociceptive thermal thresholds. The rise in paw lifts was much more pronounced in the CON+CCI animals and steadily increased throughout the experiment until d21 (d4: $5.82 \pm 0.66$, d7: $5.21 \pm 0.94$, d10: $8.68 \pm 1.64$, d14: $10.77 \pm 1.85$, d21: $11.56 \pm 1.73$). The comparison with its control CON yielded a high statistical significance ($p<0.001$) for all time points. In MS+CCI the number of paw lifts slightly increased after induction of neuropathy (d4: $1.71 \pm 0.64$, d7: $2.14 \pm 0.70$, d10: $2.17 \pm 0.92$) without gaining statistical significance when comparing to the MS group. From d14 on the thermal sensitivity further increased reaching a significant level (d14: $2.53 \pm 0.73$, $p<0.05$; d21: $4.43 \pm 0.74$, $p<0.001$). Rats that underwent early life stress (MS+CCI) developed clearly less thermal hyperalgesia under conditions of neuropathic pain than animals that grow up normally (CON+CCI). This difference could already be seen early after induction of CCI and lasted until the end of the experiment. Statistical tests revealed significances between the two groups of $p<0.01$ for d4, $p<0.05$ for d7 and $p<0.001$ for d10, d14 and d21. Altogether these results suggest that animals that were subjected to MS present reduced and delayed development of thermal hyperalgesia in neuropathic pain states.
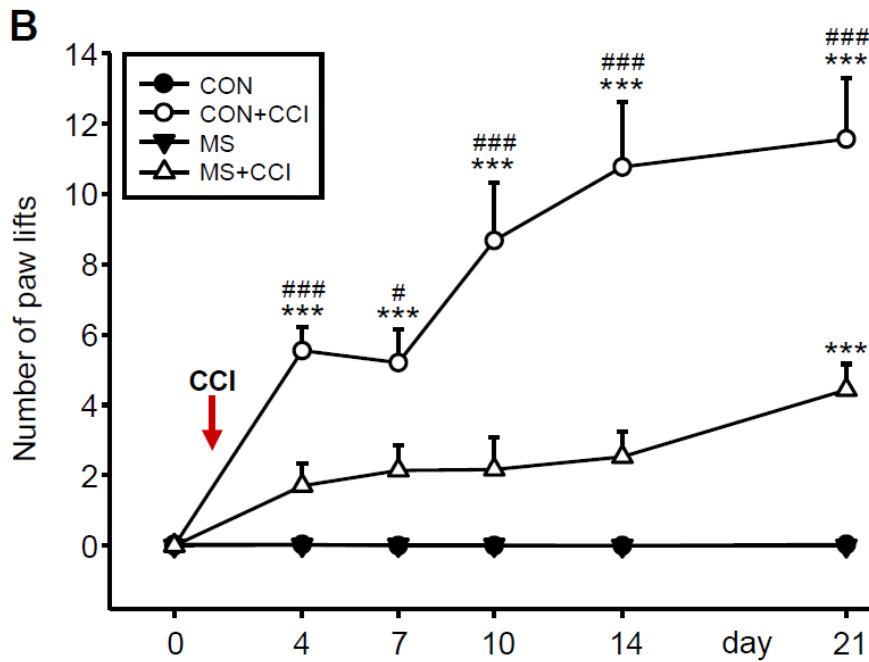
*Figure 14: Maternal separation stress reduces CCI-induced mechanical and cold hypersensitivity. Thermal pain thresholds were evaluated by using the cold plate test. The number of lifts of the ipsilateral (right) paw was recorded before and up to 21 days after induction of CCI. No paw lifts could be observed in the baseline testing of all four groups before CCI surgery. The two groups, CON (black circles) and MS (black triangles), that were not exposed to neuropathic pain remained insensitive to cold stimuli throughout the testing period. Control animals undergoing CCI surgery (CON+CCI, white circles) rapidly developed a pronounced cold allodynia that steadily increased until the end of the experiment at day 21. Rats with a history of early life stress that were exposed to neuropathic pain (MS+CCI, white triangles), exhibited a slight but insignificant increase in cold sensitivity during the first 10 days of the testing period and exhibited significant cold allodynia only in the late phase of CCI at d14 and d21. Data are expressed as mean $\pm$ SEM per group per day. * represents a significant difference between CON and CON+CCI or MS and MS+CCI for the individual time point (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001). # indicates a significant difference between CON+CCI and MS+CCI (#p < 0.05, ##p <0.01, ###p < 0.001).*

## 5.4.2. Impact of early life stress and neuropathic pain on the expression of spinal biochemical markers

### 5.4.2.1. Glucocorticoid receptor regulation

CON and MS presented comparable GR mRNA levels ($1.03 \pm 0.25$ and $1.16 \pm 0.16$ resp.) (Fig. 15A), suggesting that maternal separation stress per se did not

affect the expression of the glucocorticoid receptor. The induction of CCI resulted in a significant downregulation of GR mRNA at d4 in CON+CCI (0.72 ± 0.11; p<0.01 compared to CON) as well as in MS+CCI rats (0.72 ± 0.41; p<0.001 compared to MS). At a later state of neuropathy, on d21, GR mRNA expression returned to normal levels in the CON+CCI group (1.02 ± 0.85; p<0.001 to CON+CCI 4d) and in MS+CCI animals (0.99 ± 0.09; p<0.001 to MS+CCI 4d). The two-way ANOVA revealed significant main effects only for CCI, not for stress or interaction (Table 3).

*Table 3: Analysis of variance (ANOVA):* Summary of F-values of the behavioral (two-way repeated measures) and biochemical (two-way) studies

Two-way repeated measures ANOVA

|  | interaction | time | group |
|---|---|---|---|
| Von Frey test | $F_{(15,225)} = 6.84$ | $F_{(5,225)} = 13.09$ | $F_{(3,45)} = 41.88$ |
| Cold plate test | $F_{(15,225)} = 14.38$ | $F_{(5,225)} = 26.09$ | $F_{(3,45)} = 33.25$ |

p < 0.0001 for all values

Two-way ANOVA

|  | interaction | CCI | stress |
|---|---|---|---|
| GR | $F_{(2,37)} = 1.195$<br>P=0.3141 | $F_{(2,37)} = 26.35$<br>P<0.0001 | $F_{(1,37)} = 0.4873$<br>P=0.4895 |
| NR1 | $F_{(2,36)} = 8.798$<br>P=0.0008 | $F_{(2,36)} = 0.9035$<br>P=0.4145 | $F_{(1,36)} = 13.78$<br>P=0.0007 |
| NR2a | $F_{(2,36)} = 9.486$<br>P=0.0005 | $F_{(2,36)} = 8.985$<br>P=0.0007 | $F_{(1,36)} = 17.45$<br>P=0.0002 |
| NR2b | $F_{(2,37)} = 1.896$<br>P=0.1645 | $F_{(2,37)} = 2.397$<br>P=0.1050 | $F_{(1,37)} = 19.47$<br>P<0.0001 |
| EAAT2 | $F_{(2,36)} = 0.7511$<br>P=0.4791 | $F_{(2,36)} = 20.60$<br>P<0.0001 | $F_{(1,36)} = 5.740$<br>P=0.0219 |
| EAAT3 | $F_{(2,36)} = 11.14$<br>P=0.0002 | $F_{(2,36)} = 8.542$<br>P=0.0009 | $F_{(1,36)} = 44.50$<br>P<0.0001 |
| Iba1 | $F_{(2,36)} = 1.013$<br>P=0.3734 | $F_{(2,36)} = 47.00$<br>P<0.0001 | $F_{(1,36)} = 14.23$<br>P=0.0006 |
| GFAP | $F_{(2,36)} = 2.701$<br>P=0.0807 | $F_{(2,36)} = 6.238$<br>P=0.0047 | $F_{(1,36)} = 6.033$<br>P=0.0190 |
| IL-1β | $F_{(2,36)} = 3.310$<br>P=0.0479 | $F_{(2,36)} = 11.87$<br>P=0.0001 | $F_{(1,36)} = 5.701$<br>P=0.0223 |
| IL-6 | $F_{(2,36)} = 3.259$<br>P=0.0500 | $F_{(2,36)} = 24.10$<br>P<0.0001 | $F_{(1,36)} = 0.9615$<br>P=0.3334 |
| GDNF | $F_{(2,36)} = 4.952$<br>P=0.0126 | $F_{(2,36)} = 1.895$<br>P=0.1651 | $F_{(1,36)} = 6.573$<br>P=0.0147 |
| NGF | $F_{(2,36)} = 23.18$<br>P<0.0001 | $F_{(2,36)} = 0.0808$<br>P=0.9225 | $F_{(1,36)} = 0.02879$<br>P=0.8662 |

### 5.4.2.2. Regulation of glutamate receptors and transporters

In comparison to CON, MS per se significantly increased the mRNA expression of all three examined subunits of the NMDA receptor, NR1 (CON: $1.01 \pm 0.07$, MS: $1.60 \pm 0.06$; $p<0.05$), NR2a (CON: $1.01 \pm 0.09$, MS: $1.65 \pm 0.05$; $p<0.001$) and NR2b (CON: $1.07 \pm 0.15$, MS: $1.54 \pm 0.06$; $p<0.01$) (Fig. 15B, 15C, 15D).

In the initial post-surgical phase, at d4, CCI had no influence on the mRNA levels of NR1 ($1.12 \pm 0.08$), NR2a ($1.08 \pm 0.6$) and NR2b ($0.99 \pm 0.1$) in CON+CCI rats. In contrast, MS+CCI animals reacted with a significant decrease of NR1 ($1.07 \pm 0.11$; $p<0.05$ to MS) and NR2a ($0.94 \pm 0.07$; $p<0.001$ to MS) mRNA levels and a clear tendency to reduced mRNA expression in NR2b ($1.15 \pm 0.17$) at this time point, now being comparable to CON+CCI.

At post-CCI day 21, animals from the CON+CCI group presented a trend to reduced NR1 ($0.78 \pm 0.05$; n.s. to CON, but $p<0.01$ to CON+CCI 4d) and significantly decreased NR2a levels ($0.74 \pm 0.06$; $p<0.05$ to CON), but no significant change in NR2b ($0.86 \pm 0.05$) mRNA expression.

In MS+CCI animals a tendency to recover could be observed on d21. The mRNA levels of all three subunits increased slightly (NR1: $1.24 \pm 0.17$, NR2a: $1.19 \pm 0.21$, NR2b: $1.43 \pm 0.15$), returning nearly to basal values, except for NR2a which was still significant different from MS ($p<0.05$). Nevertheless, for NR1, NR2a and NR2b a significant difference ($p<0.01$, $p<0.05$ and $p<0.01$ resp.) to the CON+CCI group could be assessed. Generally, significant main effects were revealed for stress (NR1, NR2a, NR2b), CCI (NR2a) and interaction (NR1, NR2a) (Table 3).

EAAT2 mRNA levels did not significantly differ between non-injured CON and MS animals (CON: $1.02 \pm 0.1$; MS: $0.93 \pm 0.48$) (Fig 15E). Post-CCI, at d4, CON+CCI and MS+CCI groups presented a similar and significant reduction of EAAT2 mRNA levels when compared to the respective controls (CON+CCI: $0.67 \pm 0.03$, $p<0.001$ to CON; MS+CCI: $0.63 \pm 0.04$, $p<0.01$ to MS). In the later

phase of the neuropathic state, at d21, EAAT2 mRNA levels of CON+CCI returned approximately to basal values (0.89 ± 0.05), thus being no longer significantly different from CON, but remaining different from CON+CCI at d4 (p<0.001). In the same line the EAAT2 mRNA expression in MS+CCI animals slightly increased at d21 (0.73 ± 0.05), leading also to a loss of significant difference to MS.

Regarding EAAT3, early life stress had a clear impact on the mRNA expression (Fig. 15F). The mRNA level increased to 1.38 ± 0.01 in MS and was hence significantly higher than seen in CON (1.00 ± 0.03; p<0.001). Induction of neuropathic pain had no effect on the CON+CCI group at d4 (1.13 ± 0.06), but led to a significant decrease of EAAT3 mRNA levels in the maternally separated animals MS+CCI (1.13 ± 0.06; p<0.01 to MS), that now were similar to CON+CCI. At d21 post-surgery the EAAT3 mRNA level dropped in the CON+CCI group (0.8 ± 0.05; n.s. to CON but p<0.001 to CON+CCI 4d), whereas the level slightly increased in the MS+CCI animals (1.20 ± 0.06), thus being no longer significantly different from MS. The small downregulation in CON+CCI 21d and the minor upregulation in MS+CCI 21d finally resulted in a highly significant difference between these two groups (p<0.001). Significant main effects were obtained for stress (EAAT2, EAAT3), CCI (EAAT2, EAAT3) and interaction (EAAT3) (Table 3).
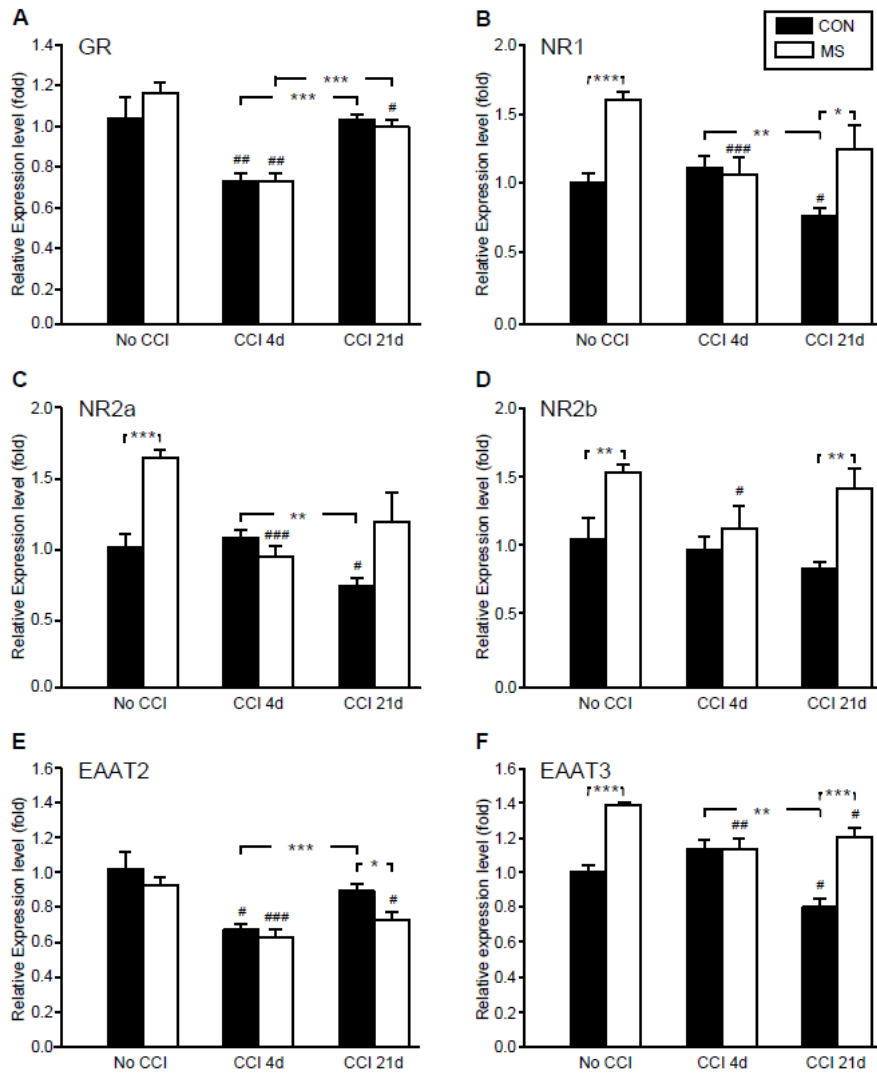
*Figure 15: Regulation of actors of spinal glutamatergic synapse function. Gene expression in the spinal cord was examined for the glucocorticoid receptor (GR) (A), the NMDA receptor subunits NR1 (B), NR2a (C), NR2b (D) and the glutamate transporters EAAT2 (E) and EAAT3 (F). Mean mRNA levels where assessed in control (CON, black bars) and maternally separated animals (MS, white bars) under three different conditions: without CCI surgery (no CCI), CCI lasting four days (CCI 4d) and CCI lasting 21 days (CCI 21d). (A) GR expression did not differ between CON and MS animals in the three conditions. GR mRNA was downregulated at 4 days after induction of CCI and recovered in the late phase at 21 days. (B-D) The three NMDA receptor subunits followed a similar regulation scheme: mRNA upregulation in MS animals per se, downregulation in MS 4 days after CCI but no change in CON, and downregulation in CON after 21 days of CCI when MS started to recover. (E, F) The regulation of mRNA expression was different in the glial EAAT2 and the neuronal EAAT3 transporter. The level of EAAT2 mRNA did not differ between CON and MS under the conditions of no CCI and CCI 4d. Only in the CCI 21d a small difference was obtained. CCI surgery downregulated EAAT2 mRNA at 4d followed by a recovery at 21d at least in CON. The regulation of EAAT3 mRNA resembles the scheme seen for the NMDA subunits: upregulation in MS per se, downregulation in MS 4 days after CCI but no change in CON, and downregulation*

*in CON after 21 days of CCI. Data are expressed as relative expression level (fold) of "CON, no CCI" (=1) and are shown as mean ± SEM. # represents a significant difference between "CON, no CCI" and "CON+CCI 4d" or "CON+CCI 21d" or between "MS, no CCI" and "MS+CCI 4d" or "MS+CCI 21d" (#p < 0.05, ##p < 0.01, ###p < 0.001). * indicates a significant difference between groups for other comparisons than the ones covered by # (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001).*

### 5.4.2.3. Glial cell activation

The expression of Iba1 (ionized calcium-binding adaptor protein-1) mRNA in microglia was affected by early life stress (Fig. 16A). The MS group exhibited a significantly reduced mRNA level ($0.74 \pm 0.01$) as compared to CON ($1.00 \pm 0.05$; $p<0.05$). Shortly after CCI surgery, the Iba1 mRNA expression significantly increased in the CON+CCI ($2.69 \pm 0.08$; $p<0.001$ to CON) as well as in the MS+CCI group ($1.84 \pm 0.14$; $p<0.001$ to MS). The difference between the two groups did stay statistically significant ($p<0.05$), as seen between CON and MS. At the early chronification phase of neuropathic pain (d21), the Iba1 mRNA level decreased in the CON+CCI group ($1.84 \pm 0.03$; $p<0.001$ to CON+CCI 4d) and was still significantly different from the basal level in CON ($p<0.01$). The expression in the MS+CCI group, did however not change as compared to d4 and hence remained significantly different from the non-injured MS ($p<0.01$). At this time point the difference between CON+CCI and MS+CCI became smaller and lost significance. In summary, maternal separation stress led to a decrease of microglia activation under normal conditions (no neuropathy) and to a reduced upregulation of Iba1 mRNA under CCI conditions.

In contrast to the upregulation of the microglia marker Iba1 observed in animals subjected to early life stress, MS per se had no impact on the mRNA expression of the astrocytic marker GFAP (glial fibrillary acidic protein) (CON: $1.03 \pm 0.11$, MS: $1.07 \pm 0.06$) (Fig. 16B). CCI led to a statistically significant upregulation of GFAP mRNA in CON+CCI at d4 post-surgery ($1.43 \pm 0.08$; $p<0.05$ to CON), but not in the MS+CCI group ($1.24 \pm 0.9$). Nevertheless, the difference between the CON-CCI and the MS+CCI groups did not reach statistical significance in the early phase of neuropathy. Later on, at d21, the

GFAP mRNA stayed upregulated in the CON+CCI animals ($1.44 \pm 0.07$; p<0.05 to CON) and the MS+CCI group remained unaffected by the surgery ($1.09 \pm 0.08$). In this state, a significant difference between the two groups could be observed (p<0.05). Taken together, MS prevented the upregulation of GFAP mRNA triggered by CCI. The two-way ANOVA revealed significant main effects for stress and CCI for Iba1 as well as for GFAP (Table 3).

### 5.4.2.4. Regulation of pro-inflammatory cytokines

The IL-1β mRNA expression was slightly, but not significantly reduced in MS per se (CON: $1.02 \pm 0.12$, MS: $0.77 \pm 0.04$) (Fig. 16C). On post-CCI day 4, IL-1β mRNA was clearly upregulated in CON+CCI ($2.69 \pm 0.82$; p<0.01 to CON) but only a tendency to higher levels was observed in MS+CCI animals ($1.15 \pm 0.13$), leading to a significant difference between the two groups (p<0.05). The IL-1β mRNA level started to decrease in CON+CCI animals at d21 after CCI surgery ($1.79 \pm 0.08$; n.s. to CON and n.s. to CON+CCI 4d) but further increased in MS+CCI ($1.83 \pm 0.25$; p<0.01 to MS), resulting in the loss of a significant difference between the CON+CCI and MS+CCI group. The data do hence suggest that the early life stress tended to decrease the expression of IL-1β under normal conditions, and particularly delayed the upregulation seen in neuropathic states. Statistical significant main effects were obtained for stress, CCI and interaction (Table 3).

Regarding the IL-6 mRNA expression, no difference was found between CON ($1.01 \pm 0.9$) and MS ($1.17 \pm 0.09$). Four days after induction of CCI the IL-6 mRNA level increased considerably in CON+CCI ($12.59 \pm 1.44$; p<0.001 to CON) and in MS+CCI ($8.28 \pm 1.50$; p<0.01 to MS). Although the CON+CCI and MS+CCI groups respectively presented a 12 fold and an 8 fold increase of IL-6 mRNA levels, this difference did not turn out to be statistically significant due to high levels of variance in the two groups. In the later phase of the experiment, at d21, the IL-6 mRNA levels decreased significantly for

CON+CCI (4.08 ± 0.59; p<0.001 to CON+CCI 4d) and also dropped in MS+CCI (5.98 ± 0.55) being no longer significantly different from CON resp. MS. The statistical analysis revealed that animals with a history of maternal separation did not display any differences in IL-6 mRNA levels as compared to the respective controls. A significant main effect was only found for CCI Table 3).
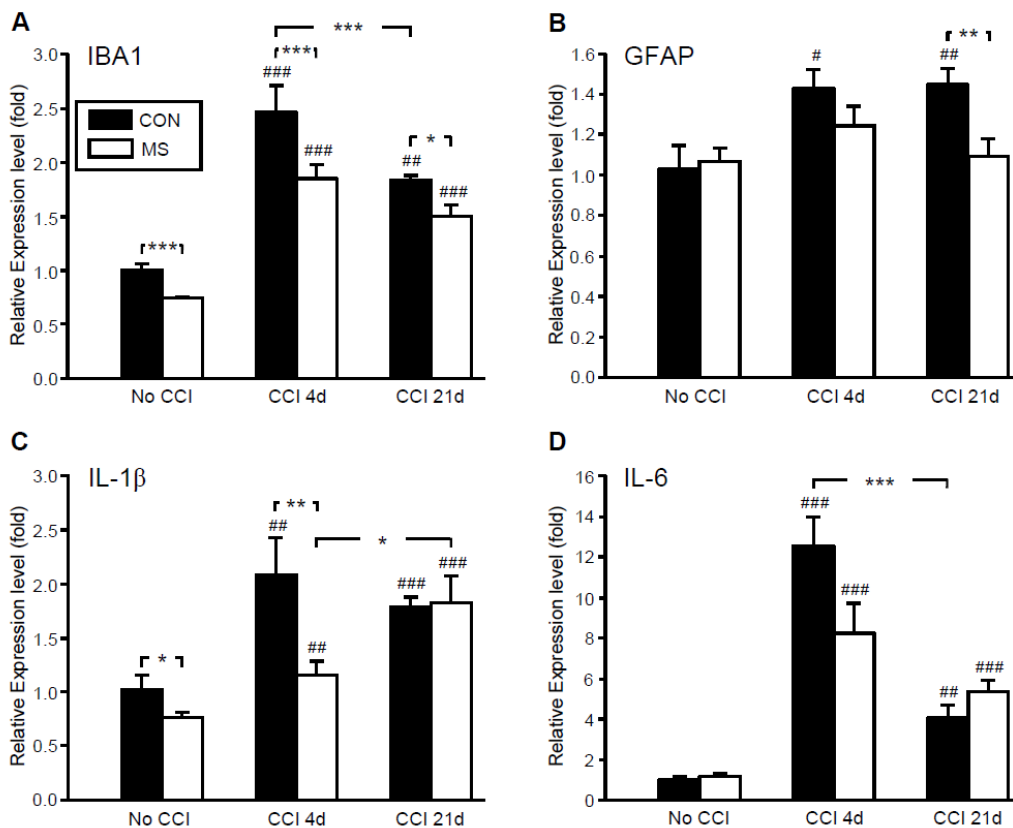


*Figure 16: Regulation of markers of spinal immunocompetent cell activation and of pro-inflammatory cytokines. Gene expression in the spinal cord was examined for the microglial marker Iba1 (A), the astroglial marker GFAP (B) and the pro-inflammatory cytokines IL-1β (C) and IL-6 (D). Mean mRNA levels where assessed in control (CON, black bars) and maternally separated animals (MS, white bars) under three different conditions: without CCI surgery (no CCI), CCI lasting four days (CCI 4d) and CCI lasting 21 days (CCI 21d). (A) Iba1 mRNA levels were downregulated in MS as compared to CON. Induction of CCI upregulated the gene expression at 4d in both groups, the significant difference between groups remained. A beginning recovery could be observed at d21 in CON and a tendency to recover in MS. (B) GFAP mRNA levels did not differ between "CON, no CCI" and "MS, no CCI". Neuropathy surgery upregulated the gene expression only in "CON+CCI 4d and 21d" but not in MS. (C) IL-1β mRNA expression tended to be lower in "MS, no CCI" as compared to "CON, no CCI". After induction of CCI at d4, IL-1β was quickly upregulated in CON and started to increase in MS..*

*Later, at day 21, the IL-1β mRNA level already decreased in CON whereas it further increased in MS. (D) IL-6 mRNA levels did not significantly differ between CON and MS in all three conditions. The gene expression was highly upregulated in CON and MS 4 days after CCI surgery. Under long-term neuropathy conditions, mRNA levels decreased coming close to normal expression rates. Data are expressed as relative expression level (fold) of "CON, no CCI" (=1) and are shown as mean ± SEM. # represents a significant difference between "CON, no CCI" and "CON+CCI 4d" or "CON+CCI 21d" or between "MS, no CCI" and "MS+CCI 4d" or "MS+CCI 21d" (#p < 0.05, ##p < 0.01, ###p < 0.001). \* indicates a significant difference between groups for other comparisons than the ones covered by # (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001).*

### 5.4.2.5. Expression of neurotrophins

MS per se did not modify GDNF mRNA levels (CON: $1.01 \pm 0.07$, MS $1.15 \pm 0.11$) (Fig 17A). The induction of neuropathic pain had no significant influence on the expression of GDNF mRNA in CON+CCI animals ($0.94 \pm 0.10$) early after surgery at d4, however a reduction could be observed in MS+CCI ($0.88 \pm 0.04$; $p<0.05$ to MS). After 21 days of injury, a significant decrease of the GDNF mRNA level took place in the CON+CCI group ($0.79 \pm 0.03$; $p<0.05$ to CON), whereas animals with a history of early life stress reacted with an increase of this neurotrophin mRNA ($1.20 \pm 0.10$; $p<0.01$ to MS+CCI 4d) at this time point, ending up with an mRNA level numerically above but not significantly different to MS. However, in this late phase of neuropathy, a significant difference was obtained when comparing CON+CCI and MS+CCI ($p<0.01$). Statistically significant main effects were obtained for stress and interaction (Table 3).

The baseline NGF gene expression was comparable between the CON ($1.15 \pm 0.62$) and MS ($1.01 \pm 0.08$) group (Fig. 17B). Four days following CCI surgery, NGF mRNA levels increased in CON+CCI ($1.36 \pm 0.06$; $p<0.05$ to CON) and decreased in MS+CCI ($0.79 \pm 0.07$, $p<0.05$ to MS) resulting in a highly significant difference between the two groups ($p<0.001$). After 21 days, both groups roughly returned to control levels (CON+CCI: $0.85 \pm 0.07$; $p<0.001$ to CON+CCI 4d; MS+CCI: $1.26 \pm 0.10$, $p<0.01$ to MS+CCI 4d) but remained significantly different from each other ($p<0.01$). A significant main effect was obtained for interaction (Table 3).
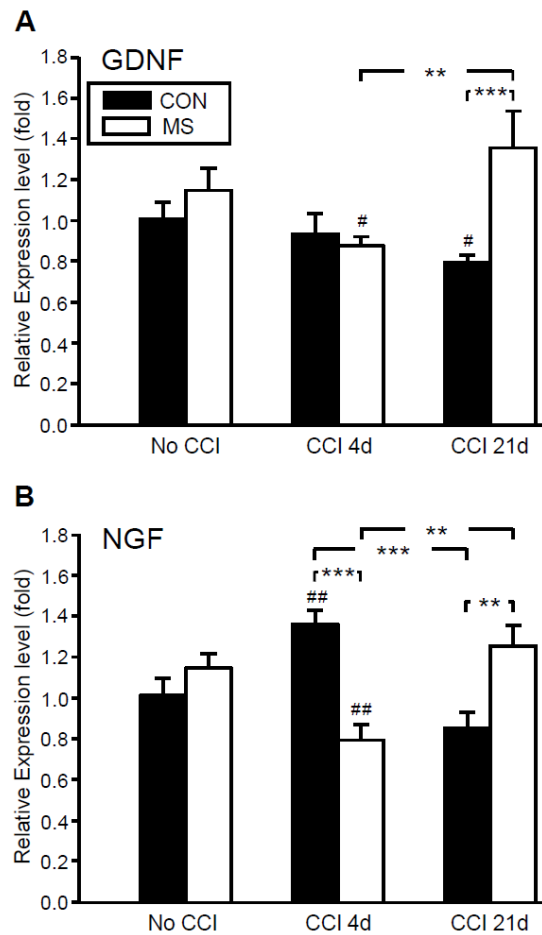
*Figure 17: Regulation of spinal neurotrophins. Spinal gene expression was examined for the neurotrophins GDNF (A) and NGF (B). Mean mRNA levels where assessed in control (CON, black bars) and maternally separated animals (MS, white bars) under three different conditions: without CCI surgery (no CCI), CCI lasting four days (CCI 4d) and CCI lasting 21 days (CCI 21d). (A) GDNF mRNA expression did not differ between CON and MS under the conditions of no CCI and CCI 4d. In the long-term CCI condition, GDNF mRNA level differed between CON and MS, due to an upregulation in MS animals. (B) NGF mRNA levels were not different in "CON, no CCI" and "MS, no CCI" animals. The induction of CCI triggered an increase in CON and a decrease in MS 4 days post-surgery. At d21, a recovery occurred, which partly overshoot and consequently resulted in a difference in mRNA expression between CON and MS. Data are expressed as relative expression level (fold) of "CON, no CCI" (=1) and are shown as mean ±SEM. # represents a significant difference between "CON, no CCI" and "CON+CCI 4d" or "CON+CCI 21d" or between "MS, no CCI" and "MS+CCI 4d" or "MS+CCI 21d" (#p < 0.05, ##p < 0.01). * indicates a significant difference between groups for other comparisons than the ones covered by # (\*\*p < 0.01, \*\*\*p < 0.001).*

## 5.5. Discussion

The present study shows that early life stress related to MS in rodents does not affect basal thermal and mechanical nociceptive thresholds per se but has a protective effect on both modalities under neuropathic conditions. This behavioral change was accompanied by molecular alterations in the spinal cord. To our knowledge the impact of MS on mediators involved in the spinal processing of neuropathic pain has not been studied so far.

### 5.5.1. MS reduces CCI-induced hyperalgesia/allodynia

In humans, adverse early life events are associated with enhanced risks of developing mental or physical health problems including chronic pain disorders later in life (Heim et al., 2010; Heim and Binder, 2012; Lupien et al., 2009). This has been shown to be related to a permanent alteration of the hypothalamo-pituitary-adrenal (HPA) axis (Sapolsky and Meaney, 1986), as well as of neural (Lippmann et al., 2007; Mintz et al., 2005; Rana et al., 2014) and immune functioning (Bilbo and Schwarz, 2009; Carpenter et al., 2010; Wieck et al., 2013). In rodents, normal development during the critically vulnerable stress hyporesponsive period (SHRP) in the first two post-natal weeks essentially depends on maternal care (Caldji et al., 1998). Disruption by prolonged and or repeated sequences of MS leads to alterations comparable to those observed in humans (Schmidt, 2010). Furthermore, the first week of the rodent's life corresponds developmentally to the third trimester of human gestation (Lupien et al., 2009) making MS an adequate model for the stress premature infants are exposed to.

In rats, early life stress has been associated with an attenuation of drug-induced analgesic effects (Dickinson et al., 2009; Kalinichev et al., 2001), increased pain sensitivity in normal conditions (Alvarez et al., 2013; Green et al., 2011) but also in the framework of several experimental and clinical pain states such as chronic bowel syndrome (O'Mahony et al., 2009), visceral hyperalgesia (Chung

et al., 2007; Tsang et al., 2012) and neuropathic pain (Zeng et al., 2008). While rats undergoing early life stress have mostly been reported to present increased nociceptive responses to mechanical and/or thermal nociceptive stimulation (Chung et al., 2007; Tsang et al., 2012; Uhelski and Fuchs, 2010), we did not observe any change in basal nociceptive thresholds, but a reduced CCI-induced hypersensitivity for both modalities. It should be noted here that MS does not in every case lead to the development of psychiatric diseases (Rana et al., 2014) or enhanced pain sensitivity (Weaver et al., 2007) later in life. Resilience, an adaptive process allowing physiological (homeostatic) and behavioral adaptation to stress, has also been observed in rodent models of early life stress such as MS (Macrì et al., 2011). Pfau et Russo (2015) propose that the resilience observed in rodents undergoing early life stress follows a U-shaped curve. Exposure to a medium amount of stress could hence lead to resilience while very low or very high levels would lead to vulnerability. It should be noted here that the term "resilience" is generally considered to represent a positive adaptation of stress processing to a context specific stressor while "vulnerability" is commonly used to describe stress-related alterations in susceptibility to health disorders like chronic pain syndromes later in life (Alvarez et al., 2013). The fact that early life stress leads to alterations of brain structures is involved both in stress and in pain processing (Prusator & Greenwood-Van Meerveld, 2016) may impede a clear differentiation between these two terms. Hence, we suggest that MS could lead to a form of resilience to CCI-induced hypersensitivity independently to its effects on vulnerability or resilience to further stress processing.

Furthermore, differences in methodology across studies such as rearing of the dams could lead to differences in experimental outcomes. Transportation from the animal supplier to the client is a routine practice but the stress induced by the shipping is rarely considered. Despite the few studies investigating the implications of the shipping in stress paradigms, increased blood corticosterone

levels, decrease of social behavior and locomotor activity up to 16 days after transportation have been observed (Arts et al., 2012). These results suggest that shipping can have long lasting physiological and behavioral effects. This would especially be true for developmental and high plasticity periods such as gestation (Lupien et al., 2009; Weinstock, 2008). Nevertheless, several studies showing an increased sensitivity following early life stress were performed in the offspring of females that were pregnant at the time point of shipment (Alvarez et al., 2013; Chung et al., 2007; Green et al., 2011; Kalinichev et al., 2001; Nishinaka et al., 2016; Tsang et al., 2012). The animals to be tested could hence have accumulated pre- and post-natal stress. Variations in experimental outcomes could thus at least partly be related to our rearing the dams in our facility.

### 5.5.2. MS alters spinal glutamatergic transmission and transport

The glucocorticoid receptor (GR) is a key regulator of the amplitude of the HPA response to stressors (Strüber et al., 2014) and of neuropathy-induced plastic changes (Wang et al., 2004, 2005, 2006). In the present study we observed that spinal GR expression was not different between CON and MS groups, neither under basal conditions, nor shortly (4d) and later (21d) after the CCI surgery. There is a paucity of studies investigating the impact of circulating corticosterone on spinal GR expression and to our knowledge no study has explored the impact of chronic stress on spinal GR expression. However, Patacchioli and colleagues (1998) showed no effect of 21 days of corticosterone treatment on spinal GR mRNA. Therefore, it is difficult to establish a parallel between our spinal GR mRNA results and a hypothetical HPA axis alteration by MS. In our hands only CCI had an impact on GR mRNA expression levels. Indeed, CON and MS groups presented a decrease of GR expression 4 days after CCI and recovered after 21 days. CCI-induced reduction of GR expression was

not expected since Wang and collaborators (2004, 2005, 2006) reported that CCI induced a time-dependent increase of GR protein and mRNA expression parallel to the development of painful behaviors. The pattern of expression found in CON and MS animals following CCI could be due to injury-related pain and distress, where the resulting hypothetical increase of corticosteroid concentration might have been compensated by a decrease of GR expression. Subsequently, as the animals recovered from the injury, the GR mRNA expression returned to levels comparable to those of non-injured rats. The finding that only MS rats displayed a strong increase of neuropathy-related pain thresholds while both CON and MS animals presented similar reduction of GR expression during the CCI phase may point to an alternative CORT-dependent mechanism of pain inhibition potentially emerging in the MS animals. In these rats, the production of spinal neurosteroids might have enhanced GABAergic and hence inhibitory transmission (Zell et al., 2015).

Since corticosterone may regulate glutamate receptors (Wang et al., 2005) and transporters (Wang et al., 2006) through GR activation under neuropathic pain conditions we investigated NMDA receptor and transporter subunit mRNA expression levels.

Experimental neuropathic pain models resulted in enhanced NR1 and NR2 expression levels ipsilateral to injury (Abe et al., 2005; Wang et al., 2005). We report a differential effect of CCI on animals that underwent MS as compared to CON. Although these results are not directly indicative of spinal nociceptive transmission, the functional properties of the NMDA receptor depend on patterns of subunit associations reflecting enduring plastic changes in synaptic efficacy. The association of NR1 and NR2a subunits does e.g. provide a higher opening probability, a reduced sensitivity to glutamate and a faster decay of excitatory postsynaptic current (EPSC) than the NR1/NR2b NMDA subunit association (Paoletti et al., 2013). This association has been proposed to be, at least to some extent, responsible for the central sensitization involved in the

development of neuropathic pain (Wilson et al., 2005; Wu and Zhuo, 2009). In adult male Wistar rats, a decay of NR1 protein has previously been reported by Wilson and colleagues (2005) 16 days after CCI. In that study, the authors did not report a decrease of NR2a but we cannot exclude the possibility that the decrease we observed at post-CCI day 21 occurred after the 16th day post-CCI. Whereas several studies suggest a primordial role of NR2b in the establishment and maintenance of neuropathic pain (Karlsson et al., 2002; Wilson et al., 2005; Wu and Zhuo, 2009) we did not observe any drastic change in the NR2a/NR2b ratio following CCI. MS animals presented a general augmentation of all three NMDA receptor subunit mRNA levels that would suggest an enhanced nociceptive transmission and a resulting decrease of pain thresholds. Shortly after the CCI surgery, NR1 and NR2a subunit mRNA expression seemed to decrease to a larger extent than NR2b mRNA. This would suggest a change of NMDA subunit proportions toward more NR1/NR2b association in MS animals. Furthermore, 21 days after CCI NR1 and NR2b subunits expression recovered to MS basal levels. The observation that MS+CCI rats seemed to display a greater NR1/NR2b subunit association than CON+CCI might indicate a mechanism underlying delayed development of neuropathic allodynia/hyperalgesia in MS animals when compared to CON.

Neuronal (EAAT3) and glial (EAAT2) glutamate transporters are involved in nociceptive processing in healthy individuals since injection of blockers elicits a dose-dependent spontaneous nociceptive behavior (Liaw et al., 2005), but also in neuropathic animals since their protein and mRNA expression are modified following CCI (Sung et al., 2003; Wang et al., 2006). In the present study, MS animals presented higher mRNA levels of EAAT3, but not EAAT2, suggesting an increased glutamate re-uptake in MS and therefore a reduced spinal transmission of noxious information. In association with the increase of NMDA receptor subunit expression, this result could explain the lack of difference in nociceptive thresholds between MS and CON animals. In CON, CCI tended to

decrease the EAAT3 mRNA expression 21 days after surgery. A reduction of EAAT3 expression was also reported by Sung and colleagues (2003) who proposed that this was due to a loss of primary afferents resulting from the CCI, considering that most of glutamate transporters are located at presynaptic sites (Danbolt, 2001).

Concerning EAAT2, we and other investigators observed a decreased expression at 4 but not 21 days after CCI in CON animals (Napier et al., 2012; Xin et al., 2009). It is probable that this recovery was due to a compensatory mechanism aimed at re-establishing a "normal" re-uptake of glutamate by glial cells. Furthermore, since glial glutamate transporters are believed to account for 90 % of glutamate clearance in the CNS (Danbolt, 2001), this result could be in line with the initiation of a possible recovery of a normal sensitivity observed at post-CCI day 21 in CON animals. In agreement with Gosselin and colleagues (2010), MS did not impact the EAAT2 expression in non-injured conditions. Likewise EAAT2 mRNA expression followed a comparable pattern in MS and CON animals 4 and 21 days after CCI. This lack of group differences suggests that the EAAT2 glutamate transporter does not considerably contribute to CCI-related alterations in mechanical and thermal hypersensitivity as seen between CON and MS in our behavioral experiments.

### 5.5.3. Glial activation is depressed in animals with early life stress history

Nerve injury is associated with a strong activation of glial cells (Mika et al., 2013). This activation results in the release of pro-inflammatory cytokines and growth factors involved in the establishment of central sensitization and in the development of neuropathic pain (Marchand et al., 2005). In this context microglia and astrocytes do not seem to be engaged in a similar manner. Iba1 and GFAP were respectively chosen as markers of microglia and astrocytes because they are both constitutively expressed and their expression is increased

upon activation (Ito et al., 1998; McMahon et al., 2005), allowing us to evaluate basal and MS- or CCI-related levels. The pronounced and immediate microglia activation after injury (Tanga et al., 2004) is believed to play a more prominent role in the establishment of mechanical hypersensitivity (Raghavendra et al., 2004). Our results showing a more pronounced Iba1 mRNA expression 4 days after neuropathy followed by a recovery to lower levels at 21 days are in agreement with previous studies (Mika et al., 2010). However, since there is a delay between the behavioral establishment of a significant mechanical hyperalgesia and allodynia and the qPCR-related findings, it seems plausible that we could have missed the peak of microglia activation. Furthermore, variability can occur due to differences in neuropathy models. Indeed, it has been shown that the activation of immunocompetent cells may partly depend on the location and type of the nerve lesion (Colburn et al., 1999; Hu et al., 2007). MS animals presented lower microglia activation than CON animals during normal and neuropathic conditions. Despite an increase of Iba1 mRNA levels following CCI, microglial activity seemed dampened in MS animals. This is consistent with the higher mechanical threshold observed from day 7 to 14 after CCI. The lack of difference in mechanical thresholds between CON+CCI and MS+CCI animals 21 days after surgery could be another indication that microglia was predominantly involved in the establishment of mechanical hypersensitivity but not in its maintenance. Astrocytic activation is weaker but long lasting (Mika et al., 2013). Maintenance of neuropathic states may depend on this sustained activation. Our result is consistent with the existing literature since the CON+CCI group presented an early but mild GFAP mRNA level increase which was sustained until post-CCI day 21. It is interesting to note that the astrocytic activation was also dampened in the MS+CCI group during neuropathic pain.

Glial activation plays a capital role in neuropathic pain through the release of pro-inflammatory cytokines (Ledeboer et al., 2005) and neurotrophins (Li et al.,

2003). We focused on the interleukins 1-beta (IL-1β) and 6 (IL-6) due to their well-documented involvement in neuropathic states. Upon nerve injury, IL-1β is released concomitantly with the induction of allodynia and hyperalgesia through its actions on NMDA and GABA currents (Kawasaki et al., 2008; Wolf et al., 2006). IL-1β also induces the secretion of other inflammatory mediators such as itself, IL-6, TNF-α or NGF therefore contributing to neuropathic pain (Marchand et al., 2005).

CCI alone induced a clear increase of IL-1β mRNA levels 4 days after the surgery and started to recover at 21 days. On the contrary, in MS the onset and extent of this upregulation was delayed. IL-6 on the other hand presented a similar expression pattern in CON and MS 4 and 21 days after induction of neuropathic pain.

It is interesting to note that, in concordance with the Iba1 and GFAP expression results, MS had an overall dampening effect on the regulation of pro-inflammatory cytokines in the early phase of neuropathy, but not later at the beginning state of chronification. This could be of importance because IL-1β and IL-6 increase are both associated with the development of abnormal sensitivity during neuropathic pain due to their capacity to enhance not only NMDA receptor currents but also AMPA receptor-mediated post-synaptic potentials (Liu et al., 2013; Schäfers and Sorkin, 2008). The MS-related dampened immunocompetent reactivity observed in our hands could explain the enhanced thermal and mechanical thresholds of these animals seen during the early neuropathic pain states. Although a longer observation period of neuropathy may be required, these results seem to indicate that MS delays or reduces the appearance of neuropathy-related pain hypersensitivity.

### 5.5.4. MS affects CCI-induced alterations in growth factor mRNA expression

Neurotrophins such as NGF are additional pro-algesic mediators released in the spinal cord upon nerve injury (Li et al., 2003). The increase of NGF levels shortly after CCI surgery observed in this study could result from glial activation-related secretion of IL-1β (Spranger et al., 1990). This would be in line with the decreased thermal threshold observed shortly after the CCI surgery in CON animals since NGF is known to be retrogradely transported to the periphery resulting in a sensitization of nociceptors (Ji et al., 2002). In addition, TRPM8 a cation channel able to sense both innocuous and noxious cold, notably after nerve injury (Xing et al., 2007), has been shown to be upregulated in a NGF-dependent manner (Babes et al., 2004). In the present study, NGF mRNA expression was decreased in MS animals shortly after CCI potentially resulting in a higher cold threshold as compared to controls. The return to basal mRNA levels at day 21 post-CCI may consequently have lowered the threshold to cold stimuli. However, the increase of NGF mRNA levels cannot solely explain the course of cold hyperalgesia/allodynia since CON+CCI animals presented a decrease of NGF mRNA levels at 21 days post CCI while remaining highly sensitive.

The implication of GDNF in neuropathic pain is controversial due to its potent analgesic effect following intrathecal administration (Boucher et al., 2000) while it can also sensitize nociceptors leading to mechanical hyperalgesia (Bogen et al., 2008). The slight decrease in MS animals and the lack of change in CON animals 4 days after surgery suggest that GDNF did not participate to a great extent to the establishment of neuropathic pain. However, given the mechanical threshold of both groups 21 days after surgery, we hypothesize that GDNF might have displayed a pro-algesic effect. Indeed, the decrease of GDNF mRNA expression in the CON group during neuropathy is concomitant with a reduction of mechanical allodynia/hyperalgesia and the increase of GDNF

mRNA in MS animals would agree with the ongoing decrease in abnormal mechanical thresholds.

## 5.6. Conclusion

Our study surprisingly shows that MS protects from an increase or at least delays the occurrence of neuropathy-related pain hypersensitivity. The assessed biochemical markers do not fully reveal the biochemical cascades involved in the MS-related modification of pain thresholds under neuropathic conditions. Considering that most nociceptive primary afferents project to the dorsal horn of the spinal cord (Basbaum et al., 2009), it is conceivable that an analysis of biochemical markers restricted to the dorsal quadrant would yield stronger effects after CCI than one that includes dorsal and ventral quadrants as was done here. Further studies are required to confirm these results and to eventually elucidate the mechanisms involved in the described stress-related resilience to neuropathic pain. In this framework it will be important to extend the biochemical investigations to the respective protein levels. Importantly, MS has been shown to have differential effects on males and females. Moreover the additionally observed sexual dimorphism in the framework of neuropathic pain has recently been shown to be mediated by sex-based differences in immune responses (Sorge et al., 2015). It thus remains essential to investigate the impact of MS on neuropathic pain as potentially underlying sex-dependent mechanisms. Electrophysiological and pharmacological studies should further add to the elucidation of these relationships.

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# Chapter VI: Impact of early life stress on inflammatory pain

## 6.1. Abstract

Early life stress (ELS) is claimed to constitute a major risk factor for developing psychopathological disorders in later life neurobiological disorders. Since brain structures involved in the processing of stress and of pain are overlapping, exposition to stressors could at least under some circumstances, be expected to enhance the vulnerability for pain syndromes. It is however recognized that not all individuals are impacted in a similar manner and that some could even benefit from stress experiences. In this context we have previously observed that ELS can reduce mechanical and cold hypersensitivity caused by nerve injury during adulthood. The goal of the present study was to test the hypothesis that ELS could be a factor of resilience against an enhancement of noxious sensitivity in the framework of enduring inflammatory pain.

Rats were exposed to the maternal separation (MS) paradigm, an established model of early life stress. At adulthood, corticosterone levels and anxiety-related behaviors were evaluated. Subsequently noxious mechanical and heat sensitivity as well as paw edema were measured prior and during an inflammation of the hind paw induced by the injection of Complete Freunds Adjuvant (CFA). Four groups of animals were thus compared: CON (no manipulations), MS (undergoing maternal separation stress), CON+CFA (no stress but CFA injection) and MS+CFA (undergoing maternal separation and CFA injection).

In the open field test no stress related behavior was observed in MS animals. Corticosterone levels were also not changed by the early life manipulation. Concerning pain sensitivity, results partly confirm our previous neuropathy-related results. Regardless of the group, animals showed identical mechanical thresholds prior to inflammation but after CFA-injection MS tended to display reduced mechanical hyperalgesia. Results on noxious heat sensitivity were more complex. Indeed, MS animals presented shorter latencies of nocifensive behaviors compared to CON. In CON but not in MS animals, the repetitions of noxious heat sensory testing led to a decrease of the latencies of pain-related

behavior. Finally, when normalized to their non-inflamed conspecifics (CON+CFA to CON and MS+CFA to MS), MS+CFA had a tendency to display less hyperalgesia than CON+CFA animals.

Altogether this study tends to confirm that long lasting stress-related alterations of neurobiological circuits could have a positive impact on chronic pain-related hypersensitivity.

**Neonatal maternal separation stress leads to a dampening of inflammation-related mechanical and thermal hypersensitivity in juvenile rats.**

Julien Genty*, Milène Tetsi Nomigni, Fernand Anton, Ulrike Hanesch

Research group Stress, Pain and Pain Modulation,

Institute for Health and Behavior,

University of Luxembourg,

162a, avenue de la Faïencerie,

L-1511 Luxembourg,

Luxembourg

*Corresponding author:*

Julien Genty

162a, avenue de la Faïencerie,

L-1511 Luxembourg,

Luxembourg

Phone:   +352 466644 6340

julien.genty@uni.lu

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**HIGHLIGHTS:**

1)   Maternal separation (MS) did not lead to a change of anxiety-related behavior and corticosterone levels.

2) Under baseline conditions MS rats displayed a more pronounced noxious heat sensitivity

3) While hot plate latencies decreased over time in control animals, they remained constant in MS

4)   MS attenuated the development of inflammatory pain-related hypersensitivity

## 6.2. Introduction

While chronic stress has been recognized to be one of the major causes for developing anxio-depressive and metabolic disorders, not everyone shows comparable levels of vulnerability [1–3]. In this context, the hypothalamo-pituitary-adrenal axis (HPA) and its final effector cortisol in humans or corticosterone (CORT) in rats, serve as major actors for the organisms' adaptability to environmental challenges [4]. In this respect the perinatal period, particularly the two weeks after birth, has been shown to be very sensitive since the HPA axis is still immature. A stress at this moment of life such as abuse or neglect can have a tremendous impact for later life, notably through long-term programming of the HPA axis [5,6]. Early life stress (ELS) does not only lead to alterations of the HPA axis, it does also perturb the maturation of the immune system [7] and the expression of the associated hippocampal pro-inflammatory cytokines and peripheral CD8+ T cells, two mediators of the immune defense [8,9]. A well-established animal model, the maternal separation (MS) paradigm has commonly been used to investigate the relationship between early life stress and the potential predisposition to psychopathology [10]. The studies have partly confirmed a causal relationship of early adverse experiences and the development and/or worsening of pathologies such as chronic inflammatory diseases in later life [11,12].

The capacity of stress to modulate pain sensitivity has been established over 30 years ago, for instance in the framework of stress-induced analgesia (SIA). More recent findings suggested that stress may under some conditions also lead to enhanced pain sensitivity (stress-induced hyperalgesia, SIH, for review see [13]). In this context, brain structures as well as mediators simultaneously involved in pain and stress processing have been identified [14,15]. Furthermore stress-related psychopathology is commonly accompanied by comorbid chronic pain disorders [16,17]. Since CORT has strong anti-inflammatory effects, differential susceptibility to chronic pain conditions involving to a great extent neuro-inflammatory mechanisms, is thought to be

largely mediated by differences in HPA axis reactivity [18]. Nevertheless, there is a paucity of studies on the long-term consequences of ELS on spinal nociceptive processing in later life. This is surprising since the spinal dorsal horn constitutes the CNS input station that transmits nociceptive information to supra-spinal brain structures and thus is a crucial site for pain modulation [19]. Previous findings from our group suggested that repeated MS carried out during the first two postnatal weeks may lead to the decrease of neuropathy-related pain hypersensitivity [20]. Considering the crucial role of immunocompetent cells in the induction of chronic pain disorders [21], we also hypothesized that this result could depend on MS effects on the immune system.

The goal of the present study was to determine whether early life stress-related decrease of pain sensitivity is specific for neuropathy or can also be observed in the framework of inflammatory pain. For this purpose we assessed mechanical and thermal pain sensitivity prior to and after induction of inflammation via intraplantar injection of Complete Freunds Adjuvant (CFA). We also aimed to evaluate the impact of our MS protocol on HPA axis activity and on anxiety-related behavior.

## 6.3. Material and methods:

### 6.3.1. Animals

Male and nulliparous female Sprague-Dawley rats were purchased from Harlan laboratories (Netherland) and reared in our facility. Their breeding provided the subjects for the following experiments. The animals were kept under standardized housing conditions: room temperature $22 \pm 1°C$, relative humidity of $60 \pm 10$ %, 12h dark/light cycle (07:00-19:00) and with food and water provided ad libitum. Male rats used throughout the experiments were kept pairwise in 42x26x20 cm Plexiglas cages from weaning to the end of the experimental protocol.

Animals were divided into four groups (n=10 per group) according to the stress and pain conditions: control group CON (underwent no manipulations), CON+CFA (no MS, CFA injection), MS (MS, no CFA injection), MS+CFA (MS and CFA injection).

All animal manipulations were carried out in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and met the ARRIVE guidelines. Experimental procedures involving animals were approved by the Animal Experimentation Ethics Committee (AEEC) of the University of Luxembourg (Project ID 15-SPM-01-UH) and the "Ministère de l'Agriculture, de la Viticulture et de la Protection des consommateurs".

## 6.3.2. Maternal separation

Gestating females were isolated from cage mates few days prior labor and checked twice per day (morning and the evening) for birth. The day the pups were first observed was determined as post-natal day 0 (P0). At P1 pups were briefly removed, counted and if necessary the litter was reduced to 12 pups per dam. Litters were randomly attributed to either the MS or the CON groups. CON pups were left undisturbed except for cleaning. The MS procedure was carried out from P2 to P12. Pups were removed from the dam's home cage, placed on heated pad at 33°C (+/- 2°C) and left undisturbed for 3 hours/day. After the 10 days of maternal separation (from P13 on), MS pups and dams were treated identically as CON animals. Both MS and CON litters were weaned at P21 and did not undergo other manipulation until habituation.

## 6.3.3. Experimental design

Before the initiation of the behavioral experiments, animals were habituated to the testing room, the testing devices and the experimenter for one week. Procedures depicted below started at 8 weeks of age. Following habituation all

groups underwent blood sampling at 8:00h for determining their plasmatic corticosterone concentration. To limit further stress, animals were returned to their home cage and left undisturbed that day. The next morning, animals were transferred to the open field room and left there for 24h to habituate to the testing room. After terminating the open field test, animals were returned to their home cage and left undisturbed until the next morning. Then all groups were tested for baseline mechanical thresholds and baseline noxious heat sensitivity three days in a row and the paw volume of both hind-paws was assessed. At experimental day 0, inflammatory pain was induced by injection of CFA. Subsequently, mechanical thresholds, thermal sensitivity and paw edema were recorded at post-injection days 4, 7, 10, 14 and 21. Following the last testing, rats were sacrificed.

### 6.3.4. Blood Corticosterone measurements:

Blood sampling was carried out at 8:00h at the beginning of post-natal week 8. Rats were loosely held in a towel and a small incision was made at the distal part of their tail, about 15-20 mm from the tip. Blood was collected using a 300 µL capillary EDTA-coated tube (Microvette CB300, Sarstedt, Essen, Belgium). Samples were immediately placed on ice before and then centrifuged at 4C° for 10 minutes to separate the plasma. It was collected and stored at -20C°until further analysis. Plasmatic CORT concentration were measured using an Enzyme-Linked Immunosorbent Assay (ELISA) kit (Assay Designs, USA). Analysis was performed according to the "small-sample" protocol provided by the manufacturer. The CORT concentrations were determined with a plate reader (Sunrise Magellan, Austria) linked to an appropriate software (Magellan Software, V6.4 standard, Austria).

### 6.3.5. Open Field test:

This test was performed 24 hours after the start of the habituation period between 9:00h and 12:00h. The test room had similar conditions as the housing

room but the light was dimed to 40 lux in the center of the maze. The open field is a rectangular arena (1m x 1m) with walls (35 cm high) to prevent escape. It was divided in sixteen equal squares and the central area corresponded to the 4 central squares. A video camera (the Imaging Source) attached to the sealing was used to record the behavioral responses during the entire 10 minutes of test. Parameters were recorded and analyzed using a video tracking system (ANY-Maze Video Tracking System). The total distance travelled was used as a measure of locomotor activity. The number of entries and the time spent exploring within the central area were considered as indexes of anxious behavior.

### 6.3.6. Complete-Freund adjuvant injection:

Following baseline testing, we induced persistent inflammatory pain in the respective groups by a subdermal injection of CFA (100 µl, 1mg/ml Mycobacterium) into the middle plantar region of the left hind paw. For this procedure, rats were anaesthetized with isoflurane (4.5% for induction, 2.2% for maintenance) using a Univentor 400 apparatus (Zejtun, Malta). The contralateral (right) hind paw served as a control for effects of the injection itself and was thus injected with an equal volume (100 µl) of 0.9% saline solution.

### 6.3.7. Evaluation of paw edema:

We used a plethysmographic device (TSE volume meter 602060, TSE Systems GmbH, Hombury, Germany) to measure alterations of paw volume (edema) before and at different time points after CFA-induced inflammation in the four different groups of rats. The animals were held in one hand and the hind paw was immersed (up to the ankle) into a cuvette filled with distilled water. The volume of dispersed water was measured by a sensor that was connected to a computer. The measurements were performed on each test day after the assessment of pain thresholds. Each hind-paw was measured three times and the values were averaged to obtain a mean score per day.

### 6.3.8. Assessment mechanical threshold

In order to measure mechanical allodynia, a dynamic plantar aesthesiometer (Ugo Basile) was used. For acclimation, animals were brought in the testing room at least one hour before the start of the experiment and placed 10-15 minutes before testing in Plexiglas chamber (19.5 x 19.5 x 14 cm) with a wire mesh floor. To test for the mechanical pain threshold, a blunt filament was applied to the mid plantar surface of the hind paw and pressure was increased until a withdrawal reaction occurred. The applied force was preset prior the experiment to a slow linear increase of 50 grams over 35 seconds (1.43 g/s) and a cut-off point of 50 g. The force applied at the moment of withdrawal was defined as mechanical pain threshold. For each rat, both hind-paws were tested alternately three times and the values obtained were averaged to give the mechanical pain threshold per day. The baseline was calculated as the mean of the three consecutive testing days.

### 6.3.9. Assessment of noxious heat sensitivity

The noxious heat sensitivity was assessed at least 30 minutes after the measurement of mechanical thresholds. The animals were placed in a plastic cylinder (30 cm of height) on a heated plate (19 cm of diameter, $50 \pm 0.1$ C°; Ugo Basile, Italy) and the time until they displayed nocifensive behavior was measured. Only jumping and/or lifting or licking of the hind-paw was regarded as nocifensive behavior. To avoid damage to the tissue and pain, a cut-off time limit of 20 s was chosen. The animals were immediately removed from the hot plate when they displayed pain or when the 20 s time limit was reached. The test was carried out twice per animal with an interval of at least 10 min and both scores were averaged to give the latency per animal per time point.

### 6.3.10. Statistical analysis

All results are presented as mean ± SEM. Normality was assessed with the Levene's test or Schapiro-Wilk test. A two way (Stress condition x time)

analysis of variances (ANOVA) with repeated measures was performed to determine statistical significance for the paw edema, mechanical thresholds and hot plate measures. Bonferroni multiple comparison post hoc tests then assessed group differences. The different open field parameters and the measures of plasmatic CORT concentration were analyzed using a two-tailed t-test.

## 6.4. Results

### 6.4.1. Locomotor activity and anxiety-like behavior

The open field paradigm is commonly used to measure general locomotor activity and willingness to explore by assessing the time spent moving or the distance covered. It is also used to assess anxiety by including additional measures of time spent in the center of the field and the number of entries in this central zone. Therefore, prior to any manipulation, except the maternal separation in the respective groups, rats were subjected to this test to examine possible distinctions in locomotor activity and anxiety-like behavior between non-stressed and early life stressed individuals. The test revealed no differences in the total distance moved between CON (66.26 ± 3.48 m) and MS (65.91 ± 2.95 m) rats suggesting no locomotor deficit in maternally stressed animals (Fig. 1, left). Additionally, no significant differences were observed regarding the time spent (CON: 22.09 ± 5.06 s; MS: 29. 87 ± 6.43s) (Fig. 1, middle) or the number of visits (CON: 9.25 ± 1.96; MS: 12.69 ± 2.19) (Fig. 1, right) in the central zone.
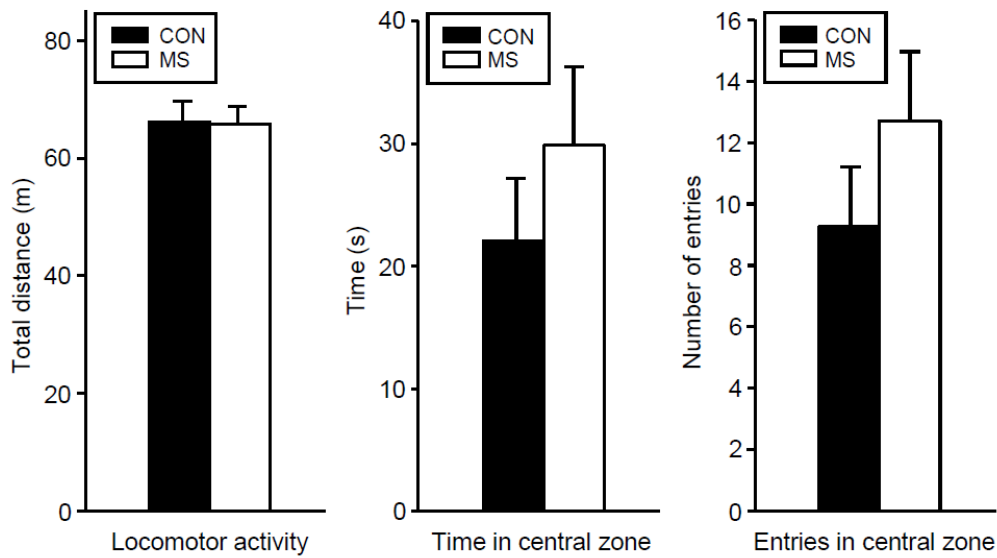
*Figure 18: Locomotor activity and anxiety-like behavior of 8-week-old non-stressed (CON, black bars) and maternally separated (MS, white bars) rats were assessed with the open field paradigm after the habituation period. Left: Total distance (m) covered by CON and MS during the 10 min of the test; no difference was found between groups. Middle: Time (s), CON and MS spent in the central zone of the field. Both groups did not significantly differ in their behavior. Right: Number of entries of CON and MS animals into the central zone of the field. The groups showed no significant different explorative behavior. Data are expressed as mean ±SEM.*

## 6.4.2. HPA-axis activity in early life stressed and non-stressed animals

At an age of 8 weeks, after the habituation period and before the start of the behavioral tests and the induction of inflammation, blood was taken and the serum corticosterone levels were determined. No significant differences in corticosterone concentration were seen between the non-stressed CON (27.38 ± 9.13 ng/l) and the early life stressed MS rats (25.01 ± 6.37 ng/l) (Fig. 2).
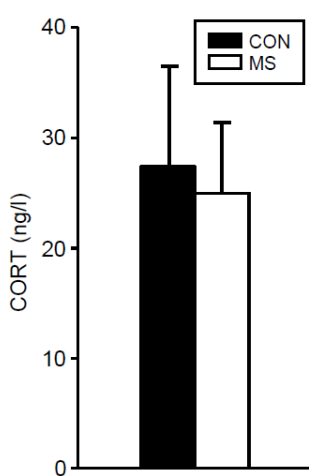
*Figure 19: Effect of maternal separation stress on blood serum corticosterone levels in early adulthood. Blood was taken to measure corticosterone concentration after the habituation period and before any manipulations like behavioral tests and induction of inflammation. The corticosterone levels of the maternal separated (MS, white bar) animals did not differ from those of the control (CON, black bar) rats. Data are presented as mean ± SEM.*

### 6.4.3. Inflammation-induced development of paw edema

Volumes of the left (ipsilateral) and the right (contralateral) hind-paw of each animal were measured and the relative paw volume expressed as ratio left/right was calculated.

At baseline, before induction of inflammation, the relative hind paw volumes did not show significant differences between any of the four groups (CON: 1.00 ± 0.01, MS: 1.01 ± 0.01, CON+CFA: 1.02 ± 0.02, MS+CFA: 1.02 ± 0.02) (Fig. 3). The relative paw volumes of animals (CON and MS) that were not injected with CFA remained at the same level throughout the 21 days of experiment without exhibiting significant inter-group differences (CON: d4: 1.02 ± 0.02, d7: 0.99 ± 0.01, d10: 0.98 ± 0.00, d14: 0.98 ± 0.01, d21: 0.97 ± 0.00; MS: d4: 0.99 ± 0.00, d7: 0.99 ± 0.01, d10: 1.01 ± 0.02, d14: 0.99 ± 0.00, d21: 0.99 ± 0.00). In the groups receiving CFA injection (CON+CFA and MS+CFA) a pronounced edema developed in the ipsilateral (left) hind-paw. The relative paw volume peaked at day 4 (CON+CFA: 1.82 ± 0.05, p<0.001 vs. CON; MS+CFA: 1.84 ± 0.08, p<0.001 vs. MS) and decreased from there on until the end of the experiment at day 21. At all-time points measured, CON and CON+CFA as well as MS and MS+CFA significantly differed in their relative

paw volume (p<0.001 for all time points and both comparisons). However, no significant differences between CON+CFA (d7: 1.76 ± 0.05, d10: 1.63 ± 0.03, d14: 1.56 ± 0.03, d21: 1.50 ± 0.03) and MS+CFA (d7: 1.72 ± 0.04, d10: 1.69 ± 0.05, d14: 1.56 ± 0.05, d21: 1.39 ± 0.03) were found, although a trend to less edema in the MS+CFA group could be observed at day 21.
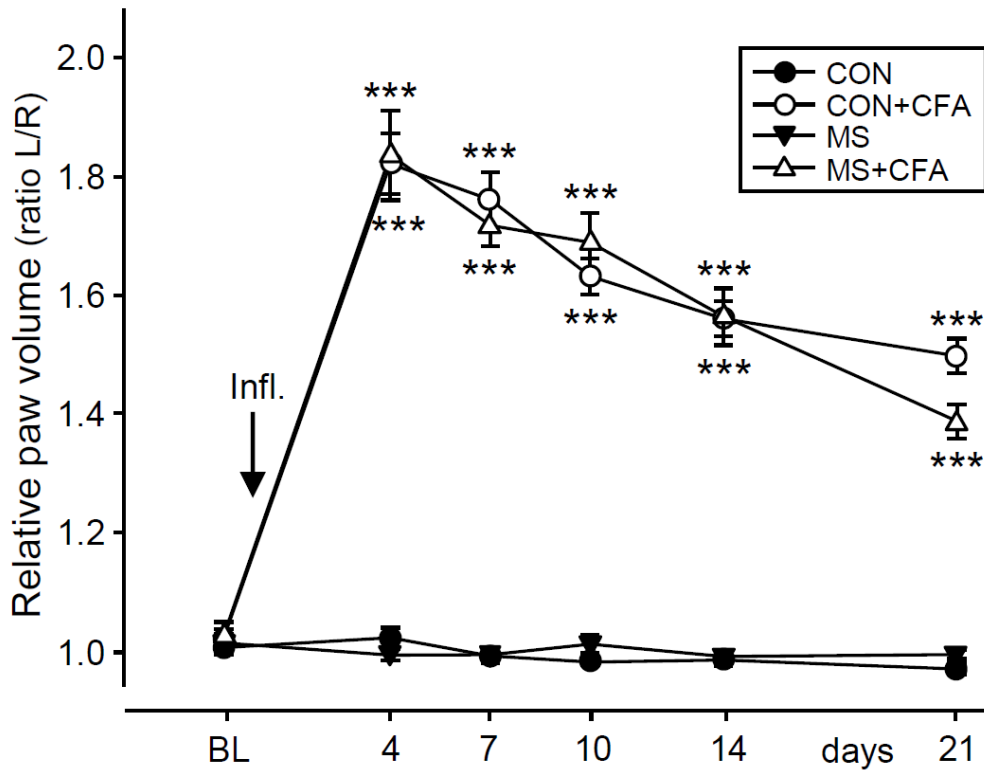


*Figure 20: Development of inflammation-induced paw edema. The relative paw volume of the left hindpaw (expressed as ratio left paw/right paw) was assessed before the induction of inflammation (baseline, BL). No difference was obtained in the four groups (CON: black circles, CON+CFA: white circles, MS: black triangles, MS+CFA: white triangles). Injection of CFA in the respective groups (CON+CFA, MS+CFA) produced a pronounced swelling which peaked at day 4 in CON+CFA as well as in MS+CFA, leading to significant differences between CON and CON+CFA (***p<0.001) and between MS and MS+CFA (***p<0.001) at each time point throughout the experiment. The relative paw volume did however not significantly differ between CON+CFA and MS+CFA, although a tendency to less edema in MS+CFA is visible at day 21. Data are expressed as mean ± SEM.*

### 6.4.4. Mechanical pain thresholds differ in inflamed animals that were subjected to early life stress

A two-way (condition x time) repeated measures ANOVA revealed significant differences in mechanical pain thresholds following CFA injection ($F_{(15.180)}$ = 8.40, $p<0.0001$ time x group interaction). The Bonferroni multiple comparison post hoc test was applied to check for group differences. At baseline, mechanical sensitivity was comparable between all four groups (CON: $26.34 \pm 1.09$, MS: $27.16 \pm 0.59$, CON+CFA: $25.42 \pm 0.84$, MS+CFA: $27.57 \pm 0.79$) (Fig. 4). In CON and MS animals that were not inflamed, the mechanical pain thresholds stayed at around baseline level throughout the 21 days of experimentation. No significant difference between these groups were seen (CON: d4: $25.20 \pm 0.99$, d7: $24.96 \pm 1.11$, d10: $24.43 \pm 1.76$, d14: $27.00 \pm 0.90$, d21: $25.79 \pm 0.80$; MS: d4: $26.51 \pm 0.91$, d7: $26.08 \pm 0.55$, d10: $27.73 \pm 1.04$, d14: $27.38 \pm 0.66$, d21: $27.43 \pm 0.77$). The injection of CFA into the left hind-paw in CON+CFA and MS+CFA animals significantly decreased the mechanical pain threshold of the ipsilateral paw already at day 4 (CON+CFA: $14.73 \pm 0.78$, MS+CFA: $17.05 \pm 2.15$), peaked at day 7 (CON+CFA: $9.95 \pm 0.90$, MS+CFA: $12.86 \pm 0.91$) and started to recover from day 10 to the end of the experiment (CON+CFA: d10: $12.07 \pm 1.16$, d14: $13.86 \pm 1.42$, d21: $16.33 \pm 1.11$; MS+CFA: d10: $15.42 \pm 0.74$, d14: $17.89 \pm 0.90$, d21: $21.13 \pm 1.00$). However, their mechanical pain thresholds remained significantly lower than their non-inflamed counterparts CON resp. MS throughout the entire inflammation period ($p<0.001$ for both groups and for all time points). Comparing the two inflamed groups, CON+CFA and MS+CFA, the maternally separated animals seemed to be less affected by the inflammation. From day 4 to 10, there was a clear trend to higher pain thresholds in the MS+CFA group that became significant at days 14 ($p<0.05$) and 21 ($p<0.01$).
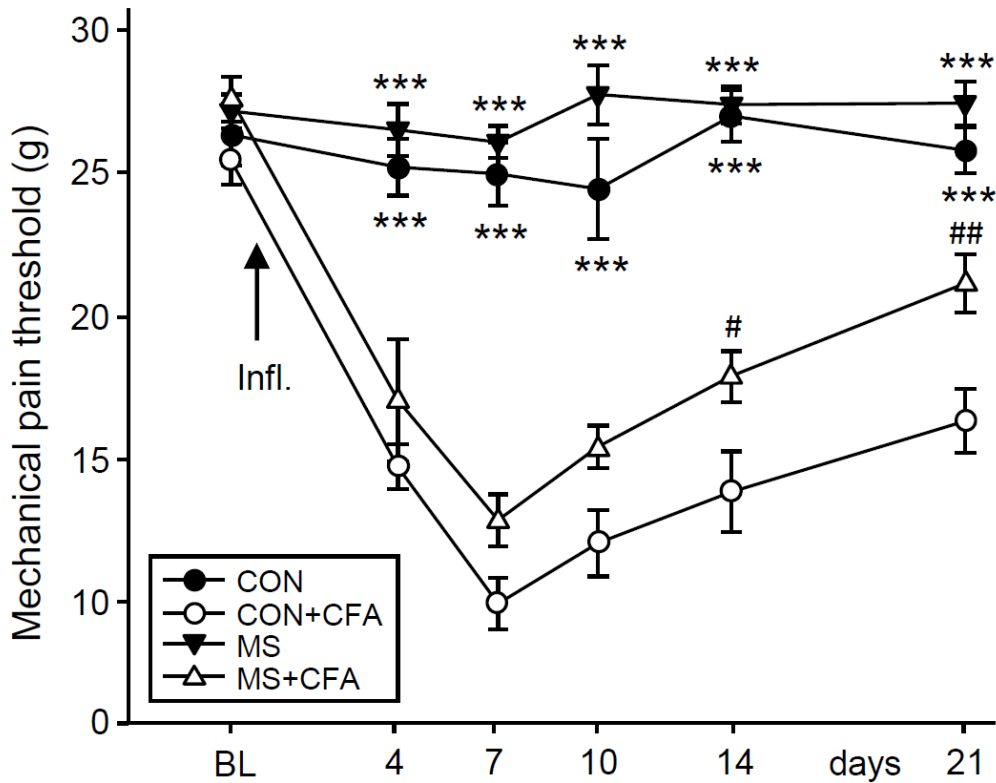
*Figure 21: Effect of early life stress on inflammation-induced mechanical allodynia/hyperalgesia. Mechanical pain thresholds were assessed before (baseline, BL) and at different time points after the induction of inflammation (Infl.). At baseline no differences between groups (CON: black circles, CON+CFA: white circles, MS: black triangles, MS+CFA: white triangles) were obtained. Induction of inflammation by injection of CFA into the left hindpaw of CON+CFA and MS+CFA animals resulted in a decrease of pain thresholds in these groups, peaking at day 7 and partially recovering until the end of the experiment at day 21. Significant differences between CON and CON+CFA (\*\*\*p<0.001) and between MS and MS+CFA (\*\*\*p<0.001) where obtained at each time point throughout the experiment. When comparing the two inflammation groups, MS+CFA seemed to be less affected by the inflammation than CON+CFA. In the early phase of inflammation (day 4 to 10) there was a tendency in MS+CFA to develop less mechanical allodynia/hyperalgesia. In the later phase there was a significant difference between these groups (day 14: #p<0.05, day 21: ##p <0.01). Data are shown as mean ± SEM.*

### 6.4.5. Thermal pain sensitivity differs MS animals

The two-way repeated measures ANOVA revealed a significant effect of time ($F_{(5, 180)} = 43.86$, $p<0.0001$), group ($F_{(3, 36)} = 19.09$, $p<0.0001$) and interaction ($F_{(15, 180)} = 4.197$). Differences between groups were then evaluated by running Bonferroni multiple comparison post hoc test. Contrary to what was obtained for mechanical pain thresholds, CON ($16.61 \pm 0.48$) and MS ($11.49 \pm 0.55$) rats displayed significantly different latencies to exhibit signs of nocifensive behavior at baseline with MS being more sensitive ($p<0.001$) (Fig.

5A). Repeated testing in the course of the experiment "sensitized" the CON rats to the manipulations and resulted in a drop of latency until day 21 (d4: 13.46 ± 1.49, p<0.05 to MS; d7: 11.21 ± 1.12, d10: 10.88 ± 0.89, d14: 10.69 ± 0.88, d21: 9.39 ± 1.24). The MS rats that started with significantly lower latencies, did not change the nocifensive reaction to heat during the experiment (d4: 9.39 ± 0.77, d7: 9.61 ± 0.91, d10: 9.37 ± 0.93, d14: 9.15 ± 0.99, d21: 8.81 ± 1.01). At the end of the testing period, CON and MS exhibited nearly identical latencies.
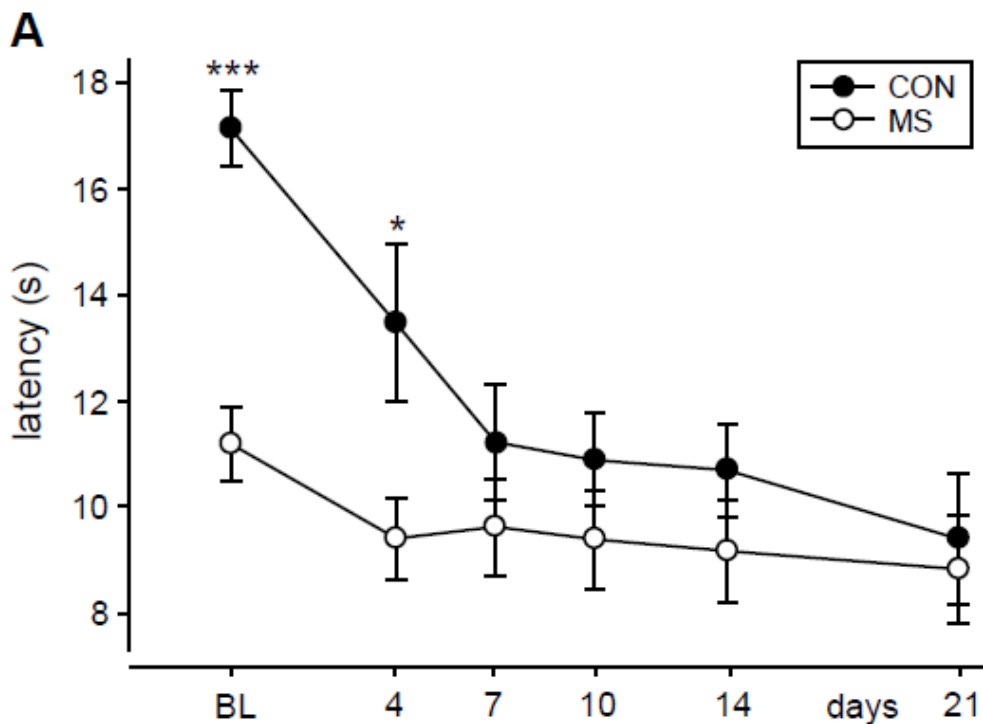


*Figure 22: Effect of early life stress on thermal pain sensitivity, (A). Thermal pain sensitivity was assessed by recording the time (= latency) animals spent on a hot plate (50 ± 0.1 °C) until they displayed nocifensive behavior like jumping or lifting/licking of the hindpaw. At baseline (BL), the latency of non-stressed control animals (CON, black circles) differed significantly from that of maternal separated rats (MS, white circles). Repeated testing sensitized CON to noxious heat, whereas MS rats stayed unaffected by the continuous measurements. Data are expressed as mean ± SEM. \*p < 0.05, \*\*\*p<0.001.*

In order to exclude the habituation phenomenon described above and to be able to judge the impact of inflammation on controls resp. maternally separated animals, we calculated the ratio of CON+CFA to CON and of MS+CFA to MS

and expressed it as percentage. This allowed us to compare the thermal sensitivity of CON+CFA and MS+CFA rats.

The statistical analysis revealed that the ongoing inflammation $(F (5, 90) = 14.79, P < 0.0001)$ and the group $(F (1, 18) = 16.03, P = 0.0008)$ had significant effects, but no significant interaction was found. The induction of inflammation induced a decrease of the latencies in CON+CFA (d4: $64.17 \pm 5.92$, d7: $44.27 \pm 4.16$, d10: $43.41 \pm 3.65$, d14: $52.09 \pm 4.48$, d21: $72.50 \pm 6.03$) as well as in MS+CFA (d4: $94.92 \pm 10.31$, d7: $65.27 \pm 10.06$, d10: $63.07 \pm 8.49$, d14: $62.72 \pm 5.36$, d21: $72.50 \pm 6.03$) animals (Fig. 5B). A tendency for MS+CFA was seen to be less sensitive to thermal stimuli than CON+CFA. The difference was however only significant at day 4 (p<0.01).
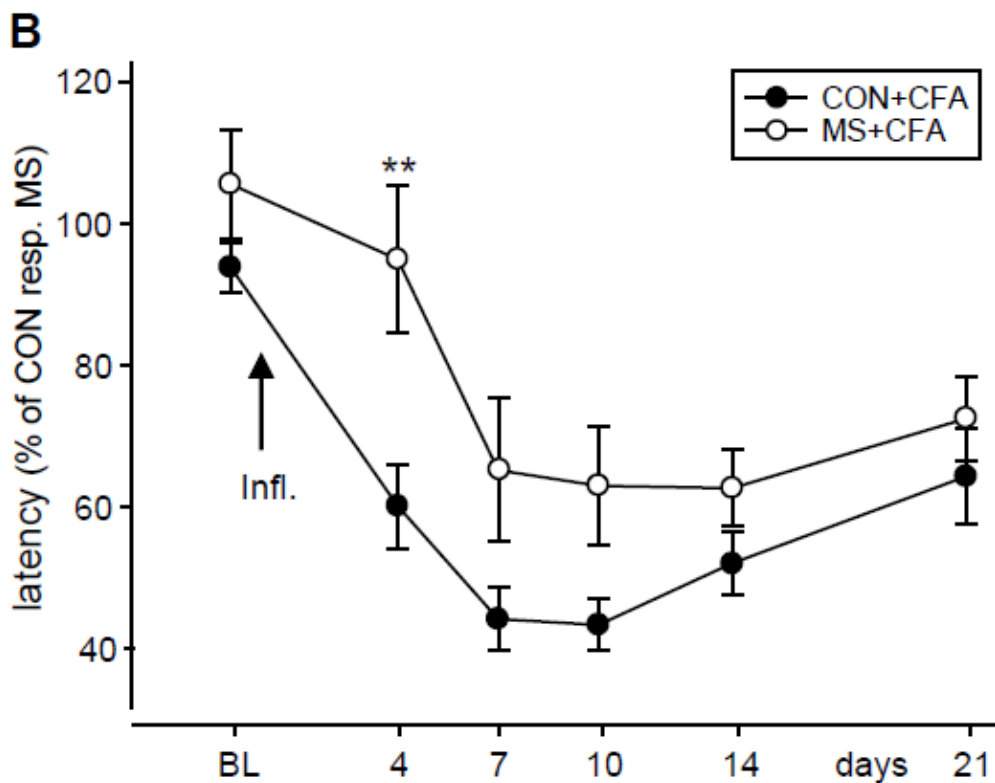


Figure 23: Effect of early life stress on thermal pain sensitivity, (B). The impact of CFA-induced inflammation (Infl.) was different in controls (CON+CFA, black circles) and maternally separated rats (MS+CFA, white circles). The MS+CFA animals were significantly less affected by CFA-injection in the early phase of inflammation, which was moving on to a tendency (days 7 and 10) and finally vanishing in the sub-chronic phase (days 14 and 21). To exclude the sensitization phenomenon seen in (A) when comparing non-stressed and maternally stressed animals, the ratio of CON+CFA to CON resp. MS+CFA to MS was calculated and expressed in percentage (%). Data are shown as mean ± SEM. **p < 0.01

## 6.5. Discussion

There has been an ongoing debate regarding the potential influence of enduring stressors, especially when occurring in early life, on the vulnerability to chronic pain development [14]. In this framework, we investigated the behavioral and endocrine consequences of MS for ongoing pain. In our hands, MS did not lead to phenotype changes indicative of a comorbidity between anxiety/stress and inflammatory pain. On the contrary, our results suggest that MS can lead to alterations of baseline and CFA-induced inflammatory pain independently of changes in anxiety-like behaviors and basal plasmatic corticosterone levels.

### 6.5.1. Stress-related phenotype

The open field test is classically used to evaluate anxiety in rodents. It relies upon the inner conflict between exploration of unknown environment and fear of exposed and well-lit areas [22]. Classically, rodents displaying anxious features show less exploratory behavior [23,24]. Here we observed that CON and MS animals were not presenting any differences regarding explorative behavior and time spent in the potentially anxiety-inducing well-lit central area.

Adverse early-life experiences have often been associated with a dysregulation of the HPA axis and anxio-depressive states in later life [6,25,26]. The HPA axis activity has been extensively studied in the context of early life stress and in the etiology of stress-related disorders such as anxiety and depression [27]. As it is the final effector, corticosterone is classically measured to evaluate possible alterations of the HPA axis. In our hands, plasmatic corticosterone levels were comparable in CON and MS animals confirming previous results from our laboratory (unpublished results). However, despite multiple studies showing heightened corticosterone levels in later life in animals exposed to MS [28], it is increasingly recognized that corticosterone levels and stress do not linearly relate [29].

Taken together, the lack of anxiety-related behaviors during the open-field test coupled with the comparable blood corticosterone levels between CON and MS animals suggest that early life stress, as performed with repeated short episodes of MS may not have a long term influence on the stress-related neuro-circuitry function. Therefore, it would be interesting to complement this result with measurements of the glucocorticoid receptor expression in structures involved in the regulation of the HPA axis activity (e.g. hippocampus, pituitary gland).

### 6.5.2. MS reduces CFA-induced mechanical hyperalgesia

CFA has been shown to induce mechanical allodynia rapidly after its injection and generally to lead to the development of rheumatoid arthritis, an inflammatory chronic pain condition affecting all levels of the pain system, from peripheral to central nervous system [30–32]. The effects of CFA administration have been commonly divided in early (roughly the first 10-14 days) and late phases (from 14 days and on) [33]. Our current results seem to confirm previous findings. Indeed, CON and MS rats did not present any differences in baseline mechanical thresholds [20]. However, MS animals tended to be less sensitive to the CFA-induced decrease of mechanical thresholds during the early phase and displayed considerably higher thresholds in the later phase of inflammation. Hence, it seems that the impact of MS on pain processing remains minor until a new insult such as inflammation unravels its long-term consequences. In the present study we did not observe "classical" stress-induced alterations of neuroendocrine circuits nor of anxiety-related behaviors suggesting that our MS protocol might not have led to long-standing consequences. However, stress-related alterations do not only apply to eventual changes in neural structures [2,34,35]. The immune system also is of great interest in this context. Indeed, MS has been shown to act on the immune system which does e.g. result in enhanced circulating interleukin levels in the early phase after MS [8] but also later in life [36]. This will have aggravating consequences for pain syndromes

such as irritable bowel syndrome or neuropathy [20,37].We did not evaluate circulating interleukins. However, we can speculate that the long-term effects of our MS procedure would have been "too mild" to lead to direct immune system-mediated adverse repercussions. In physiological conditions MS would then not result in an activation of HPA axis associated with anxio-depressive states. However, after an insult, the "minor" differences of neuro-immune functioning between MS and CON animals could lead to different inflammatory pain thresholds and hypothetically to dissimilar stress-related neuro-endocrine circuits and/or behaviors. This remains to be tested in future studies, however the measure of stress related parameters often rely on movement and the impairment of locomotor activity induced by pain models such as neuropathy or hind paw inflammation, might limit the evaluation of anxio-depressive symptoms.

### 6.5.3. MS modulates noxious thermal sensitivity during normal and inflamed conditions

Contrary to the von Frey measures, the hot plate test revealed shorter latencies of nocifensive behavior, hence hypersensitivity to 50°C heat, in the MS group. These results may seem confounding since von Frey results suggested that CON and MS had similar baseline thresholds for mechanical noxious stimulation while thermal noxious stimuli elicited a quicker nocifensive behavior in MS animals. However, noxious mechanical and heat stimuli involve different peripheral processing mechanisms [37]. Furthermore, contrary to the paw lifting behavior used to determine mechanical thresholds, licking and jumping used in the hot plate test as experimental endpoints are considered to be supra-spinally integrated responses [38].

We can also observe the reduction in hot plate latencies in CON animals that did not undergo CFA-inflammation. This phenomenon has already observed been in early studies using repeated testing protocols [39,40]. The behavioral

tolerance [41] expressed in this way was suggested to be related to the test endpoint and to daily and/or weekly test repetitions. Since jumping and licking are supra-spinally mediated responses to noxious stimulation, it was suggested that this habituation phenomenon [42] leads to a reduction of the tonic descending serotoninergic inhibition [39]. Other investigators have more recently proposed that first exposures to the hot plate lead to a potent stress induced analgesia. Stress and concomitant stress-induced analgesia would then decrease with the successive tests leading to a shortening of the nocifensive behaviors latencies [41]. Interestingly, MS animals did not display a reduction of hot plate latencies comparable to the CON group. This could reflect an inability of rats exposed to early life stress to habituate to the repeated hot plate tests. Considering the overlapping structures involved in pain and stress processing and phenomenon like stress induced analgesia [43], we speculate that MS could have altered rats' capacity to display stress induced analgesia.

In order to bypass the problem of having the behavioral tolerance added to the CFA-induced decrease in noxious heat sensitivity, we used respectively the CON and MS groups as "references" for the CON-CFA and MS-CFA groups. As a result, MS animals display a slower development of CFA-induced hyperalgesia than CON. This is in line with the CFA-induced changes of mechanical thresholds despite differences in the time course. Indeed, MS animals displayed a reduced sensitivity to CFA–induced mechanical allodynia/hyperalgesia during the late phase inflammation whereas this was the case in the early phase for noxious heat. To our knowledge, the long-term impact of MS on CFA-induced allodynia/hyperalgesia has not been investigated yet. However, it has been observed that analgesics may differentially affect mechanically and thermally induced pain. Administration of amitriptyline 9 days after CFA injection induced a full recovery of thermal hyperalgesia but had not effect on mechanical allodynia [44]. Amitriptyline is a tricyclic antidepressant and mainly acts via inhibition of serotonin and noradrenalin

reuptake. The differential effect of MS on mechanical and thermal allodynia/hyperalgesia could point alterations of different levels of nociceptive processing, conceivably serotoninergic descending inhibition and spinal cord nociceptive transmission. Furthermore, early life stress has been shown to modulate the neuro-immune system [7,9,45].

The immune system crucially participates in the establishment of peripheral and central sensitization, particularly in the framework of inflammatory pain. Reduced immune function and/or the availability of peripheral pro-inflammatory cytokines could e.g. decrease the CFA-induced hypersensitivity. However, since we did not observe any difference in paw edemas, peripheral effects accounting for the effects of MS on pain sensitivity are quite unlikely.

## 6.5. Conclusion

Altogether, the present study tends to confirm our previous results on neuropathic pain. Early life stress did thus lead to a reduction of pain hypersensitivity in young adulthood, under conditions of neuropathy as well as of inflammation. The observed differences in the respective time courses suggest differential mechanisms underlying the regulation of neuropathy- and inflammation-related pain sensitivity. The present data add an additional another level of complexity since MS animals displayed an enhanced sensitivity to noxious heat during baseline conditions but a reduced one during the early phase of inflammation. According to our results, changes of pain sensitivity did not seem to depend on HPA axis alteration or anxiety-related behavior. The programming of pain sensitivity by early life stress might thus depend on substrates other than the well-established stress-related neuro-circuitry.

**Author Contributions**

**Conflict of interest**

**Funding**

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# Chapter VII: Testing the cumulative and mismatch hypotheses

## 7.1. Abstract:

The cumulative and match/mismatch hypotheses of stress are still under discussion regarding the effects of early life stress on vulnerability or resilience to psychopathology such as depression and frequent comorbid pain states under conditions of additional stress in later life. Previous results from our laboratory suggested that rats exposed to maternal separation (MS), a common model of early life stress, leads to a reduced vulnerability for neuropathy- and inflammation related- pain). In the present study we investigated the respective effects of an additional stressor (second hit applied in young adult rats) on the vulnerability to inflammatory pain.

Sprague Dawley pups were divided into 4 groups: controls (no stress), MS, social stress (SS) and finally the accumulation of MS+SS. At young adult age, psychobiological (blood corticosterone level, sucrose preference and forced swim test) as well as pain- and inflammatory-related (von Frey hair test and hot plate test) parameters were evaluated prior and following the induction of paw inflammation with complete Freund's adjuvant (CFA). Following 21 days of inflammation, spinal glutamatergic transmission, immunocompetent cells, pro-inflammatory cytokines and growth factors levels were evaluated using qPCR.

None of the stress conditions had an obvious impact on corticosterone levels and sucrose preference. However, while MS and SS displayed increased immobility in the forced swim test, the combination of both stressors brought immobility back to CON levels. Regarding pain sensitivity, no stress application seemed to have an influence on basal (prior to inflammation) mechanical thresholds and heat sensitivity. All stress groups displayed lower mechanical thresholds than CON at day 4 after the induction of paw inflammation but the respective values were comparable at 7, 10, and 14 days. On day 21, MS rats seemed to be more sensitive to mechanical stimulation than all the other groups. Regarding noxious heat sensitivity, MS+SS animals were generally less sensitive, this difference was however only significant at 10 and 21 days after CFA-injection. qPCR

results are mitigated. Despite the finding that stress conditions differentially affected different players of glutamatergic transmission, astrocyte activity and NGF expression, our biochemical results cannot readily be related to the behavioral observations, precluding a congruent conclusion.

Despite some alterations specific to stress conditions we are not thus not able to corroborate or dismiss either the cumulative or match/mismatch hypothesis.

**The cumulation of stress in early life and young adulthood does not lead to enhanced inflammatory pain sensitivity and depression-related behavior in rats**

Julien Genty*, Milène Tetsi Nomigni, Fernand Anton, Ulrike Hanesch

Research group Stress, Pain and Pain Modulation,

Institute for Health and Behavior,

University of Luxembourg,

162a, avenue de la Faïencerie,

L-1511 Luxembourg,

Luxembourg

* Corresponding author:

Julien Genty

162a, avenue de la Faïencerie,

L-1511 Luxembourg,

Luxembourg

Phone:+352 466644 6340

julien.genty@uni.lu

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## 7.2. Introduction

While stress normally engenders autonomic, neuroendocrine and neuro-immune mechanisms aimed at preserving health under conditions of enhanced strain, it may lead to deleterious reactions if threatening life conditions are sustained for prolonged periods of time. In this context, a major research emphasis was recently put on the long-term consequences of early life stress (ELS) on the processing of novel stress exposure throughout adulthood. Several concepts aimed at predicting these programming effects have been advanced, ranging from the cumulative stress hypothesis to the match/mismatch hypothesis and finally the three-hit concept of vulnerability and resilience [1] According to the two-hit-related match/mismatch theory ELS can have beneficial consequences if the respective individual is exposed to similar (matching) adversities in adulthood while a mismatching environment may lead to enhanced vulnerability for psychopathological diseases [2].

Until recently, most of the studies investigated the brain mechanisms underlying the impact of ELS on vulnerability/resilience to stress-related cognitive or emotional impairment later in life [2–4]. Only few studies focused on altered vulnerability for potential comorbid pathologies such as chronic pain [5–7] that is commonly associated with negative emotions and that may at least partly be processed via overlapping brain structures and pathways [8]. Although there is some data on the effects of ELS on neuropathic pain and inflammatory pain sensitivity and potentially involved brain circuits [5,9] even less is known about alterations of biochemical pathways that might also be involved in the regulation of nociceptive processing at the level of the spinal cord. This region is however of major importance since it constitutes the point of entry and distribution of noxious information throughout the central nervous system [10]. We have previously observed that maternal separation (MS), a rodent model of ELS could per se have a protective effect regarding the establishment of neuropathic and inflammatory pain conditions in later life [11].

Considering the above mentioned concepts on ELS and consequences for stress reactivity and affective disorders in later life, we sought to determine whether ELS-treated rats displayed altered vulnerability for inflammatory pain and concomitantly altered processing of negative emotions when exposed to ongoing social stress (SS) in young adulthood. The second goal of our study was to investigate the ELS- and SS-dependent regulation of spinal biochemical markers involved in the processing of stress and/or nociception. For this purpose we assessed stress-related alterations in the mRNA expression of spinal glucocorticoid receptors (GR) and of mediators involved in glutamatergic transmission. GR may play a critical role in the framework of chronic pain since it has been shown to be involved in injury-induced NMDA receptor upregulation [12,13]. The NMDA receptor is an ionotropic glutamate receptor crucial for central sensitization [14], its sub-units NR1 and NR2 have been shown to undergo genomic and non-genomic regulation following acute or persistent inflammatory stimuli [15,16]. Furthermore, metabotropic group 1 glutamate receptors (mGluR), particularly mGluR1 and 5, have been shown to be implicated in increased excitability and central sensitization [17]. In addition it has been shown that in terms of the two-hit hypothesis ELS primes brain microglia, leading to an enhanced response to subsequent stress [18]. Since microglia activation also plays a critical role in inflammation [19,20], we included the measurement of ionized calcium binding adaptor molecule 1 (Iba1) mRNA as a marker for microglial activity. In the same line of reasoning, astrocytes are also activated and are known to upregulate the expression of glial fibrillary acidic protein (GFAP) [20]. It is has been shown that chronic stress may activate spinal glia cells [21] and it is well established that activated glial cells have the ability to release several mediators, including pro-inflammatory cytokines that may in turn enhance the sensitivity of nociceptive neurons [22–25]. Among these mediators, spinal tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) are of major importance as their

release in the spinal cord leads to exaggerated pain experience and persistent pain [26,27].

Finally, in addition to the described markers, we investigated the mRNA expression of the neurotrophic factors nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) that have both been discussed to be implicated in chronic stress/depression as well as in nociception [28].

## 7.3. Material and Methods

### 7.3.1. Animal housing

Male Sprague Dawley rats taken for the experimental procedures described below were obtained from our in-house breeding facility. All animals were kept under standardized housing conditions in a temperature ($22 \pm 1°C$), humidity ($60 \pm 10\%$) and light/dark cycle (12h, 7 am to 7 pm) controlled room. Food and water were provided ad libitum. Unless indicated differently, animals were handled twice per week during the cleaning of the cages.

### 7.3.2. Breeding

Sprague Dawley males and females used in the breeding were purchased from Harlan Laboratories (Netherland). After a week of habituation to the housing conditions, two females were mated with a male for 4 days. Gestating females were housed individually 15 days after the end of mating. From day 18 after the first day of breeding on, we checked for litters daily at 8:30 am and 7:00 pm. When the litter was observed, the day of birth was defined as post-natal day 0 (P0).

### 7.3.3. Experimental procedure

The experimental design of this study is graphically summarized in Figure 1.

Four groups of rats were used: CON (underwent neither MS nor SS, n=23), MS (underwent MS but not SS, n=16), SS (underwent SS but not MS, n=12) and MS+SS (underwent the combination of MS and SS, n=12).

Apart from MS, rats were not subjected to any manipulation until the age of 6 weeks. At that stage they were habituated to the experimenter and experimental devices. At 7 weeks of age, the experimental procedures started with blood sampling to measure basal corticosterone levels and behavioral tests to obtain baseline values. Mechanical (von Frey test) and thermal (hot plate test) noxious sensitivity as well as depressive (sucrose preference test) behavior were assessed. Once the pre-stress baseline was established, the animals in the relevant groups were subjected to the social stress paradigm. Blood sampling and the two bottle choice sucrose preference test were performed during the SS period in order to evaluate the effect of stress across time. After the 6 weeks of SS, all animals underwent a second and similar baseline testing (=post-stress baseline). One supplementary test was added in order to complete the assessment of depressive like behaviors by using the forced swim test (FST). Subsequently, all animals were injected with Complete Freund's Adjuvant (CFA) to induce chronic inflammatory pain and noxious sensitivity was tested at post-injections days 4, 7, 10, 14 and 21. Finally, animals were euthanized and L4-L6 levels of the spinal cord were sampled to assess mRNA expression of spinal biomarkers involved in the modulation and the transmission of noxious signals.
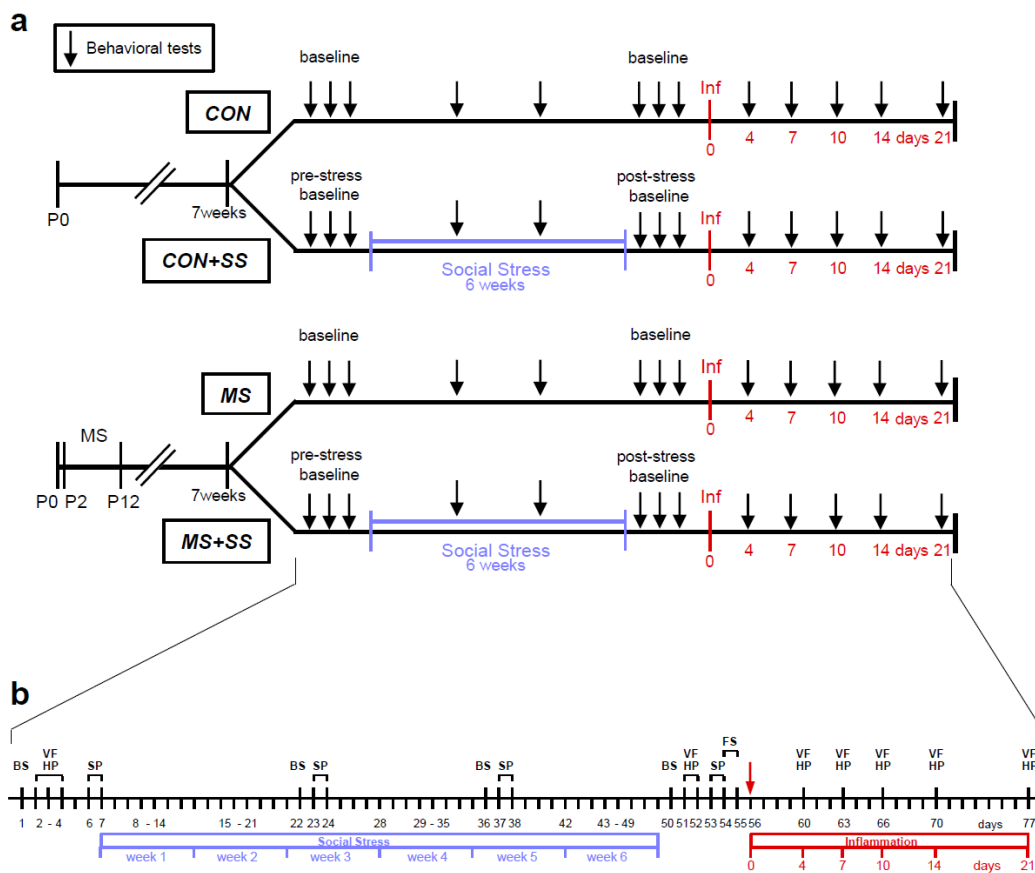
*Figure 24: Experimental design.* The course of the experiment is depicted in a) and the experimental procedures carried out are outlined in detail in b) by giving the exact timeline. After birth (P0), rats were separated into two experimental groups: the control group that was left undisturbed until the age of 7 weeks and the MS group that underwent the maternal separation from postnatal day 2 (P2) to 12 (P12). At an age of 7 weeks, each of the two groups were further divided into a non-stressed group (CON resp. MS) and a stress group (CON+SS resp. MS+SS) that underwent the social stress paradigm. Baseline values were collected for blood corticosterone levels (blood sampling, BS), basal mechanical (von Frey test, VF) and thermal (hot plate test, HP) sensitivity and for depressive behavior (sucrose preference test, SP). The animals in the CON+SS and the MS+SS groups were then subjected to the social stress for 6 weeks. Blood was taken and sucrose preference was measured during the social stress period after week 2 and 4. After the stress, BS, VF, HP and SP were performed to reveal a post-stress baseline. Additionally, the animals were subjected to a forced swim test. Following that, inflammation was induced by Complete Freunds Adjuvant (CFA) in all four groups. Mechanical and thermal noxious sensitivities were further measured on days 4, 7, 10, 14 and 21. After the last testing, animals were sacrificed.

### 7.3.4. Maternal Separation

Early life stress consisting of a maternal separation (MS) procedure was performed on at least 4 different litters. Every day, from P2 to P12, pups and dams were brought in an adjacent room with similar housing conditions and separated from each other. Pups were transferred to another cage equipped with a heated pad having a temperature of $33 \pm 2°C$ and left undisturbed for 3 hours. After each separation, they were returned to their home cage with the dam. Rats from the experimental groups CON and SS that were not selected for the MS procedure were left undisturbed during this period.

### 7.3.5. Social stress

The social stress (SS) paradigm used was based on studies performed in mice by Schmidt and colleagues [29,30] and on a model initially developed in rats by Mormede and colleagues [31]. Young siblings from 4 different litters were housed 3 per cage until they reached the age of 8 weeks. The group composition in the cages was then changed twice per week during cage cleaning, for a period of 6 weeks. The redistribution of the rats was performed in a manner to ensure a minimum of interactions between already familiarized rats and siblings. At the end of the SS period, rats were brought together with their original siblings. Animals that did not undergo the SS procedure were left undisturbed.

### 7.3.6. Pain sensitivity assessment

Mechanical thresholds and thermal sensitivity testing were performed successively on the same day with a minimum of 30 min interval to minimize phenomenon of nociceptor sensitization.

**Mechanical noxious sensitivity**

Mechanical thresholds were assessed by using an electronic version of the Von Frey test (dynamic plantar aesthesiometer; Ugo Basile, Milan, Italy). Animals were transferred to the testing room between 7:15 and 7:30 am and left

undisturbed for at least one hour before each rat was individually placed in a Plexiglas chamber with wire mesh floor. Both hind paws were tested 3 times alternatively with at least a 5 minute interval. A device-controlled pressure was progressively applied on the plantar hind paw surface by a blunted metallic filament (ramp of 50 g in 35 s). The pressure that evoked a withdrawal of the tested hind paw was recorded automatically and was considered as the mechanical nociceptive threshold. Each paw was tested three times and the results were averaged for each day of test. For the pre-stress baseline animals were tested on 3 consecutive days and the thresholds obtained per day were averaged and the mean was defined as the mechanical sensitivity threshold of the animal.

**Thermal noxious sensitivity**

Thermal noxious sensitivity was evaluated as the latency to show nocifensive behavior when placed on a temperature controlled ($50 \pm 1°C$) hot plate. The hot plate apparatus (Ugo Basile, Milan, Italy) is composed of a flat metal plate (19 cm in diameter) surrounded by a Plexiglas cylinder (30 cm of height). For the test, animals were placed on the hot metal plate within the cylinder and left undisturbed until they presented signs of noxious behavior. At the first clear signs of painful behavior, the test was stopped and the animals removed from the hot plate. Tests were recorded using a camera (Sony HDR-CX220E) and the latency to display a nocifensive behavior was measured by using a stopwatch.

### 7.3.7. Depressive-like behavior assessments

**Sucrose preference test: two bottle choice**

Anhedonia is one of the traits observed in patients suffering from depression. In animal models of depression, the two bottle choice paradigm is commonly chosen to evaluate this behavior. This test was performed before, during (week 2 and 4) and after the SS period. Each rat was housed individually for a 24 hour period. During this period, they were given the possibility to drink from two

bottles, one containing tap water and the other a 0.8 % sucrose solution. In order to avoid a side preference effect, the bottles were randomly distributed between left and right sides. After the 24 hours of test, animals were returned to their home cage. Food, water and sucrose solution were weighed before and after the 24 hours in order to obtain the raw consumption of food and liquids. The preference was then calculated as the volume of sucrose solution ingested over the total volume consumed during the 24 hours of test.

$$\% \text{ Preference} = [(\text{Sucrose Intake/Total Intake}) \times 100]$$

For anhedonia to be confirmed, the sucrose consumption had to be below 60%. Values were removed if a bottle presented a damage provoking a leakage of sucrose solution or water.

**Forced swim test**

We used a modified version of the forced swim test [32]. The forced swim test was performed only once at the end of the SS period since it puts a strain on the animals. It consisted in placing the rats in glass cylindrical tanks (diameter: 24cm, height: 45cm) containing water ($23.5 \pm 0.5°C$, $35 \pm 2$ cm of water depth) on 2 consecutive days. The water was changed after each swim test. As for other behavioral tests, animals were brought to the experimental room at least one hour before the beginning of the testing session. On the first test day, animals were submitted to a 15 minutes swim (pre-test) session that was recorded with a camera (Sony HDR-CX220E). The session on the second day consisted in a shorter forced swim (5 min). As previously, this test was video recorded and the time rats spent immobile, climbing or swimming was acquired. An animal was considered to be immobile only when floating or performing very slight movements allowing it to keep its head above water level. After the test sessions, rats were dried in a towel and returned to their home cage.

### 7.3.8. Induction of peripheral inflammation and evaluation of CFA-induced edema

Rats were briefly anesthetized with isoflurane (4.3% for induction and 2.5% for maintenance) using an anesthesia unit (Univentor 400, Zejtun, Malta). Inflammation was induced by injecting 0.1 ml of CFA (suspended in oil/saline emulsion 1:1) into the plantar surface of the left hind paw. The contralateral hind paw was injected with an equal volume of saline.

The CFA-induced hind paw edema was evaluated by measuring the paw circumference after the behavioral tests at day 4, 7, 10, 14 and 21 after the injection. Rats were loosely immobilized in a towel, the hind paw was held and a second experimenter measured the circumference by the use of a string. Three measures per paw and measurement day were performed and averaged. In order to normalize the paw size among rats and avoid weight and body size effects, the ratios of the circumferences of ipsi- to contralateral side were computed for each animal and expressed in percent.

### 7.3.9. Blood sampling and measurement of plasma corticosterone levels

In order to evaluate the impact of maternal separation stress and of social stress, as well as their combination, we determined the blood corticosterone concentration before, during and after the SS period. Blood was taken from awake rats in the morning, between 8 and 9 am. To reduce stress reactions, animals were loosely held in a towel and a small incision in the distal part of the tail was performed by the use of a scalpel. About 300 µl of venous blood was collected with an EDTA-coated capillary tube (Microvette CB300, Sarstedt, Essen, Belgium). The blood samples were immediately placed on ice and then centrifuged at 12000 rpm for 10 min at 4°C. The segregated plasma was collected in 0.5 ml tubes and stored at -20°C until further analysis.

Plasma corticosterone levels were measured using an ELISA (Enzyme-Linked Immunosorbent Assay) kit (Assay Design, USA). The "small samples volume"

protocol was used according to the manufacturer's provided information. Data were acquired using the Sunrise Magellan (Austria) plate reader and analyzed with the corresponding software package (Magellan Software, v6, 4 standard, Austria).

### 7.3.10. Tissue sampling and RNA extraction

After 21 days of CFA-induced peripheral inflammation animals were deeply anesthetized with isoflurane and decapitated. Spinal cord levels L5-L6 were removed and divided into ipsilateral and contralateral side. Total RNA was extracted by the acid guanidium–thiocyanate–phenol–chloroform method using TRIzol® reagent (Life Technologies, Halle, Belgium). After centrifugation, the aqueous phase was collected and the RNA was precipitated with isopropanol. The pellet was rinsed with 70% ethanol, air dried, dissolved in RNase free water and finally stored at -80 °C until further analysis. RNA quality was assessed with either the Experion system or the Experion Automated Electrophoresis Station (Bio-Rad Laboratories, Nazareth, Belgium) using StdSens chips (Bio-Rad). RNA concentration was measured by using the Nanodrop 2000 spectrophotometer quantification system (Isogen Life Sciences, Netherlands).

### 7.3.11. Reverse transcription and Real-Time qPCR

Using the Improm-II reverse transcription kit (Promega, Leiden, Netherlands), 500 ng of total RNA was reverse transcribed into cDNA with 0.5 µg/ml of oligo dT15 primer in a C1000 Touch thermocycler (Bio-Rad, Nazareth, Belgium).

Real-Time qPCR experiments were performed on a CFX 96 real time system (Bio-Rad, Nazareth, Belgium) with 12.5 ng of cDNA in a final volume of 20 µl by the use of PerfeCTa® SYBR® Green SuperMix (VWR, Leuven, Belgium) containing 2X reaction buffer with optimized concentrations of MgCl2, dNTPs, AccuStart Taq Polymerase, SYBR Green I dye, stabilizers and forward and reverse primers at 2 µM (for the list of primers see Table 1).

Primers were designed with the Beacon Designer™ software. Their sequence specificity was tested using the Basic Local Alignment Search Tool at NCBI and they were validated on spinal cord samples. The following steps were performed: polymerase activation at 95 °C for 3 min, 40 cycles of amplification at 95 °C for 10 sec, annealing at 61 °C for 30 sec, recording of melting curves between 65 °C and 95 °C in 0.5 °C intervals. All samples were run in triplicate and no-template controls were added as negative controls. Relative expression levels were estimated using the ΔΔCt-method with β-actin as the reference. Threshold cycle values (Cq) were used to calculate the amount of target gene mRNA in relation to the reference gene mRNA (β-actin). Therefore, ΔCq represents the difference between the number of cycles that were necessary to detect the PCR products of the target and the reference genes. ΔΔCq indicates the difference between the ΔCq of the experimental groups (CON+SS, MS and MS+SS) and the ΔCq of the control animals (CON). The data were expressed as $2-\Delta\Delta Cq$ and the mean of the left (ipsilateral) inflamed side was computed for each group.

### 7.3.12. Statistical analysis

Data are presented as mean ± SEM. Homogeneity of variance was tested using Levene's or Shapiro-Wilk test. The sucrose preference, corticosterone level and hot plate data were analyzed by a Kruskal-Wallis test followed by a Dunn's multiple comparison test. For the forced swim test we used a one-way analysis of variance (ANOVA) and a Tukey's multiple comparison post hoc test to check for differences between groups. The statistical analysis for the paw circumference measurements and the von Frey experiments were carried out using a two-way (time x condition) repeated measures ANOVA followed by Tukey's multiple comparison post hoc test.

For all gene expression experiments, the ipsilateral spinal cord of CON served as control and the relative expression level was set to 1. The expression levels

of the treatment groups MS, SS and MS+SS were expressed as fold of CON. For statistical analysis, these relative expression levels (fold) were compared using the one-way ANOVA followed by Tukey's multiple comparison post hoc test.

## 7.4. Results

### 7.4.1. Behavioral assessments

**Sucrose preference was not affected by stress**

At the beginning of the experiment, under baseline conditions, the group that was assigned to MS+SS conditions unexpectedly showed a significantly lower sucrose preference as compared to CON ($p<0.001$) and to MS ($p<0.01$) (Fig. 2a). Since sucrose preference is commonly considered as anhedonia when being below 60%, this result cannot be interpreted as anhedonic behavior. Over the course of the experiment, no differences between groups were observed in this respect.

**MS and SS animals showed depression-like behavior in the forced swim test**

The time (s) rats spent immobile during the testing session was significantly impacted by the different manipulations ($F_{3, 59} = 10.168$, $p<0.001$) (Fig. 2b). The post hoc analysis revealed a significant increase of immobility in the MS ($178.21 \pm 6.42$; $p<0.01$) and SS ($198.30 \pm 10.37$; $p<0.001$) groups when compared to CON ($141 \pm 6.3$). Combining the two stressors, MS+SS, did not result in a further increase of immobility but, in contrast, reduced the time spent immobile to control levels ($152.63 \pm 10.79$).
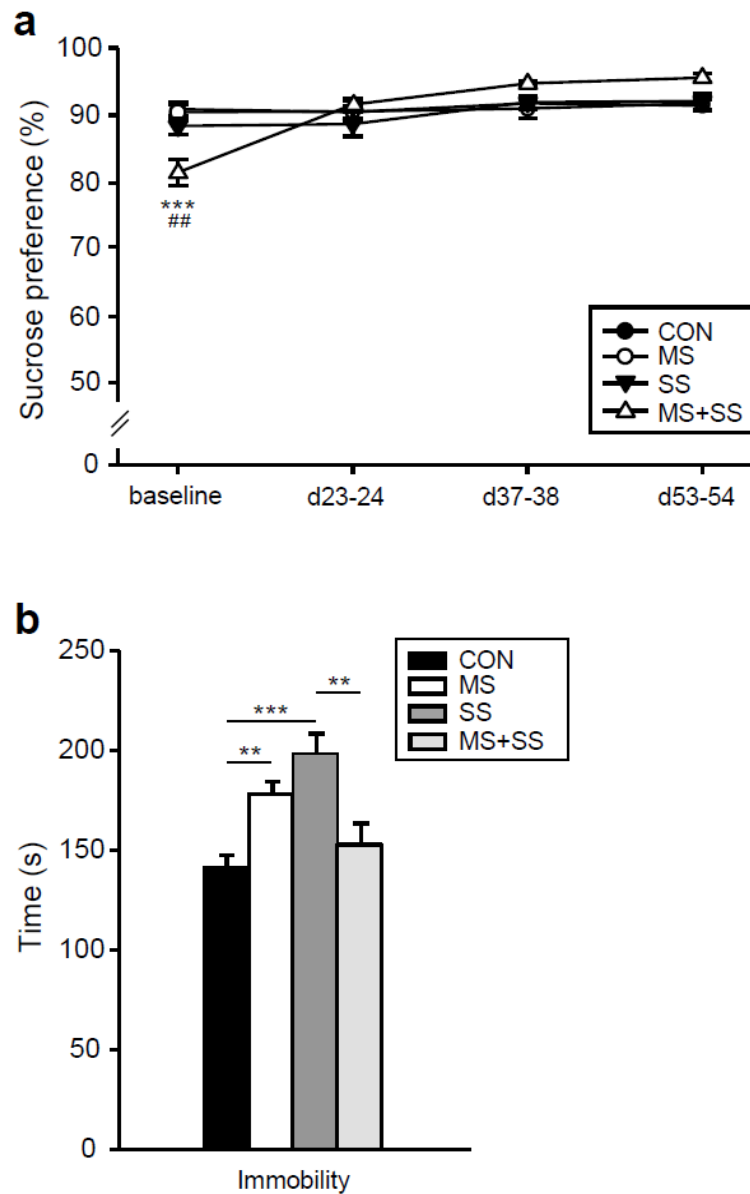
*Figure 25: Depression-like behavior. **a)** Sucrose preference was measured before (baseline), during (d23-24 and d37-38) and after (d53-54) the social stress period. The four groups, control (CON, black circles), maternally separated (MS, white circles), social stress exposed (SS, black triangles) and the combined maternal separation and social stress subjected group (MS+SS, white triangles) exhibited similar preference levels throughout the stress period. At baseline the MS+SS group unexpectedly showed significantly lower sucrose preference, although it cannot be interpreted as anhedonic behavior. Data are expressed as mean ± SEM. \* represents a significant difference between CON and MS+SS (\*\*\*p < 0.001). # indicates a significant difference between MS and MS+SS (##p <0.01). **b)** Forced swim test: the time (s) rats spent immobile was measured after the social stress period at d54-d55. MS (white bar) and SS (dark gray bar) animals exhibit a significant higher immobility than CON (dark bar) whereas the combination of the two stressors (MS+SS, light grey bar) reduced the immobility time to control levels. Data are expressed as mean ± SEM. (\*\*p<0.01, \*\*\*p<0.001)*

**Early life stress (MS) and social stress (SS) only slightly alter inflammation-induced development of mechanical allodynia/hyperalgesia**

Mechanical pain thresholds, assessed by a dynamic plantar aesthesiometer, did not differ between groups at the beginning of the experiment prior to stress application in the respective groups (Fig. 3a). Post-stress, CON, MS and MS+SS exhibited slightly increased mechanical thresholds, which could be interpreted as habituation to the testing conditions. In SS animals such an increase could not be observed, however the statistical analysis did not reveal a significant difference to the other groups. After CFA injection all animals progressively developed a strong tactile allodynia/hyperalgesia on the ipsilateral side peaking at day 7 and then slowly recovering without coming back to baseline levels at day 21. At day 4 inter-group comparisons revealed a significantly lower mechanical threshold of CON when compared to MS ($p<0.05$), SS ($p< 0.001$) and MS+SS ($p<0.05$). From day 7 to 14, no differences between groups were observed and at day 21 MS had a significantly lower mechanical threshold than CON ($p<0.01$), pointing to a slower recovery of the maternally separated animals.

**There is no pronounced difference in inflammation-induced development of thermal hyperalgesia under MS and SS conditions**

In the hot plate test no significant difference in noxious heat sensitivity was found between any of the groups at baseline (Fig. 3b). After the social stress period, a slight decrease in thermal sensitivity was observed in MS and SS animals, possibly due to habituation. However, it turned out not to be of statistical significance. The induction of inflammation rapidly and lastingly decreased the latency for the animals to present nocifensive behaviors. At day 10 post-injection, significant differences in thermal sensitivity were only seen between CON and MS+SS ($p<0.05$) and at day 21 between CON and MS+SS

(p<0.01) and between MS and MS+SS (p<0.05). In both cases, the MS+SS animals showed slightly less thermal hyperalgesia than the other groups.
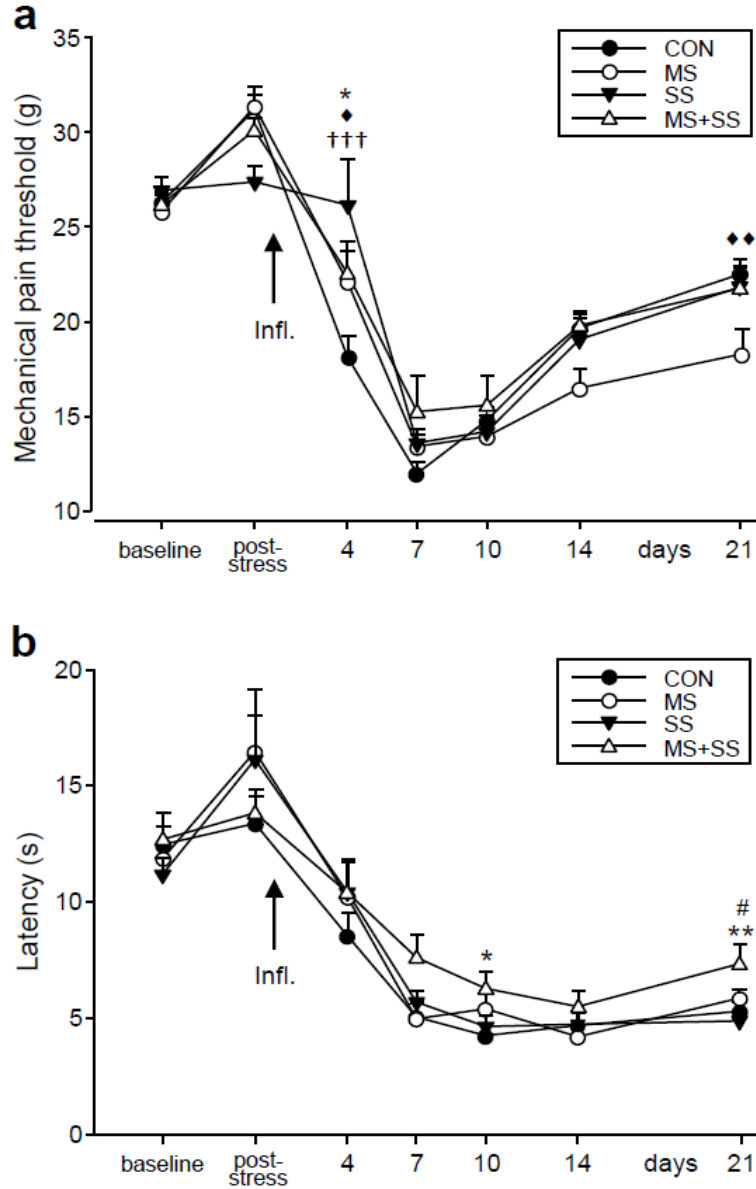


*Figure 26: Effect of early life stress (MS) and social stress (SS) on inflammation-induced mechanical and thermal allodynia/hyperalgesia. a) Mechanical pain thresholds were assessed before (baseline) and after the social stress period (post-stress). In both cases no significant difference was found between groups (CON: black circles, MS: white circles, SS: black triangles, MS+SS: white triangles), although there was a tendency in the SS group to be more sensitive after the stress. Induction of inflammation by injection of CFA into the left hindpaw resulted in a decrease of pain thresholds in all groups, peaking at day 7 and partially recovering until the end of the experiment at day 21. The onset of mechanical allodynia/hyperalgesia was significantly delayed in all three stress groups (MS, SS, MS+SS) at day 4 when compared to CON. The recovery (day 21) was only*

*significantly delayed in MS animals. Data are expressed as mean ± SEM. * represents a significant difference between CON and MS+SS (\*p < 0.05), ♦ between CON and MS (♦p <0.05, ♦♦p <0.01) and † between CON and SS (†††p <0.001). b) Thermal pain thresholds did not differ between groups (CON: black circles, MS: white circles, SS: black triangles, MS+SS: white triangles) before (baseline) and after the social stress period (post-stress). Injection of CFA into the left hindpaw induced thermal hyperlagesia in all groups persisting until the end of the experiment at day 21. There was a tendency for the MS+SS animals to show less hyperalgesia in the course of the experiment reaching significance at day 10 and 21. Data are expressed as mean ± SEM. * represents a significant difference between CON and MS+SS (\*p < 0.05, \*\*p<0.01) and # between MS and MS+SS (#p <0.05).*

## 7.4.2. The development of CFA-induced paw edema is different in MS and SS animals only in the early phase of inflammation

No significant difference in paw circumference (data computed as percentage of the contralateral, non-injected hind paw) was found between any of the 4 groups before induction of inflammation (Fig. 4). CFA injection produced a significant swelling of the paw in every group (p<0.001 for all time points) as revealed by a two-way repeated measures ANOVA ($F_{5, 348}$ = 194.1, p <0.0001). A significant group effect was also seen ($F_{3, 348}$ = 5.045, p = 0.002). In CON (130.8 ± 0.83) and SS (136.8 ± 2.17; p<0.05 vs. CON) the paw circumference peaked on day 4 and in MS (133.3 ± 2.01) and MS+SS (132.9 ± 1.70) on day 7 post-inflammation, pointing to a small delay in the development of edema in maternally separated animals. Consequently, significant differences between groups were seen at day 7, when the maximum swelling was reached in the MS and MS+SS animals, whereas in CON and SS a recovery was already observed (MS vs. CON: p<0.01, MS+SS vs. CON: p<0.05; MS vs. SS: p<0.001, MS+SS vs. SS: p<0.001). At day 10, the edema decreased also in the MS and MS+SS groups. Significant differences where however still be seen between MS and CON (p<0.05) and between MS and SS (p<0.01). On later days the paw circumferences leveled off to about 120% in all four groups.
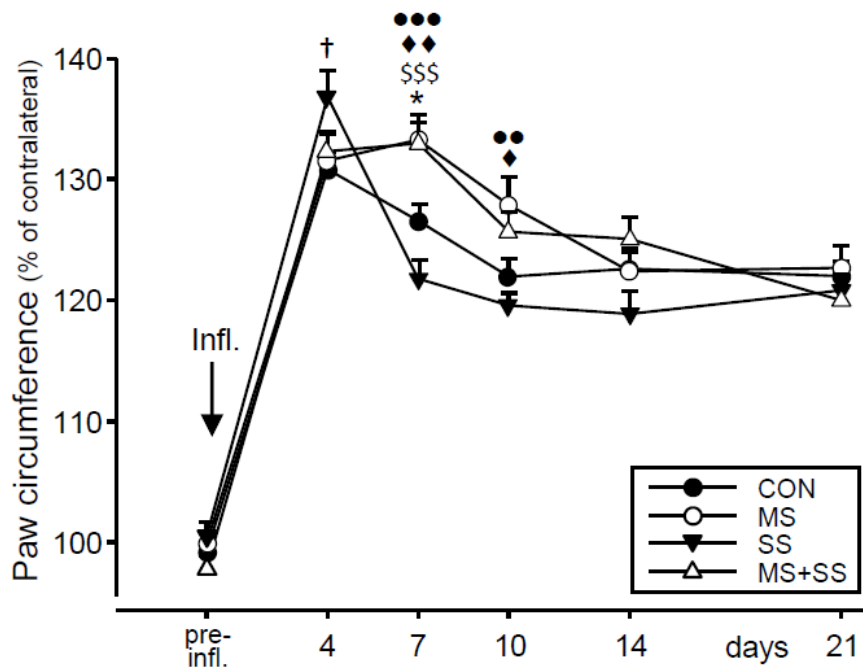
*Figure 27: Development of inflammation-induced edema. The circumference of the left hindpaw (expressed as % of the circumference of the right hindpaw) was assessed before the induction of inflammation (pre-infl.). No difference was obtained in the four groups (CON: black circles, MS: white circles, SS: black triangles, MS+SS: white triangles). Injection of CFA produced a pronounced swelling which peaked at day 4 in the CON and SS groups and at day 7 in MS and MS+SS leading to significant differences in the early phase of edema development between CON and SS (†p<0.05) at day 4, between CON and MS (◆◆p<0.01), CON and MS+SS (\*\*p<0.05), SS and MS+SS ($$$p<0.001), MS and SS (●●●p<0.001) at day 7 and between CON and MS (◆p<0.05) and MS and SS (●●p<0.01) at day 10. Data are expressed as mean ± SEM.*

### 7.4.3. There is no clear correlation between stress conditions and corticosterone levels

Baseline measurements revealed a small tendency to lower corticosterone levels in maternally separated animals (MS and MS+SS groups) (Fig. 5). However, this difference turned out to be not significant when compared to CON whereas it was significant as compared to SS (p<0.05 for MS and MS+SS) although the two groups, CON and SS, where not subjected to different manipulations at this time point. Two weeks after the start of the social stress paradigm, the corticosterone levels increased in all experimental groups, but no inter-group differences could be seen. In CON and MS+SS the ongoing stress resulted in a

further increase of the blood corticosterone concentration 4 weeks after the beginning of the social stress period, leading to a significant difference between the SS and MS+SS groups (p<0.05). After the 6th week of social stress, no further changes were observed. The following inflammation period of 21 days either had no influence on the corticosterone levels in CON animals or the concentration turned back to the pre-inflammation level after this period. In the groups with a history of stress manipulation, the blood corticosterone concentration was likewise increased at that time point and a higher intra-group variation was noticeable. Significant differences were revealed for SS vs. CON (p<0.05) and for MS+SS vs. CON (p<0.01). No significant difference was obtained for MS vs. CON, probably due to the mentioned intra-group variation.
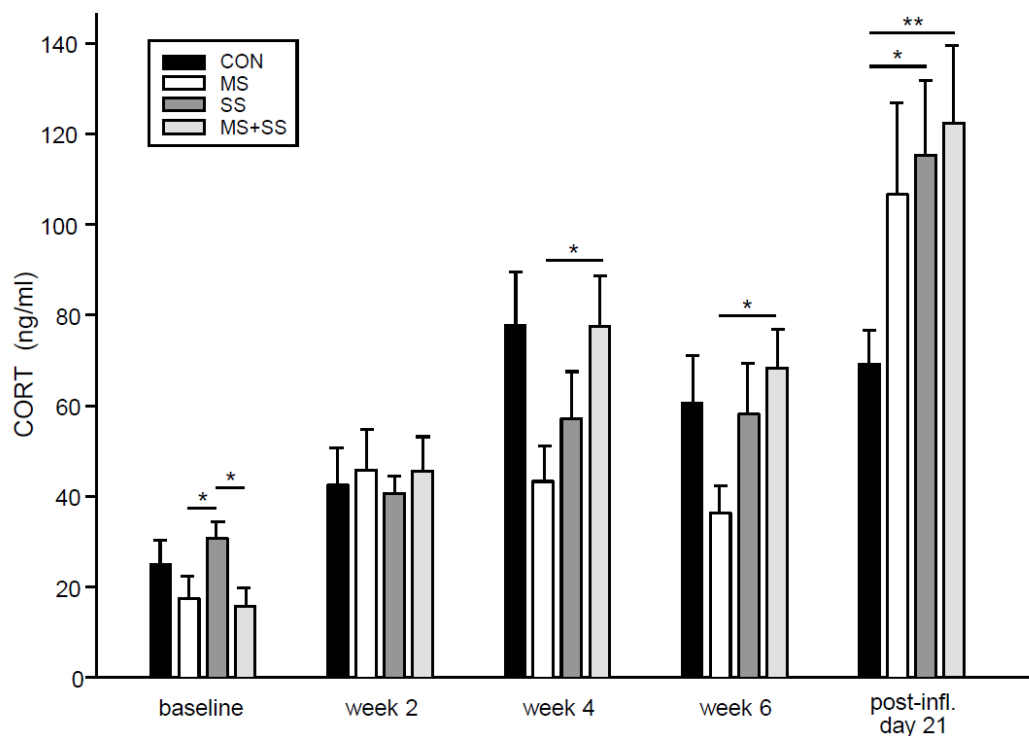


Figure 28: Effect of early life (MS) and social stress (SS) and their combination (MS+SS) on blood serum corticosterone levels. Blood was taken and corticosterone levels were measured before (baseline), during (end of week 2 and 4) and after (week 6) the social stress period as well as 21 days after the onset of inflammation. At baseline, the maternal separated animals (MS, white bars and MS+SS, light grey bars) tended to have lower blood corticosterone concentration when compared to controls (CON, black bars) and to SS (dark grey bars).

*Two weeks after the start of social stress in the respective groups, the corticosterone levels increased in all animals, independent of the stress condition. After week 4, the corticosterone concentration further increased in CON, SS and MS+SS but not in MS and roughly stayed at that level after week 6. The CFA-induced inflammation increased the concentration of serum corticosterone as well as the intra-group variability in the stress groups MS, SS and MS+SS but not in CON. Data are expressed as mean ±SEM. (\*p<0.05, \*\*p<0.01).*

### 7.4.4. Are spinal pain- and stress-related biomarkers differentially affected by early life or/and social stress under inflammatory conditions?

**Stress-mediated alterations of the mRNA expression of spinal glucocorticoid receptor (GR) and receptors of the glutamatergic system**

*Glucocorticoid receptor:* The regulation of the GR mRNA expression did not seem to be affected by any of the stress conditions. No significant difference between groups was found (Fig. 6).
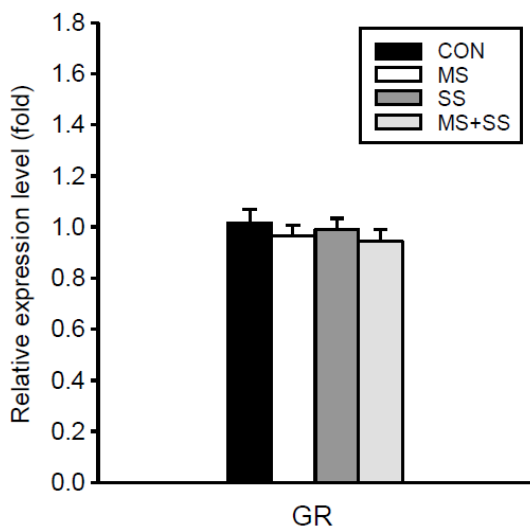


*Figure 29: Regulation of the spinal glucocorticoid receptor (GR). Gene expression of GR was examined in the ipsilateral (left) spinal cord segment L5/L6 of controls (CON, black bars), maternally separated rats (MS, white bars), animals subjected to social stress (SS, dark grey bars) and rats that were exposed to both stressors (MS+SS, light grey bars), 21 days after induction of inflammation. GR mRNA expression was not influenced by any of the three stress conditions. Data are expressed as relative expression level (fold) of CON (control group = 1) and are shown as mean ±SEM.*

*Glutamatergic system:* In order to evaluate the implication of the glutamatergic system in the alteration of noxious sensitivity following different stress conditions and concomitant inflammation we focused on the regulation of mRNA expression of the NMDA receptor subunits NR1 and NR2a. We also

investigated the effects on the group I metabotropic receptors mGluR1 (GRM1) and mGluR5 (GRM5). After 21 days of inflammation, the NR1 mRNA levels were upregulated in all the experimental groups as compared to the controls ($1.01 \pm 0.05$) and reached statistical significance in MS ($1.30 \pm 0.08$, $p<0.05$) and MS+SS ($1.25 \pm 0.05$, $p<0.05$), but not in SS ($1.22 \pm 0.05$) despite a strong tendency (Fig. 7a). NR2a expression was affected in a different manner as it was upregulated in MS ($1.32 \pm 0.07$, $p<0.01$), downregulated in SS ($0.79 \pm 0.04$, $p<0.05$) and not significantly altered in MS+SS ($1.17 \pm 0.06$) when compared to CON (Fig. 7b).

Concerning the metabotropic glutamate receptors, there was a clear tendency for mGluR1 mRNA to be upregulated in the MS group ($1.22 \pm 0.03$) when compared to CON (Fig. 7b). This increase reached significance in comparison with SS ($0.92 \pm 0.06$, $p<0.05$) and MS+SS ($0.97 \pm 0.03$, $p<0.05$). In the case of mGluR5 mRNA there was no significant alteration in the stress groups relative to CON. Only a significant inter-group difference could be found for SS vs. MS+SS ($p<0.05$) (Fig. 7b).
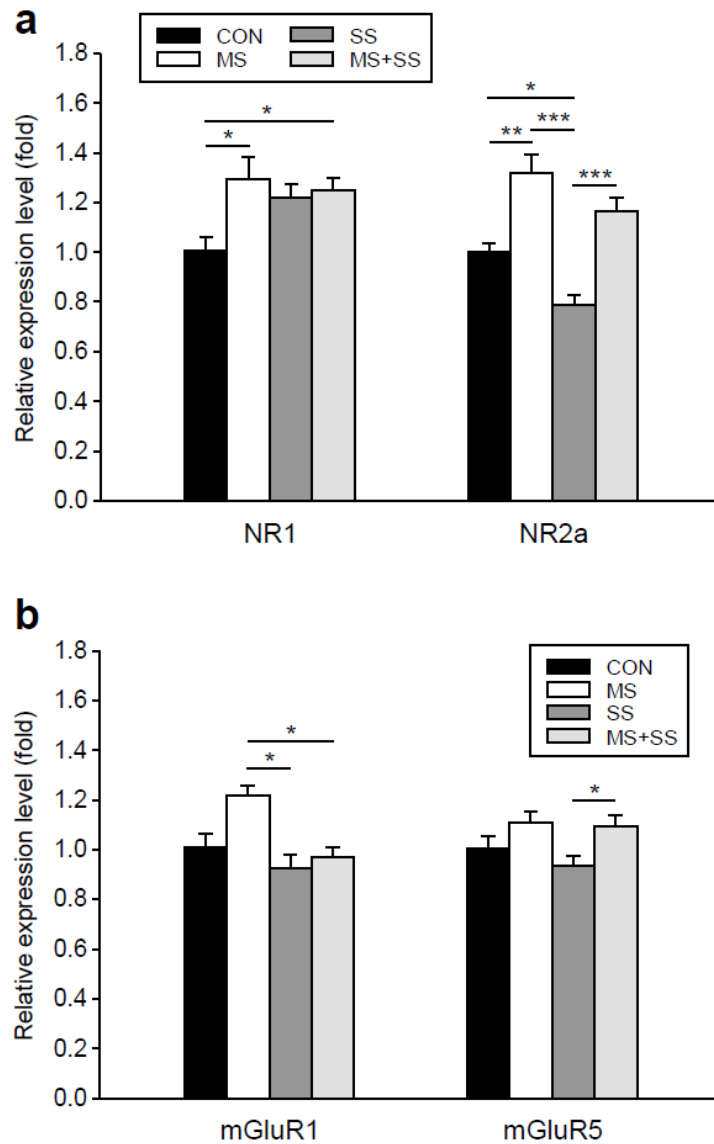
Figure 30: Regulation of spinal ionotrophic and metabotrophic glutamate receptors. The gene expression of two subunits of the ionotrophic NMDA receptor, NR1 and NR2a (a), and of the group I metabotropic receptors mGluR1 and mGluR5 (b) was examined in the ipsilateral spinal cord segment L5/L6 of controls (CON, black bars), maternally separated rats (MS, white bars), animals subjected to social stress (SS, dark grey bars) and rats that were exposed to both stressors (MS+SS, light grey bars), 21 days after induction of inflammation. Data are expressed as relative expression level (fold) of CON (control group = 1) and are shown as mean ± SEM. a left: NR1 mRNA was slightly upregulated in all three stress groups reaching significance in MS and MS+SS animals. a right: NR2a mRNA was upregulated in MS, downregulated in SS and not significantly altered in MS+SS. b left: mGluR1 mRNA showed an upregulation trend in the MS group. SS and MS+SS conditions had no influence on the mGluR1 mRNA expression. b right: No significant alteration of the mGluR5 mRNA level was found in the three stress conditions when compared to CON. (*p<0.05, **p<0.01, ***p<0.001)

**Stress-mediated alterations in the mRNA expression of activation markers of immunocompetent cells and of pro-inflammatory cytokines**

*Activation of astrocytes and microglia:* To assess differences in the activation level of glial cells between the experimental groups, the mRNA expression of the astrocyte marker GFAP and the microglia marker Iba1 was examined. 21 days after CFA-induced hind paw inflammation a significant reduction of GFAP mRNA expression level was observed in animals subjected to social stress, SS ($0.81 \pm 0.08$, $p < 0.05$) and MS+SS ($0.83 \pm 0.02$, $p < 0.05$) but not in MS ($0.99 \pm 0.11$) when compared to CON ($1.01 \pm 0.04$) group (Fig. 8a). No significant differences between groups were observed for the regulation of Iba1 mRNA expression (Fig. 8a).

*Pro-inflammatory cytokines:* After 21 days of inflammation, no statistically significant inter-group differences could be found in the mRNA expression levels of IL-1$\beta$ and IL-6. In the case of TNF-$\alpha$, an upregulation trend was observed in the MS group ($1.39 \pm 0.17$). Due to a pronounced intra-group variance, no statistical significances were obtained (Fig. 8b).
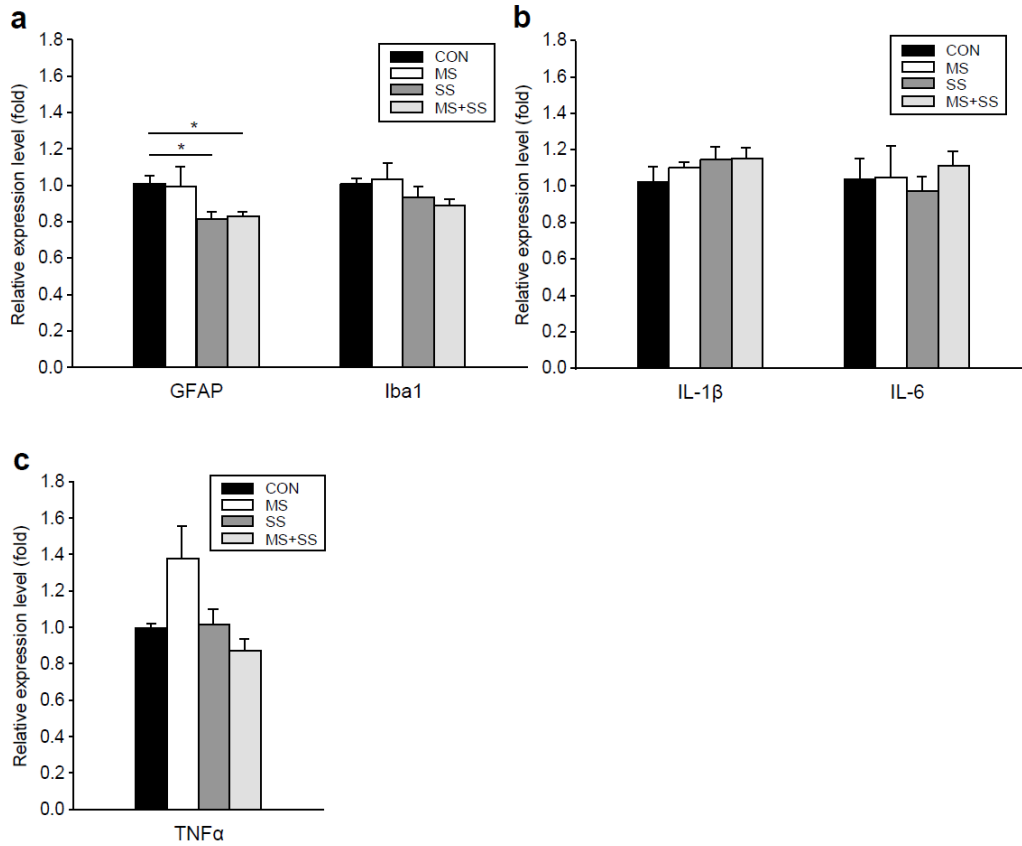
*Figure 31: Regulation of glial activation markers and pro-inflammatory cytokines. The gene expression of the astrocyte marker GFAP and the microglia marker Iba1 (a), and of the pro-inflammatory cytokines IL-1β, IL-6 and TNFα (b, c) was examined in the ipsilateral spinal cord segment L5/L6 of controls (CON, black bars), maternally separated rats (MS, white bars), animals subjected to social stress (SS, dark grey bars) and rats that were exposed to both stressors (MS+SS, light grey bars), 21 days after induction of inflammation. Data are expressed as relative expression level (fold) of CON (control group = 1) and are shown as mean ± SEM. a left: GFAP mRNA was downregulated in SS and MS+SS animals, but not in the MS group (\*p<0.05). a right: the three stress conditions (MS, SS, MS+SS) had no influence on Iba1 mRNA expression. b: the mRNA expression of IL-1β (left) and IL-6 (right) was not altered by stress. c: TNFα mRNA expression was slightly, but not significantly upregulated in MS. Social stress (SS) and the combination of early life and social stress (MS+SS) had no effect on the regulation of the TNFα gene.*

**Stress-mediated alterations in the mRNA expression of the growth factors GDNF and NGF**

The three examined stress conditions did not differentially regulate spinal GDNF mRNA levels. No significant inter-group differences were obtained (Fig. 9). Concerning the expression of NGF mRNA, the stress groups did not exhibit any significant difference to the CON group ($1.02 \pm 0.07$) although an upregulation tendency was observed in MS ($1.17 \pm 0.05$) and SS ($1.17 \pm 0.05$) groups (Fig. 9). An opposite trend was seen in MS+SS animals ($0.93 \pm 0.04$) which led to the expression of significant differences of this group to MS ($p < 0.05$) and SS ($p < 0.01$).
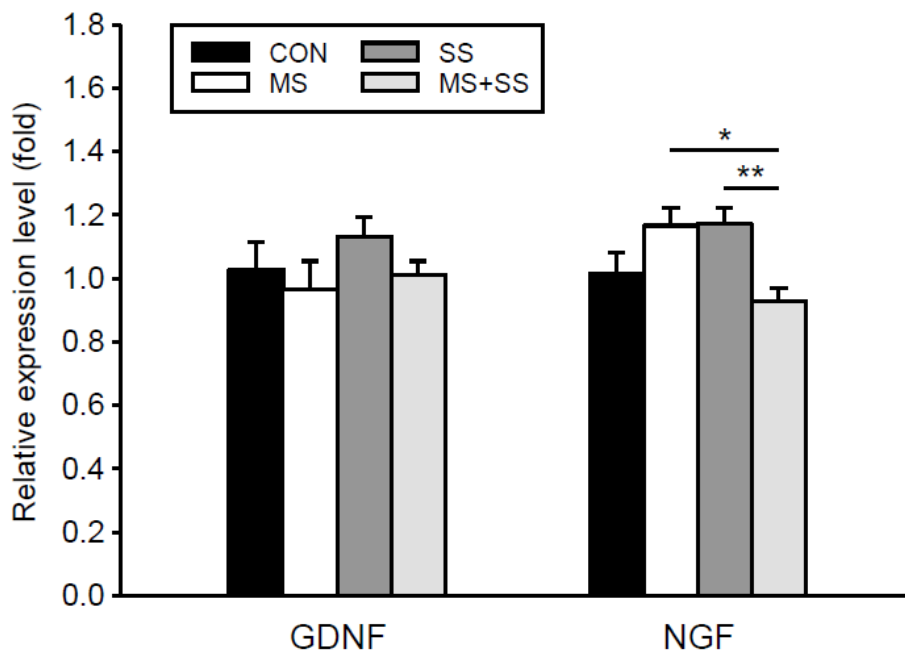


*Figure 32: Regulation of spinal growth factors. The gene expression of the growth factors GDNF (left) and NGF (right) was examined in the ipsilateral spinal cord segment L5/L6 of controls (CON, black bars), maternally separated rats (MS, white bars), animals subjected to social stress (SS, dark grey bars) and rats that were exposed to both stressors (MS+SS, light grey bars), 21 days after induction of inflammation. Data are expressed as relative expression level (fold) of CON (control group = 1) and are shown as mean ± SEM. Left: the different stressors had no effect on the mRNA expression of GDNF. Right: None of the three stress conditions resulted in significant alterations of NGF mRNA expression when compared to the controls (CON). Slight upregulation in MS and SS animals and slight downregulation in the MS+SS group led to significant differences between MS and MS+SS and between SS and MS+SS. (\*p<0.05, \*\*p<0.01)*

## 7.5. Discussion:

Considering the high comorbidity between stress-related disorders and chronic pain we sought to determine if individuals exposed to ELS and then undergoing a prolonged period of social stress during adulthood could be more susceptible to chronic inflammatory pain.

In order to induce early life stress in rats we chose a maternal separation (MS) model that we previously used in combination with neuropathic pain in young adulthood [11]. MS has been shown to enhance vulnerability for psychiatric diseases (O'Mahony et al, 2009) and chronic pain conditions ([33–35]), but it may also lead to resilience against the same pathologies [11,36]

**Impact of MS and/or SS on anxio-depressive-like disorders**

In adult animals, commonly used chronic stress paradigms using repeated exposure to adverse physical stimuli such as restraint do not readily mimic the etiology of stress-related disorders found in humans since the increased prevalence of psychiatric disorders is mainly related to adverse psycho-social conditions [29,37]. Other experimental approaches like the resident intruder and visible burrow system paradigms may hence be more relevant for the comprehension of coping strategies and vulnerability/resilience mechanisms and were used in the present study [38].

We did not observe significant differences in sucrose preference between any of the groups after 2, 4 and 6 weeks of the accordingly applied social stress. Surprisingly however, MS+SS animals presented a significantly reduced sucrose preference compared to all the other groups at baseline, prior the start of the SS period. Therefore this result can probably be ascribed to the variability inherent in our experimental design. In spite of the significantly reduced sucrose preference of the MS SS animals, they did not reach the reduction to 65 % commonly used as the threshold value for anhedonia [39,40] which was hence not displayed by our animals.

The forced swim test has long been considered as an animal paradigm inducing depression and it has traditionally been used for screening potential antidepressant molecules [41]. However, recent views claim that the FST provides a model for responses to an acute uncontrollable stress rather than for depression [42]. Our results show that animals undergoing MS or SS presented enhanced immobility compared to CON animals. Immobility is considered to reflect behavioral despair or the development of passive coping when facing an uncontrollable stress [43]. Previous experiments demonstrated that micro-injections of GABA, lidocaine or antidepressant in structures involved in the processing of stress and pain such as the amygdala [44] or the periaqueductal gray [45] lead to reduction of immobility time in the FST. Furthermore, chromatin reorganization and changes in histone regulation have been observed after chronic stress in CORT-sensitive meso-limbic structures. Also, these genomic and non-genomic modifications are suggested to underlie behavioral despair after additional acute FST [41,46]. Since these structures are involved in the affective dimension of pain [47], the enhanced immobility observed in MS and SS groups could also reflect an altered pain sensation or unpleasantness. Interestingly however, animals undergoing the combination of MS and SS displayed a comparable total time of immobilization behavior as the CON rats pointing to the possibility that the combination of MS and SS may have led to more resilience to stressful situations as also expressed by the enhancement of active coping in the FST.

Taken together our anhedonia and FST-related results may seem confusing since other authors observed anhedonia associated with an increased swimming in the FST [48]. However other investigators showed anhedonia and FST-immobility could be dissociated [49]. Moreover, it has been emphasized that anhedonia and immobility in the FST following a chronic stress were linked to submissive behavior [50]. We did not test submission in our experiment. However we believe that the social instability model we used here, mingling cage mates and hence dominance/submission relationships is hardly reconcilable with tests of

susceptibility to submission because individuals are not put in a situation where they are forcefully dominated or dominating. Indeed, in our social stress paradigm, a rat displaying submissive behavior on one week might be dominant rather than submissive on the next week when the composition of the cage partners is changed. Furthermore it should be noted that despite the overall non-anhedonia displayed in the FST, we observed some rare case of anhedonia at specific time-points that always recovered in the following tests. Using a defeat paradigm, Willner and colleagues [51] found that loss of social rank induces a decrease of sucrose preference. This could suggest that an "ectopic" decrease of sucrose preference may be indicative of a formerly dominant rat now in turn being dominated by other companions while the recovery of sucrose preference could be indicative of a re-establishment of the social status. Pure social defeat stress may hence induce a more homogenous stress than the model proposed by Strekalova and colleagues [50] that induces impairment that is less dependent of rats' submissive traits. Another possibility explaining the discrepancies between studies is the impairment of locomotor activity since the FST also relays on motricity [52]. We did however never observe such an impairment in MS or SS in the open field test nor the zero-elevated maze (non-published observations).

**Stress-induced modification of CORT blood concentration**

Corticosterone was measured to assess possible ELS-induced changes in hypothalamic-pituitary-adrenal (HPA) axis activation during social stress and at the end of the chronic inflammatory period. Early life stress has been shown to lead to higher CORT levels in later life but this is not a uniform finding as factors like genetic background, type of stress and later experiences can differentially influence the HPA axis [1,2]. In our hands, MS tended to reduce CORT levels as shown in the baseline measurement. It is important to note that the baseline measurements were done at the end of the late rodent adolescence period (P46 to P59) [53]. At this stage of life the HPA axis is still sensitive [54,55] to

maturation-related phenomena such as puberty that could influence CORT levels in MS animals [56]. The lack of difference between the CON and SS groups before and throughout the social stress period is surprising but could be related to the timing of the social stress. Indeed, social stress performed during adolescence have been shown to cause lasting alterations of behavioral [57] and neuro-endocrine [29] features. However, in our experiments rats underwent social stress during the late adolescence/beginning of adulthood, thus possibly after the high adolescence-related vulnerability window and consequently may have displayed lower alterations of their HPA axis. Early experiments have focused on social stress in adult rats, however only a confrontation with unfamiliar and aggressive males in combination with the presence of females provoked social instability and was linked to increased adrenal weight and CORT levels [31]. As we did neither assess our animals for dominant behaviors nor introduce a competition for females, social stress could have been too mild to induce elevated CORT levels. After 21 days of inflammation we observed a significant increase of CORT in SS and MS+SS groups and a strong tendency in MS, when compared to CON group. This could suggest that the CFA-induced increase of CORT levels may be potentiated by a history of SS but not MS. CORT levels have been shown to increase with the onset of rheumatoid arthritis triggered by CFA injection [58] but results are not always congruent [59]. It should be kept in mind here that CFA-induced arthritis aggravating factors may depend upon factors like rat gender and animal supplier [60,61] that may explain a lack of CORT increase in some animals. Since the loss of CORT diurnal rhythm has also been reported following CFA injection [58] measures done prior to and after 21 days of inflammation may be difficult to compare. Nevertheless, our result seems to indicate that social stress leads to an enhanced HPA axis activity during a chronic inflammatory state. Indeed, despite a trend toward an increase observed in MS, only SS and the combination of MS+SS lead to significant differences. However, the high level of variability of our ELISA data

preclude a confirmation of a heightened HPA axis activity during chronic inflammation in the MS group.

The possible sustained activation of the HPA axis observed (through elevated CORT levels) may inhibit or enhance immune responses as it leads to alterations in the release of pro- and anti-inflammatory cytokines [21]. On days 4 and 7 after CFA-induced inflammation, we observed that MS animals presented the most pronounced edema. Due to the anti-inflammatory effect of CORT [62,63], this result could be due to the (insignificant) reduction of CORT levels observed in MS group after the 6 weeks of SS period. However, despite comparable levels of CORT in CON, SS and MS+SS groups at the end of the SS period, CFA-induced paw edemas differed. Recently, plasma corticosteroid binding globulin (CBG), has proposed to play a key role in the susceptibility to inflammation as this protein can deliver CORT to inflammation sites [64,65]. Therefore, in spite of equivalent CORT levels, distinct CBG levels could have led to different CORT reservoirs readily available to reduce paw swelling. The development of inflammation depends on the clinical and preclinical state [60] of the immune system which can be influenced by environmental factor such as stress. As our study only evaluates biochemical markers during inflammation, it is premature to speculate on the direct link between circulating CORT and CFA-induced paw edema.

**Modulation of mechanical and thermal CFA-induced hyperalgesia by chronic stress**

The present study confirms prior results [11,57] as we do not see differences in thermal and mechanical sensitivity between CON groups and all three stress groups before inflammation. However the measurement of noxious sensitivity during periods of ongoing inflammation may lead to misleading results. CFA-induced inflammation has been divided in different early and late phases [20,66] and animals with different stress experiences may display differential reactivity

at these different stages. In our study, all stress groups displayed lower mechanical but comparable thermal sensitivity as compared to CON during the early or subacute phase. While acute stress is commonly accepted as producing a so called "stress-induced analgesia" (SIA) [67], the situation is less clear for chronic stress. It has e.g. been shown that MS or SS may either induce analgesia or hyperalgesia [68].

Despite possible FST-engendered stress-induced analgesia (SIA) [67,69] the von Frey results obtained in the present study seem to support previous reports from our laboratory. Indeed, previous data [11] suggest an MS-related protective effect against CCI-induced mechanical hyperalgesia in the developmental phase. Considering the common neuro-inflammatory mechanisms involved in neuropathy [70,71] and inflammation [20] it is conceivable that the analgesic effects observed in our studies share common mechanisms. Moreover, chronic stress as performed via MS [2] but also SS [72] has been shown to alter HPA axis function and CORT secretion which could lead to a "long term SIA" [73] during the early phase of chronic pain.

Interestingly, the MS group displayed decreased mechanical thresholds at day 21 after CFA injection. A similar shift, from analgesia in the early phase to hyperalgesia in the late phase was previously observed around day 21 [57]. In that particular study, SS rats displayed an enhanced cold allodynia 28 days after CCI while being less sensitive shortly after CCI. A similar phenomenon was observed in MS animals undergoing CCI as their mechanical sensitivity kept decreasing until post-CCI day 21 whereas CON rats started to recover [11]. Longer observation periods may be required to confirm that these results indicate a shift from analgesia to hyperalgesia, possibly due to a general allostatic load [68,74].

Noxious thermal sensitivity did not present the same pattern of modulation by stress as seen for the mechanical sensitivity. Indeed, despite CFA eliciting the expected decrease of paw withdrawal latency (PWL), no significant differences were seen during the early phase of the inflammation between any of the groups.

However, MS+SS group displayed a tendency to be less sensitive to noxious heat that became more evident during the later phase of inflammation. In order to understand how and why the stress seems to impact the noxious modalities in a differential way, further experiments are needed. Also, it is important to consider other levels of regulation. For instance, Banik and colleagues [75] investigated paw edema latency and paw swelling following CFA injection in different rat strains. Interestingly, Sprague Dawley rats turned out to be the less sensitive to CFA. This was represented by a lesser incidence and longer latency to develop arthritis, accompanied by a greater paw volume variation and less pronounced edema. Here we did not observe a clear impact on paw swelling, however this could be related to the lack of precision of our paw measurement procedure. It is nevertheless conceivable that interindividual differences in susceptibility and/or the longer latency of arthritis establishment together with the enhanced variability inherent to the multiple manipulations could have led to mitigated results. Furthermore, other investigators have observed a link between blood and paw tissue content of pro-inflammatory cytokines [75,76] and susceptibility to inflammation and inflammatory pain. Indeed, interleukins can activate TRPVs [77] and differences in interleukin levels displayed by more or less susceptible animals could lead to different heat sensitivities

Some issues that could have enhanced the variability of our data and hence mitigated the results should be addressed. Early life stress and social stress have been shown to possibly lead to vulnerability [30,78] however interindividual variability exists [79,80]. Much research has been done on susceptibility and resilience and has been mainly linked to genetic predispositions [81] and epigenetic mechanisms [36,82]. As we did not investigate epigenetic mechanisms in this study, we cannot rule out this possibility. Also the severity of CFA-induced arthritis has been shown to fluctuate across and among rat strains. Indeed, in Sprague Dawley rats and despite a high dose of CFA (1.2 mg) paw edema was reduced, appeared later and was less consistent (in only 38% of the cohort) than in Lewis or Fisher that had received lesser doses [75]. We did

not observe such fluctuations of paw edema in our Sprague Dawley population. However, slight differences in paw edema and hence sensitivity at measurement days could have existed, enhancing variability within groups. Furthermore the small number of animals does not allow us to define clear clusters of animals susceptible or resilient to develop a mild, moderate or severe inflammation.

## Spinal GR expression is not affected by stress under CFA-induced inflammation

Spinal GR expression was previously studied in the context of neuropathic pain. Furthermore GR upregulation seemed to be required for CORT-mediated spinal sensitization [12,13,83]. In our hands GR mRNA expression did not change throughout 21 days of inflammation. This result is in agreement with our previous data, since we did not observe any change of spinal GR expression in animals undergoing CCI, MS nor the combination of both [11].

## Modulation of glutamatergic receptors by stress in an inflammatory context

Accumulating evidence show that chronic stress and glucocorticoid levels have a significant effect on glutamatergic synapses [84]. NMDA receptors are a crucial component for the establishment and maintenance of central sensitization involved in chronic pain phenomenon [85]. However, alterations in the expression of NMDA subunits depend on brain structures and on stress parameters [86]. Our data indicate that the spinal cord mRNA content of the NR1 subunit of the NMDA receptor was equivalent after 21 days of inflammation in all three groups of stressed animals, suggesting comparable NR1-dependent glutamatergic [87]. This result might hint at an enhanced spinal sensitization in stressed animals as compared to controls, resulting in higher noxious sensitivity. However, the properties of NMDA receptors are largely defined by their subunit composition [88]. Indeed, NR2a containing receptors are characterized by a fast deactivation and hence mediate short depolarization

[89]. Our results showed that while MS increased and SS decreased NR2a mRNA expression, the combination of both stressors resulted in expression levels that were comparable to the ones observed in CON. Even though, the enhanced NR2a expression seen in MS could concord with the reduced mechanical sensitivity. Nevertheless, other sub-units such as NR2B, shown to play a crucial role in CFA-induced hyperalgesia should be included [15,90].

Pharmacological activation of Group I metabotropic glutamate receptors such as mGluR 1 and 5 can elicit spontaneous nocifensive behaviors [91] and potentiates the responses of AMPA and NMDA receptors [92]. Furthermore, their activation following peripheral inflammation plays an important role for central sensitization as they do in turn activate phospholipase C [93] and trigger the intracellular ERK/MAPK cascade [94] thus directly or indirectly modulating spinal plasticity [95].

On one hand, mGluR1 has been implicated in models of chronic pain due to its Importance in central sensitization. Indeed, mGluR1 and 5 agonists but not selective mGluR5 agonists have been shown to enhance spino-thalamic tract (STT) cell responses to innocuous mechanical stimuli in monkeys undergoing capsaicin-induced inflammation [17]. On the other hand, selective agonists of mGluR5 have been shown to produce mechanical hyperalgesia while antagonists elicit a dose dependent reversal of CFA-induced inflammatory hyperalgesia [96]. Furthermore selective mGLuR5 antagonists induce a reversal of mechanical hyperalgesia in CFA model of inflammation [97]. We did not observe any significant differences in mGluR5 gene (GRM5) expression in any of the stress groups during ongoing inflammation. Also, mGluR1 expression was only different between MS and the groups that underwent SS (SS and MS+SS). However, behavioral results collected after 21 days of inflammation indicate that MS animals only displayed significant differences to CON (mechanical thresholds) and MS+SS (thermal sensitivity). Hence, mGluRs levels are difficult to relate to the noxious behavior with the noxious behavior

even though we cannot completely rule out their implication in the regulation of mechanical and thermal sensitivity between groups.


**Astrocytes but not microglial activation are modulated by stress in the late phase of inflammation**

Following CFA-induced paw inflammation microglial cells display a rapid and intense activation at the L5 level of the spinal cord as shown by Raghavendra and colleagues [20] even if this result is not always consistent following intraplantar CFA injection [59,98]. Chronic stress has been shown to have an impact on microglia. Indeed, rats undergoing a similar protocol of MS (3h/days during the SHRP) as used in the present study displayed enhanced microglial activity [99]. Furthermore, neonatal rats experiencing paw incision at 3 days of age presented enhanced hyperalgesia following incision at adult age. This was accompanied by a more intense Iba1 staining in the spinal cord at 7, 14 but not 28 days after incision, suggesting that the neonatal incision is amplifying microglial response to later injuries [100]. At adult age, chronic restraint stress led to an increased number of iIba1-positive cells in various stress-sensitive brain regions [101] while chronic unpredictable stress induced an initial phase of activation and proliferation followed by apoptosis of hippocampal microglia cells [102]. Here, we did not observe any differences in Iba1 mRNA expression between any of the groups 21 days after CFA-injection. This result could be due to two reasons 1) as the spinal cord is not a "stress-sensitive" CNS region, the various stresses might not have impacted this tissue 2) it is also conceivable that microglial activation could have already subsided due to the late measurement after the onset of inflammation.

Contrary to microglial cells, astrocytes are not rapidly activated after CFA injection. However their activation was sustained from day 4 to at least day 14 after CFA paw injection as seen by the GFAP and S100β astrocytic marker mRNA levels [20]. Like microglial cells, astrocytes have been shown to be sensitive to both early life and adult chronic stress. Indeed, early life stress seems

to induce a rapid (after 24h) downregulation of astrocytic markers GFAP and S100β in the anterior cingulate cortex (ACC), a crucial region for pain processing [103]. Also, chronic unpredictable stress or repeated administration of CORT led to GFAP downregulation in the prefrontal cortex and hippocampus [104,105]. Here we showed that SS and the combination of MS and SS but not MS per se reduced GFAP mRNA expression in the spinal cord 21 days after CFA-injection. GFAP expression is an indicator of astrocyte reactivity and is involved in the maintenance of their structure. The reduced GFAP mRNA levels seen in SS and MS+SS could be indicative of an enhanced astrocyte reorientation, a reorganization of the astrocytic cytoskeleton or astrocytic death following nociceptor activation [106]. In order to determine if our results represent a difference in astrocytic morphology or a reduction of the astrocyte population it would be necessary to complete our protocol with other markers such as S100β or other techniques such as immunostaining. Furthermore, astrocytes directly participate in the homeostasis of the glutamatergic synapse [84,107] and are able to modulate spinal nociceptive processing via the release of cytokines [20]. Concerning the MS and MS+SS animals this could be of particular interest as they respectively display different mechanical and thermal sensitivities 21 days after CFA injection.

**Stress does not change the expression of spinal pro-inflammatory cytokines after 21 days of CFA-inflammation**

Clinical and preclinical settings often associated chronic stress exposure with major depressive disorders and enhanced pro-inflammatory cytokine levels, most consistently IL-1β and L-6 [108,109]. Preclinical studies have shown that pro-inflammatory cytokines are mediating the long-term effects of chronic stress exposure [102,110–112]. We did not observe any significant differences between the 4 groups in any of the three cytokines measured after 21 days of inflammation. These results are surprising since TNF-α, IL-β and IL-6 expression has been shown to be changed by social [113–116] and by post-natal

stress [9,117]. One possibility is that inflammation may have raised to the same level possible pre-existing differences in cytokine mRNA. These previous were investigating cytokine expression in the spleen or plasma [9,113–117]. Despite evidence supporting the ability of peripheral cytokines to signal through the blood brain barrier [118] it seems that spinal TNF-α, IL-β and IL-6 levels do not directly correlate with enhanced circulating cytokines [21], a priming of CNS glia could nevertheless exist. As microglia-induced immune activation and cytokine production are believed to be critical for the onset of arthritis [119,120]. Hence, differences to be expected during the initial phase of inflammation could have waned 21 days after CFA-injection.

**NGF but not GDNF is influenced by stress under CFA-induced chronic inflammation**

Neurotrophic factors have been shown to be crucial for C-fiber nociceptor development and survival upon insults [121,122]. In this context, two C-fiber sub-classes have been classified according to the neurotrophic factor affecting them. The tyrosine kinase A receptor (Trk A) expressing neurons depend on NGF while the Trk rearranged during transfection (Ret) neurons depend on GDNF [123]. Stress has been found to affect NGF levels however results are not always congruent. Adult animals undergoing chronic stress have been shown to display decreased protein levels in the prefrontal cortex, the hypothalamus and the hippocampus [124–127]. On the contrary, early maternal separation in rats induced NGF increases in those same structures a few days after the stress protocol [128,129] or following a stressful event at adult age [130] suggesting that individuals undergoing MS might have a better coping ability. However, spinal NGF has been shown to mediate neuronal plasticity involved in visceral hypersensitivity [35]. Also, socially stressed rats undergoing chronic constriction injury (CCI) displayed higher NGF levels and enhanced pain sensitivity than rats only undergoing CCI [57]. In our hands, MS and had a tendency to increase NGF mRNA levels after 21 days of inflammation. This

effect was not found in animals that experienced both stressors. However, 21 days after CFA-injection, MS+SS rats displayed longer nocifensive behavior latencies on the hot plate than MS and CON group. Hence, it is difficult to draw any explicit conclusions about a possible cause-effect relationship between NGF gene expression and stress-related behaviors.

GDNF delivered intrathecally has been shown to be a potent analgesic agent [131,132]. However, endogenous GDNF is believed to play a role in sensitization and the decrease of mechanical thresholds [133,134]. Nevertheless, we did not observe any significant differences between any of the groups which could suggest that GDNF was not involved in the CFA-induced central sensitization.

## 7.6. Conclusion

This study aimed to test the commonly expressed assumption that the combination of early life adversity and adult exposure to social stress predisposes rats to affective disorders and comorbid pain states. While we did indeed observe more pronounced maintenance of inflammatory reactions in rats exposed to the two stressors, this group of animals did not display any alterations in mood and pain related behavior. In addition, the measurement of biochemical markers of stress- and/or pain processing did not reveal any congruent differences between the experimental groups. In spite of potential limitations related to sample size and measurement time points we believe that these negative results may allude to a more complicated interdependence of stress, affect and pain processing than initially assumed. Future studies should be aimed at a systematic evaluation of potentially relevant parameters.

## 7.7. References

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# Chapter VIII: Conclusion and outlook

Clinical and experimental studies have demonstrated a strong relationship and comorbidity between stress-related disorders and pain conditions. Stress and pain are both relying on common brain structures such as the limbic system and the hypothalamus. In addition to the brain sustained stress can affect endocrine and immune systems. Early life stress is believed to be associated with long lasting alterations of neuro-endocrine and neuro-immune systems and a higher risk to develop anxio-depressive disorders. In this context, only few studies focused on the effect of early life stress on adult vulnerability to pain and on underlying spinal cord mechanisms. Hence, during my Ph.D. I investigated the long term effect of early life stress on pain sensitivity later in life and on potentially involved spinal mediators.

In the first study, I submitted rats to a maternal separation procedure and evaluated, once they reached adult age, their mechanical and cold noxious sensitivity during baseline and neuropathic conditions. Rats undergoing maternal separation had a comparable noxious sensitivity during normal conditions. However, following the induction of neuropathic pain, rats experiencing early life stress were less sensitive than controls and the appearance of mechanical allodynia/hyperalgesia seemed to be delayed. Behavioral results were accompanied by various alterations of spinal biomarkers, notably of immunocompetent cells and pro-inflammatory cytokines.

These results led me to determine if the protective effect of maternal separation was specific to the neuropathic pain condition or if it could be reproduced under conditions of inflammatory pain. Furthermore, I wanted to characterize MS-related alterations of the HPA axis through measurement of blood corticosterone and of stress-related behaviors. In order to induce inflammatory pain, we chose the complete Freund's adjuvant model due to its temporal characteristics allowing us to compare this study with the previous one. Our results did not reveal any differences in corticosterone levels nor in anxiety-related behaviors as observed in the open field test. Similarly to the first study, maternal separation

did not impact mechanical thresholds under normal conditions. Since cold allodynia is a typical and quite specific symptom of neuropathic pain, I changed the tested thermal modality from cold to heat. In this case, baseline sensitivity was changed. Indeed, MS animals presented shorter latencies to exhibit nocifensive behaviors in reaction to noxious heat. Furthermore, this test revealed the incapacity of MS animas to habituate to the test which could possibly be ascribed to alterations of higher brain centers normally inducing stress-induced analgesia.

Finally, considering theories relating to early life stress, we aimed to investigate the cumulative and match/mismatch hypothesis of vulnerability and resilience to stress. The goal of the third project was thus to determine if a stress experienced at adult age in addition to early life stress would lead to enhanced or reduced pain sensitivity. For this purpose rats were submitted to maternal separation and later on, at adult age to social instability stress. In order assess relevant stress-related psychological disorders, rats also underwent the zero elevated plus maze (data not published), the sucrose preference test and the forced-swim test. Then, nociceptive mechanical and thermal sensitivities were evaluated as previously done. The results of these experiments were mitigated and I was not able to reproduce previously obtained findings regarding pain sensitivity during physiological (no differences in noxious heat sensitivity) and inflammatory conditions. The multiple animal groups and repeated testing, sometimes generating intense stress (e.g. isolation during sucrose preference, forced swimming) may have led to high levels of variability and to erratic effects.

The results obtained during my Ph.D. thesis still require to be deepened in order to be able to have an insight of the mechanisms involved in the effects of MS on pain sensitivity. First, an analysis of spinal protein content in complement to the mRNA evaluation seems necessary. It could also be interesting to determine the immediate alterations caused by early life stress. Indeed, to my knowledge no study ever determined the effect of repeated MS on the still immature spinal

microglia, astrocytes, nociceptive neurons and the crosstalk between immunocompetent and neural cells. Another point could be the better characterization of MS-related HPA axis alteration. Even though we did not obtain any significant differences regarding the CORT levels, MS can lead to various alterations of HPA axis reactivity during times of stress. A short restraint stress associated with blood sampling could be helpful in this respect.

Nevertheless our overall results seem to point out two major leads: 1) MS seems to alter immunocompetent cells which could have repercussions for the establishment of sustained and/or enhanced noxious sensitivity later on, 2) differences in heat sensitivity and lack of habituation in the hot plate test suggest that descending pain control pathways might be altered.

## 8.1. Does early life stress-related resilience of neuropathic pain critically depend on altered microglial reactivity?

### 8.1.1. Scientific background

The goal of this project is to characterize the long-term consequences of maternal separation for pain-related behavior and to investigate to what extent neuro-immune system alterations can relate to these behaviors. It was shown recently that early life stress has to the capacity to impact immune system responsiveness (Roque et al. 2014, Roque et al. 2015) and is associated with many disorders ranging from depression to irritable bowel syndrome (O'Mahony et al 2009) in later life. Mechanisms by which immunocompetent cells such as microglia are capable of enhancing nociceptive processing remain incompletely understood. However it seems that microglial activation is accompanied by a loss of inhibitory tone in the spinal cord, therefore enhancing spinal nociception (Coull et al. 2005, Sorge et al. 2015). Hence, microglial cells are an essential component of the onset of several chronic pain conditions (Raghavendra et al. 2003, Ledeboer et al. 2005). The first study of my thesis suggested a pain hyposensitivity of MS animals (compared to CON animals)

during neuropathy. Beside the change in growth factor expression other molecular markers of nociceptive processing were affected. Indeed, the behavioral results were associated with reduced spinal Iba-1 (marker of microglial cells) and IL-1β mRNA levels. This could be particularly interesting since pro-inflammatory cytokines released by activated microglia are capable of enhancing spinal nociceptive processing (Ren and Torres 2009, Yan et al. 2014). We hypothesize that the reduced noxious sensitivity we observed could be related to a dampened immune system reactivity. In this framework, upon injury or inflammation, MS-induced inhibition of microglial cells would prevent or delay the full extent of injury-induced hyperalgesia. The first objective was to determine if the behavioral differences observed previously may originate from an altered microglia reactivity. In order to evaluate the potential impairment of microglial cell in the neuropathy-induced phenotype observed in MS animals we undertook to activate microglia pharmacologically using Amphotericin b (AmpB) (Motoyoshi et al., 2008; Döring et al., 2015). Secondly, we aimed to investigate the molecular basis of MS-induced modulation of neuropathic painful behaviors using immunohistochemistry and RT-qPCR.

### 8.1.2. Methods

As described in previous studies, baseline mechanical and thermal noxious sensitivity were evaluated using the von Frey hair and cold plate tests in MS and CON animals. Then, both MS and CON rats received intraperitoneal injections of amphotericin B (activator of microglial cells) or sodium deoxycholate (vehicle) prior and during the neuropathic pain period. The neuropathy was induced using the chronic constriction injury model described in chapter 3. Animals were then retested 4 and 7 days after CCI-surgery in order to see if the pharmacological activation of microglial cell can reinstall a "normal" neuropathy-induced hypersensitivity in MS animals. Finally, part of the animals were sacrificed by overdose of pentobarbital (60mg/ml) and perfused

transcardially. The spinal cord was then be dissected and immunochemistry using anti Iba-1 and anti F4-80 was performed in order to put in evidence the total number of cells and the number of cells activated by the neuropathy. The second part of MS and CON animals were used to examine spinal mRNA and protein expression that was associated with microglial cells and the onset of neuropathic hypersensitivity. This could lead to the identification of key molecular targets for future therapies alleviating the neuronal long-term consequences of maternal separation.
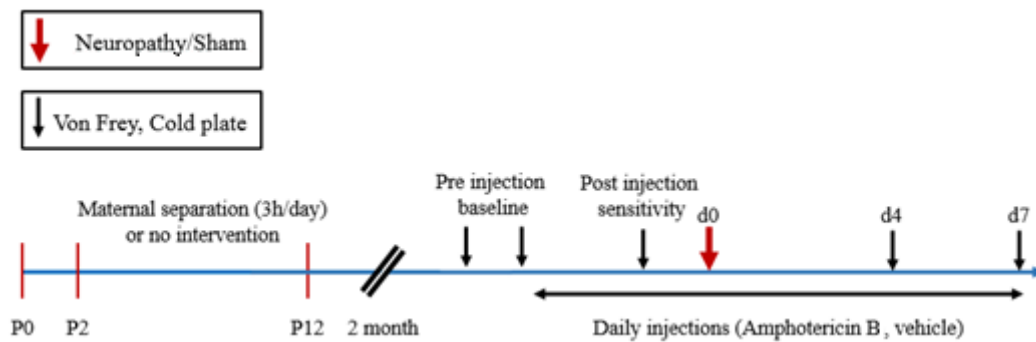


*Figure 33: Experimental timeline*

## 8.1.3. Preliminary results:
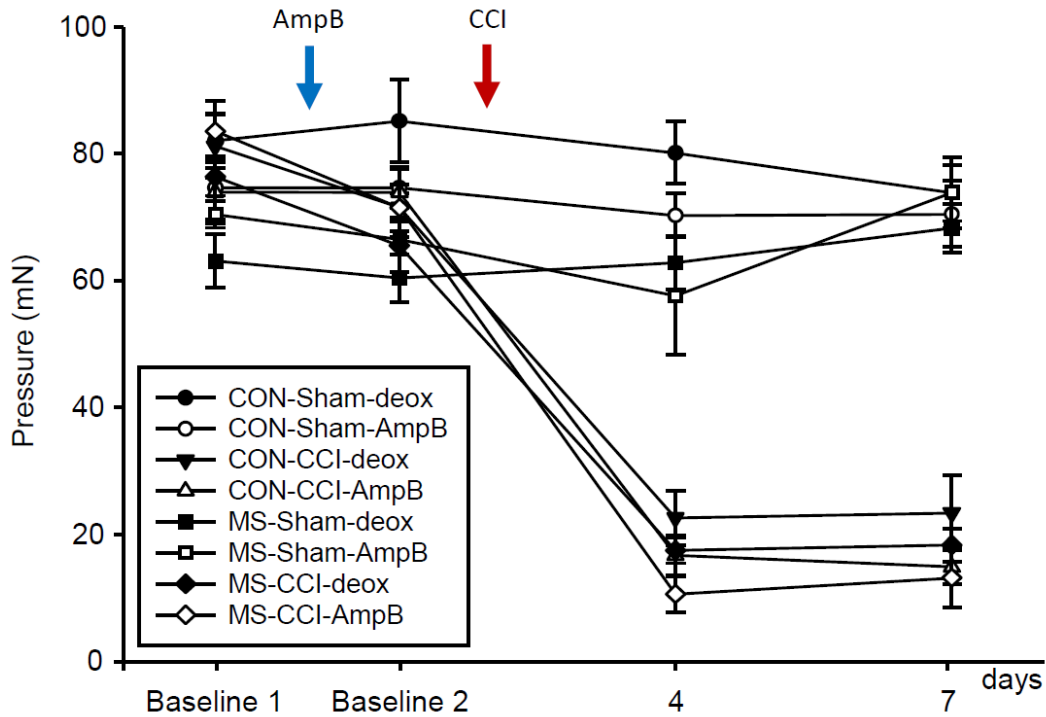


*Figure 34: Mechanical thresolds of MS and CON rats undergoing (or not) AmpB injections and/or CCI surgery. Black circle: CON-sham-deox, white circle: CON-sham-AmpB, black square: MS-sham-dox, white square: MS-sham-ampB, Black triangle: CON-CCI-deox, white triangle: CON-CCI-ampB, Black diamond: MS-CCI-deox, white diamond: MS-CCI-ampB. Data are shown as mean ± SEM.*

Mechanical pain thresholds were measured by the Von Frey test before and during the injection of amphotericin b or its vehicle (sodium deoxycholate) as well as during the 7 days of neuropathy. Regardless of the stress condition or the injection regime, sham operated animals did not see their mechanical threshold change throughout the testing period. Animals undergoing CCI surgery displayed reduced mechanical thresholds as a consequence of the CCI, however the amphotericin treatment seemed not to have a great impact.
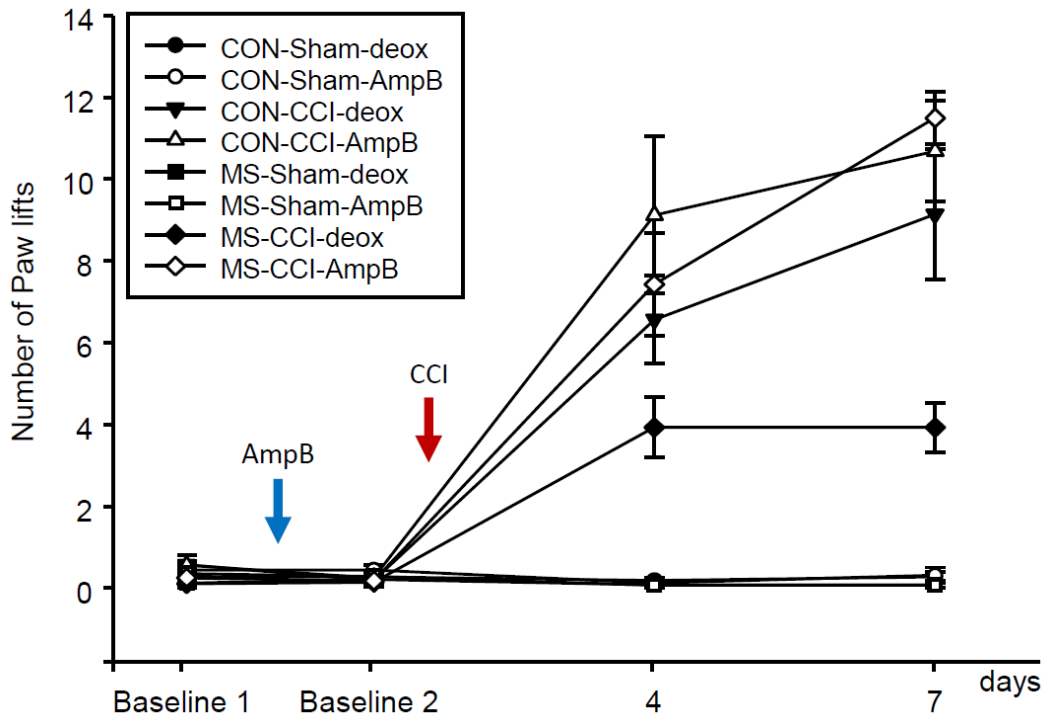
*Figure 35: Thermal (cold) sensitivity of MS and CON rats undergoing (or not) AmpB injections and/or CCI surgery. Black circle: CON-sham-deox, white circle: CON-sham-AmpB, black square: MS-sham-dox, white square: MS-sham-ampB, Black triangle: CON-CCI-deox, white triangle: CON-CCI-ampB, Black diamond: MS-CCI-deox, white diamond: MS-CCI-ampB. Data are shown as mean ± SEM.*

Sensitivity to an above threshold cold stimuli was not affected by solely stress and/or drug treatment throughout the experiments. At days 4 and 7 following CCI surgery, CON animals injected with the vehicle (sodium deoxycholate) displayed an increase of the number of paw lifts reflecting a higher sensitivity to 5°C stimulus. MS-CCI-deox animals displayed a lesser increase of paw lifts following the CCI surgery suggesting a reduced CCI-induced cold hypersensitivity. Finally the amphoterin B treatment significantly increased the sensitivity to cold of MS-CCI-ampB but not CON-CCI-ampB animals.
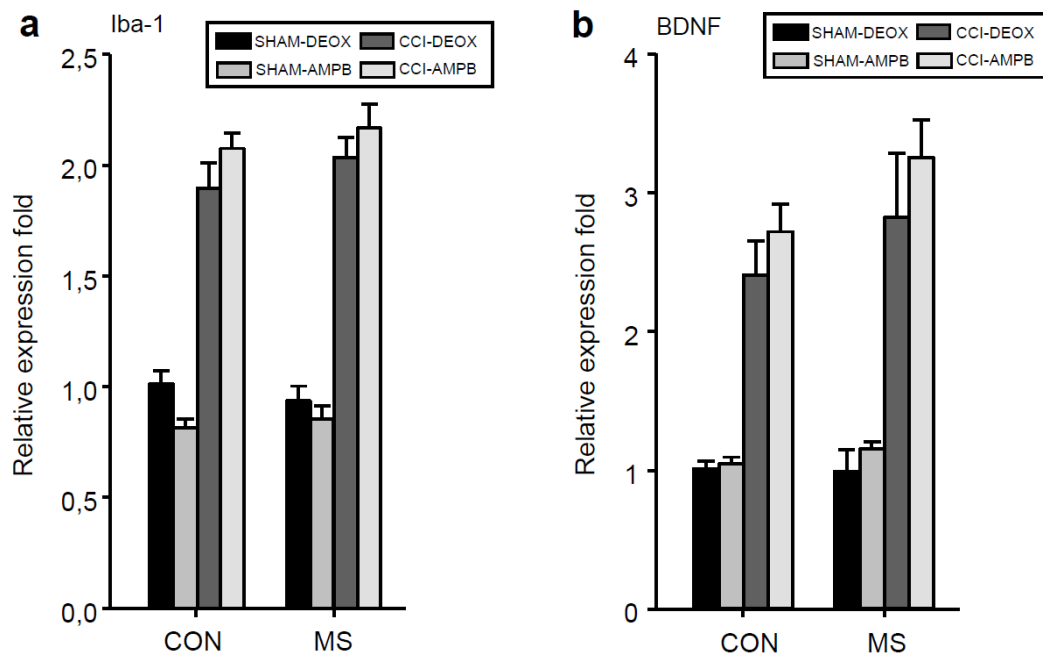
*Figure 36: Regulation of microglial cell activation and BDNF expression. Gene expression in the spinal cord was examined for the microglial marker Iba1 (A) and the growth factor BDNF (B). mRNA levels where assessed in CON and MS animals under 4 conditions (sham-deox, sham-ampB, CCI-deox, CCI-ampB). Data are shown as mean ± SEM.*

Analysis of Iba-1 and BDNF expression revealed that MS and or ampB have no impact on their own or combined. Only the induction of neuropathy was able to increase significantly Iba-1 and BDNF mRNA levels.
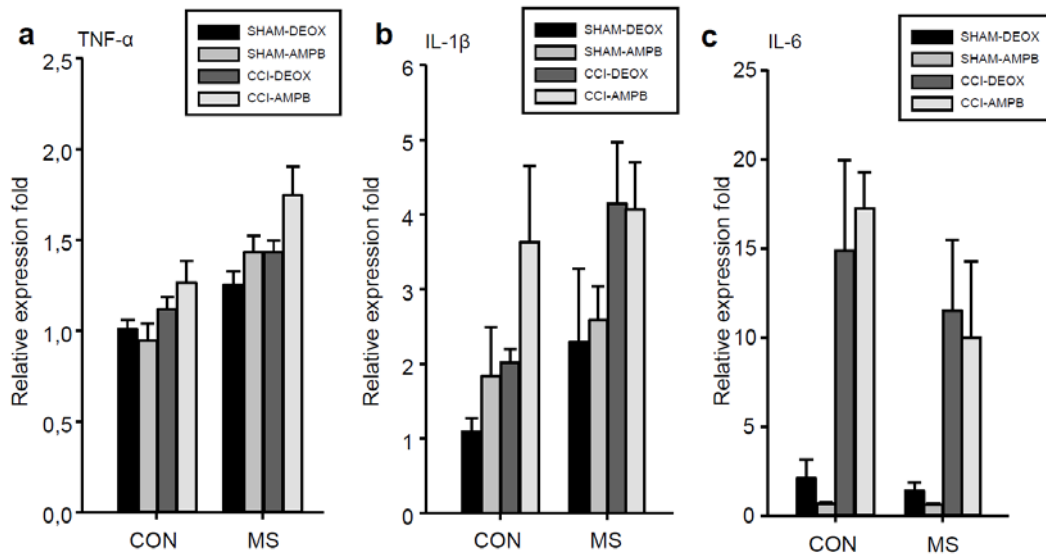
*Figure 37: Regulation of microglial cell activation and BDNF expression. Gene expression in the spinal cord was examined for the TNF-α (A), the interleukin 1ß (B) and the interleukin 6 (C).*

## 8.2. Impact of early life stress on electrophysiological response behavior of spinal nociceptive neurons and on descending pain control pathways in young adult rats.

### 8.2.1. Scientific background

In rodents, the maturation of nociceptive system components such as spinal glutamatergic transmission occurs during the first weeks of life (Bardoni et al 1998; Li et al 1998). Also the descending modulatory pain pathways that will comprise inhibitory as well as facilitatory components mature functionally during the first three weeks (Fitzgerald and Koltzenburg, 1986; Kwok et al., 2014). They are inefficient at birth, then excitatory before they gain their "normal" modulatory activity in the course of the early postnatal development. Cortical and subcortical structures such as the anterior cingulate cortex (ACC), the prefrontal cortex (PFC), the ventral tegmental area (VTA) and the nucleus accumbens (NaC), controlling emotional and cognitive processes, are tightly connected to descending modulatory pathways (Ossipov et al 2014). In this framework it may be expected that neonatal maternal separation, an emotional

and physical stressor, permanently affects descending modulation, possibly leading to alterations of normal/nociceptive (Schwaller and Fitzgerald, 2014) and neuropathic pain sensitivity (De Felice et al. 2011). Some of our experimental results (e.g. hot plate test) may suggest that higher brain center processing could be involved in the differences observed between CON and MS. Furthermore, the transition from acute to chronic pain, as seen e.g. in neuropathic pain states or chronic inflammation, involves plastic remodeling of nociceptive processing at the level of the spinal dorsal horn and at supra-spinal levels, a phenomenon called central sensitization (Kuner and Flor, 2017). Furthermore, it is now clear the balance between inhibition and facilitation existing during healthy conditions is disturbed in chronic pain and can mediate spinal central sensitization with descending facilitatory effects dominating under pathological conditions (Kim et al, 2015). However the exact process by which nerve injury or chronic inflammation lead to the enhancement of nociceptive information transmission is not yet fully understood, especially when pathologies linked to stress and pain are co-occurring. Our objective thus is to establish whether maternal separation has an impact on spinal nociceptive transmission using in vivo electrophysiology under normal and chronic pain conditions and to determine if alterations in descending modulation are involved. Then, we plan to use repetitive electrical stimulation of peripheral nociceptors to induce the wind-up phenomenon which is considered to constitute a simple form of sensitization of dorsal horn neurons. This approach will allow us to elucidate differences in spinal plasticity between rats undergoing early life stress and controls. Finally, a reversible cold block of descending pathways will cancel the potential differences in descending pain control between these two groups of animals.

### 8.2.2. Method:

This study uses the in vivo extracellular recording technique allowing us to characterize the electrophysiological response behavior of dorsal horn neurons in a dynamic manner during physiological and pathological pain conditions. In order to perform these experiments, deeply anesthetized animals undergo a surgical procedure aiming to expose their spinal cord. In order to isolate a single neuron, a stainless steel electrode is inserted and penetrates into the dorsal horn in steps ranging from 10 to 50 μm. In the same time, the ipsilateral hind paw is stimulated by light taps to find responding neurons and to assess their receptive fields. Once a neuron is detected, it can be characterized according to its response to natural stimulations (brush, pressure, and heat), its depth (determined from the surface of the dorsal horn, nociceptive specific neurons in the superficial laminae and WDR in the deeper ones), latency of firing (Aβ, Aδ and C fibers responses) and action potential wave form.

Altogether, this setup allows us to investigate spinal conduction but also dorsal horn plasticity (via the described induction of wind up-by repeated electrical stimulations) and the respective functional state of descending pathways.

### 8.2.3. Objectives

The experimental protocol we use will allow us to investigate several hypotheses in the same animal. First, we will record from dorsal horn neurons in CON and MS rats while stimulating the rat hind paw mechanically. Using a standardized forceps developed for that particular purpose, 5 different pressures will be applied (stimulus response function). This will give us first indications concerning the underlying neuronal excitability possibly accounting for prior behavioral results obtained.

Secondly, wind-up (temporal summation of discharge behavior of dorsal horn neurons, an experimental model of central sensitization), will be induced

through a train of electrical impulses (1Hz) applied to hind paw primary afferents.

Subsequently, the responses to mechanical stimuli (mechanical stimulus-response functions) are reassessed, allowing us to evaluate to what extent MS modifies spinal cord processing and plasticity during pathological conditions.

Wind-up is a short lasting state (Eide, 2000) allowing us to perform additional recordings after a "recovery period" of about 15-20 min without contamination by eventual wind-up related functional plasticity. Hence, following an appropriate resting time, we will proceed to a reversible block of nerve fiber conduction (application of cold Tyrode) at the lower cervical/upper thoracic levels of the dorsal and dorsolateral spinal cord. This will allow us to transiently eliminate descending modulatory inputs without altering nociceptive transmission at the recording site. A third mechanical stimulus-response function will be performed informing us about purely spinal nociceptive processing.

Finally, animals will again receive electrical stimulation trains in order to re-induce the wind-up phenomenon under conditions of cold block. A mechanical stimulus-response function will be performed as explained previously. The blockade of descending modulatory influences will allow us to determine their involvement in the establishment of spinal sensitization in control animals and furthermore, it will give us a clue to what extent they are modified or are modifying the spinal sensitization in MS animals.

This first set of experiment will give us a great deal of information regarding the possible alterations of spinal nociceptive transmission in MS animals. However, most of the behavioral differences regarding pain sensitivity obtained in the first studies of my Ph.D. thesis were during chronic pain conditions. Hence, after assessing the impact of MS on spinal nociceptive transmission, plasticity and descending modulatory pathways (questions 1 to 4) in normal conditions, we will use the same setting and protocols to investigate pathophysiological

(neuropathic) pain processing in animals that underwent a chronic constriction injury (CCI, a rodent model of neuropathic pain).

*"As for me, all I know is that I know nothing"*

Socrates
470-399 BC

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# Supplement

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# Chronic Social Stress Time-Dependently Affects Neuropathic Pain-Related Cold Allodynia and Leads to Altered Expression of Spinal Biochemical Mediators

*Glenn-Marie Le Coz, Julien Genty, Fernand Anton and Ulrike Hanesch\**

*Laboratory of Neurophysiology and Psychobiology, Institute for Health and Behavior, University of Luxembourg, Luxembourg, Luxembourg*

Clinical data have shown that chronic exposure to stress may be accompanied by an enhancement of inflammation-related pain sensitivity. In this context, little is however known on the impact of stress on neuropathic pain. In the present study we addressed this issue by combining the chronic constriction injury (CCI) model with an ongoing social stress (OSS) paradigm. Cold plate and von Frey tests were performed in 48 rats divided into four groups: OSS exposed to OSS, CCI subjected to chronic nerve constriction, OSS+CCI with a combination of neuropathy and stress and CON, a control group lacking any manipulation. While we did not observe any stress-related differences in mechanical sensitivity throughout the observation period, CCI rats were more sensitive to cold stimulation than OSS+CCI in the initial phase of neuropathy. A switch was observed at a later stage, leading to a hypersensitivity of the OSS+CCI compared to the CCI rats. At this time point we investigated the spinal mRNA expression of neuron and glia related molecules potentially involved in neuropathic pain and stress. The combination of psychosocial stress and neuropathic pain seemed to enhance glial cell activation, pro-inflammatory cytokine and neurotrophic factor mRNA levels, rather than glutamatergic transmission. Our data show that long lasting social stress may lead to time-dependent alteration of neuropathy-related cold pain sensitivity while mechanically-induced pain remains unchanged.

Keywords: neuropathy, social stress, cold sensitivity, mechanical sensitivity, biochemical pathways

## INTRODUCTION

While exposure to stress may lead to the classically described phenomenon of stress-induced analgesia (SIA, for review see Butler and Finn, 2009), increasing amounts of data suggest that it may under certain conditions also lead to an enhancement of pain, denominated as stress-induced hyperalgesia (SIH, see Jennings et al., 2014). In this context, research has laid a more pronounced emphasis on inflammatory as compared to neuropathic pain, a clinical entity that remains difficult to treat (Finnerup et al., 2010). In this context comorbidities

between chronic stress and pain may also have to be considered (Sharp and Harvey, 2001; Shipherd et al., 2007). Recent preclinical studies have also shown that exposure to chronic stress may lead to an exacerbation of pain sensitivity (Shi et al., 2010; Bravo et al., 2013; Burke et al., 2013).

Several biochemical pathways and mediators known to be involved in the processing of stress may also play a major role in regulating neuropathic pain. Stressors such as early life stress (Burke et al., 2013) and stress-related catecholamine release (O'Connor et al., 2003; Johnson et al., 2005) have e.g., been described to lead to neuroinflammatory reactions encompassing the peripheral and central release of pro-inflammatory cytokines like IL-1β and IL-6. These substances have been shown to play a crucial role in the enhancement of nociceptive processing (for review, see Watkins et al., 2001). Another major player in the context of the present article is glutamate, the principal excitatory neurotransmitter in the central nervous system (CNS). Glutamatergic transmission is significantly modulated by stress-related release of corticosteroids. On one hand stress may exacerbate neuropathic pain via glucocorticoid-dependent enhancement of NMDA receptor activation and glutamate release (Imbe et al., 2006; Alexander et al., 2009; Popoli et al., 2012). On the other hand it has recently been shown that corticosterone may also mediate analgesia via the spinal production of neuroactive metabolites that enhance GABAergic inhibitory transmission (Zell et al., 2015). With regard to neurotrophic factors, stress-induced release of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) is involved in the neurobiological alterations underlying increased vulnerability to disease in humans (Cirulli and Alleva, 2009); but NGF is also a critical mediator for the enhancement and maintenance of inflammatory (Woolf et al., 1994; Watson et al., 2008) and neuropathic pain (Santos et al., 2012). Recently, dynamic epigenetic regulations of the glial cell line-derived neurotrophic factor (GDNF) promoter in the nucleus accumbens (NAc) also have been shown to play important roles in determining both the susceptibility and the adaptation responses to chronic stressful events (Uchida et al., 2011). In the context of the present article, GDNF has been demonstrated to have a beneficial effect in neuropathic pain states (Boucher et al., 2000).

Stress-related activation of the HPA axis leads to the release of glucocorticoids (GC). Alterations of adrenocortical reactivity have been shown to impinge on the further coping with stress (Heim et al., 2000) as well as on nociceptive processing (Straub et al., 2002; Geiss et al., 2005; Le Coz et al., 2014a,b). In a model of persistent stress in rodents, Schmidt et al. (2007) highlighted lasting adaptations of the HPA axis that could modify the biochemical cascades described above (i.e., glial cell activation, pro-inflammatory cytokines release, neurotrophic factors and glutamatergic transmission).

The effects of stress on the processing of nociception and pain may depend on a host of factors including the kind of stress (physical or psychological), the intensity as well as the temporal characteristics (Jennings et al., 2014). In order to take into account that humans are mainly exposed to persistent or intermittent psychosocial stress in modern societies we used

a psychosocial stress paradigm initially introduced in mice by Schmidt et al. (2007) but inspired by previous models in rats (Mormède et al., 1990).

Taking into account the findings and considerations described above, we hypothesized that long lasting social stress may enhance pain sensitivity related to chronic constriction injury (CCI), a well-established model of neuropathic pain (Bennett and Xie, 1988). Matching alterations of biochemical pathways concomitantly involved in the processing of stress and pain should provide clues to potentially involved mechanisms. For the biochemical analyzes we focused on the spinal cord, which constitutes the first relay station and site of complex processing and gating of nociceptive information and contains all the mentioned stress- and pain-related mediators (Golovatscka et al., 2012).

## MATERIALS AND METHODS

### Animals
Experiments were performed in 48 male Sprague Dawley rats being 3 weeks old and weighing 48–86 g at arrival. We only used male rats to avoid estrogen-related effects on glucocorticoid release. The animals (Harlan Laboratories, Netherlands) were housed three per cage in a temperature-controlled room (20–22°C) under a 12 h day-night cycle. Food and water were provided *ad libitum*. Starting 1 week before initiation of the behavioral experiments, the animals were handled daily and habituated to the testing room and devices.

All animal experiments were carried out in accordance with the European Communities Council Directive of September 22, 2010 on the protection of animals used for scientific purposes (2010/63/EU). The animal procedures were approved by the Animal Care and Use Committee of the University of Luxembourg.

### CCI Surgery
For CCI surgery at an age of 8 weeks, the animals were anesthetized with isoflurane (4.3% for induction and 2.5% for maintenance) using an anesthesia unit (Univentor 400, Zejtun, Malta). The right sciatic nerve was exposed at the level of the thigh, as described in the classical model by Bennett and Xie (1988) and three loose ligatures using natural chromic gut 4–0 (Stoelting Europe, Dublin, Ireland) were placed around the nerve with an interspace of 1 mm. The muscle layers were stitched together with 4–0 silk sutures and the skin layer was closed with surgical skin staples.

### Experimental Protocol
The rats were divided into four groups: the control group CON undergoing no manipulation ($n = 12$), the ongoing social stress group (OSS) being exposed to the stress procedure ($n = 12$), CCI subjected to chronic constriction injury ($n = 12$), and OSS+CCI combining the CCI surgery with chronic social stress ($n = 12$). The course of the experiment was as follows (see **Figure 1**): 4 weeks before the CCI surgery in the respective groups (day 0), the chronic stress protocol was started in the OSS and OSS+CCI rats and continued until the end of the experiment at day 49.
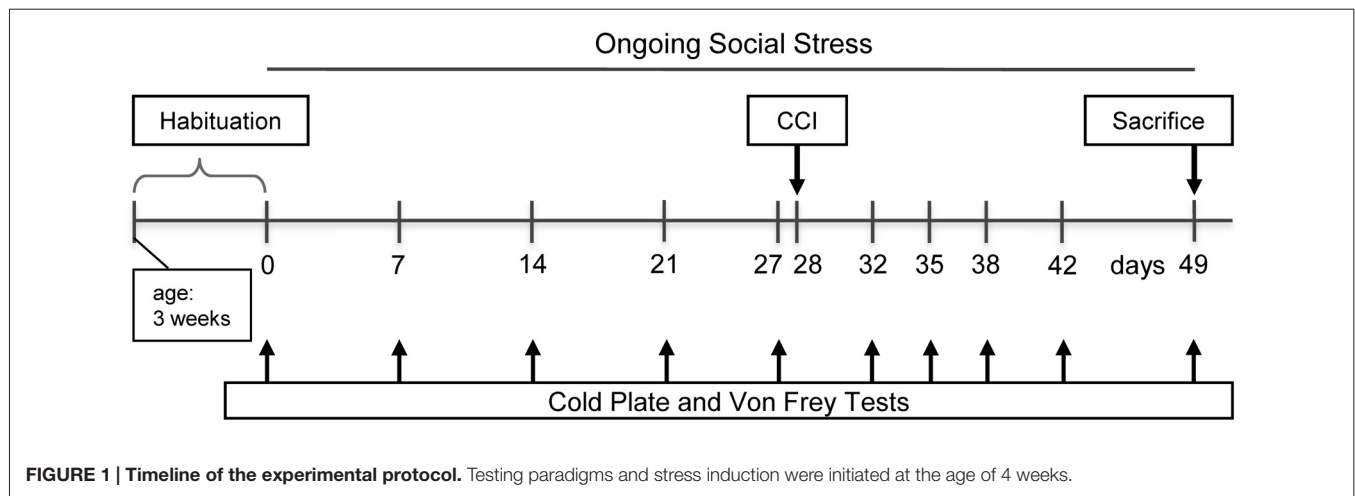
FIGURE 1 | Timeline of the experimental protocol. Testing paradigms and stress induction were initiated at the age of 4 weeks.

The social stress therefore was performed during adolescence and early adulthood in the age ranging from 4 to 11 weeks and had a total duration of 7 weeks. The cold plate and Von Frey tests were performed once a week (days 0, 7, 14, 21 and 27) in all groups in the morning hours. At day 28, the CCI and OSS+CCI rats underwent the CCI surgery. Testing for cold and mechanical sensitivity went on at days 32, 35, 38, 42 and 49 in all groups. Animals were sacrificed after the experiments at day 49 in the early afternoon and the spinal cord was removed and processed for qPCR.

## Chronic Social Stress Protocol

Rats were placed three per cage at their arrival and were enabled to habituate to this situation for 1 week before starting the stress protocol. The chronic social stress paradigm consisted in changing the composition of the adolescent cage-mates twice a week starting 4 weeks before the eventual CCI surgery until 21 days post CCI. A group size of 12 rats was chosen to avoid a recurrent composition of the cage-mates (Schmidt et al., 2007).

## Cold Plate Test

The cold plate (Hot/cold plate, Ugo Basile, Varese, Italia) was used to assess the sensitivity to thermal stimuli. A temperature of 5°C has been shown by Jasmin et al. (1998) to be optimal for testing cold hyperalgesia. Rats were placed three times on the cold plate for 3 min. The three repetitions were separated by a time out of 10 min in their respective home cages. The paw lifts were counted during the 3 min and the mean of the three sessions was calculated for each rat. Finally the mean for each group and day was calculated.

## Von Frey Monofilament Test

We used the Von Frey test to measure mechanical allodynia/hyperalgesia. Rats acclimated 10 min in Plexiglas® cages with wire mesh bottoms before the tests. Each monofilament (OptiHair, MarstockNervTest, Germany) was placed perpendicularly onto the midplantar region of the hind paw and pressure was increased until the point of deflection of

the filament was reached. The ascending and descending method of limits was applied with forces ranging from 8 mN to 256 mN in 11 logarithmic steps to determine pain thresholds (Chaplan et al., 1994). Ascending and descending series were repeated three times and the thresholds obtained in the respective series were averaged for each paw. We then referred the ipsilateral paw values to the results of the contralateral side (control) set at 100%.

## Tissue Sample and RNA Collection

At day 49 (= 21 days post surgery), rats were deeply anesthetized with isoflurane and decapitated. Levels L4/L5 of the spinal cord were removed, the right (ipsilateral) side separated from the left (contralateral) side and the total RNA was extracted with the Invitrap Spin Tissue RNA Microkit (Invitek, Germany). The RNA concentration was determined by measuring the absorbance at 260 nm, using a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

## Reverse Transcription and Real-Time qPCR

Total RNA (500 ng) was reverse transcribed into cDNA using the ImProm-II Reverse Transcription System (Promega Corporation, Madison, WI, USA). Real-time PCR reactions were performed from 10 ng of cDNA with a CFX-96 thermocycler (Bio-Rad Laboratories, Nazareth, Belgium) using SYBR green Supermix PerfeCTa (95053-02K, Quanta Biosciences, Gaithersburg, MD, USA). The primers used for the reference gene, β-actin, and the genes of interest are presented in **Table 1**.

The steps consisted of one cycle of 3 min at 95°C and 40 cycles of amplification (10 s at 95°C, 30 s at 61°C). All samples were run in triplicate. Relative expression was estimated using the $\Delta\Delta Ct$-method with β-actin as the reference. Threshold cycle values (Ct) were used to compute the amount of target gene mRNA in relation to the reference gene mRNA (β-actin). $\Delta Ct$ represents the difference between the number of cycles that were necessary to detect the PCR products of the target and the reference genes. The $\Delta\Delta Ct$ indicates the difference between the

**TABLE 1 | Sequences of primers used in this study.**

| Name | Accession | Sequence |
|---|---|---|
| b-actin | NM_031144 | 5′ GCT GAG AGG GAA ATC GTG CGT GAC 3′ |
|  |  | 5′ GGA GGA AGA GGA TGC GGC AGT GG 3′ |
| Iba1 | NM_017196 | 5′ TCC CAT CCA ACC TCT CTT CC 3′ |
|  |  | 5′ GCA GCC TCA TCG TCA TCT C 3′ |
| GFAP | NM_017009 | 5′ TGA GTC GCT GGA GGA GGA G 3′ |
|  |  | 5′ GCT GTG AGG TCT GGC TTG G 3′ |
| IL-1β | NM_031512 | 5′ GTT GAA TCT ATA CCT GTC CTG TG 3′ |
|  |  | 5′ TGG TCT TGA CTT CTA TCT TGT TG 3′ |
| IL-6 | NM_012589.1 | 5′ CCA GAG TCA TTC AGA GCA ATA C 3′ |
|  |  | 5′ CTT CTC CAT TAG GAG AGC AT 3′ |
| GDNF | NM_019139.1 | 5′ GTG TTG CTC CAC ACC GCG TCT 3′ |
|  |  | 5′ GGT CTT CGG CGG GCG CTT C 3′ |
| NGF | NM_001277055.1 | 5′ CAC GGA CAT CAA GGG CAA GGA 3′ |
|  |  | 5′ GCT CGG CAC TTG GTC TCA AA 3′ |
| EAAT3 | NM_013032.3 | 5′ TCA TAG TCG GGA AGA AC 3′ |
|  |  | 5′ AGC GGA ATG TAA CTG GAA GG 3′ |
| NR1 | NM_001270607 | 5′ GGT TGC GTG GGC AAC ACC AA 3′ |
|  |  | 5′ CCG TCC GCA TAC TTA GAA GA 3′ |
| NR2a | NM_012573.3 | 5′ CAG ATA ACA ATA AGA ACC ACA AG 3′ |
|  |  | 5′ AAC ATC GCT ACA GTC CTT 3′ |

$\Delta$Ct of the individual groups (CCI, OSS, OSS+CCI) and the $\Delta$Ct of the control (CON) animals. The data were then expressed as $2^{-\Delta\Delta CT}$. The presented data are the mean values of the ipsilateral side for each group.

## Data Analysis

Statistical analyzes for the behavioral tests (cold plate and Von Frey) were carried out using a two-way (time × condition) analysis of variance (ANOVA) followed by a Tukey's multiple comparison *post hoc* test to check for differences between groups. Data are presented as mean ± SEM. The qPCR data were analyzed by using one-way ANOVA followed by Scheffé's multiple comparison *post hoc* test. They are presented as mean ± SD. A summary of the statistical analysis is given in **Table 2**.

The level of significance was defined as $p < 0.05$. Statistical tests were performed with IBM SPSS Statistics version 21 (IBM corporation, Somers, NY, USA).

**TABLE 2 | Analysis of variance (ANOVA)—summary of *F*-values of the behavioral (two-way) and biochemical (one-way) studies.**

**Two-way ANOVA**

|  | Interaction | Time | Condition |
|---|---|---|---|
| Cold plate test | $F_{(27,425)} = 38.79$ | $F_{(9,425)} = 113.80$ | $F_{(3,425)} = 278.20$ |
| Von Frey test | $F_{(27,437)} = 40.63$ | $F_{(9,437)} = 118.06$ | $F_{(3,437)} = 360.46$ |

<div align="center">

$p < 0.0001$ for all values

</div>

**One-way ANOVA**

| | | |
|---|---|---|
| GFAP | $F_{(3,20)} = 6.00$ | $p = 0.004$ |
| Iba1 | $F_{(3,20)} = 19.65$ | $p < 0.0001$ |
| IL-1β | $F_{(3,20)} = 32.27$ | $p < 0.0001$ |
| IL-6 | $F_{(3,19)} = 16.38$ | $p < 0.0001$ |
| GDNF | $F_{(3,18)} = 35.11$ | $p < 0.0001$ |
| NGF | $F_{(3,18)} = 11.39$ | $p < 0.0001$ |
| NR1 | $F_{(3,18)} = 9.27$ | $p = 0.001$ |
| NR2a | $F_{(3,18)} = 3.25$ | $p = 0.046$ |
| EAAT3 | $F_{(3,18)} = 6.36$ | $p = 0.004$ |

# RESULTS

## Development of Cold Hypersensitivity Following CCI Surgery and Chronic Social Stress

Four weeks (day 0) before the CCI surgery in the CCI and OSS+CCI animals (performed at day 28), at the beginning of the stress paradigm, we started to assess the cold sensitivity in all groups (**Figure 2A**). The measurements were carried out once a week (days 0, 7, 14, 21, 27). Paw lifts could be observed in none of the four groups indicating that there was no effect of the social stress itself on thermal pain sensitivity in the OSS and OSS+CCI groups.

The CON and the OSS groups did not display any cold hyperalgesia throughout the observation period. The groups undergoing CCI at day 28 (CCI and OSS+CCI) developed increasing cold sensitivity throughout the next 21 days of observation. However, the extent of paw lifts differed between the two groups at the different time points. Whereas shortly after surgery (day 32) the CCI animals exhibited a statistically significant higher cold sensitivity compared to the OSS+CCI group (5.5 ± 0.6 vs. 3.5 ± 0.6; $p = 0.0052$) they were significantly less sensitive at the end of the experiment at day 49 compared to OSS+CCI (11.2 ± 0.8 vs. 14.3 ± 1.3; $p < 0.0001$). However, as can be seen in **Figure 2A**, a "switch" in sensitivity seemed to take place 7–14 days after the surgery (day 35: CCI 7.6 ± 0.7, OSS+CCI 6.1 ± 0.7; day 38: CCI 8.4 ± 1.1, OSS+CCI 8.2 ± 0.8; day 42: CCI: 8.9 ± 1.5; OSS+CCI: 9.8 ± 0.9).

Obviously, the cold sensitivity of the CCI and OSS+CCI rats was significantly higher compared to CON and also to OSS animals after the CCI surgery ($p < 0.0001$ for all time points).

## Onset and Maintenance of Mechanical Hypersensitivity Following the CCI Surgery and Stress Protocol

Throughout the observation period and as displayed in **Figure 2B**, the CON and OSS groups did not display any differences in mechanical sensitivity as compared to basal levels (values are given in **Table 3**).

The CCI and OSS+CCI groups showed a strong increase in pain sensitivity from 4 days post-surgery (day 32) until the end of the experiment on day 49. We did however not find any significant differences between these two groups.

Statistical differences were only observed from day 32 to day 49 ($p < 0.001$) between the CON and OSS groups on one hand compared to the CCI and OSS+CCI rats on the other hand.

## Impact of Ongoing Social Stress and CCI Surgery on Spinal Glial Cell Activation

We examined the spinal mRNA expression of two glial cell markers, GFAP for astrocytes and Iba1 for microglia at the end of the experiment at day 49, when the cold sensitivity was highest in CCI and OSS+CCI (**Figure 3**). We aimed to see if chronic social stress was modifying the neuropathy-mediated activation. In this respect, we focused on changes in the mRNA expression in the right spinal cord levels L4/L5 corresponding to
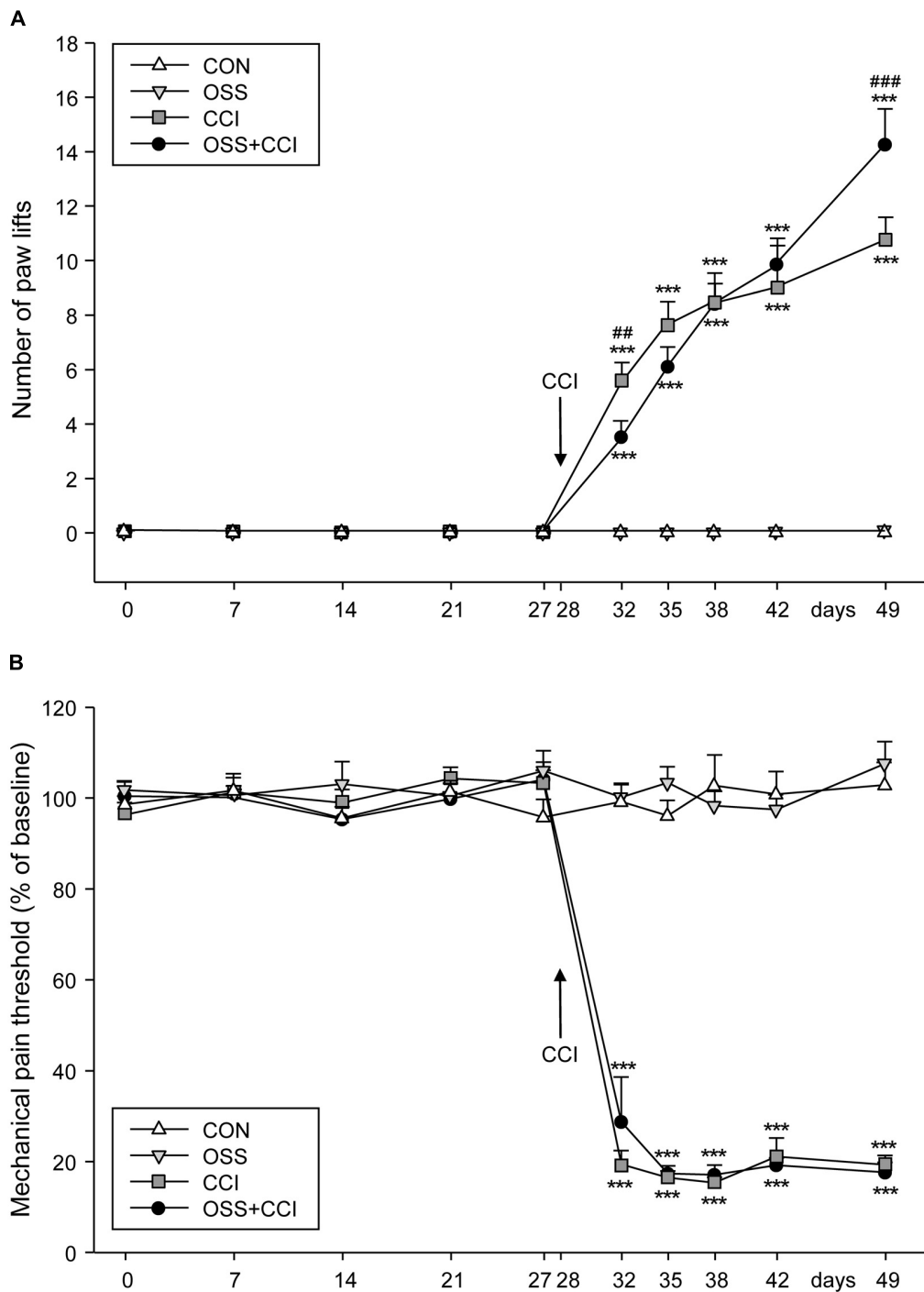
**FIGURE 2 | Effect of ongoing social stress (OSS) and chronic constriction injury (CCI) on thermal pain sensitivity measured with the cold plate test and on mechanical allodynia/hyperalgesia. (A)** The number of right paw lifts (ipsilateral to constriction injury) per 3 min is expressed as mean ± SEM per group per day. In the 28 days preceding the CCI surgery, no paw lifts were observed in the four groups (n = 12 per group): CON (no manipulation, white triangles), OSS (exposed to ongoing social stress, light gray triangles), CCI (subjected to constriction injury, dark gray squares) and OSS+CCI (constriction injury under social stress conditions, black circles). After CCI surgery at day 28, cold hypersensitivity developed in the CCI and OSS+CCI groups with CCI being more sensitive in the first days and less sensitive after 49 days post-surgery than OSS+CCI. **(B)** Paw withdrawal response was measured by the Von Frey test. The CON and OSS groups did not display any differences in pain sensitivity compared to baseline during the entire experiment. The CCI and OSS+CCI rats demonstrated an important increase of pain sensitivity following the CCI surgery but no differences were observed between these two groups at any time point. Data are shown as mean ± SEM per group per day. # represents a significant difference between the CCI and the OSS+CCI group for the individual time point ($^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$). Asterisks indicate a significant difference between the CCI or OSS+CCI group and the OSS as well as the CON group ($^{***}p < 0.001$).

**TABLE 3 | Von Frey thresholds—data presented as % of baseline for each group.**

| Group/Days | 0 | 7 | 14 | 21 | 27 | 32 | 35 | 38 | 42 | 49 |
|---|---|---|---|---|---|---|---|---|---|---|
| CON | 98.6 ± 1.8 | 101.7 ± 2.9 | 95.6 ± 2.5 | 101.4 ± 2.4 | 95.8 ± 3.8 | 99.2 ± 3.7 | 96.2 ± 3.4 | 102.8 ± 6.8 | 100.9 ± 5.0 | 102.9 ± 5.0 |
| OSS | 101.8 ± 2.0 | 100.6 ± 2.2 | 103.1 ± 4.9 | 100.5 ± 2.7 | 106.1 ± 4.4 | 100.2 ± 3.1 | 103.4 ± 3.5 | 98.3 ± 3.1 | 97.5 ± 3.3 | 107.6 ± 4.8 |
| CCI | 96.3 ± 2.7 | 101.3 ± 1.4 | 98.9 ± 3.0 | 104.3 ± 2.5 | 103.3 ± 2.5 | 19.4 ± 3.0 | 16.5 ± 1.5 | 15.4 ± 1.2 | 21.1 ± 4.1 | 19.3 ± 2.1 |
| OSS+CCI | 100.5 ± 3.0 | 100.2 ± 5.2 | 95.4 ± 3.8 | 99.9 ± 3.4 | 104.2 ± 3.7 | 28.8 ± 9.8 | 17.4 ± 1.6 | 17.2 ± 2.1 | 19.3 ± 1.6 | 17.7 ± 2.1 |



**FIGURE 3 | Influence of social stress and CCI on the mRNA expression of markers for the activation of astrocytes (GFAP) and microglia (Iba1) in the ipsilateral spinal cord levels L4/L5 at day 49.** Data are expressed as relative expression level (fold) of CON (control group = 1, white column) and are shown as mean ± SD ($n$ = 6 per group). **Left**: GFAP mRNA expression was increased only in the groups subjected to chronic social stress: OSS (light gray column) and OSS+CCI (constriction injury under social stress conditions, black column), but not in the group that solely underwent CCI (dark gray column). **Right**: Iba1 mRNA expression was increased in the group subjected to constriction injury (CCI) and in the OSS+CCI group where stress enhanced the effect of the constriction injury. No effect was noticeable in the stress group OSS. *$p$ < 0.05, **$p$ < 0.01, ***$p$ < 0.001.

## Spinal mRNA Expression of the Pro-Inflammatory Cytokines IL-1β and IL-6 Following CCI and Chronic Social Stress

In this experiment, we observed a slight, but not significant increase in the spinal mRNA expression of IL-1β in the stress (1.81 ± 0.42; $p$ = 0.068) and CCI (1.47 ± 0.58; $p$ = 0.503) groups (**Figure 4**). The combination of CCI and OSS however ended up with a pronounced and highly significant upregulation of this pro-inflammatory cytokine (3.47 ± 0.45; $p$ < 0.001). Furthermore there was a statistically significant increase of IL-1β mRNA in the OSS+CCI group as compared to OSS ($p$ < 0.001) and CCI ($p$ < 0.001).

Regarding IL-6, we did not find any impact of the chronic social stress paradigm on the spinal mRNA expression (0.97 ± 0.25; $p$ = 1.00; **Figure 4**). In contrast, the expression was highly upregulated following CCI surgery (8.63 ± 3.42; $p$ < 0.001). Combination of constriction injury with social stress did not further enhance the expression level (7.26 ± 3.45; $p$ = 0.003). Additionally, we found statistically significant differences between OSS and CCI ($p$ < 0.001) as well as between OSS and OSS+CCI ($p$ = 0.002) but not for CCI vs. OSS+CCI ($p$ = 0.82).

the side of CCI surgery. For all mRNA expression experiments, the ipsilateral spinal cord of the CON group served as control (relative expression level = 1) and the expression levels of the three treatment groups were expressed as fold of CON. For statistical analysis, we compared the expression levels of the experimental groups OSS, CCI and OSS+CCI to CON and the obtained $p$-values are given together with the respective means.
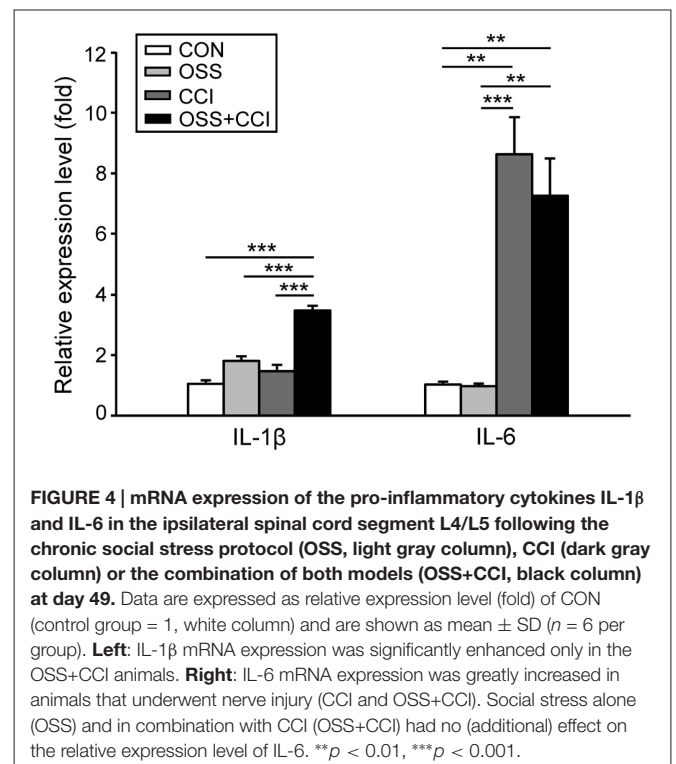
Regarding the activation of astrocytes, there was a significant upregulation in the OSS group (1.49 ± 0.39; $p$ = 0.050), a small but not significant increase in the CCI rats (1.35 ± 0.28; $p$ = 0.237) and an even more distinct increase in the OSS+CCI animals (1.66 ± 0.12; $p$ = 0.006). These data exhibit an amplification of CCI-mediated astrocyte activation under conditions of OSS.

In the case of microglial activation we observed a different pattern. The stress protocol alone did not impact the spinal Iba1 mRNA expression (1.3 ± 0.21; $p$ = 0.434) while CCI led to a significant activation of microglia (1.66 ± 0.41; $p$ = 0.008). CCI injury under conditions of stress in the OSS+CCI group, however, amplified the mRNA expression of the microglial marker (2.20 ± 0.25; $p$ < 0.001). Statistical analysis revealed also significant differences between OSS and OSS+CCI ($p$ < 0.001) as well as between CCI and OSS+CCI ($p$ = 0.03).



**FIGURE 4 | mRNA expression of the pro-inflammatory cytokines IL-1β and IL-6 in the ipsilateral spinal cord segment L4/L5 following the chronic social stress protocol (OSS, light gray column), CCI (dark gray column) or the combination of both models (OSS+CCI, black column) at day 49.** Data are expressed as relative expression level (fold) of CON (control group = 1, white column) and are shown as mean ± SD ($n$ = 6 per group). **Left**: IL-1β mRNA expression was significantly enhanced only in the OSS+CCI animals. **Right**: IL-6 mRNA expression was greatly increased in animals that underwent nerve injury (CCI and OSS+CCI). Social stress alone (OSS) and in combination with CCI (OSS+CCI) had no (additional) effect on the relative expression level of IL-6. **$p$ < 0.01, ***$p$ < 0.001.

## Effect of CCI and Chronic Social Stress on the mRNA Expression of the Neurotrophic Factors GDNF and NGF

Chronic social stress during adolescence/early adulthood slightly but not significantly increased the spinal expression level of GDNF mRNA ($1.42 \pm 0.29$; $p = 0.139$; **Figure 5**), whereas CCI surgery significantly decreased the mRNA level ($0.58 \pm 0.21$; $p < 0.001$). The stress component in the OSS+CCI group greatly outweighed the decreasing effect of the constriction injury and finally led to a two-fold increase ($2.06 \pm 0.32$; $p < 0.001$) in the mRNA expression level of GDNF. Statistical analysis also unravelled significant differences between OSS and CCI ($p < 0.001$), OSS and OSS+CCI ($p = 0.004$), as well as between CCI and OSS+CCI ($p < 0.001$).

The expression of spinal NGF mRNA was unaffected by the chronic social stress procedure ($1.30 \pm 0.32$; $p = 0.515$) and by the CCI surgery ($0.91 \pm 0.11$; $p = 0.967$; **Figure 5**). However, the combination of both manipulations resulted in a significant upregulation of the NGF mRNA ($1.85 \pm 0.45$; $p = 0.004$). The expression level was also significantly different from OSS ($p = 0.044$) and CCI ($p < 0.001$).

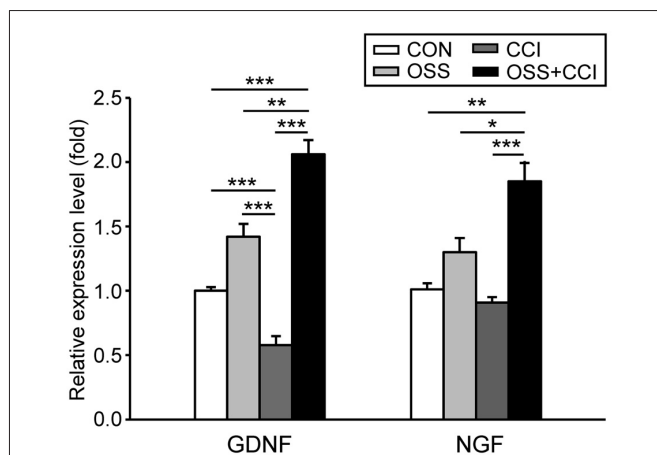## Implication of the Glutamatergic System in CCI and Chronic Social Stress Mechanisms

To investigate the involvement of the glutamatergic system in the development and maintenance of neuropathic pain and
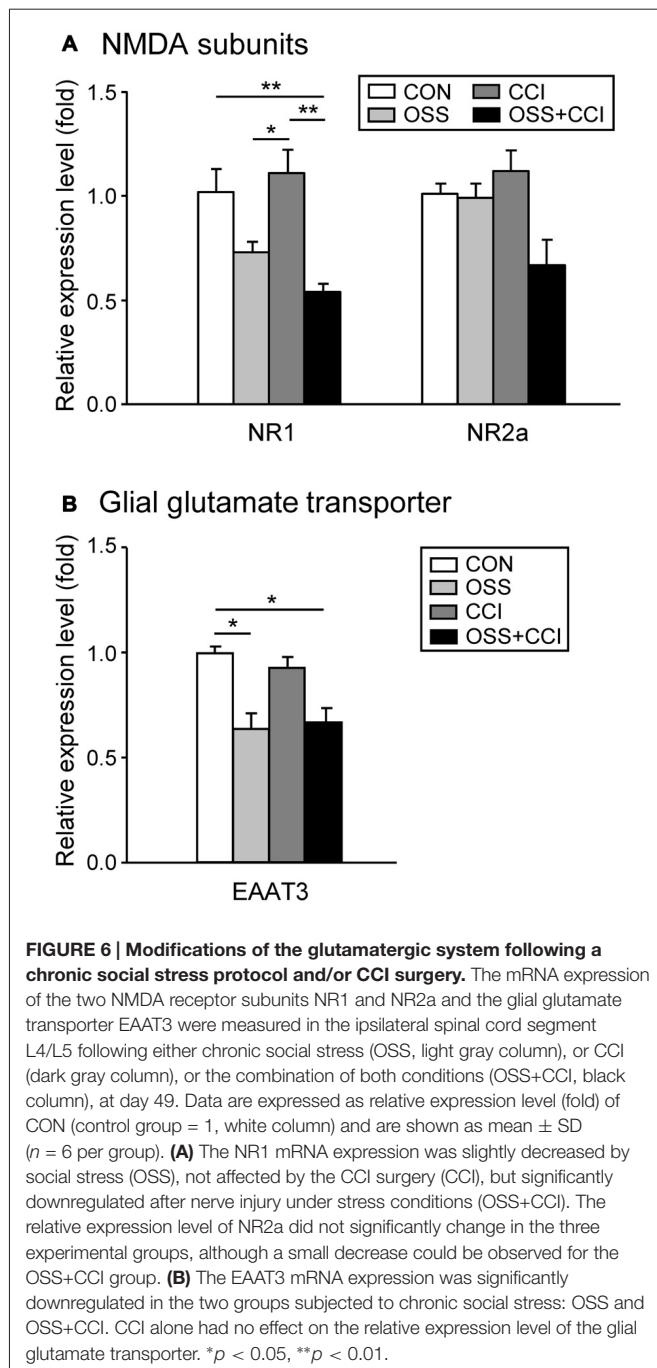


**FIGURE 5 | Effect of chronic social stress and nerve injury on the mRNA expression of the neurotrophic factors glial cell line–derived neurotrophic factor (GDNF) and nerve growth factor (NGF) in the ipsilateral spinal cord level L4/L5 at day 49.** Data are expressed as relative expression level (fold) of CON (control group = 1, white column) and are shown as mean ± SD ($n = 6$ per group). **Left**: GDNF mRNA expression was slightly but not significantly increased in the group subjected to OSS (light gray column) and significantly downregulated after nerve injury (CCI, dark gray column). However, after CCI under conditions of social stress (OSS+CCI, black column) the relative expression levels of GDNF mRNA were significantly upregulated. **Right**: NGF mRNA expression did neither change after exposure to chronic social stress (OSS) nor following CCI surgery (CCI). Nerve injury under chronic social stress (OSS+CCI) did however lead to a significant upregulation of NGF mRNA expression. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

in the processing of chronic social stress, we measured the mRNA expression of NR1 and NR2a, two common subunits of the NMDA receptor and of EAAT3, a glial transporter of glutamate.

The spinal NR1 mRNA expression was slightly, but not significantly decreased in the OSS group ($0.73 \pm 0.14$; $p = 0.243$; **Figure 6A**). The CCI surgery alone had no effect on NR1 expression levels ($1.11 \pm 0.31$; $p = 0.908$) but induction of neuropathy under stress conditions caused a significant downregulation of NR1 mRNA transcription ($0.54 \pm 0.10$; $p = 0.020$) exceeding the decrease related to stress alone. This reduction in the OSS+CCI group turned out to be also statistically significant compared to CCI ($p = 0.002$). Although the expression level in the OSS group was not significantly different from the CON, it differed from the CCI group ($p = 0.039$).

Regarding the NR2a subunit of the NMDA receptor, we did not find any significant alteration in the mRNA expression, although a small decrease was apparent for the OSS+CCI group.

OSS significantly downregulated the spinal mRNA expression of EAAT3 ($0.64 \pm 0.21$; $p = 0.046$; **Figure 6B**). On the other hand, neuropathy had no impact on the regulation of this transporter ($0.93 \pm 0.13$; $p = 0.954$). In the OSS+CCI group the impact of the social stress predominated and led likewise to a significant downregulation of EAAT3 mRNA in the ipsilateral spinal cord level L4/L5 ($0.62 \pm 0.21$; $p = 0.033$). Glutamate might hence have accumulated in the synaptic cleft, reinforcing synaptic transmission.

## DISCUSSION

In the present study the major finding resulting from the combination of preclinical models of neuropathic pain and psychosocial stress was a switch in cold pain sensitivity from initially dampened to exacerbated allodynia. For mechanical sensitivity no differences were observed between stressed and non-stressed neuropathic rats. Biochemical results revealed an activation of astrocytes in a stress condition but also of microglia under conditions of combined stress and neuropathic pain, a differential regulation of pro-inflammatory cytokine mRNA, IL-1β being more prone to the combination of stress and neuropathy while the expression of IL-6 was mainly enhanced in neuropathic conditions, a synergistic effect of stress and neuropathy regarding the expression of neurotrophic factor mRNA, and finally a decrease of the transporter EAAT3 and NMDA receptor subunit NR1 in the stress group and in the group exposed to both stress and chronic constriction injury.

### Selection of the Chronic Social Stress Model

As mentioned in the introduction, our goal was to focus on the impact of psychosocial stress on neuropathic pain sensitivity. While some studies using psychological stressors observed an enhancement of pain sensitivity (Rivat et al., 2007; Burke et al., 2013), others failed to identify similar effects (Bravo et al., 2012, 2013). These divergent findings may partly be related

**FIGURE 6 | Modifications of the glutamatergic system following a chronic social stress protocol and/or CCI surgery.** The mRNA expression of the two NMDA receptor subunits NR1 and NR2a and the glial glutamate transporter EAAT3 were measured in the ipsilateral spinal cord segment L4/L5 following either chronic social stress (OSS, light gray column), or CCI (dark gray column), or the combination of both conditions (OSS+CCI, black column), at day 49. Data are expressed as relative expression level (fold) of CON (control group = 1, white column) and are shown as mean ± SD ($n$ = 6 per group). **(A)** The NR1 mRNA expression was slightly decreased by social stress (OSS), not affected by the CCI surgery (CCI), but significantly downregulated after nerve injury under stress conditions (OSS+CCI). The relative expression level of NR2a did not significantly change in the three experimental groups, although a small decrease could be observed for the OSS+CCI group. **(B)** The EAAT3 mRNA expression was significantly downregulated in the two groups subjected to chronic social stress: OSS and OSS+CCI. CCI alone had no effect on the relative expression level of the glial glutamate transporter. *$p < 0.05$, **$p < 0.01$.

to methodological differences with respect to the approaches chosen for stress induction.

In the present study, we did therefore aim at using a rodent model that would as closely as possible match ongoing psychosocial stress situations that humans are commonly exposed to. The model we chose was introduced in mice by Schmidt et al. (2007) and derived from a model initially developed in rats by Mormède et al. (1990). Briefly, the group composition of male rat cage mates was changed in regular intervals starting at the age of 4 weeks until the end of the

experimental protocol at 11 weeks. The stress hence started during the adolescence period, meaning in puberty, a period of life that is highly adaptive and during which substantial remodeling occurs in areas involved in emotional and learning processes (Tsoory et al., 2007). Furthermore, this period is also of much importance regarding social interaction and acquisition of social skills that will induce stability in adulthood (Sachser et al., 1998). Hence stressing the animals in this precise period when the behavior and neuroendocrine system are fine-tuned can have important repercussions in adulthood. Finally, contrary to many stress models used in the literature, this ongoing social stress is continuous (most of the stressors are only applied during some time of the day), inescapable for the animal, applicable to a large number of animals in order to have an adequate rotation schedule and less offensive than most other models such as social defeat or chronic unpredictable stress.

In order to have objective markers of the impact of stress we measured plasmatic corticosterone levels once a week during all the experiment. However we were not able to demonstrate any statistical differences between the groups (data not shown). This is in accordance with several studies (Norman et al., 2010b; Shi et al., 2010) and can be explained by an adaptation of the HPA axis over time. Regarding the body weight of the animals also, it is known that chronic stress can induce a reduction (Shi et al., 2010; Bravo et al., 2012). We did however not observe any differences between the four groups. In this respect Schmidt et al. (2007) have claimed that the animals have a different weight regulation during the adolescent period where the stress paradigm has been started and that during this period the regulation of growth is less influenced by environmental factors. In addition adolescent animals might compensate more easily for weight loss than adult animals.

Finally, we only used male rats. It is however established that sex can have an influence on pain sensitivity (for review, see Mogil, 2012) but also on stress impact (Burke et al., 2013). We did not observe any stress model-related differences in mechanical sensitivity for the male rats we used, in accordance with Burke et al. (2013) who did however observe a reduction in mechanical pain threshold and a sensitization of the contralateral paw in female Wistar rats. These findings point to the importance of considering sex-related effects.

## Development and Maintenance of Cold and Mechanical Hypersensitivity

Regarding the assessment of pain sensitivity, the cold plate test has been shown to reliably reveal cold hypersensitivity, a characteristic of CCI-induced neuropathic pain (Berrocoso et al., 2011). We chose this paradigm because exacerbated cold sensitivity is more often observed than heat hypersensitivity in clinical neuropathic conditions (Attal et al., 2008). The test was performed regularly from the beginning of the stress protocol (day 0) to 21 days after the eventual CCI surgery (day 49). Before CCI, no paw lifting was observed for any group, confirming that we observed neuropathy-related allodynia. CCI and OSS+CCI rats started to display cold plate related pain behavior a few days post-surgery, with the stress seeming to have a protective effect in the OSS+CCI group as compared to the CCI group.

Acute and chronic stressors may differentially affect pain. Under acute conditions, "stress-induced analgesia" (SIA) is commonly observed (for review, see Amit and Galina, 1998) while chronic stress may more regularly be accompanied by hyperalgesic states (Suarez-Roca et al., 2006; Jennings et al., 2014). Our results from days 32 and 35 could reflect SIA. At day 38, the two groups had equivalent sensitivities when finally, at day 49, we observed a switch, the OSS+CCI rats displaying a hypersensitivity to cold as compared to the CCI group. This phase could correspond to the SIH phenomenon that to our knowledge has not yet been described for neuropathic pain conditions. While these results may seem contradictory to observations by Aghajani et al. (2012) who did not detect any impact of a social instability model on chronic pain in mice, other studies using cold stress (Imbe et al., 2006) or social defeat (Marcinkiewcz et al., 2009) demonstrated increased nociceptive responses. Other stress paradigms such as repeated swim stress (Suarez-Roca et al., 2006; Quintero et al., 2011) or chronic restraint stress (da Silva Torres et al., 2003; Bardin et al., 2009) also ended up with a long lasting hyperalgesia. It should however be kept in mind that these physical stressors may not match our conditions of ongoing social stress. Ultimately, the described reversion in behavioral sensitivity came quite as a surprise to us at this time point and experiments using a longer observation period than the initially scheduled 49 days are required to confirm the significance of this effect.

Concerning our failure to identify differences in mechanical sensitivity between CCI and OSS+CCI groups, it is interesting to note that Bardin et al. (2009) as well as Bravo et al. (2012, 2013) reported similar findings in a model of chronic mild stress and in a model of social isolation. Whereas the temperature of the cold plate test (5°C) used in the present study quite selectively stimulates cold nociceptors of the C-fiber type (Simone and Kajander, 1996), the von Frey filaments may activate a larger range of nociceptors belonging both to the C- and A-delta fiber categories (Martin et al., 1997). In the framework of the present study, it could hence be speculated that the ongoing psychosocial stress primarily affected the processing of C-fiber rather than A-delta fiber mediated nociceptive processing. In line with this reasoning, there is evidence for an interaction of stress hormones with nociceptive C fibers (Chen and Levine, 2005). Selective pharmacological inhibition of C- but not A-delta fiber-mediated nociception has recently been described by You et al. (2009). Alternatively, stress-mediated effects may have depended on factors like stimulus modality, sensory transduction, stimulation surface and duration. It should also be noted that for both stimulation modalities we did not observe any neuropathic pain-related effects at the contralateral paw, findings that are congruent with earlier studies (Pitcher and Henry, 2004; Benbouzid et al., 2008; Zeng et al., 2008).

## Glial Cell Activation and Pro-Inflammatory Cytokine Expression

Regarding astrocytes and microglia, we observed different patterns of activation pointing to distinct roles in the mediation of pain and stress signals. In agreement with previous investigators (Lambert et al., 2000), we noticed a stress-related enhancement of GFAP mRNA expression. This expression was similar in the OSS+CCI group, indicating that astrocyte activation might primarily have depended on stress- rather than on neuropathy-related mechanisms. While the CCI group displayed an activation of microglia, the greatest increase in mRNA expression was observed for OSS+CCI. The finding that microglial activation is usually accompanied by a behavioral hypersensitivity (Watkins et al., 2001; Zhuo et al., 2011) is in accordance with the OSS+CCI group expressing more pronounced cold hyperalgesia at day 49.

Microglial and astrocytic activation induces the release of pro-inflammatory cytokines (for review, see Watkins et al., 2001). This is in agreement with the enhanced expression of IL-1β mRNA noticed in the OSS+CCI group. In addition, psychosocial stress-related microglia activation has previously been observed in different brain areas such as the hippocampus, amygdala and prefrontal cortex, leading to the production of IL-1β (Wohleb et al., 2011; Hinwood et al., 2012). Also, in a model of isolation stress combined with a model of cardiac arrest surgery (Norman et al., 2010b), and under conditions of maternal deprivation combined with spinal nerve ligation (SNL) neuropathy model (Burke et al., 2013), mRNA for Il-1β was shown to be upregulated in the hippocampus. The expression of IL-6 mRNA was significantly increased to a similar extent in the CCI and OSS+CCI groups and thus seemed to be selectively implicated in inflammatory processes inherent to CCI-related neuropathic pain. Our finding is in accordance with hippocampus-related data from Burke et al. (2013) but contradictory to previous results describing a stress-mediated release of this cytokine in mice (Norman et al., 2010a; Aghajani et al., 2012). This could be due to the use of different stress paradigms or to species differences. The fact that in our hands IL-1β mRNA expression was most pronounced in the OSS+CCI rats may point to a combined induction by stress- and pain-related mechanisms.

## Implication of Neurotrophic Factors

Our major observation concerning the spinal mRNA expression pattern of NGF and GDNF was that stress led to enhanced levels, while a diminished expression was exhibited by the CCI group. In the OSS+CCI rats, the respective expression changed into the same direction as for the stress group, albeit to a more pronounced extent.

GDNF is involved in cell survival, differentiation and migration. Its serum level is reduced in patients with major depression (Miller, 2011) and decreased in the dorsal root ganglia (DRG) after nerve injury (Nagano et al., 2003). It has also been demonstrated that GDNF has a beneficial effect on neuropathic pain. A continuous administration ameliorated pain responses and suppressed pain behavior following SNL or CCI (Boucher et al., 2000; Nagano et al., 2003; Chou et al., 2014). These data are in accordance with our observations in the CCI group. The most important increase noticed in the OSS+CCI group at day 49, when the respective animals were hypersensitive to the cold

plate, seems to indicate that GDNF could have an additional stress-related function that could be more pronounced than the neuropathy-related effects *per se*. In addition, an increase in GDNF could result from the activation of astrocytes (Bian et al., 2012). This observation could corroborate our data, the GFAP marker mRNA displaying the same pattern of expression as GDNF.

Regarding NGF, controversial findings have been described. While some studies demonstrated a stress-related decrease of NGF mRNA expression in DRG or hippocampus (Nagano et al., 2003; Alfonso et al., 2004), others revealed an increase under such conditions (for review, see Miller, 2011). On the other hand, NGF has been shown to be a major contributor to the production of inflammatory hyperalgesia and maintenance of neuropathic pain (Matsuura et al., 2013). An increased expression has been observed in a CCI model of neuropathic pain (Nagano et al., 2003; Santos et al., 2012) and a single injection of NGF has been shown to induce hyperalgesia (Lewin et al., 1993). In the present study we observed almost no change in the mRNA expression of NGF in the CCI group. We did however find an increase in the OSS group that was even more pronounced in the OSS+CCI group. In our setting, NGF could hence have been more implicated in stress mechanisms than in pain processing itself. The fact that we observed comparable bilateral patterns of mRNA expression for GDNF and NGF (data not shown) seems to support this hypothesis.

Since it has also been shown that the pro-nociceptive effect of NGF is blocked by MK-801, an NMDA receptor antagonist (Herzberg et al., 1997), we investigated the implication of glutamatergic transmission via the mRNA expression of a glutamate transporter and two subunits of the NMDA receptor.

## Involvement of the Glutamatergic System

Regarding the glutamate transporter EAAT3 and the NR1 subunit of the NMDA receptor, comparable patterns of mRNA expression, namely a downregulation, were noticed in the OSS and OSS+CCI groups.

It has been shown that the glutamatergic system has a predominant implication in the relationship between the HPA axis, activated by stress mechanisms, and neuropathic pain (Le Coz et al., 2014a). The NMDA receptor may be implicated both in neuropathic pain and in stress mechanisms (Gould and Tanapat, 1999). Repeated cold stress leads to an enhanced sensitivity of the NMDA receptor and to a facilitation of glutamate release in the spinal cord and hippocampus (Imbe et al., 2006; Quintero et al., 2011). This is also true in conditions of neuropathic pain (Wang et al., 2005). We did however observe the opposite effect for OSS and no change for CCI. Since the mRNA expression of NR1 was even more decreased in the OSS+CCI rats, the stress-related mechanisms might have overruled the nociception-related ones.

To our knowledge, there are no published data on transporter expression in a context of psychosocial chronic stress. Our observations seem to indicate a more important impact of the chronic social stress than of the CCI procedure on the spinal expression of EAAT3 mRNA. One study showed that a repeated

restraint stress enhanced glutamate release and uptake (Imbe et al., 2006). This increase can be interpreted as an augmentation in the expression of transporters, but in the present study we observed the opposite effect regarding EAAT3. Gosselin et al. (2010) also observed a decrease in glutamate transporter (EAAT1) in the spinal astrocytes of rats undergoing maternal separation. These discrepancies may be related to the use of different stress paradigms.

As previously described, a link is possible between the neurotrophic factor NGF and the NMDA receptor. An activation of NMDA receptors will induce an increase of NGF expression (Herzberg et al., 1997). In the OSS+CCI group we did however observe an increase of the NGF mRNA expression and a decrease of the NR1 subunit of the NMDA receptor. Also, we did not detect any differences regarding the mRNA expression of the NR2a subunit of the NMDA receptor. These discrepancies may be due to the stress and pain models used in the present study. Other subunits that could be involved should be considered in further investigations.

## Concluding Remarks

Our exploratory study confirms that exposure to long lasting social stress starting during adolescence may enhance neuropathic pain-related cold sensitivity in adulthood. At the level of the spinal cord, the combination of psychosocial stress and neuropathic pain may have an important incidence on glial cell activation, on the release of pro-inflammatory cytokines and of neurotrophic factors and to a lesser extent on glutamatergic transmission. Further studies will have to include protein level measurements. For correlative purposes, the observation period should be extended and the measurements of biochemical markers should include earlier time points preceding and including the observed switch of pain sensitivity. The potential role of ongoing stress on descending pain control pathways will also have to be considered.

## AUTHOR CONTRIBUTIONS

## FUNDING

## ACKNOWLEDGMENTS

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