Universidade do Minho
Escola de Ciências da Saúde

Hugo Miguel do Vale Leite Santos de Almeida

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Tese de Doutoramento em Ciências da Saúde
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TÍTULO TESE: Impact of chronic pain in emotional and cognitive behaviour in the rat: the effects of age and lateralization

ORIENTADORES: Professor Doutor Armando Almeida e Professor Doutor Nuno Sousa

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"Illness is the doctor to whom we pay most heed: to kindness, to knowledge we make promises only: pain we obey"

Marcel Proust *in Le temps retrouvé*

Aos meus avós...

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Abstract

Multiple areas along the neuroaxis mediate pain modulation and perception. Dysfunction at any of these stations can have devastating consequences that result in pain chronification and in the surfacing of emotional and cognitive disturbances. In the past decade, it was demonstrated that these comorbidities frequently associated with chronic pain also manifest in the animal model, paving the way for further research on the underling pathophysiology. However, conflicting observations have been published probably as a result of experimental heterogeneity. This prompted us to further characterize emotional and cognitive behaviour alterations in a rodent model of chronic neuropathic pain – the spared nerve injury (SNI).

In the first set of experiments, the effect of chronic neuropathic pain was studied in the context of ageing. We observed an age-related increase of anxiety-like behaviour in the elevated-plus maze (EPM), which was further augmented in young and old males, but not mid-aged SNI animals. On the contrary, only SNI mid-aged animals had a depressive-like phenotype in the forced-swimming test (FST) when compared to age-matched controls. SNI mid-aged animals had also an impaired ability to perform a reversal learning task when compared with the respective age-matched controls. In this task, the SNI lesion affected neither young nor old groups, although in the last, controls themselves had a poorer performance. In fact, in this cognitive domain ageing was a major determinant of incapacity. Ageing was also demonstrated to have a mild negative influence in the performance of a spatial working memory (WM) but not in a long-term spatial memory – Morris water maze (MWM). The SNI lesion had no observable effect in both WM and MWM.

In the second set of experiments, the effect of pain lateralization was accessed in young rats. Left-sided (but not right-sided) SNI was shown to be anxiogenic in the EPM. On the contrary, right-sided (but not left-sided) SNI was detrimental in all prefrontal cortex (PFC)-dependent cognitive paradigms, namely WM, reversal learning (in the attentional-set shifting task; ASST) and response inhibition (impulsivity). Neither right- nor left-side SNI affected the performance in the MWM.

These observations indicate that both the age of the animal at the pain onset as well as the location of pain are determinant in the behavioural outcome on emotional and cognitive paradigms. Additionally, our behavioural observations suggest that the PFC has a major role in the observed emotional and cognitive shifts occurring after SNI installation.

Resumo

A modulação e percepção da dor dependem da contribuição de várias áreas ao longo do neuro-eixo. A disfunção deste eixo pode resultar em consequências devastadoras que incluem a cronificação da dor e a manifestação de perturbações emocionais e cognitivas. Na última década, demonstrou-se que estas comorbilidades associadas a estados de dor crónica também se manifestavam no modelo animal, o que abriu novas perspectivas de investigação sobre os mecanismos subjacentes. No entanto, dados contraditórios têm vindo a ser publicados quanto à natureza destas manifestações. Este cenário esteve na base dos estudos desta tese, que pretenderam aprofundar a caracterização das alterações do comportamento emocional e cognitivo num modelo de dor crónica neuropática – o modelo SNI (*spared nerve injury*).

No primeiro conjunto de experiências, o efeito da dor crónica foi estudado no contexto do envelhecimento. Com o envelhecimento observamos um aumento do comportamento do tipo ansioso no paradigma *elevated-plus maze* (EPM) e que este efeito era potenciado nos animais novos e velhos submetidos à lesão neuropática SNI. Pelo contrário, apenas o grupo de meia-idade submetido ao SNI apresentava um comportamento do tipo depressivo no teste do *forced-swimming* (FST) quando comparado com o respectivo controlo da mesma idade. O mesmo grupo SNI de meia-idade mostrou também uma capacidade reduzida de aprendizagem reversa, o que não se verificava nos grupos de animais mais novos ou mais velhos embora, nestes últimos, os próprios controlos tenham tido um mau desempenho. De facto, neste paradigma o aumento da idade revelou-se um factor determinante de insucesso. O envelhecimento estava também associado a uma deterioração do desempenho numa tarefa de memória de trabalho (WM; *working memory*) não havendo no entanto qualquer influência na tarefa de memória de longo prazo (MWM; *Morris water maze*). A lesão SNI não teve qualquer influência no desempenho destes paradigmas pelos animais dos diferentes grupos etários.

No segundo conjunto de estudos, avaliou-se o efeito da lateralidade da dor em animais jovens. A lesão SNI esquerda, (mas não a direita), resultou no aumento do comportamento do tipo

ansioso no EPM. Pelo contrário, a lesão SNI direita, (mas não a esquerda), induziu um pior desempenho nos paradigmas de comportamento cognitivo, nomeadamente em todos aqueles com um componente prefrontal (*prefrontal cortex*; PFC): WM, aprendizagem reversa (no contexto de um paradigma de *attentional-set shifting task*; ASST) e controlo da resposta impulsiva. A lesão SNI, independentemente do lado onde era instalada, não teve qualquer efeito na execução do MWM.

Os nossos dados indicam que, quer a idade do individuo aquando da instalação da neuropatia, quer o lado do corpo onde esta se localiza, influenciam o desempenho em paradigmas de comportamento emocional e cognitivo. Resulta também das nossas observações que o PFC tem um papel determinante nas alterações comportamentais observadas após a instalação da neuropatia.

Abbreviations list

5-csrtt	5-choice serial reaction time task
8-arm	8-arm radial maze
ACTH	Adrenocorticotropic hormone
Amy	Amygdala
ASST	Attentional-set shifting task
CD	Compound discrimination
CGRP	Calcitonin gene-related peptide
CPP	Conditioned place preference
CRF	Corticotropin releasing factor
CRPS	Complex regional pain syndrome
CVLM	Caudal ventrolateral medulla
DB/LB	Dark/light box paradigm
DRt	Dorsal reticular nucleus
EDS	Extradimensional shift of the attentional-set shifting task
EPM	Elevated-plus maze
FRAP	Fluoride-resistant acid phosphatase activity
FST	Forced swimming test
gp120	human immunodeficiency virus type 1 glycoprotein 120
GR	Glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal-axis
IASP	International Association for the Study of Pain
IB4	*Griffonia simplicifolia* isolectin B4
IDS	Intradimensional shift steps 1-2 of the attentional-set shifting task
IL	Infralimbic prefrontal cortex
IL-1β	Interleukin 1 beta
mb	Marble burying test
mPFC	Medial prefrontal cortex
MWM	Morris water maze
NeuPSIG	Neuropathic Pain Special Interest Group of the IASP
NK1r	Neurokinin 1 receptor
OFC	Orbitofrontal cortex
PAG	Periaqueductal gray
PFC	Prefrontal cortex
PVN	Paraventricular nucleus
R (1-4)	Reversal steps 1-4 of the attentional-set shifting task
RVM	Rostral ventromedial medulla
SNI	Spared nerve injury
SP	Substance P
WHO	World Health Organization
WM	Working memory

Contents

Chapter 1

Introduction

1. INTRODUCTION

1.1. Pain

"It was once thought the mechanisms that subserve pain would be entirely revealed if we applied noxious stimuli to the skin and then mapped the pathways taken by the nerve impulses through the spinal cord and brain. Unfortunately, pain mechanisms are not as simple as this."

in The Challenge of Pain (Melzack and Wall, 1982)

Written accounts on pain origins and treatments are as almost old as the invention of written word itself. Pain was regarded by ancient peoples as something controlled by superior forces on which the individual had little or no control. In fact, the word "Pain" has its roots in both Greek and Latin words *poine* and *poena*, meaning penalty or punishment (Finger, 1994). It was not until the seminal works of Max von Frey (1852-1932) and Friedrich Kiesow (1858-1940) on the role of free nerve endings as "pain receptors" that pain started to be recognized has a separate modality, subserved with distinct physiological machinery (Finger, 1994). By the same period, Alfred Goldscheider (1858-1935) among others, defended the idea that the same receptors would simultaneously convey innocuous touch and pain sensation, the resulting outcome differing as a function of the stimulus intensity. Specificity and pattern theories, as they become recognized, bear strengths and weaknesses. On the one hand, the specificity model had a solid physiological basis but simultaneously imposed a fixed relation between stimulus intensity and pain perception. Innumerable descriptions of severely wounded soldiers from the battle field claiming to feel no pain (Beecher, 1959) or the evocation of pain triggered by light touch in patients suffering from causalgias[1] would indicate differently (Baron *et al.*, 2010). On the other hand, the pattern model accounted for summation phenomena but totally disregarded the role of free nerve endings. Melzack and Wall extensively discussed these points in their breakthrough article and advanced a new theory: the gate-control theory (Melzack and Wall, 1965). This model hypothesized that peripheral information related to light touch and pain sensation was modulated by specific interneurons located in the substantia

[1] Term coined by Silas Weir Mitchell (1829-1914), from the Greek words *Kausus* (heat) and *algos* (pain).

gelatinosa of the spinal cord dorsal horn (the gate) before reaching higher centers in the brain (figure 1). The ascending output from the dorsal horn resulted from the reciprocal interaction between these spinal interneurons and large-diameter (innocuous) or small-diameter (nociceptive) primary afferent fibers. In the first case this would result in an overall decrease of transmission ("closing the gate") and in the second in a net increase ("opening the gate"). The gate-control theory was subsequently reanalysed, reformulated and restated in the light of new data incorporating, for instance, the contribution of descending information from the brain (Wall, 1978; Melzack and Wall, 1982; Lima, 1996; Wall, 1996). Importantly, the gate-control theory was the first pain model that integrated the notion of central modulation.

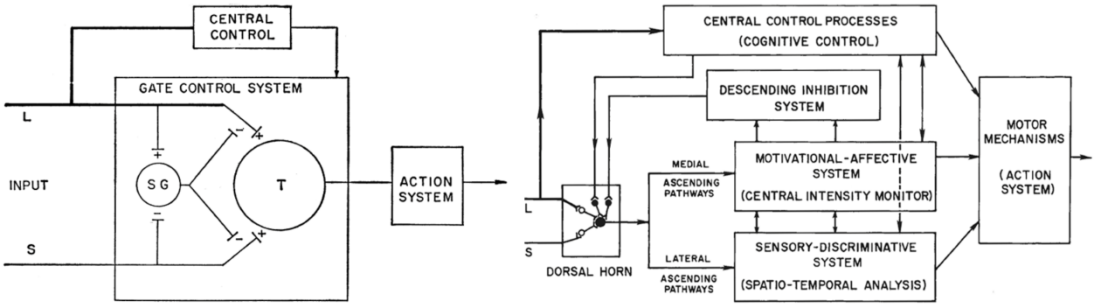

Figure 1. The gate-control theory. (left) Explanatory diagram as published in the original article by Melzack and Wall (1965), showing the interaction between two peripheral systems of fibers, large-diameter (L) and small-diameter (S) primary afferent neurons, and the substantia gelatinosa, on the first central transmission cells (T). The model provided a theoretical framework for the central modulation of the output of T based on balance between the two concurring systems. (right) A more complex version of the model integrating multiple ascending systems and the contribution of descending controls, was envisaged by Melzack and Casey (1968).

Pain is currently defined by the International Association for the Study of Pain (IASP) Committee on Taxonomy as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Merskey *et al.*, 1979; Lindblom *et al.*, 1986). As a sensorial experience pain is unique in many aspects. For instance, unlike other sensations that tend to adapt after continuous and uniform stimulation, pain does not adapt to the continuous presence of the nociceptive stimulus, tending in fact to get progressively worse (Cervero, 2009). IASP definition reflects not only this specificity but also the heterogeneity associated with pain manifestations.

1.2. Anatomofunctional organization of the nociceptive system

"I have elsewhere put forward a view that there has been evolved in the skin ""a special sense of its own injuries"". There is considerable evidence that the skin is provided with a set of nerve-endings whose specific office it is to be amenable to stimuli that do the skin injury, stimuli that in continuing to act would injury it still further"

Sherrington, 1903

1.2.1. Nociceptors

The set of nerve-endings mentioned by Sherrington (1903) would be coined a few years later by himself as *nociceptors* (Sherrington, 1906). Up-to-date literature defines the nociceptor as a primary afferent nerve fiber that is suited to encode relevant properties of the noxious stimulus (Djouhri *et al.*, 2006; Treede, 2009; Dubin and Patapoutian, 2010). The complete absence of these structures in the brain or cartilage determines that pain cannot be elicited from these organs. On the contrary, pain is nearly the only sensation that can be elicited from the cornea, dura mater and dental pulp, indicating a predominance of nociceptors over other afferent terminals (Mense, 2009).

The nociceptor is a pseudounipolar cell and shares with other primary sensory neurons the same basic organization (Woolf and Ma, 2007). They are predominantly unmyelinated (C-fibers) or thinly myelinated (Aδ-fibers) but thick, heavily myelinated fibers (Aα/β), normally responsible for transmission of innocuous information, have also been reported to conduct noxious information (Djouhri and Lawson, 2004; Todd, 2009). Conditions associated with loss of function in thinly myelinated or unmyelinated fibers interfere with pain sensation while sparing light touch sensation, whilst pain is preserved in conditions selectively affecting large myelinated Aβ fibers (Raja *et al.*, 1988; Scherer, 2006; Verhoeven *et al.*, 2006). A similar effect can be elicited by ischemic compression of a limb resulting in the blockade of large myelinated fibers with concomitant loss of tactile but not pain sensation (Landau and Bishop, 1953). The different degree of myelination of these two populations, C and Aδ, imposes that the latter conducts nerve impulse faster than the former therefore transmitting the so called "first pain", i.e., the sharp pain that immediately follows a noxious pinch, pinprick or heat.

"Second pain", on the other hand, is transmitted by the unmyelinated nociceptors in the form of a more diffuse, dull and burning sensation that persists after the "first pain" (Julius and Basbaum, 2001; Meyer *et al.*, 2005; Basbaum *et al.*, 2009; Porreca, 2010). The unmyelinated C-fiber population is morphofunctionally diverse. It has been shown that it consists of two major neurochemical groups (Hunt and Rossi, 1985; Snider and McMahon, 1998; Craig, 2003; Mense, 2009; Todd, 2009). One group expresses neuropeptides like calcitonin gene-related peptide (CGRP), substance P (SP), galanin and somatostatin, whereas the other contains fluoride-resistant acid phosphatase activity (FRAP) and binds the plant lectin *Griffonia simplicifolia* isolectin B4 (IB4) (Nagy and Hunt, 1982). These two neural populations named peptidergic and non-peptidergic, respectively, differ in their terminations at the dorsal horn. Peptidergic afferent fibers project to lamina I and outer lamina II whereas non-peptidergic terminate mainly in inner lamina II (Coimbra *et al.*, 1974; Braz *et al.*, 2005; Basbaum *et al.*, 2009). Ascending pathways from these sites are segregated suggesting differential contributions to supraspinal pain processing (Braz *et al.*, 2005). Peptidergic and non-peptidergic populations are functionally distinct in terms of their electrophysiological proprieties (Stucky and Lewin, 1999) and, when selectively ablated, noxious heat and noxious mechanical pain sensitivity dissociate to a certain extent (Cavanaugh *et al.*, 2009). A third population of nonpeptidergic unmyelinated afferents that do not bind IB4 and is sensitive to noxious cold has also been identified (Dhaka *et al.*, 2008).

On its peripheral extremity, the nociceptor is usually an unencapsulated free nerve ending that branches from the main axon (Dubin and Patapoutian, 2010). This is the case for all C-fibers but not for Aδ-fibers as some, for instance, innervate D-hairs afferents (Mense, 2009; Todd, 2009). The repertoire of transduction molecules expressed at the periphery confers to the nociceptor the modality (thermal, mechanical or chemical) and the range of sensibility (Basbaum *et al.*, 2009). In contrast to innocuous receptors, nociceptors can frequently be activated by more than one type of stimulus, i.e. they are polymodal (Ringkamp and Meyer, 2009). Coherently with its primary function, the threshold of a nociceptor is set below the tissue damage intensity signalling imminent tissue damage (Treede, 2009). Adequate stimuli include extreme temperatures (> 40°C–45°C or < 15°C), intense pressure and an array of potentially harmful substances (Raja *et al.*, 1988; Dubin and Patapoutian, 2010). These nociceptive morphofunctional units are phylogenetically conserved across animal species

suggesting a paramount biological asset for species survival (Andrew and Greenspan, 1999; Lewin and Moshourab, 2004; Foulkes and Wood, 2008).

1.2.2. Ascending nociceptive transmission

Primary afferents terminate in the spinal cord dorsal horn arranged in a modality specific manner (Todd, 2009). Marginal zone and substantia gelatinosa, which according to Rexed's nomenclature (1954) correspond to spinal cord laminae I and II, receive the majority of nociceptive afferents, with a smaller part terminating in the deep dorsal horn (lamina V). Deeper laminae III-V receive mainly primary afferents involved in low-threshold signalling, namely, tactile, hair and proprioceptive sensation (Todd, 2009). Conversely, inputs from descending projections arising from various areas of the brain (see section 1.2.3.) target dorsal horn neurons and participate in the endogenous modulation of the spinal nociceptive transmission (Millan, 2002; Gebhart, 2004; Vanegas and Schaible, 2004; Almeida *et al.*, 2006; Pertovaara and Almeida, 2006). Dorsal horn spinal cord neural population is composed by two main types of neurons classified according to their axonal projections: i. neurons with short axons that communicate locally designated as interneurons; ii. neurons with long axons that communicate supraspinally, designated as projection neurons (Todd, 2009). Projection neurons are not uniformly distributed within a spinal cord segment. The vast majority concentrates in lamina I and the remaining disperses in deeper laminae and gray matter around the central canal, although segmental heterogeneity has also been observed (Al-Khater *et al.*, 2008). Most of these lamina I projection neurons express the neurokinin 1 receptor (NK1r) for SP. Similarly, laminae III and IV neurons whose dorsal dendrites extend to lamina I express NKr1 as well (Ding *et al.*, 1995; Li *et al.*, 1996; Marshall *et al.*, 1996; Li *et al.*, 1997; Li *et al.*, 1998; Todd *et al.*, 2000; Spike *et al.*, 2003). This is anatomically coherent with the termination site of peptidergic primary afferent terminals mentioned in the previous section (1.2.1.). Coherently, saporin derived ablation of NK1r expressing cells leads to a decreased mechanical and thermal hyperalgesia in several models of inflammatory and neuropathic pain (Mantyh *et al.*, 1997; Nichols *et al.*, 1999), in accordance with the expected role of lamina I afferent terminals on pain.

Based on supraspinal termination targets of spinal projection neurons, a number of tracts are defined that include the spinothalamic, spinoreticular, spinomesencephalic, cervicothalamic, spinohypothalamic and spinothelencephalic (Basbaum and Jessel, 2000; Tracey, 2004; Lima, 2009). The spinothalamic tract is in all aspects the most studied tract being therefore the archetype of the nociceptive ascending pathway. It is classically divided in two major ascending systems, the lateral (or neospinothalamic) responsible for the sensory-discriminative properties of pain and the medial (or paleospinothalamic) related with its affective-motivational aspects, which terminate in the posterior lateral sensory nuclei and in the medial nuclei of the thalamus, respectively (see figure 2; Melzack and Casey, 1968; Treede *et al.*, 1999; Treede, 2002; Lima, 2009). Lateral thalamic neurons project thereafter to the primary and secondary somatosensory cortex, whereas medial thalamic send information, among other cortical areas, to the anterior cingulate cortex (Treede *et al.*, 1999; Treede, 2002; Lima, 2009). Spinal cord and thalamic neurons that are part of the lateral tract have small receptive fields and encode accurately the noxious stimulus properties (Kenshalo *et al.*, 1979; Kenshalo *et al.*, 1980; Peschanski *et al.*, 1980), while those neurons integrating the medial system possess large receptive fields and the activity pattern does not encode the stimulus properties (Giesler *et al.*, 1981). These anatomofunctional properties of the lateral and medial systems reflect their involvement in the sensory-discriminative and affective-motivational aspects of the nociceptive ascending transmission.

Nociceptive ascending pathways are traditionally believed to target the contralateral brain. This is mostly true for the spinothalamic pathways described above but cannot be generalized to all the ascending systems. The vast majority of ascending tracts project in a bilateral fashion, with variable contralateral or ipsilateral contribution (Lima, 2009). Moreover, it became clear in studies using retrograde tracers that the sidedness of a particular tract can actually vary according to the spinal cord origin level and/or laminae (Al-Khater *et al.*, 2008). The functional implications of this lateralized organization have been a matter of debate particularly in those aspects referring to frequency, threshold, modulation and functional impact of pain. Merskey and Watson in 1979 based on a systematic review of the literature available at the time, concluded that pain, when lateralized, occurred mostly on the left side (Merskey and Watson, 1979). The authors attributed the lateralized effect to a diminished efficiency of the right hemisphere in processing sensory information. Subsequently, Hall and colleagues tested this

hypothesis and concluded that pain frequencies were identical on both sides (Hall *et al.*, 1981). Ensuing research favored this view (Campbell *et al.*, 1985; Margolis *et al.*, 1985) the sole exception being the trigeminal neuralgia that presents a clear bias toward the right side (Katusic *et al.*, 1990). This result is not, however, extensible to other facial pain conditions (Lam and Remick, 1988; Harness and Chase, 1990). Similarly, side-related differences in the threshold to painful stimulation were initially reported (Wolff and Jarvik, 1964; Wolff *et al.*, 1965; Haslam, 1970; Murray and Safferstone, 1970; Gobel and Westphal, 1987) but these did not find support in other studies (Newton and Mumford, 1972; Seltzer *et al.*, 1992; Schiff and Gagliese, 1994; Gagliese *et al.*, 1995). Lateralized organization of pain pathways has however been suggested to have implications for pain-related emotional arousal, with left pain resulting in greater emotional disturbance (Schiff and Gagliese, 1994; Gagliese *et al.*, 1995).

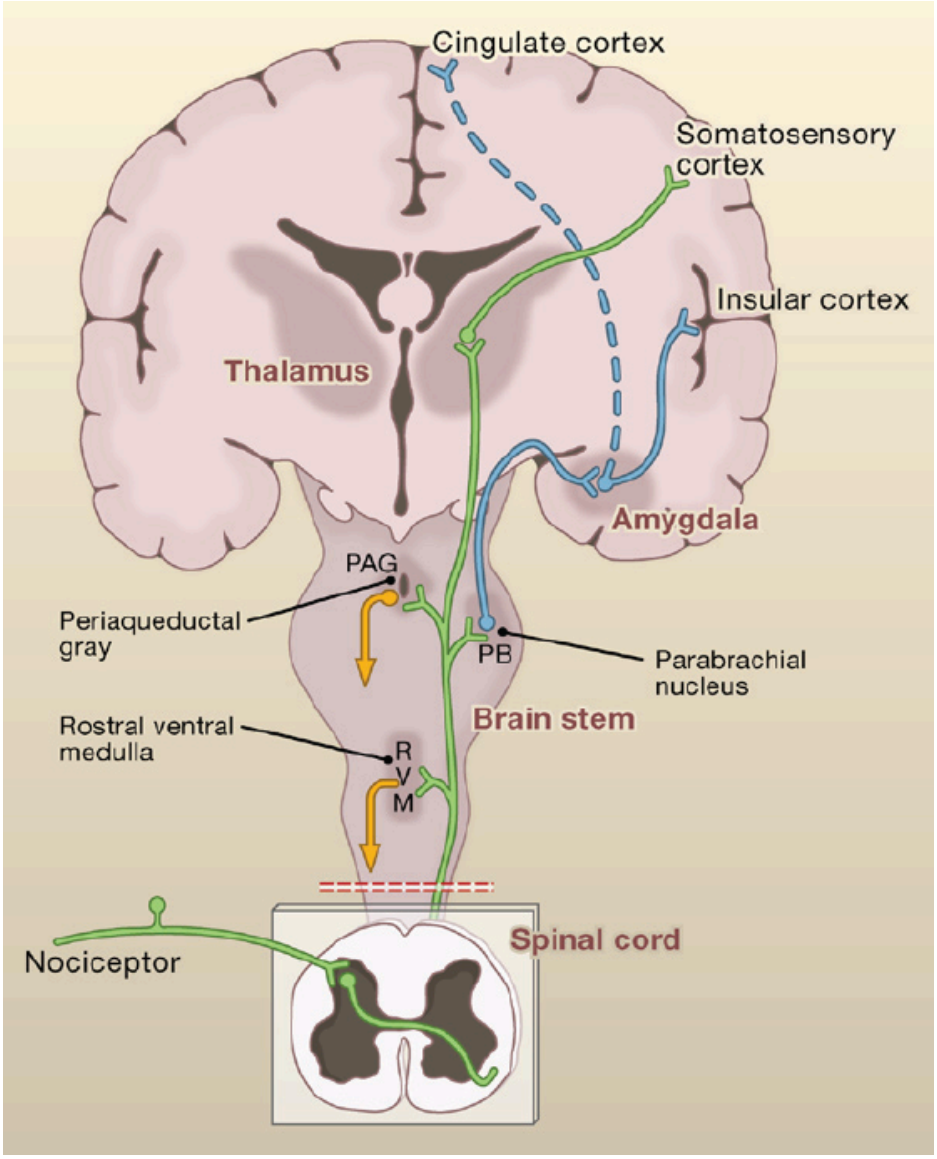

Figure 2. Main ascending and descending pain pathways (Basbaum *et al.*, 2009). Primary afferent terminals transmit nociceptive information from the periphery to projection neurons at the dorsal horn of the spinal cord. Information reaching the somatosensory cortex via thalamus and prefrontal cingulate/insular cortices via amygdala (Amy), originates in different populations of projection neurons. These pathways are related respectively with the sensory/discriminative and emotional/affective components of pain. The ascending nociceptive input is modulated by the periaqueductal gray/rostral ventromedial medulla (PAG/RVM) descending axis that acts upon the spinal projection neurons and terminal axons of peripheral sensory fibers.

Progressively, a small number of brain areas have been shown to present a marked pain processing lateralization. This is the case of frontal operculum (acute painful laser radiant heat pulses in the human; Schlereth *et al.*, 2003) and amygdala (Amy) (persistent inflammatory pain in the rat; Carrasquillo and Gereau, 2008; Ji and Neugebauer, 2009). In both cases, independently of stimulation side, the activation/processing in the brain was biased toward the left and right sides, respectively.

1.2.3. Descending modulation of pain

Beecher observations of extensively wounded soldiers (Beecher, 1959) with minimal pain complaints anticipated a non canonical relation between injury and perceived pain as predicted by the specificity theory. By the same period, the first observations of cortical modulation of spinal cord and trigeminal sensory information (Hagbarth and Kerr, 1954; Hernandez-Peon and Hagbarth, 1955) started to unveil the existence of an endogenous pain control system. In a seminal work, Reynolds reported that complete analgesia could be elicited through electrical stimulation of the periaqueductal gray (PAG) allowing an open abdomen surgery in rat without the support of any chemical anaesthetic (Reynolds, 1969). Soon it became clear that other sites were also involved in descending inhibition of pain, including the Raphe Magnus nucleus and adjacent reticular formation [rostral ventromedial medulla (RVM)], which were shown to project directly to the spinal dorsal horn and to mediate electrical/pharmacological induced analgesia (Basbaum *et al.*, 1976; Basbaum *et al.*, 1977; Basbaum and Fields, 1979). In fact, direct application of morphine (a paradigmatic opioid analgesic) in sites where analgesia could be elicited by electric stimulation produced the same effect (Pert and Yaksh, 1974; Jacquet and Lajtha, 1976). Furthermore, the specificity of the effect was ascribed to opioid μ-receptor

as it was reverted with opioid antagonist naloxone (Akil *et al.*, 1976). The same effects of PAG stimulation/naloxone inhibition were also observed in humans within a few years after these pioneering finding (Adams, 1976). The set of information collected led to the conceptualization of a descending modulatory system centred in the PAG-RVM-spinal cord axis and functionally inhibitory in its overall effect on pain perception (Basbaum and Fields, 1978, 1984). In the following years it became clear that supraspinal modulation of pain was in every aspect far more complex than anticipated by the initial findings (Stamford, 1995; Lima and Almeida, 2002; Millan, 2002; Gebhart, 2004; Almeida *et al.*, 2006; Ossipov *et al.*, 2010). Other supraspinal areas involved in descending modulation are now recognized not only in the brainstem, such as the dorsal reticular nucleus (DRt; Almeida *et al.*, 1996; Almeida *et al.*, 1999) or the caudal ventrolateral medulla (CVLM; Tavares and Lima, 2002; Pinto-Ribeiro *et al.*, 2011), but also in the hypothalamus (Pinto-Ribeiro *et al.*, 2008) or cortex (Zhang *et al.*, 2005). Importantly, descending modulation is presently known also to operate in a facilitatory fashion (Lima and Almeida, 2002; Porreca *et al.*, 2002; Almeida *et al.*, 2006) in addition to its well-known antinociceptive action (Pertovaara and Almeida, 2006).

1.3. Chronic neuropathic pain

"We have some doubt as to whether this form of pain [causalgia] ever originates at the moment of the wounding; but we have been so informed as regards two or three cases. Certain it is that, as a rule, the burning arises later, but almost always during the healing of the wound. Of the special cause which provokes it, we know nothing, except that it has sometimes followed the transfer of the pathological changes from a wounded nerve to unwounded nerves, and has then been felt in their distribution, so that we do not need a direct wound to bring it about. [...] The part itself is not alone subject to an intense burning sensation, but becomes exquisitely hyperaesthetic, so that a touch or a tap of the finger increases the pain. Exposure to the air is avoided by the patient with a care which seems absurd, and most of the bad cases keep the hand constantly wet [...]. As the pain increases, the general sympathy becomes more marked. The temper changes and grows irritable, the face becomes anxious, and has a look of weariness and suffering [...]. At least the patient grows hysterical, if we may use the only term which covers the facts."

in Gunshot Wounds and Other Injuries of the Nerves (Mitchell *et al.*, 1864)

Pain is a main motivation for seeking medical assistance. When persistent, it can be highly incapacitating to the individual and impact on the society. A survey by the World Health Organization (WHO) in 14 countries concluded that prolonged pain (present most of the time for a period of 6 months or more during the prior year) has prevalence rates between 5,5% and 33% and a combined result of 21,5% (Gureje *et al.*, 1998; Bond *et al.*, 2006). Chronic pain is triggered by injury or disease but may be perpetuated by factors other than the initial cause and therefore to outlast it (Loeser and Melzack, 1999). According to the nature of the underlying cause it can be classified as nociceptive, inflammatory or neuropathic according to the underlying cause (Ashburn and Staats, 1999), although in some cases a clear classification cannot be attained as the triggering factors may be multiple (e.g. complex regional pain syndrome (CRPS); Baron *et al.*, 2010).

Neuropathic pain is defined by the International Association for the Study of Pain (IASP) as a pain initiated or caused by a primary lesion or dysfunction in the nervous system (Merskey and Bogduk, 1994, 1997). The Neuropathic Pain Special Interest Group (NeuPSIG) of the IASP has, however, recently proposed a new definition: pain arising as a direct consequence of a lesion or disease affecting the somatosensory system (Treede *et al.*, 2008; Jensen *et al.*, 2011). The replacement of *dysfunction* by *disease* accounts for the fact that the former also included pain caused by neuroplastic changes as a result of intense stimulation and therefore not necessarily neuropathic. Additionally, *nervous system* is replaced by *somatosensory system*, excluding this way, for instance, pain associated with muscular spasticity resulting from lesions in the central motor pathways (Hansson, 2010; Haanpaa *et al.*, 2011). Neuropathic pains encompass a number of heterogeneous conditions (table I) with diverse aetiologies and anatomical locations ranging from the peripheral nociceptor to higher centers in the brain (Dworkin *et al.*, 2003; Dworkin *et al.*, 2007; Jensen and Finnerup, 2007). Despite the diversity of presentations, several common features can nevertheless be identified. These include concomitant partial or total loss of sensation (anesthesia) and pain in the same defined anatomical area, ongoing, paroxysmal or evoked pain, after sensations, abnormal summation and autonomic features (Dworkin *et al.*, 2003; Jensen and Finnerup, 2007; Hansson, 2010). Negative signs reflecting nervous system damage like partial or complete sensory loss and positive signs like allodynia, hyperalgesia, dysesthesia and hyperpathia, which reflect

hiperexcitability, can coexist simultaneously (Woolf and Mannion, 1999; Jensen and Finnerup, 2007; Bouhassira and Attal, 2011) and in diverse combinations (Maier *et al.*, 2010).

Table I. Common types of neuropathic pain (Dworkin *et al.*, 2003)

Peripheral
Acute and chronic inflammatory demyelinating polyradiculoneuropathy
Alcoholic polyneuropathy
Complex regional pain syndrome
Entrapment neuropathies (e.g. carpal tunnel syndrome)
HIV sensory neuropathy
Iatrogenic neuralgias (e.g. post mastectomy or post thoracotomy)
Idiopathic sensory neuropathy
Nerve compression or infiltration by tumour
Nutritional deficiency-related neuropathies
Painful diabetic neuropathy
Phantom limb pain
Postherpetic neuralgia
Postradiation plexopathy
Radiculopathy (cervical, thoracic or lumbosacral)
Toxic exposure-related neuropathies
Trigeminal neuralgia
Posttraumatic neuralgias
Central
Compressive myelopathy from spinal stenosis
HIV myelopathy
Multiple sclerosis-related pain
Postischemic myelopathy
Postradiation myelopathy
Poststroke pain
Posttraumatic spinal cord injury pain
Syringomyelia

1.3.1. Animal models of chronic neuropathic pain

Animal models of neuropathic pain have been proven to be valuable tools in the research of pain mechanisms. The clinical conditions leading to neuropathic pain present a high degree of variation (table I) implying therefore that the pathophysiological mechanisms need different

modelling. This is reflected in the significant number of animal models of chronic neuropathic pain that have been developed in the past decades (see table II; Bennett, 1993; Kim *et al.*,

Table II. Animal models of chronic neuropathic pain

Model	Key references
Complete sciatic nerve transection	Wall *et al.*, 1979b
Chronic nerve (sciatic) compression	Mackinnon *et al.*, 1984 (rat); 1985 (primate)
Chronic sciatic constriction injury (CCI)	Bennett and Xie, 1988; Grace *et al.*, 2010 (graded)
Partial sciatic nerve ligation (PSNL)	Seltzer *et al.*, 1990; Malmberg and Basbaum, 1998 (mouse); Hulse *et al.*, 2008 (mouse; saphenous)
Spinal nerve ligation (SNL)	Kim and Chung, 1992 (rat, L5/6); Carlton *et al.*, 1994 (primate, L7)
Sciatic cryoneurolysis	DeLeo *et al.*, 1994
Experimental lumbar radiculopathy	Kawakami *et al.*, 1994
Inferior caudal trunk injury	Na *et al.*, 1994
Trigeminal neuralgia (infraorbital nerve)	Vos *et al.*, 1994 CCI; An *et al.*, 2011 (cobra venom)
Toxic exposure-related neuropathies	Aley *et al.*, 1996 (vincristine); Authier *et al.*, 2003 (cisplatin); Authier *et al.*, 2009 (various);
Photochemically-induced ischemia of the sciatic	Gazelius *et al.*, 1996
Models of inherited neuropathies	Martini, 1997; Meyer Zu Horste and Nave, 2006
Sciatic nerve neuritis	Eliav *et al.*, 1999
Viral-related neuropathies	Fleetwood-Walker *et al.*, 1999 (Chronic varicella-zoster virus infection); Wallace *et al.*, 2007 (human immunodeficiency virus type 1 glycoprotein 120)
Spared nerve injury (SNI)	Decosterd and Woolf, 2000; Lee *et al.*, 2000
Partial sciatic nerve transection	Lindenlaub and Sommer, 2000
Sciatic inflammatory neuritis	Chacur *et al.*, 2001
Malignant sciatic neuropathy	Shimoyama *et al.*, 2002
Nonarteritic anterior ischemic optic neuropathy	Bernstein *et al.*, 2003 (rat); Chen *et al.*, 2008 (primate)
Avulsion of the brachial plexus	Rodrigues-Filho *et al.*, 2003
Diabetic sensory neuropathy models	Calcutt, 2004
Chronic post-ischemia pain (hindpaw)	Coderre *et al.*, 2004
Common peroneal nerve ligation	Vadakkan *et al.*, 2005
Saphenous partial ligation	Walczak *et al.*, 2005
Spinal nerve transection	Hasnie *et al.*, 2007
Sciatic nerve cuffing	Benbouzid *et al.*, 2008;
Auditory neuropathy	Matsumoto *et al.*, 2008
Thalamic syndrome	Wasserman and Koeberle, 2009
Spinal cord injuries	Nakae *et al.*, 2011
Optic nerve crush	Tang *et al.*, 2011
Autoimmune (antibody-mediated) neuropathy models	Willison, 2011
Partial injury of the median and ulnar nerves	Yi *et al.*, 2011

1997; Bennett *et al.*, 2003; Wang and Wang, 2003; Dowdall *et al.*, 2005; Authier *et al.*, 2009; Decosterd and Berta, 2009; Jarvis and Boyce-Rustay, 2009; Klusakova and Dubovy, 2009; Bennett, 2010; Colleoni and Sacerdote, 2010; Khan and Hargreaves, 2010; Jaggi *et al.*, 2011; Nakae *et al.*, 2011). The first model of peripheral neuropathy consisted in the complete denervation of the distal limb simulating a condition of anesthesia *dolorosa* (Wall *et al.*, 1979a; Wall *et al.*, 1979b). Self-mutilation of the deafferented areas (autotomy) was used as an index of pain as evoked responses could not be obtained in the affected areas (Wall *et al.*, 1979a; Kauppila, 1998). Models subsequently developed involved in its majority traumatic injury of peripheral nerves, nerve roots or spinal cord, caused by sectioning, cuffing, ligation, freezing, inflammation and/or ischemia (Decosterd and Berta, 2009; Mogil, 2009). Additionally, drug toxicity and systemic metabolic disorders have also been used to trigger neuropathies. Some of the most common used models of chronic neuropathic pain are presented in table II.

1.3.2. Central plasticity in chronic pain states

In the above citation of the seminal work Gunshot Wounds and Other Injuries of the Nerves (Mitchell *et al.*, 1864) published close to the end of the American Civil War (1861-1865), Silas Wier Mitchell and colleagues described for the first time some peculiar characteristics of a pain syndrome later named causalgia, observed in young soldiers that had been hit by projectiles. These included a (i) temporal mismatch between the initial injury and the pain onset, (ii) the persistence of pain long after the healing of the primordial injury, (iii) a drop in the pain threshold and (iv) a paradoxal manifestation of pain in unaffected dermatomes. A role of the central nervous system in this and other painful conditions was largely disregarded for more than one century, except for two works that postulated its influence on the amplification of ischemic cardiac pain (Sturge, 1883) and on the manifestation of secondary hyperalgesia in an experimental pain study in human volunteers (Hardy *et al.*, 1950). The breakthrough discovery came in 1983 in a series of electrophysiological studies of the responses of spinal cord flexor motoneurons to peripheral stimulation (Woolf, 1983). Central sensitization, as the described phenomenon became known, provides a rational framework for the temporal, spatial and threshold alterations like those observed by Mitchell more than one century before (Latremoliere and Woolf, 2009). The biological importance of central (and also peripheral)

sensitization can be ascertained by its conservation across virtually all animal phyla (Woolf and Walters, 1991; Walters, 2009). In normal conditions, central sensitization has a benign character, as it lowers pain thresholds in injured tissues and surrounding areas, favouring protective behaviours. However, in pathological conditions involving sustained inflammation and lesions to the nervous system, the benign character is lost, and pain serves no protective and/or biologically relevant role. This form of centrally established plasticity decouples the peripheral stimulus from its regular central processing to a point that pain no longer reflects the existence of a peripheral noxious event but solely a new organization of the central circuitry (Woolf, 2011).

Peripheral and central sensitization differ in many respects both at the mechanistic level as well as in their manifestations (Latremoliere and Woolf, 2009). Perl and colleagues (1976) were the first to describe that nociceptor peripheral terminals can become sensitised following injury. This phenomenon was then observed by others in different conditions (Meyer and Campbell, 1981; LaMotte *et al.*, 1982; LaMotte *et al.*, 1983; Torebjork *et al.*, 1984; Kocher *et al.*, 1987; Campbell *et al.*, 1988a; Bishop *et al.*, 2010). Peripheral sensitisation manifests as a drop in threshold and consequent amplification of nociceptors response when these are exposed to damaged tissue and inflammatory mediators. This form of sensitization depends on nociceptor activation at the periphery, the effect being therefore restricted to the affected area and is the core mechanism underlying heat primary hyperalgesia (Hucho and Levine, 2007; Latremoliere and Woolf, 2009; Ringkamp and Meyer, 2009). On the contrary, manifestations of central sensitization can disperse to areas unrelated with the primary injury as in the cases described by Mitchel (Woolf, 2011). Mechanical allodynia is one of such manifestations. In this case, inputs that normally parallel the nociceptive pathway, including low-threshold mechanoreceptors, are co-opted, resulting in Aβ fiber-mediated pain (Woolf and Salter, 2000). In fact, blood-brain barrier impermeable lidocaine analogues, contrary to regular lidocaine, fail to abolish mechanical allodynia when injected systemically evidencing its central mediation (Chen *et al.*, 2004).

The cascade of events that follow peripheral nerve lesion (or sustained inflammation) and eventually leads to central sensitization can be summarized as follows (Saade and Jabbur, 2008): 1. spontaneous activity is generated (Wall and Gutnick, 1974; Devor, 2009) both in

injured and intact fibers (Wu *et al.*, 2001; Lee *et al.*, 2003); 2. locally (and centrally) pro-inflammatory molecules are released, (Campana *et al.*, 2006; Campana, 2007; Schäfers *et al.*, 2007); 3. phenotype switch occurs in the form of expression of new receptors and ionic channels giving the nociceptor a sensitised profile (Waxman, 1999) and, additionally, an otherwise "silent" or "sleeping" C-fiber population is recruited (Schmidt *et al.*, 1995); 4. peripheral and central fibers reorganize (McMahon and Kett-White, 1991; Woolf *et al.*, 1995); 5. receptive fields and sensory modalities of both injured and intact fibers modify as a consequence (Campbell *et al.*, 1988b; Gracely *et al.*, 1992; Liu *et al.*, 2000; Pitcher and Henry, 2004). The resulting augmented afferent barrage to the spinal cord drives central sensitization (Latremoliere and Woolf, 2009; Ossipov and Porreca, 2009). In fact, pharmacological block of the afferent activity prevents the development of behavioural pain symptoms (Xie *et al.*, 2005). Additionally, descending facilitation from the brain also contributes to central sensitization at spinal cord level (Heinricher *et al.*, 2009; Ossipov and Porreca, 2009). The RVM has been the most studied in this context. The surgical interruption of RVM-spinal cord communication (Ossipov *et al.*, 2000; Burgess *et al.*, 2002; Gardell *et al.*, 2003), its pharmacological inactivation with lidocaine (Pertovaara *et al.*, 1996; Burgess *et al.*, 2002) or the selective ablation of μ-opiod receptor positive RVM neurons (Porreca *et al.*, 2001; Burgess *et al.*, 2002; Gardell *et al.*, 2003) abolished behavioural signs of ongoing pain (Ossipov and Porreca, 2009). This is not an exclusive action of the RVM as other areas, namely the medullary pronociceptive dorsal reticular nucleus (DRt) (Almeida *et al.*, 1996; Almeida *et al.*, 1999; Lima and Almeida, 2002) were also shown to contribute to sensitization (Sotgiu *et al.*, 2008).

Central sensitization is the consequence of a number of functional, chemical, and structural plastic alterations. These are extensively characterized in the spinal cord and, to certain extent, in the brain (Saade and Jabbur, 2008; Zhuo, 2008; Jaggi and Singh, 2011). Similarly to what is observed in the spinal cord in different animal models of chronic pain, signs of abnormal neuronal activity have been recorded in the brain, namely in the RVM (Goncalves *et al.*, 2007), thalamus (Guilbaud *et al.*, 1990; Miki *et al.*, 2000), Amy (Ikeda *et al.*, 2007) and prefrontal cortex (PFC; Zhuo, 2008). Measurements of metabolic activity anatomically correlate with electrophysiological data (Mao *et al.*, 1993; Neto *et al.*, 1999) and, again, in notable parallel with similar observations in the spinal cord (Schadrack *et al.*, 1999). Data from brain imaging

studies in human patients with chronic pain support an increased activity in the PFC but, paradoxically, not in the thalamus (Seifert and Maihofner, 2009; Apkarian, 2010). At the morphological level, structural abnormalities have been reported both in animal (Seminowicz *et al.*, 2009; Millecamps *et al.*, 2010) and human studies (May, 2008). Generally, these reflect a decreased gray matter density and have been detected in brainstem, thalamus, PFC and somatosensory cortex. On the contrary, an increase in the striatum and in the orbitofrontal cortex (OFC) gray matter was reported in patients with fibromyalgia (Schmidt-Wilcke *et al.*, 2007). Alterations in brain neurochemistry have also been observed both in the animal model and (Millan, 1999) in the human disease (Grachev *et al.*, 2000). Of particular interest in the context of pain, μ-receptor availability was found to be decreased in the Amy, accumbens and dorsal cingulate in patients with fibromyalgia (Harris *et al.*, 2007) and in the Amy of mice (Narita *et al.*, 2006). Concerning the activation of the glial population, which is a neuropathy hallmark in the spinal cord, in the brain findings are contradictory. While some groups report an increase of both microglial and astrocytic markers in the brainstem, thalamus and forebrain (Raghavendra *et al.*, 2004), others found no alterations (Zhang *et al.*, 2008).

The morphological and functional alterations described above impact not only in areas with an established role in pain modulation but also in areas that are classically associated with the modulation of emotional and cognitive behaviour, like the Amy and the PFC. In fact, anxiety, depression and cognitive impairments are frequent comorbidities associated with human chronic pain suggesting a causality relation (Moriarty *et al.*, 2011). Experimental animal models, however, have produced contradictory results concerning the emergence of these traits. This can largely be attributed to the experimental factors varying among the reports, some of these underlying putatively important aspects of the pathophysiology. Yalcin and colleagues, for instance, have recently established that the duration of the neuropathy is a determinant factor in the expression of emotional alterations, with anxiety-like behaviours appearing first, followed then by depressive-like behaviours suggesting that the process has a well defined kinetic (Yalcin *et al.*, 2011). Additionally, given the organization of the ascending pathways described previously (see 1.2.2.) one might postulate that the left/right origin of pain can differentially affect areas that are functionally lateralized like the Amy and the PFC, having therefore a selective functional impact. Despite this, left and right neuropathies have been indiscriminately used in the published reports. On the contrary, factors like gender and age of

experimental subjects are the same in all studies (only young male animals have been used). Although being a great advantage for comparison terms, this leaves uncovered important aspects of the human pathology. This is particularly pertinent concerning ageing because of the increased prevalence of pain, notably pain associated with musculoskeletal degenerative conditions, in the elderly (Jones and Macfarlane, 2005). It should, however, be acknowledged that the relation is complex and some pain conditions are in fact age-independent (e.g. stomach and head) or decrease with age (e.g. abdominal and some types of orofacial pain) (Jones and Macfarlane, 2005). The neuropathy prevalence is also positively correlated with aging both in humans and in rats (Gagliese and Farrell, 2005) although results concerning with its algesic quality are contradictory (Gagliese and Melzack, 1997, 2000; Gagliese and Farrell, 2005; Gagliese and Melzack, 2005). Importantly, the risk of affective disorders (Vink *et al.*, 2008; Wolitzky-Taylor *et al.*, 2010) and cognitive deterioration (D'Esposito, 1999; Rosenzweig and Barnes, 2003; Jones *et al.*, 2006) is also increased in the aged individual augmenting the chances of co-occurrence with pain.

1.4. Aims

The general aim of the present thesis was therefore to further extend the characterization of an animal model of neuropathic pain beyond its somatosensory disturbances, namely in respect to the manifestation of emotional and cognitive disturbances. For the reasons raised above, the influence of the experimental subject age at the time of the neuropathy induction (chapter 2.1.) and its lateralization (chapter 2.2.) were studied. Concerning the cognitive impact of the neuropathy, which is by far less studied than the emotional impact, a diversified battery of tests were employed in order to ensure a good characterization of the executive function domains affected. Additionally, considering that some of these domains have well established anatomical substrates we aimed to gain some insight into the areas of increased susceptibility.

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Chapter 2

Experimental work

Chapter 2.1.

Leite-Almeida H, Almeida-Torres L, Mesquita AR, Pertovaara A, Sousa N, Cerqueira JJ, Almeida A.

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The impact of age on emotional and cognitive behaviours triggered by experimental neuropathy in rats

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ABSTRACT

Chronic pain syndromes encompass several clinical entities that frequently affect the individuals' emotional and cognitive behaviours which, in turn, can also alter pain perception. Additionally, both pain perception and motivational-affective behaviours change with increasing age. In order to evaluate the influence of age upon the interaction between chronic pain and affective/cognitive behaviours, 3-, 10- and 22-month-old rats with 1 month neuropathy (*spared nerve injury*, SNI model) were compared with age-matched sham-operated controls in the open field (OF; locomotor and exploratory behaviours), elevated plus-maze (EPM; anxiety-like behaviour), forced swimming (FST; depressive-like behaviour), working memory water maze (WM; spatial short-term memory), Morris water maze (MWM; spatial reference memory) and spatial reversal (behavioural flexibility) tests. Locomotor and exploratory activities decreased steadily with age and were further reduced by SNI. Aging was associated with increased anxiety-like behaviour, which was potentiated by SNI in both 3- and 22-month-old rats. The performance in the FST was affected by SNI but only in mid-aged animals. Cognitive performances in the MWM and spatial reversal tests deteriorated with age; however, the SNI lesion was only detrimental in the reversal task to mid-aged animals. Our data demonstrate that the influence of neuropathic pain on affective and cognitive behaviours is age dependent and varies with the behavioural domain that is tested. Importantly, mid-aged animals seem to be more susceptible to depression and cognitive deterioration associated to chronic pain than young and old groups.

1. Introduction

Pain is a multidimensional experience with sensory-discriminative and motivational-affective components [77] and thus variation in pain sensitivity across life span is not a linear phenomenon among different people. It depends on pain modality, gender, pain-related past experiences and the contribution of several unidentified factors. Moreover, the construction of solid models for age dependence of pain sensitivity has been hampered by the existence of contradictory data (see reviews by [28–30]).

Alterations in the neuronal substrates underlying pain sensation and in the respective subsidiary glial arrays might in part explain differences in age-dependent pain behaviour. Reported alterations include: (i) anatomical and morphological senescence events [2,7,25,61,79]; (ii) variations in neurotransmitters and receptors implicated in pain sensation and modulation [6,17,20,21,34,39,45,46,51,59,60,64]; (iii) changes in neuronal electrophysiological properties as response latency, intensity, pre- (background) and post- (after discharges) stimuli activity and receptive field sizes [45,50,84]; and (iv) modifications in the glial activation status [90,91] and glia-to-glia coupling [41]. Similarly, emotional and cognitive behaviours are also sustained by plastic neuronal structures that display alterations with aging [33,75]. Among others, dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis occurs during normal aging in both aged human and animal subjects [73]. This new hormonal milieu impacts on several central nervous system areas, notably the hippocampus and the prefrontal cortex (PFC) at morphological [15,88] and electrophysiological [14,49] levels (see also [13,86]), providing a plausible contribution for augmented anxiety levels, depressive symptoms and cognitive impairments in aged subjects.

Chronic pain syndromes comprise a vast and heterogeneous group of clinical entities that are frequently accompanied by debilitating mood disorders such as anxiety and depression [58,69]. Despite this, there are only a handful of animal studies using models of prolonged pain to assess the impact of chronic pain on emotional behaviour [35,37,53,66,67,92] and none has analysed the

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influence of age in pain/emotional links. In the present work, we report how 1 month of spared nerve injury (SNI) model neuropathic pain [24] affects the behavioural performance of 2 (young)-, 9 (mid-aged)- and 21 (old)- month-old rats in different emotional and cognitive tasks.

2. Materials and methods

2.1. Animals

Fifty male Wistar-Han rats (Charles River Laboratories, Barcelona, Spain) aged, at the beginning of the protocol, 8 weeks (young animals, $n = 21$), 9 months (mid-age animals, group $n = 18$) and 21 months (old animals, $n = 11$) were used. Weight ranges for young, mid-aged and old animals were 230–300 g, 500–600 g and 650–850 g, respectively. Animals were housed in plastic cages in groups of 2 to 3 with food and water available *ad libitum* in a standard *vivarium* with controlled temperature (22 ± 1 °C) and a 12 h light/dark cycle (lights on at 8 a.m.). All experiments were performed during the light period of the cycle, starting at 9:00 and extending as far as 18:00 depending on the duration of each protocol (see below). Acclimatization to the testing room was allowed for approximately 1 h. All procedures with animals followed the normatives stated by the European Community Council Directive 86/609/EEC and the ethical guidelines for the study of experimental pain in conscious animals [104]. At least one category C FELASA certified experimenter (see the Federation of European Laboratory Animal Science Associations web page http://www.felasa.eu/ for more information) was present when animals were manipulated. Detailed information on experimental conditions is given as supplemental material according to the guidelines established by others [80].

2.2. Surgery and experimental design

An experimental neuropathy was induced using the spared nerve injury (SNI) model, as originally described by Decosterd and Woolf [24], in half of the animals of each age category, the remaining being sham operated and used as controls. Animals were deeply anaesthetized with a mixture of 1.5: 1.0 of ketamine (Imalgene®)/medetomidine (Dormitor®) at a dose of 1 mL/kg. The hairy skin of the left limb lateral surface was shaved and a longitudinally oriented incision was made. The musculature was blunt dissected and the sciatic nerve and its three terminal branches, sural, common peroneal and tibial, were exposed. The last two branches were then tightly ligated with 4–0 silk ligatures and a 1 mm portion was distally sectioned. Caution was taken not to damage or stretch the sural nerve, which should remain intact (spared nerve). Similar procedures were applied to the sham groups, except that all nerves were exposed but left untouched. Finally, muscles and skin were separately sutured. During the recovery period animals were left in individual cages for 24 h and then placed with their previous cage-mates.

During the first month after surgery, except for cage cleaning and non-scheduled occasional handling twice a week, animals were left without any kind of manipulation, to avoid additional distress. Then, a battery of behavioural paradigms was employed as represented in Fig. 1. Animals were always tested in the same pre-established order, which included testing sequentially subgroups of young SNI, young sham, mid-aged SNI, mid-aged sham, old SNI and old sham animals. Since this sequence was repeated three times until all animals were tested, the influence of confounders such as the testing hour was equally distributed through all the experimental groups. A brief summary on each test is given below (for a recent review see for example [87]).

2.2.1. Open field

The open field (OF) is probably one of the oldest behavioural paradigms in rodents (Hall 1934). It is a versatile test that permits assessment of anxiety-like exploratory and locomotor behaviours by measuring, respectively, the percentage of time spent in the centre of the OF arena (higher values being associated with less anxious profiles [78]), the amount of rearing activity and the total distance travelled [87]. The OF was performed in a square (43.2 cm × 43.2 cm) arena with transparent acrylic walls (Med Associates Inc., St. Albans, Vermont, USA) placed in a brightly illuminated room. Animals started the test at the arena's centre and were given 5 min to explore it. Location (peripheral *versus* central) and total distance travelled in the arena were automatically registered by equipment sensors. Additionally, the number and duration of rearings were manually registered by an observer located 1.5–2.5 m away from the apparatus. The inner areas, floor and walls, were cleaned with 10% alcohol and carefully dried between trials. This test spanned for approximately 6 h, starting at 9 a.m.

2.2.2. Elevated plus-maze

The elevated plus maze (EPM) was developed to test anxiolytic/anxiogenic effects of drugs in rodents [36,74] and it is presently considered the most validated and reliable test of anxiety-like behaviour. The rationale of the test results from the conflict between the drive to explore the maze and the aversion to open areas. More anxious behavioural profiles are correlated with smaller ratios between time spent in the open arms and in the closed arms (open arm avoidance index). Other EPM behavioural indexes are: (i) risk assessment (RA) (calculated according to the formula: RA = [number of explorations/(300 − open arm time in seconds)] × 60) for exploratory activity and (ii) the number of entries in EPM closed arms for locomotor activity, which is considered more valid [9,22,81] than the total (open + closed) number of arms entries (see [95] and references within). The EPM was performed in a plus-shaped maze made on black polypropylene plastic, with two opposed open arms (50.8 cm × 10.2 cm)

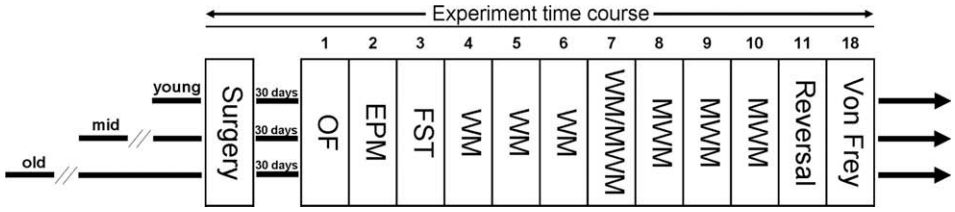

Fig. 1. Chronogram of the experimental protocol. Eight weeks (young), 9 months (mid-aged) and 21 months (old) old animals with 1 month neuropathy and correspondent controls, were submitted to a battery of daily tests according to the following sequence: OF, EPM, FST, WM, MWM and reversal. On the seventh day, WM and MWM coincided, i.e., the submerged platform (see main text) was located in the same quadrant of the swimming pool. One week after the behavioural tests, animals were probed for allodynic response, a well-known marker of neuropathy in nerve-injury models, using calibrated Von Frey filaments. EPM – elevated plus-maze; FST – forced swimming test; OF – open field; MWM – Morris water maze; WM – working memory.

and two opposed closed arms with the same dimensions but surrounded by walls (40.6 cm height; Med Associates Inc., St. Albans, Vermont, USA). The whole structure was elevated 72.4 cm from the floor and was placed in the centre of same room where the OF took place the previous day. Each animal was placed at the centre of the maze and allowed to explore for 5 min. The number of arm entries, the time spent in each arm and the number of explorations were automatically recorded. Inter-trial procedures were as described for the OF. This test spanned for approximately 6 h, starting at 9 a.m.

2.2.3. Forced swimming test

Since its development in the mid-1970s [76], the forced swimming test (FST) has been employed as a successful screening tool for assessing antidepressant-like effects of drugs in rodents. The perseverance of the animal struggling to escape from an inescapable aversive scenario (a cylinder full of water), has been proved to be inversely correlated with a depressive-like profile. Two parameters are currently employed: 1. latency to immobility (Lat_i) – the duration of the first and usually most intense period of escaping activity (inverse correlation with depressive states); 2. immobility (T_i) – total time staying afloat without trying to escape (direct correlation with depressive-like profiles). The FST was performed in a cylinder filled with water (23–24 °C) to a depth of 40 cm, such that animals could not lay their rear paws on the bottom without being totally submersed, and with a height of 30 cm above the water level, thus preventing the escape of the animals. A first trial in the FST (training) was done approximately 1 h after the EPM after which animals were allowed to rest 24 h before the second session (test). Both sessions lasted 5 min and were videotaped by a fixed camera located on the ceiling. Since two different animals were simultaneously recorded (using two FST equipments), the whole FST session lasted only 3 h, starting at 12 p.m.; this ensured homogeneity of testing times between all animals. Lat_i and T_i parameters from the second (test) trial were scored offline using the freeware Etholog software [71].

2.2.4. Cognitive tests

Cognitive tests were made in a black, circular (170 cm diameter) tank, filled with water (23–24 °C) to a depth of 31 cm in a dimly illuminated room with spatial clues on the walls, kept unaltered during the duration of the experiment. A video camera fixed on the ceiling, above the centre of the tank, captured the image to a video tracking system (Viewpoint, Champagne au Mont d'Or, France) located in an adjacent room. Four virtual quadrants [north (N), east (E), south (S) and west (W)] were then assigned on the computer and a black circular platform, 12 cm in diameter, 1 cm below water surface (therefore, invisible to the rats) was placed within one of the quadrants. The position of the platform varied according to each test (see below). In each testing, animals were given four trials to find the platform, each starting from a different quadrant. At the beginning of each trial, animals were gently placed in the periphery of the selected quadrant, approximately half-way from its limits and facing the wall of the maze. Trials were automatically ended once the animals reached the platform or 120 s had elapsed, whichever occurred first. If an animal failed to find the platform in 120 s, it was gently guided to it and allowed to remain there for 30 s before starting a new trial. Time to escape to the platform, as well as to the path, and distance swum during that period were automatically recorded.

2.2.4.1. Working memory. The working memory (WM) test is a modification [23] of the Morris water maze (MWM [65]). It measures the ability of the animal to acquire and keep on line during four consecutive trials, information about the platform location that is changed daily to a different quadrant (Table 1).

2.2.4.2. Morris water maze. The MWM test was designed to study the animal capacity to learn the platform location during four consecutive days – spatial reference memory [65]. The platform was therefore kept in the same position throughout the test (Table 1).

2.2.4.3. Reversal. The day after learning the platform location in the MWM, animals were similarly tested during four trials, but with the platform positioned in the opposite quadrant (Table 1). The rationale underlying this test is that animals displaying behavioural flexibility will rapidly learn to search the platform in its new location while impaired rats will spend more time around the old location.

2.2.5. Von Frey

One week after the end of the emotional and cognitive behavioural tests, animals were evaluated for allodynic responses, a hallmark for a neuropathic chronic pain condition. Each paw (ipsi- and contralateral) was probed with Von Frey filaments on the lateral surface (sural nerve territory), starting with softer (lower bending force) and progressively increasing to harder Von Frey filaments, each applied five consecutive times in each paw. The brisk lifting of the probed paw was considered a positive pain-like response; simultaneous similar movements of the other paw – mirror-responses – were also registered but not considered as a pain response. This test was performed on an elevated grid, where the animals were placed, inside an inverted white light-transparent plastic box to limit their movements. In order to minimise the potential stressful impact of the testing conditions, animals were allowed to acclimatize daily to the examination room, for 1 week before the test (Fig. 1).

2.3. Statistical analysis

Results are expressed as means ± standard errors. Comparisons between means were analysed with one-way or repeated measures analysis of variance (ANOVA), as appropriate. Differences between groups were analysed post-hoc with Tukey's honestly significant differences test (Tukey's HSD). Results were considered statistically significant if $p < 0.05$.

3. Results

3.1. General considerations

After the SNI/sham surgery, animals spent 1 month period without any manipulation besides cage cleaning and occasional

Table 1
Sequence and organization of the cognitive tests: working memory (WM), Morris water maze (MWM) and reversal (see main text for details on each of these tests). The location of the platform on each day and the starting quadrant in each of the four trials are referred. When coincident, this was highlighted. In the WM, animals had to learn a new platform location on a four-trial run, everyday. In the MWM, reference memory was tested by keeping the platform position unaltered for four consecutive days during each of which the starting quadrant for each trial was randomly assigned (order repetitions were not allowed). Finally, on the last day, after a 4-day period learning a location, behavioural flexibility was tested by changing the platform to a new position – approximately opposed to the previous – within the north quadrant, N* (different from that on day 5).

Day	Protocol	Platform location	Starting quadrant
4	WM	O	N S **O** E
5	WM	N	O **N** S E
6	WM	E	**E** S N O
7	WM/MWM	S	E O N **S**
8	MWM	S	N **S** E O
9	MWM	S	**S** N O E
10	MWM	S	O E **S** N
11	Reversal	N*	**N** S E O

handling, without a fixed schedule to avoid habituation. During this period, animals were monitored for abnormal signs indicative of disease (e.g. weight loss, infections, alterations in grooming and other behaviours). Only one wound infection, promptly treated with the topical application of chlorohexidin, was detected, in a mid-age SNI animal; as this resolved in less than 1 week, we found no motives for not including the animal.

All SNI rats developed signs of spontaneous pain (flinching and paw protection) almost immediately after nerve lesion and brisk pain responses were elicited when forces as low as 0.04 g were applied to the lateral area of the nerve-lesioned paw. When this paw was probed five times with the 1 g Von Frey filament, nearly 75%, 85% and 60% of young, mid-aged and old SNI animals, respectively, responded at least once (Fig. 2A). In contrast, only 1 of 11 young animals and no mid-aged nor old sham animals responded to the same filament. After stimulation with a 15 g filament, virtually all (100%) SNI animals from all age groups showed pain-like behaviour, while only 40% (young) – 60% (old) of sham-operated animals showed the same type of reaction (Fig. 2B).

3.2. Locomotor behaviour

The total distance travelled in the OF, the number of entries in the EPM closed arms and the average velocity in the water maze were used as locomotion indexes (Fig. 3). In the OF, a stepwise decrease in motor activity was apparent with increasing age ($F_{2,44} = 31.811$, $p < 0.001$). Nerve lesion also affected locomotor activity significantly ($F_{1,44} = 17.126$, $p < 0.001$), with older animals being more susceptible to its effects (interaction between age and nerve lesion $F_{2,44} = 3.606$, $p = 0.035$; Fig. 3A). Specifically, when age-matched animals were compared, SNI animals were found to travel significantly less than controls, but only in mid-aged ($p = 0.028$) and old ($p = 0.030$) groups. In the EPM, age ($F_{2,43} = 21.335$, $p < 0.001$), but not SNI ($F_{1,43} = 2.335$, $p = 0.134$), significantly reduced the number of closed arm entries and this effect was similar in both treatment groups ($F_{2,43} = 2.054$, $p = 0.141$) (Fig. 3B). Mid-aged and old animals displayed fewer entries in the closed arms when compared to young animals of the same experimental group (sham: x mid-aged $p = 0.045$; x old $p = 0.034$; SNI: x mid-aged and x old $p < 0.001$). Although it did not reach statistical significance, treatment effect was more pronounced in old animals ($p = 0.064$). In the water maze, age ($F_{2,44} = 4.976$, $p = 0.011$) and SNI ($F_{1,44} = 14.424$, $p < 0.01$) significantly decreased the average velocity but no interaction was found between these two factors ($F_{2,44} = 0.272$, $p = 0.763$) (Fig. 3C): nerve injury decreased the average swimming speed only in mid-aged and old animals (young $p = 0.066$; mid-aged $p = 0.032$; old $p = 0.014$).

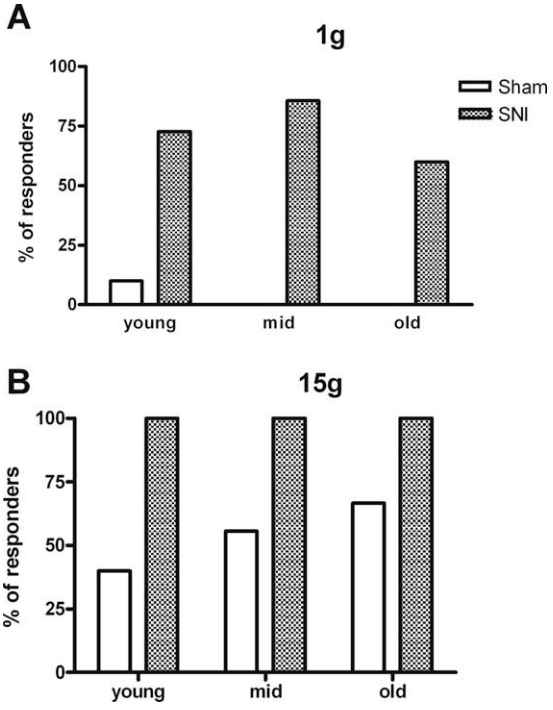

Fig. 2. Percentage of responders to 1 g (A) and 15 g (B) Von Frey probes on the ipsilateral paw. The great number of responders in the SNI group to 1 g filament probe – an innocuous stimulus for sham animals – is a clear indication of allodynic behaviour and neuropathy. For comparison purposes, responses to 15 g probe are also given. Note that sham group scores are still smaller than those obtained with 1 g probe in SNI animals. Results referring to the contra-lateral paws do not differ from sham ipsilateral paw probing and were therefore omitted.

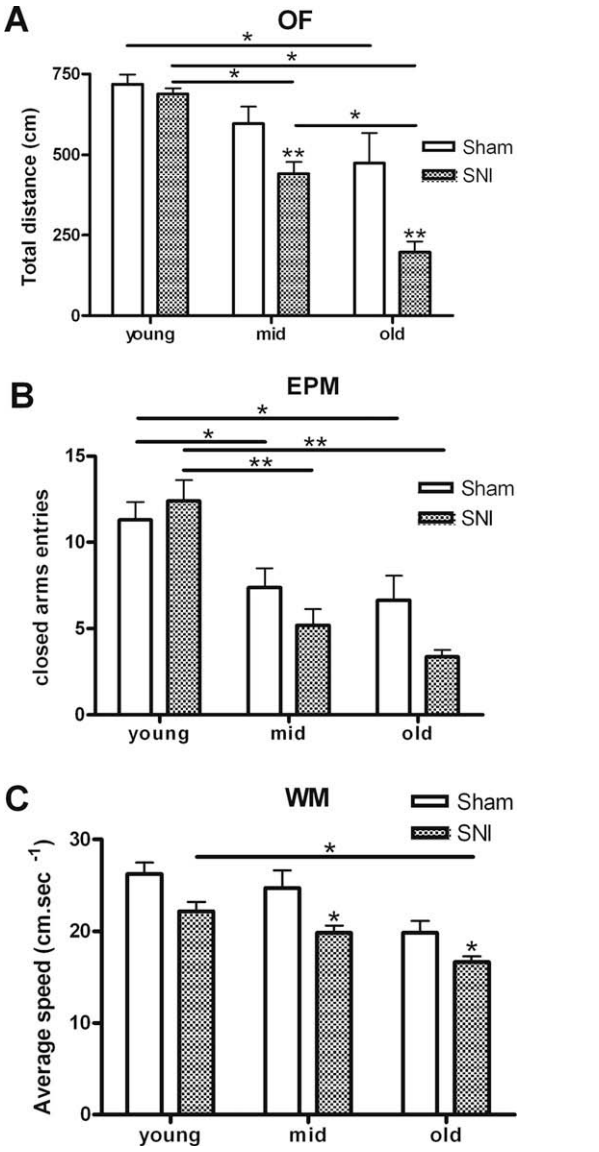

Fig. 3. Locomotory behaviour. Three indexes of locomotion were accessed: (A) total distance travelled in the OF; (B) number of entries in the EPM closed arms; (C) average velocity in the water maze. Note the aging trend similarities of locomotory activity across the three indexes. $^{*}p < 0.05$ and $^{**}p < 0.001$; comparisons within age-matched animals are indicated over SNI graph bar and comparisons along aging within surgery groups, sham and SNI, are indicated over horizontal lines. EPM – elevated plus-maze; OF – open-field.

3.3. Anxious-like and exploratory behaviours

The percentage of time spent in the EPM open arms (percentage of OA; Fig. 4A), and the RA parameter (EPM; Fig. 4B) were used as anxiety-like behavioural indexes. The total amount of time spent rearing (T_{rea}; Fig. 4C) and the absolute number of rearings (N_{rea}; Fig. 4D) that animals performed during the exploration of the OF arena were used as indexes of exploratory behaviour.

In the EPM, both percentage of OA (Fig. 4A) and RA (Fig. 4B) significantly decreased with increasing age ($F_{2,43} = 15.107$, $p < 0.001$ and $F_{2,43} = 28.922$, $p < 0.001$, respectively) and nerve lesion ($F_{1,43} = 6.287$, $p = 0.016$ and $F_{1,43} = 4.729$, $p = 0.035$, respectively). Young SNI and sham animals differed in percentage of OA ($p = 0.028$) and RA ($p = 0.041$) whereas in old animals treatment affected only percentage of OA ($p = 0.016$) (Fig. 4A and B). In the OF, the duration (T_{rea}; Fig. 4C) and the absolute number of rearings (N_{rea}; Fig. 4D) inside the OF arena were affected similarly by both age (T_{rea} $F_{2,44} = 7.725$, $p < 0.001$; N_{rea} $F_{2,44} = 15.669$, $p = 0.001$) and surgery (T_{rea} $F_{1,44} = 25.678$, $p < 0.001$; N_{rea} $F_{1,44} = 20.158$, $p < 0.001$). Within groups of age-matched animals, T_{rea} and N_{rea} were decreased by SNI surgery (young $p_{Trea} = 0.003$ and $p_{Nrea} = 0.006$; mid-aged $p_{Trea} = 0.006$ and $p_{Nrea} = 0.145$; old $p_{Trea} = 0.020$ and $p_{Nrea} = 0.005$). Additionally, a clear age-dependent attenuation of the exploratory behaviour was detected in the T_{rea} ($SNI_{youngxmid}$ $p = 0.024$; $SNI_{youngxold}$ $p = 0.003$) and N_{rea} ($SNI_{youngxold}$ $p = 0.001$; $sham_{youngxmid}$ $p = 0.005$; $sham_{youngxold}$ $p = 0.010$) (Fig. 4C and D).

3.4. Depressive-like behaviour

The period of escaping activity between the introduction of the animal in the water cylinder and the first immobilization – latency to immobilization (Lat_i; Fig. 5A) – and the amount of time that the animal stayed afloat without evident efforts to escape – immobility time (T_i; Fig. 5B), were used as indexes of depressive-like behaviour. Both factors were significantly affected by age ($F_{2,43} = 4.593$, $p = 0.016$ and $F_{2,43} = 12.376$, $p < 0.001$, respectively) but not by nerve ligation. Additionally, there was a significant interaction between age and surgery for T_i ($F_{2,43} = 3.747$, $p = 0.032$). T_i was reduced in mid-aged sham animals compared to young ($p = 0.001$) and old controls ($p = 0.001$). Interestingly, only in this age group did SNI increase T_i when compared to sham animals ($p = 0.032$), indicating a depressive-like behaviour induced by nerve lesion.

3.5. Cognitive assessment

Repeated measures analysis of WM performance demonstrated only a marginal effect of age ($F_{2,43} = 3.155$, $p = 0.053$) but not surgery ($F_{1,43} = 3.507$, $p = 0.068$) nor an interaction between these two factors ($F_{2,43} = 0.662$, $p = 0.521$; Fig. 6A–C). None of these factors influenced the performance in the Morris water maze (Fig. 6D–F). Analyses of behavioural flexibility revealed an interaction between aging and lesion ($F_{4,86} = 2.630$, $p = 0.040$), with SNI impairing this behaviour only in mid-aged animals as revealed by the higher percentage of distance swum in the old location and lower percentage of distance swum in the new location of the platform (Fig. 6G). There was, in addition, an aging effect ($F_{2,43} = 5.108$, $p = 0.010$) with older animals displaying a higher percentage of distance swum in the old location compared to young animals ($p = 0.030$). No differences were observed in the distances swum in the neutral quadrants (data not shown).

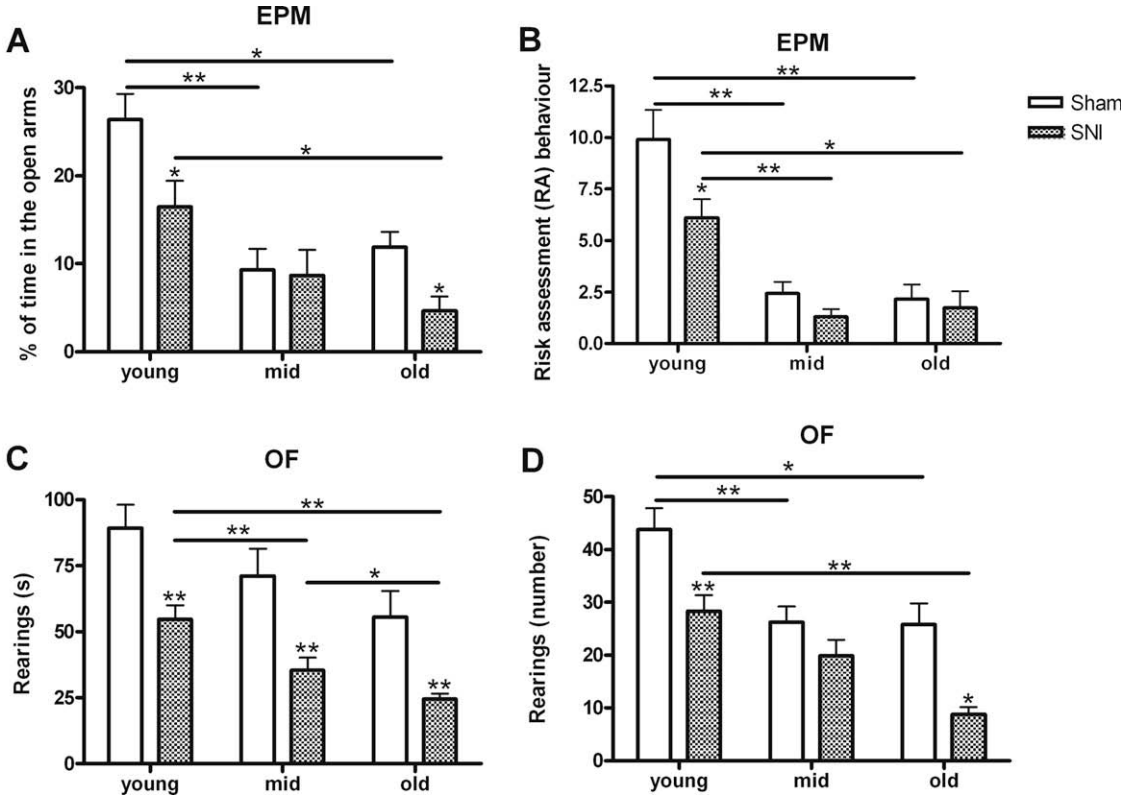

Fig. 4. Anxiety-like and exploratory behaviours. Two indexes of anxiety-like behaviour are given, namely the percentage (%) of time spent by the animals in the EPM open arms (A) and in the central area of the OF arena (C). The remaining – risk assessment behaviour (B), rearing time (D) and number of rearings (E) – are measures of exploratory behaviour. $^{*}p < 0.05$; $^{**}p < 0.001$; comparisons within age-matched animals are indicated over SNI graph bars and comparisons along aging within surgery groups, sham and SNI, are indicated over an horizontal line. EPM – elevated plus-maze; OF – open-field.

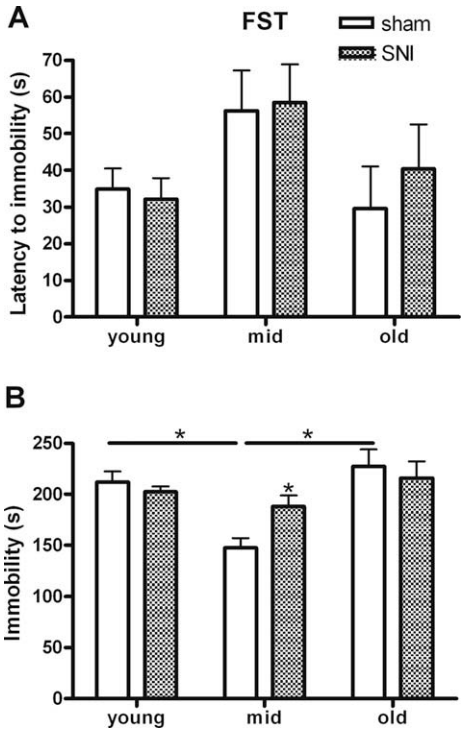

Fig. 5. Forced swimming test for depressive-like behaviour. Two indexes are given: latency to immobility (A) and immobility time (B). $^{*}p < 0.05$; comparisons within age-matched animals are indicated over SNI graph bar and comparisons along aging within surgery groups, sham and SNI, are indicated over horizontal lines. FST – forced swimming test.

4. Discussion

In this study we attempt for the first time to analyse the combined influence of aging and chronic neuropathic pain on cognitive and emotional behaviours in rats. One month after the induction of SNI, young, mid-aged and old animals went through a battery of tests and were then probed for signs of allodynia, a well-established marker of neuropathic pain. Our results demonstrate that even nearly 2 months after surgery, animals submitted to SNI displayed an increased response to light (1 g) Von Frey probing; persistence of pain related behaviours is a well-known feature of the SNI model [24]. Spontaneous pain-related behaviours such as flinching and protection of the affected paw have also been observed in SNI animals during their normal activities in the cages, possibly indicating ongoing pain. We demonstrate that after 1 month of peripheral nerve injury (SNI model) mid-aged rats, but not young nor aged animals, acquire a depressive-like behaviour and show impairment of executive function (behavioural flexibility).

4.1. Neuropathic pain increases locomotor impairment with aging

The present study shows that SNI potentiates the reduction of locomotor activity that normally occurs with increasing age. The age-related decline in motor performance is in accordance with previous studies [3,11,12,27,38,44,66,89,97]. The underlying mechanisms for this phenomenon include a contribution of age-related alterations of neuronal networks involved in motor activity (planning, execution and coordination) but also side factors such as weight gain, diminished metabolic activity as a result of cardiovascular and musculoskeletal impairments [3]. Our data also show that nerve lesion related-impairments of locomotor behaviour augment with increasing age. A possible explanation might be that young animals are able to use intact limbs more effectively to overcome the partial loss of function of the lesioned limb. A more effective functional recovery in younger animals could also provide an explanation for the near absence of functional impact of SNI 30 days after the surgery. On the contrary, the locomotor behaviour in mid-aged and old animals was reduced after the lesion. It is not known to what extent performances in movement-dependent behavioural tests such as the OF, EPM and FST are affected but principal component analyses studies demonstrated an extensive segregation between motor and anxiety-related indexes in the EPM [9,22,81] and OF [9]; as expected, the use of anxiolytic and/or anxiogenic drugs (in a certain dose range) only affected the anxiety-related factor [22]. Similar findings have been reported recently by Rice's group in a model of HIV anti-retroviral-associated pain [96]. Treated and control animals differed in their thigmotaxic behaviour in OF, the former group presenting a more anxious-like behaviour, but no differences were found regarding motor activity. Pharmacological approach with analgesics (gabapentin and morphine) and one anxiolytic (diazepam) drug significantly reduced anxious-related behaviour without any interference with motor behaviour [96] (see also [82]). Concerning the FST, this test is mostly dependent on forepaws and tail movements and therefore SNI is not expected to interfere extensively: indeed, intact and operated old animals presented no differences in this test.

4.2. Neuropathic pain affects behavioural flexibility only in mid-aged animals

To avoid possible interferences related to altered locomotion abilities in the water maze tests, we used swum distances (more related to animals' strategy) instead of velocities (related to locomotive performance) to compare animals' performances. The learning curves in the spatial reference memory obtained herein revealed that aged animals have learning/memory deficits; this is in accordance with several previous studies [31,32,98]. On the contrary, no association between aging and decreased performance was apparent in the WM test. Nevertheless, it should be noted that, by the end of both WM and MWM tests, sham and SNI animals were equally good performers indicating that the memory performance was not affected by chronic neuropathic pain. In the reversal task age-related deficits in behavioural flexibility are demonstrated, as old sham animals failed to show an adaptive behaviour when challenged with a new rule. Furthermore, in this test, SNI affected exclusively the performance of mid-aged animals, with sham rats displaying successful behavioural adaptation (similar to young sham and SNI animals) and injured animals failing to adapt their behaviour (similar to old sham and SNI animals). The herein reported selectivity of SNI effects for mid-aged rats in the reversal test is in accordance with the report by Smith and collaborators [85] showing that repeated tail pinch only activated the PFC of mid-aged rats but not of young or old rats. Indeed, behavioural flexibility is associated with the function of the medial PFC [18,83] and impairments on reversal-type tasks, such as the one used in the present study, have been associated with volumetric decreases, neuronal loss, altered dendritic morphologies and impaired LTP on the hippocampal-PFC connection [14–16]. It is remarkable that, in mid-aged rats, SNI seemed to induce a premature aging of PFC function, suggesting that, in this particular region, chronic neuropathic pain might trigger, and hasten, the plastic mechanisms involved in aging. Interestingly, previous studies on chronic pain subjects (human and animal) have identified alterations in regional cerebral blood flow [40,72], metabolic neural activity [62], gray matter density [5,54], gene expression patterns [4,68], receptor affinity/availability [48], synaptic activity [99] and glial status [55,67] in prefrontal areas. Moreover, manipulation of the PFC was shown to alter the nociceptive response in several

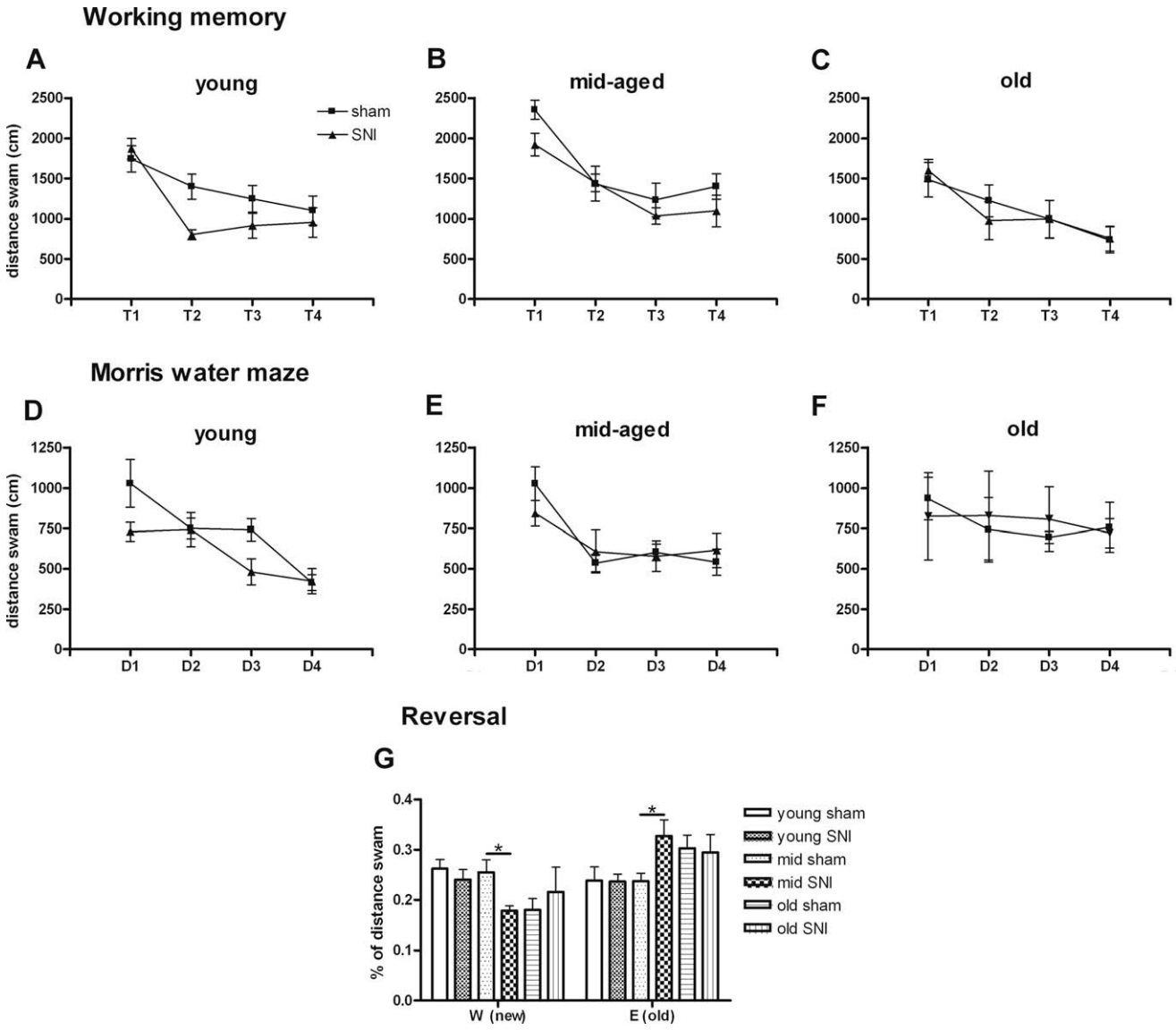

Fig. 6. Cognitive behaviours. Animals were challenged with three tasks that differed in their cognitive requirements (see main text for detailed explanation). Working memory performances of sham and SNI are compared for young (A), mid-aged (B) and old (C) animals. Similarly, Morris water maze (MWM) performances are plotted in graphs (D)–(F). Reversal task performances are plotted in graph (G). The four quadrants of the pool are assigned with letters W, N, E, S; the actual and the former platform positions are, respectively, W and E. $^{*}p < 0.05$; comparisons within age-matched animals are indicated over bar lines. MWM – Morris water maze; WM – working memory.

models of acute and chronic pain [1,10,19,26,47,56,57,70,101–103]. Altogether, these studies indicate that the PFC is a likely target for the mediation of the interaction between cognition and pain processing. The fact that performances in the MWM and WM are not affected by the SNI lesion, suggest that the hippocampal function is preserved after chronic neuropathic pain. This finding contrasts with the report that hippocampal long-term potentiation is depressed in neuropathic mice [52]. The use of distinct species and temporal differences in the neuropathy duration (7–9 days in the later study and over 1 month in the present study) hindered further comparisons.

4.3. Neuropathic pain affects emotional behaviours differently with aging

In the FST, mid-aged animals struggled for longer periods of time and spent significantly more time swimming than young and old animals (similar results were obtained in female mice [100]). Remarkably, once again differences between sham and SNI animals were only evident in mid-aged animals; rats submitted to SNI presented increased learned helplessness. A number of functions have been proved to be dependent on PFC activity including the regulation of emotional behaviour [13]. Whether these behavioural profiles observed in SNI mid-aged animals share a common neural substrate namely on the PFC, remains to be proved. However, as stated above, several structural and functional aspects of PFC have been shown to be dramatically affected by prolonged pain.

In this perspective, our data on anxiety-like behaviour appear to be paradoxical as mid-aged SNI animals are apparently spared from the anxiogenic effect of the lesion, a well-known phenomenon in young rodents [63,66,67,92]. Plastic alterations in other brain centres such as the extended amygdala (AMY) are likely to be involved. Indeed, affinity shifts of opioidergic ligands, increased glucocorticoid receptor and corticotropin-releasing factor mRNA expression, synaptic potentiation, astrogliosis and neurogenesis in the AMY [35,42,67,94] have been described after peripheral nerve injury. Concerning the observed age-related anxiogenic effects in rodents, they are in accordance with previous reports either using the EPM [8,43], the OF [93] or using both [9].

4.4. Concluding remarks

In the present work we demonstrate that some of the cardinal features of aging are anticipated by peripheral nerve injury. The near absence of SNI-evoked behavioural effects in old animals in some of the tasks is possibly due to the poor level of performance of these subjects in basal (sham) conditions – "ceiling effect" – possibly rendering our analyses ineffective to discriminate SNI effects. On the other hand, behavioural impairments caused by SNI were evident in mid-aged animals. A putatively higher algesic quality of mid-life neuropathies has been described (reviewed by [29]), but at the moment it is premature to establish a causality relation between these two observations. It also stems from our work that PFC function appears to be more susceptible to the effects of the neuropathic lesion than the hippocampal function. This observation concurs with a vast number of literature published by other authors and mentioned above, confirming the PFC as one of the principal targets for future studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pain.2009.02.024.

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Chapter 2.2.

Leite-Almeida H, Cerqueira JJ, Wei H, Ribeiro-Costa N, Martins HA, Sousa N, Pertovaara A, Almeida A

Differential effects of left/right neuropathy on rats' emotional behavior and executive function

(submitted)

Differential effects of left/right neuropathy on rats' emotional behavior and executive function

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The prefrontal cortex is severely affected in prolonged pain conditions. Since it is functionally lateralized, we reasoned that its functions could be selectively affected according to the side of pain origin. We tested this assumption in rats with a unilateral neuropathy to conclude that left-sided neuropathic pain is more anxiogenic than right-sided pain and that the latter disrupts efficient executive function and working memory while having no emotional effect.

Emotional disturbances and cognitive impairments are a common feature of prolonged pain conditions[1]. One of the brain areas most consistently shown to be selectively damaged in patients with chronic pain syndromes is the prefrontal cortex (PFC)[2,3]. Importantly, this region is involved in the expression of emotions[4] and in a wide range of cognitive functions[5], therefore appearing as a good candidate for mediating the above mentioned effects.

The PFC has a central role in working memory, attention, impulsivity and in the construction of flexible adaptive behaviors[5], collectively named executive functions. Additionally, PFC regions were also shown to modulate the expression of emotional states, including anxiety, through their projections to centers such as the bed nucleus of stria terminalis, the amygdala and the hypothalamus[5]. The extensive range of cortical and subcortical PFC targets, upon which it exerts a top-down control, is matched by a similar broad range of afferents from several cortical and subcortical regions[6], including direct projections from the spinal cord[7]. Importantly, the PFC is the only cortical area that receives such projections, which might explain its selective vulnerability to the effects of prolonged pain. In fact, it has been shown that the thalamus, which also receives spinal cord projections, is similarly affected upon prolonged pain[2].

If the plasticity observed in cortical and subcortical areas in chronic pain conditions relates totally or partially with a disturbance of the ascending pathways we can postulate that lateralized pain would have a differential behavioral impact according to its left/right origin as the PFC itself has been shown to be functionally lateralized[8].

To test this hypothesis, we assessed the behavior of young Wistar-Han rats after 1 month of either left- or right-sided neuropathic pain (spared nerve injury model[9], SNI) on a battery of tests for sensory, emotional and cognitive function. Both right and left sham-operated

animals were used as controls but their performances were clustered together since there were no differences between them.

Using established paradigms to assess both acute evoked nociception (von Frey monofilaments) and ongoing, non-provoked, pain (analgesia-derived conditioned place-preference)[10] we firstly showed that left- (SNI-L) and right-sided (SNI-R) neuropathies have similar algogenic properties. Nerve-injured animals showed an expected decrease of the monofilament-induced limb withdrawal threshold, which was of similar magnitude irrespective of lesion side (Fig. 1a). In both left- and right-sided lesion groups (Fig. 1b), but not in sham-operated animals (Supplementary Figure S1b), a single intrathecal clonidine injection produced a significant place-preference for the clonidine-paired chamber. Since increased anxiety is frequently reported in both humans with prolonged pain conditions and animal models of chronic pain, we assessed anxiety-like behavior, in a different cohort of animals, by exposing them for 5 minutes to the elevated-plus maze (EPM). Here, contrary to the previous results, we had a lateralized effect of prolonged pain exposure, with SNI-L animals spending significantly less time in the open arms when compared to both SNI-R and sham-lesioned animals (Fig. 1c). These differences do not relate to alterations in locomotor activity, since the number of closed arms entries is similar in all groups (Supplementary figure 2), suggesting therefore an increased anxiety-like behavior upon left- but not right-sided chronic pain.

Finally, to test whether the cognitive effects of prolonged pain were also lateralized, we assessed animals for several PFC-dependent executive functions including working memory (WM; assessed in the water maze), behavioral flexibility (assessed in an attentional-set shifting task; ASST) and impulse control (assessed in a delay-to-signal task; see Supplementary Methods for a detailed description). Interestingly, right-lesioned animals showed consistent impairments in all tasks, when compared to SNI-L and control rats (respectively Fig. 1d, e and

f). Importantly, no significant differences between any experimental groups were observed in a hippocampal-dependent spatial long-term memory task (the Morris Water Maze; MWM, supplementary Figure 3), suggesting a specific susceptibility of PFC-dependent cognitive domains in chronic pain conditions.

Rodent models of neuropathic pain proved to be robust enough to manifest a whole range of emotional and cognitive disturbances observed in human disease besides sensorial abnormalities (e.g. hyperalgesia and allodynia) that are considered to be hallmarks of neuropathies[1,11]. Given the well established association between prolonged pain conditions and increased anxiety, our findings that only left-lesioned rats do display a hyperanxious phenotype are striking, though in line with a previous study in human volunteers[12]. Concerning the detrimental effect of right-sided pain on cognitive function, no parallel exists in the current literature.

Results from our cognitive studies confirm a selective PFC susceptibility in chronic pain conditions as SNI-R had deteriorated cognitive performances specifically in PFC-dependent tasks (WM, ASST and impulse control) but not in the hippocampal-dependent task (MWM; supplemental fig. 3), in which performance was at the level of SNI-L and controls. Additionally, the fact that SNI-R behavioral performance in the ASST task was only impaired in the reversal steps (Rev) but not in the extradimensional shift step (EDS; supplemental fig. 4) suggests that PFC malfunction is restricted to the ventral mPFC/OFC subareas, sparing dorsomedial prelimbic and cingulate PFC subareas[13].

Apart from cognitive function, manipulations of ventral mPFC/OFC subareas have been shown to influence behavioral outcomes in anxiety paradigms and the levels of anxiety–related biomarkers such as corticosteroids. Importantly, this effect is lateralized, depending on the right PFC, expectably the area affected in SNI-L (reviewed by Sullivan and Gratton[4]). On the

contrary, the individual contribution of left/right mPFC to cognitive construction is still a matter of debate[8]. However, Luerding and colleagues found in fibromyalgia patients an impairment on non-verbal working memory that was positively correlated with the grey matter volume of the left middle frontal gyrus[14], expectedly, and according to our guiding hypothesis, the area affected in SNI-R animals.

Curiously, the ventral mPFC and OFC are the only cortical areas where direct projections from the contralateral spinal cord dorsal horn terminate (reviewed by Lima[7]). This body of evidence supports our hypothesis that peripheral nerve injury affects the ascending pathways resulting in functional impairment of these cortical terminal areas. Given the above mentioned lateralization of PFC functioning, particularly in the ventral mPFC and OFC, peripheral nerve injury has a differential and selective emotional/cognitive impact according to the side of injury.

In summary, in the present work we demonstrated for the first time that the side of a neuropathic lesion is crucial in determining the emotional and cognitive comorbidities chaperoning pain.

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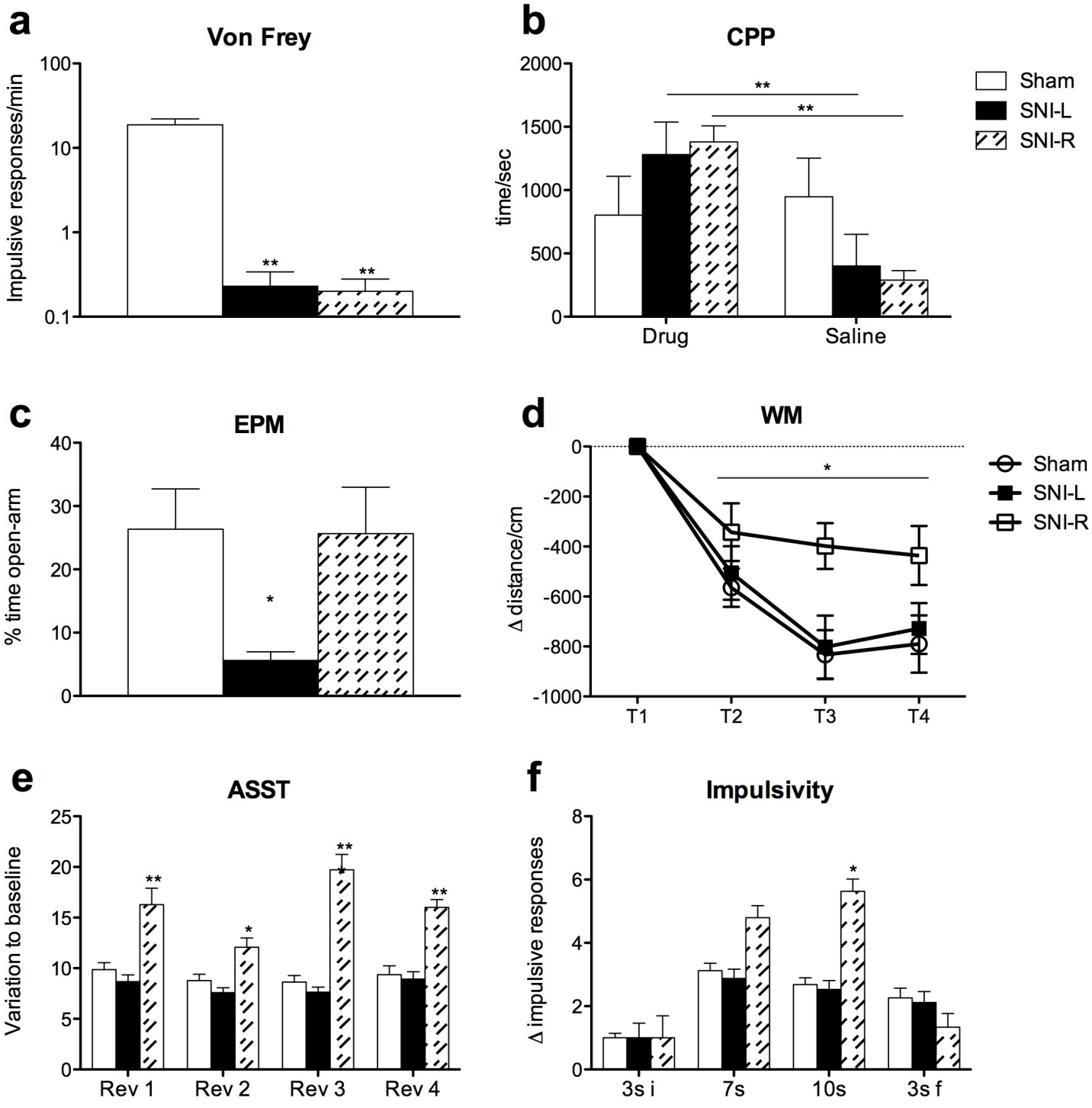
Figure 1. Left- and right-sided neuropathies are detrimental to emotional and cognitive behaviors, respectively. (a) Nerve injury produced a significant decrease in the monofilament-induced limb withdrawal threshold ($F_{2,13}$=25.98, P<0.0001), while the side of nerve injury had no significant effect on the withdrawal threshold. (b) Clonidine treatment (10 μL intrathecally) produced a significant place preference for the clonidine-paired chamber ($F_{1,16}$=26.84, P<0.0001), while the side of nerve injury (left versus right) had no influence on the clonidine-

induced place preference ($F_{1,16}$=0.00). (c) Left- but not right-sided SNI decreased the percentage of time spent in the EPM open-arm indicating anxiety-like behavior ($F_{2,37}$=3.888; P=0.03; SNI-R *versus* sham and SNI-L (P=0.05)). (d, e, f) The performance of SNI-R rats is impaired in PFC-dependent tasks. In the WM paradigm (d), the side of the SNI had an impact on animals' performance ($F_{2,48}$=4.987; P=0.01; SNI-R *versus* sham (P=0.01) and SNI-L (P=0.04)). Similarly, in the reversal steps 1-4 of ASST (e), the side of SNI had a significant effect ($F_{(2,45)}$=14.522, p<0.001; $F_{(2,45)}$=11.347, P<0.001; $F_{(2,45)}$=46.343, p<0.001 and $F_{(2,45)}$=24.661, P<0.001; respectively). SNI-R animals required a significantly higher number of trials to criteria than sham and SNI-L animals (P<0,001 in all comparisons). (f) The ability to refrain from responding when the delay-to-response was increased to 10 seconds ($F_{(2,21)}$=7,884, P=0,003) was impaired in the SNI-R group when compared to both sham (P=0,01) and SNI-E (P=0,007). Results presented as mean±SEM. *P<0.05, **P<0.001, ***P<0.0001 (Tukey's test).

Figure 1


a
Von Frey
Impulsive responses/min
100
10
1
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b
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0
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Differential effects of left/right neuropathy on rats' emotional behavior and executive function

Hugo Leite-Almeida, João José Cerqueira, Hong Wei, Nuno Ribeiro-Costa, Helena Anjos-Martins, Nuno Sousa, Antti Pertovaara, Armando Almeida

Supplementary Methods

Animals and surgery

2 months old, male, Wistar han rats were used in all experiments. Animals were kept in a room with controlled temperature (22 ± 1°C), 12 h light/dark cycle (lights on at 8 a.m.) and housed in groups of 2-3 animals in plastic cages with food and water available *ad libitum*. All procedures with animals were approved by the respective local organisms: Direcção Geral de Veterinária (Lisboa, Portugal) and The Experimental Animal Ethics Committee of the Provincial Government of Southern Finland (Hämeenlinna, Finland); and the experiments were performed according to the guidelines of European Communities Council Directive 2010/63/EU.

An experimental model of peripheral neuropathy was induced by a spared nerve injury (SNI)[1]. The unilateral axotomy and ligation of the tibial and common peroneal nerves on the left (SNI-L) or right (SNI-R) hindpaws was performed under 1,5:1,0 of ketamine (Imalgene)/medetomidine (Dormitor) at a dose of 1 mL/kg as described in detail elsewhere[1]. Briefly, the skin of the lateral surface of the thigh was incised and a section made directly through the *biceps femoris* muscle exposing the sciatic nerve and its three terminal branches. Following ligation and removal of 2–4 mm of the distal nerve stumps of the tibial and common peroneal nerves (the sural nerve was left intact), muscle and skin were closed in two layers. Sham-operated animals underwent identical surgical procedures (half of the group in the left and the remaining in the right paw) except that tibial and common peroneal nerves were left intact. After the surgery, animals were allowed to recover before the actual testing that was performed one month after the operation.

Animals participating in the conditioned place preference (CPP) experiment had an intrathecal (i.t.) catheter for drug delivery to the spinal cord. The catheter (Intramedic PE-10,

Becton Dickinson and Company, Sparks, MD, USA) was placed at the level of the lumbarspinal cord under pentobarbital anesthesia (50 mg/kg i.p.) one week before actual testing, as described in detail elsewhere[2]. Following recovery from anesthesia, the correct placing of the catheter was verified by injecting lidocaine (4%, 7–10 µl followed by a 10 µl of saline for flushing) with a 50 µl Hamilton syringe (Hamilton Company, Bonaduz, Switzerland). Only those rats that had no motor impairment before lidocaine injection but had a bilateral paralysis of hind limbs following i.t. administration of lidocaine were studied further.

Behavioral experiments

All behavioral experiments were performed during the light period of the daily cycle. Acclimatization to the testing room was allowed for approximately 1h. To avoid possible bias, in particular related with the testing period, animals belonging to different groups were tested alternately (e.g. sham, SNI-L, SNI-R,...).

Von Frey monofilaments. Development of neuropathic hypersensitivity was verified behaviorally in all animals. In the group undergoing CPP, this was done in the beginning before the installation of the i.t. catheter and was used to compare the lesion lateralization effect. In the remaining animals, hypersensitivity was assessed 1 week after the end of behavioral assessment to verify the successful development and maintenance of the neuropathy. Animals were habituated to the experimental conditions 1–2 h daily for 2 to 3 days. For assessment of tactile allodynia, the hind limb withdrawal threshold was determined by stimulating the sural nerve areas in the hind paw of the nerve-injured or sham-operated limb with monofilaments. The calibrated series of monofilaments used in this study produced forces ranging from 0.008 to 60 g (North Coast Medical, Inc. Morgan Hill, CA, USA). The monofilaments were applied to the lateral foot pad with increasing force until the rat withdrew its hind limb. The lowest force producing a withdrawal response was considered the threshold. The threshold for each hind paw of each rat was based on three separate measurements and the median of these values was considered to represent the threshold. Limb withdrawal threshold was assessed in a separate session one month after nerve injury or sham operation.

Conditioned place preference (CPP). For analysis of ongoing pain one month after the nerve injury or sham operation, rats received spinal clonidine as previously described for a single conditioning trial protocol[3]. All rats underwent a 3 day habituation, in which they were placed in automated CPP boxes (Place Preference System, San Diego Instruments, Inc., San Diego, CA, USA) with access to all 3 chambers for 30 min per day. Time spent in each of the boxes was recorded for 15 min on day 3 (Supplementary Figure S1a). Rats that spent more than 720 s in one of the conditioning chambers were eliminated from the study. The following day (day 4), all rats received a morning injection of saline and were immediately placed in the appropriate pairing chamber for 30 min. Four hours later, all rats received clonidine and were immediately placed in the opposite chamber for 30 min. Testing, in which the animals were placed drug-free in the CPP boxes with access to all chambers, occurred the following day (20 h following drug pairing). Clonidine hydrochloride (Sigma-Aldrich, St.Louis, MO, USA) was administered intrathecally at the dose of 10 µg and at the volume of 10 µl. Clonidine as well as saline control were flushed with 10 µl of saline. Clonidine and saline control injections were monitored by movement of an air bubble between the drug/saline control and the saline used for flushing.

Elevated-plus maze (EPM). The EPM was performed in a plus-shaped maze made on black polypropylene plastic, with two opposed open arms (50.8 cm x 10.2 cm) and two opposed closed arms with the same dimensions but surrounded by walls (40.6 cm height; Med Associates Inc., St. Albans, Vermont, USA). The whole structure was elevated 72.4 cm from the floor and was surrounded by 4 black walls perpendicular and at equal distances from each arm of the maze. Animals were placed at the centre of the maze and allowed to explore for 5 min. In the end of each trial all areas were cleaned with 10% alcohol and carefully dried. The number of arm entries and the time spent in each arm was registered. The percentage of the time spent in the open-arms is inversely related with anxiety-like behavior. The number of closed-arm entries was used as an index of locomotor activity.

Working memory (WM) and Morris water maze (MWM). The WM and MWM tests were performed in a circular tank (diameter 170 cm) with black walls located in the center of a room with white walls where black geometric figures have been placed as spatial references. A video camera positioned in the ceiling captured the image to a video tracking system (Viewpoint,

Champagne au Mont d'Or, France). A black, circular (diameter 12 cm) platform was placed within one of the quadrants virtually assigned in the computer. During the tests the water temperature was kept in the range 22-24°C and the level was such that it slightly covered the platform (therefore, invisible to the rats). In the beginning of each trial the animal was placed in the respective quadrant (Supplementary table 2) facing the maze walls. The trial was interrupted if the platform was found or if 120 seconds had elapsed (thereafter the animal was guided to the target). The animal was then allowed to spend 30 seconds in the platform before starting a new trial. In the WM paradigm, the daily trial-to-trial progression of the swim distance was averaged for the different platform locations, whereas in the MWM day-to-day progression (learning) was averaged across the 4 daily trials, for the same platform location.

Attentional-set shifting task[4]. The ASST was performed in a rectangular arena (40 x 70 cm) with 20 cm height walls. In one end of the arena a fixed divider created two compartments (20 x 20 cm) each containing a bowl (7 cm diameter/ 4 cm depth) filled with sawdust, whose top was at the floor level. Near the opposite end, a removable divider placed 25cm from the wall created a waiting area (25 x 40cm). 6 days before the experiment, food availability was restricted to the last hour of the day cycle. In the first 3 days animals also received in their cages, during the morning, 2 pieces of Cheerios (Nestle, Portugal) per animal. In the second 3 days, these rewards were delivered in the two bowls of the test arena, buried progressively deeper, until the animals become used to dig the media. The actual test spanned for 2 days, the first consisting of two simple discrimination tasks (odor and textures) and the last of 4 compound discrimination/reversal task pairs. Rats performed each task until a criterion of 6 consecutive correct trials was reached. Trials started by removing the divider of the waiting area and allowing the animal to explore the arena and terminated when the it excavated one of the bowls, only one of which was baited with ½ Cheerio. The location of the correct (baited) bowl varied in each trial according to a pre-established order (see Supplemental Table 3). In the odor and texture simple discrimination tasks, each bowl was marked with a different odor (aromatic oil) or texture (placed at the entrance of the choice compartments), respectively, one of which signaled the presence of the buried reward. In the compound tasks of the 2nd testing day, odors and textures (attentional dimensions) were simultaneously presented and the animal had to disregard the irrelevant dimension and focus on the relevant one. During each trial, procedures were similar to the ones described above. A first task in which odor was the

relevant cue was followed by its reversal (same odors and textures, but with the previously unbaited odor as the signal for reward), and then to a second pair of odors and textures, with odor the relevant dimension, at its reversal. Animals were then presented with a new group of odor and texture pairs, of which one texture was the relevant dimension, followed by its reversal, and a new group of odors/textures (with texture the relevant dimension) again followed for its reversal. Detailed information on the sequences used is presented in the Supplementary Table 3. The number of trials necessary to reach the criteria is used to compare the performances at each step.

Inhibitory control to variable delay. This task was performed in the classical 5-choice serial reaction time task apparatus[5], consisting in an nearly squared arena with 20cm tall walls, one being curved and having 5 apertures at the animal head level with nose-poke detectors and a light. In the opposite wall, a single aperture with similar dimensions is connected to a food dispenser. In this test, only the middle aperture out of the 5 nose-poke apertures was available (the remaining 4 were closed). The day before the first session, food was removed from animals' cages and thereon its availability was restricted to the last hour of the light cycle. Animals were trained to nose-poke the open aperture in order to receive a sugar pellet (dustless precision pellets; Bio-Serv, Frenchtown, US). Each trial in the training sessions started with the house-light on, signaling an ongoing trial. After a 3 seconds delay interval, the light-signaling aperture lighted up. A nose poke before the signal (impulsive response) interrupts the trial and initiates a "punishment" period of 5 seconds (house light off). On the other hand, a nose poke after the signal triggers a pellet deliver. Each training session spanned for 30 min or 100 trials. After nine training sessions all animals were able to accomplish 100 trials and, except for two (1 sham and one SNI-L), had less than 30% of impulsive responses. The 10th session animals were tests in a sequence of 25 trials at 3 seconds delay, followed by 70 trials at 7 or 10 seconds delay attributed randomly by the computer and returning finally to 3 seconds delay for more 25 trials. In this session premature responses were not punished. The total number of premature responses per total amount of delay time, were used to compare groups' performances.

Statistics

The data are presented as mean ± S.E.M. and analyzed using one- or two-way analysis of variance (1-w- or 2-w-ANOVA) or repeated measures of variance, accordingly, followed by Tukey's post-hoc test for multiple comparisons (comparison of three or more groups), or t-test (comparison of two groups). $P<0.05$ was considered to represent a significant difference.

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Supplementary figure 1

During pre-conditioning period, the animals failed to show preference for any of the chambers ($F_{1,26}$=0.45; p=0.51), independent of the experimental group ($F_{2,26}$=0.24; p=0.79;). Drug and saline indicate whether the chamber was paired with clonidine or saline treatment in the following day. Data presented as mean + S.E.M.

Supplementary figure 2

EPM closed-arms (CA) entries. Neither left- or right-sided SNI influences the number of CA entries ($F_{2,37}$=1.640; p=0.21) indicating that the groups do not differ regarding their locomotor activity. Data presented as mean + S.E.M.

Supplementary figure 3

The learning curves in the Morris water maze spatial reference memory task are given as the average daily gain in reference to day 1. A multivariate analysis indicates that SNI has no significant effect on the ability to learn the platform location $F_{(2,48)}$=1.628 (p=0.21). Data are presented as mean + S.E.M.

Supplementary figure 4

All steps of the ASST are presented. Except for the reversal steps (see main text) no differences were found between groups particularly in the Extra-Dimensional Shift ($F_{(2,45)}$=2.949, p=0.063) indicating that PFC medial areas are apparently spared after SNI. Data presented as mean + S.E.M.

Supplementary table 1

			Starting quadrant			
day	platform location	Protocol	T1	T2	T3	T4
1	W	WM	N	S	**W**	E
2	N	WM	W	**N**	S	E
3	E	WM	**E**	S	N	W
4	S	WM/MWM	E	W	N	**S**
5	S	MWM	N	**S**	E	O
6	S	MWM	**S**	N	O	E
7	S	MWM	O	E	**S**	N

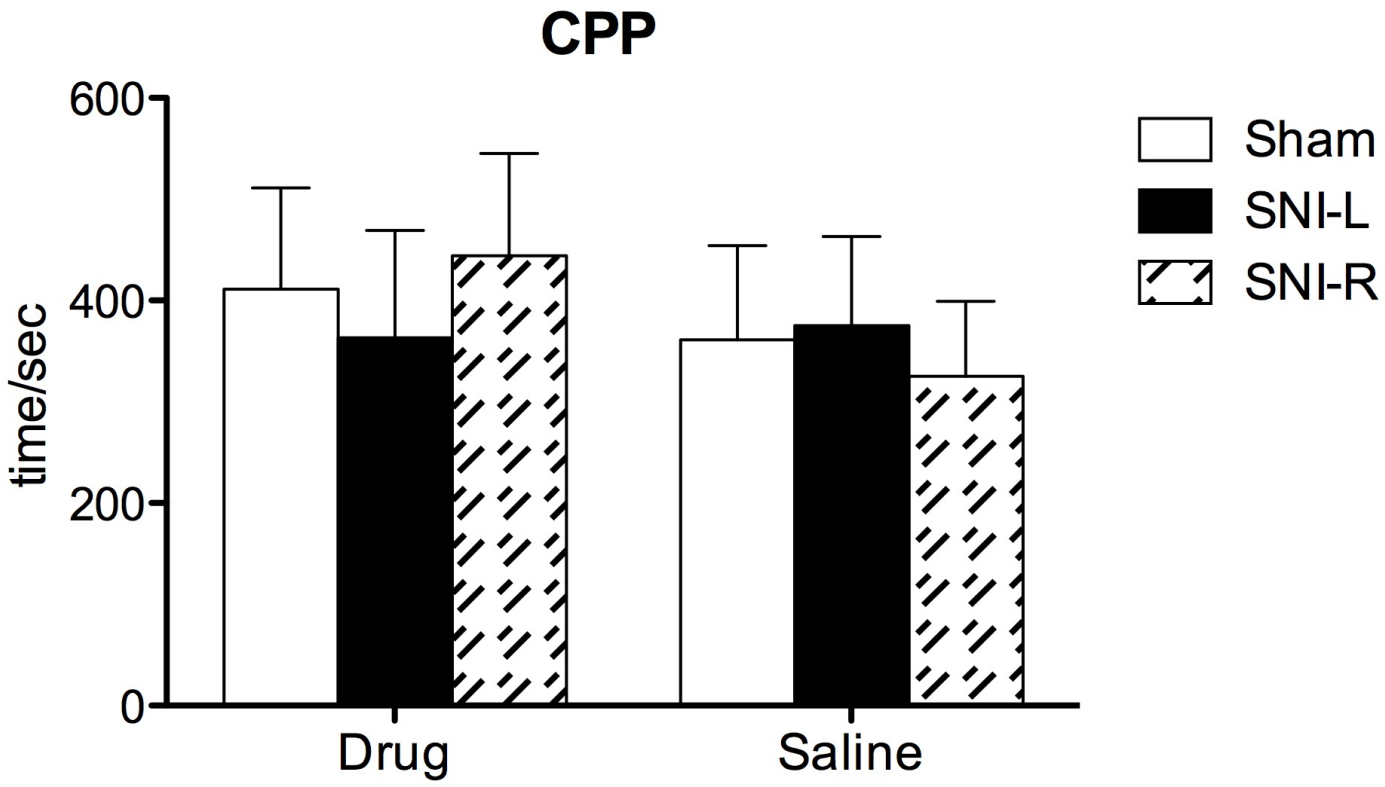
Supplementary table 1. Sequence and organization of the WM and MWM tests. Platform location on each day and the starting quadrant on each trial are given. Note that when the animal initiates the trial in the quadrant where the platform is located (bold), there exists an increased chance of finding the platform by accident. To avoid this possible bias, these trials were distributed evenly in the four days.

Supplementary table 2

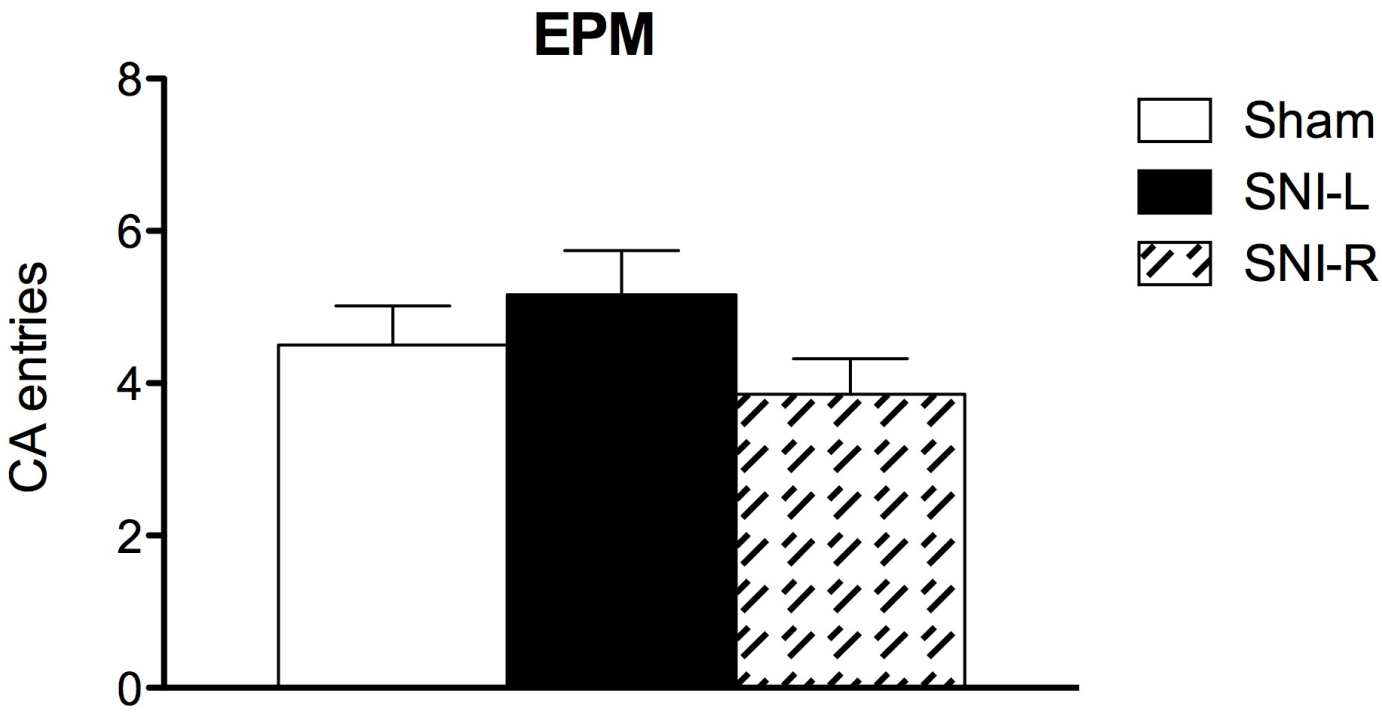
	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R
trial	1		2		3		4		5		6		7		8		9		10	
SD_{odo}	O_2	**O_1**	**O_1**	O_2	**O_1**	O_2	O_2	**O_1**	**O_1**	O_2	O_2	**O_1**	O_2	**O_1**	O_2	**O_1**	**O_1**	O_2	**O_1**	O_2
SD_{tex}	T_2	**T_1**	**T_1**	T_2	**T_1**	T_2	T_2	**T_1**	**T_1**	T_2	**T_1**	T_2	**T_1**	T_2	**T_1**	T_2	T_2	**T_1**	T_2	**T_1**
CD	**O_3**	O_4	O_4	**O_3**	O_4	**O_3**	O_4	**O_3**	**O_3**	O_4	**O_3**	O_4	O_4	**O_3**	O_4	**O_3**	**O_3**	O_4	**O_3**	O_4
	T_3	T_4	T_3	T_4	T_4	T_3	T_4	T_3	T_4	T_3	T_3	T_4	T_3	T_4	T_3	T_4	T_4	T_3	T_4	T_3
Rev_1	O_3	**O_4**	O_3	**O_4**	O_3	**O_4**	**O_4**	O_3	**O_4**	O_3	O_3	**O_4**	**O_4**	O_3	**O_4**	O_3	O_3	**O_4**	**O_4**	O_3
	T_4	T_3	T_4	T_3	T_4	T_3	T_4	T_3	T_3	T_4	T_3	T_4	T_3	T_4	T_4	T_3	T_3	T_4	T_3	T_4
IDS_1	**O_5**	O_6	**O_5**	O_6	**O_5**	O_6	O_6	**O_5**	O_6	**O_5**	O_6	**O_5**	O_6	**O_5**	O_6	**O_5**	**O_5**	O_6	**O_5**	O_6
	T_5	T_6	T_5	T_6	T_6	T_5	T_5	T_6	T_6	T_5	T_5	T_6	T_5	T_6	T_6	T_5	T_6	T_5	T_6	T_5
Rev_2	**O_6**	O_5	O_5	**O_6**	O_5	**O_6**	O_5	**O_6**	**O_6**	O_5	**O_6**	O_5	O_5	**O_6**	**O_6**	O_5	**O_6**	O_5	O_5	**O_6**
	T_5	T_6	T_6	T_5	T_6	T_5	T_5	T_6	T_6	T_5	T_5	T_6	T_5	T_6	T_6	T_5	T_6	T_5	T_6	T_5
EDS	O_7	O_8	O_8	O_7	O_7	O_8	O_7	O_8	O_7	O_8	O_8	O_7	O_7	O_8	O_8	O_7	O_8	O_7	O_8	O_7
	T_7	T_8	T_8	**T_7**	**T_7**	T_8	**T_7**	T_8	T_8	**T_7**	T_8	**T_7**	T_8	**T_7**	T_8	**T_7**	**T_7**	T_8	**T_7**	T_8
Rev_3	O_8	O_7	O_8	O_7	O_7	O_8	O_7	O_8	O_7	O_8	O_8	O_7	O_8	O_7	O_8	O_7	O_7	O_8	O_7	O_8
	T_7	**T_8**	T_7	**T_8**	T_7	**T_8**	**T_8**	T_7	T_7	**T_8**	**T_8**	T_7	**T_8**	T_7	**T_8**	T_7	T_7	**T_8**	**T_8**	T_7
IDS_2	O_9	O_{10}	O_9	O_{10}	O_{10}	O_9	O_9	O_{10}	O_9	O_{10}	O_{10}	O_9	O_{10}	O_9	O_9	O_{10}	O_{10}	O_9	O_{10}	O_9
	T_9	T_{10}	T_{10}	**T_9**	**T_9**	T_{10}	**T_9**	T_{10}	T_{10}	**T_9**	T_{10}	**T_9**	T_{10}	**T_9**	T_{10}	**T_9**	**T_9**	T_{10}	**T_9**	T_{10}
Rev_4	O_9	O_{10}	O_{10}	O_9	O_9	O_{10}	O_9	O_1	O_9	O_{10}	O_{10}	O_9	O_{10}	O_9	O_9	O_{10}	O_9	O_{10}	O_{10}	O_9
	T_9	**T_{10}**	T_9	**T_{10}**	T_9	**T_{10}**	**T_{10}**	T_9	T_9	**T_{10}**	T_9	**T_{10}**	**T_{10}**	T_9	**T_{10}**	T_9	T_9	**T_{10}**	**T_{10}**	T_9

Supplementary table 2. Odor and texture presentation at each ASST trial. If at the 10th trial the learning criteria was not successfully achieved the sequence was restarted. Odors: O_1-strawberry; O_2-camellia; O_3-lemon; O_4-papaya; O_5-rose; O_6-vanilla; O_7-lotus; O_8-cinnamon; O_9-mango; O_{10}-eucalyptus; Textures: T_1-styrofoam; T_2-sponge; T_3-sandpaper; T_4-scourer; T_5-sponge cloth 1; T_6- sponge cloth 2; T_7-cardboard; T_8- velvet; T_9-carpet; T_{10}-foam. L-left compartment; R-right compartment.

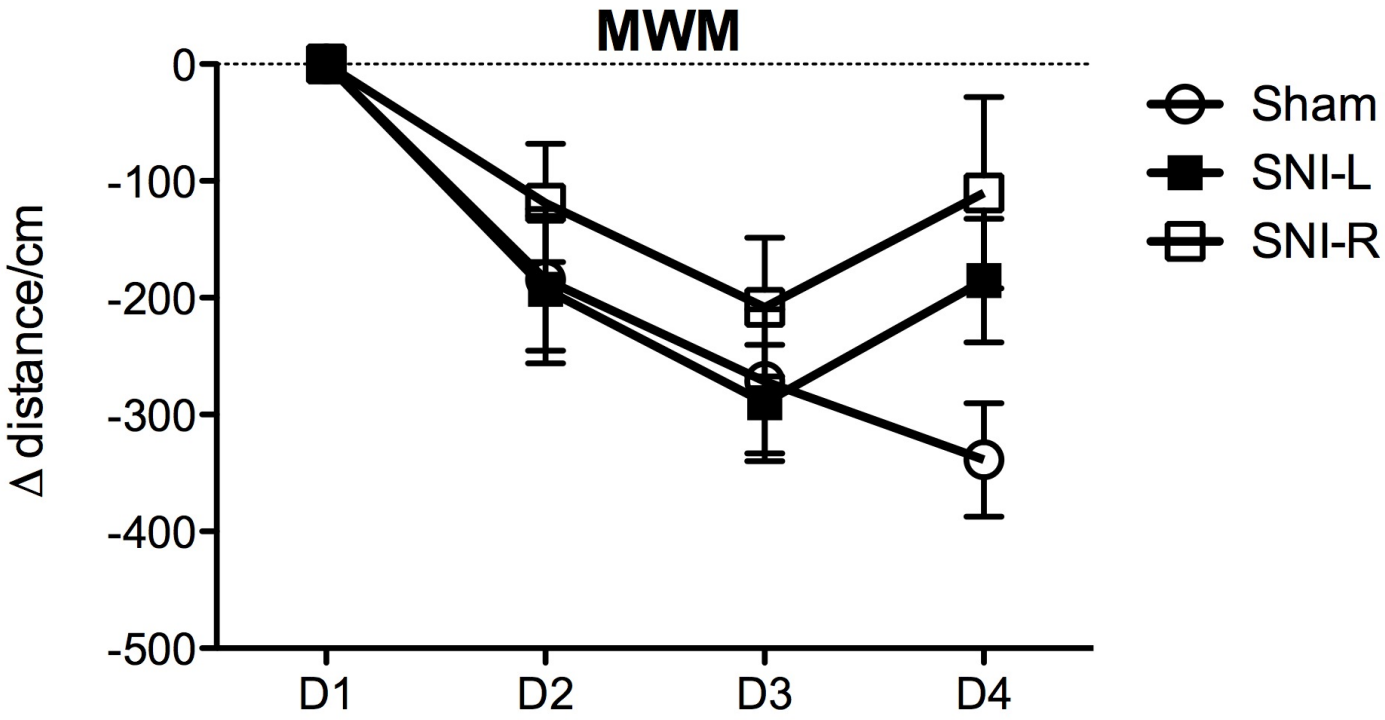
Supplementary Figure 1



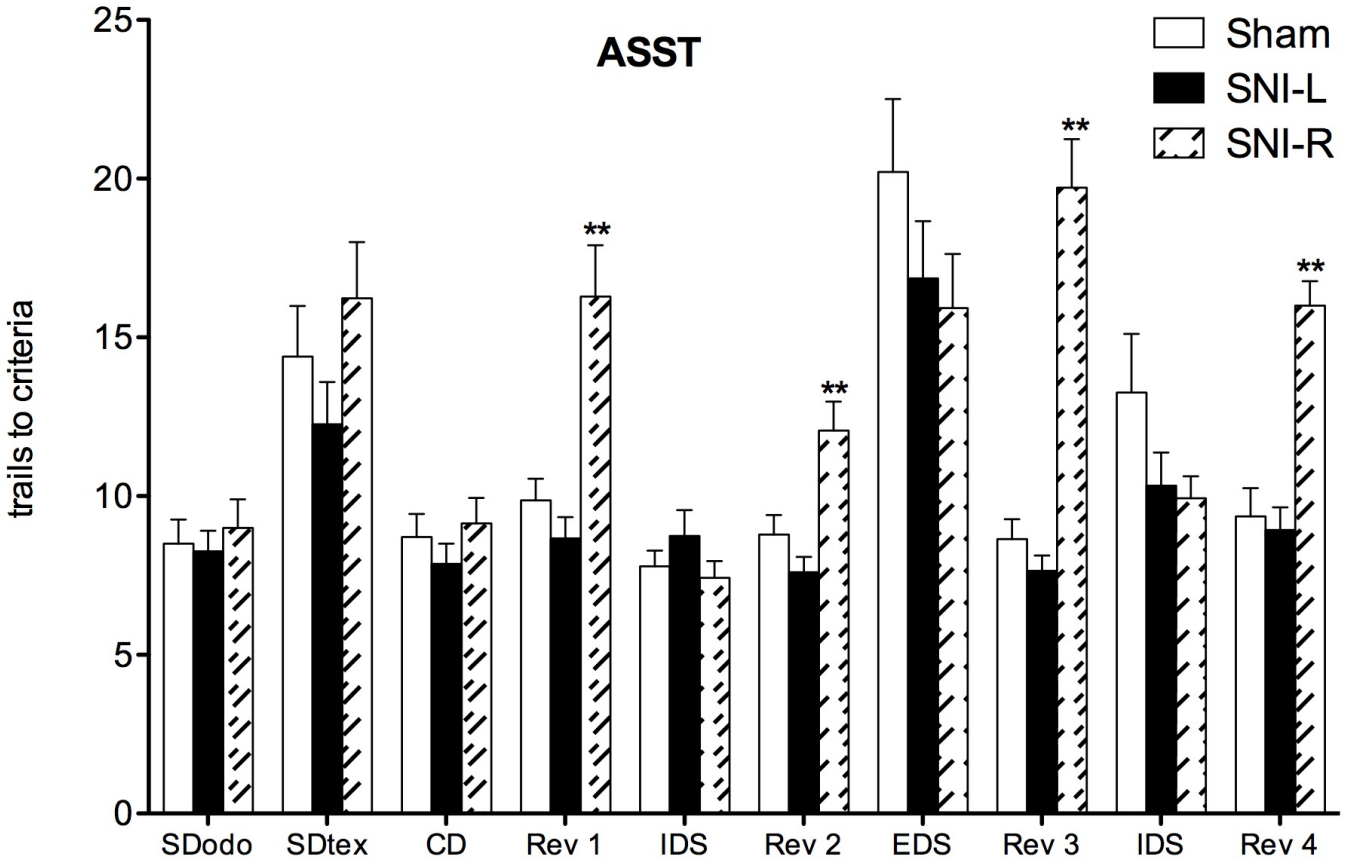
Supplementary Figure 2



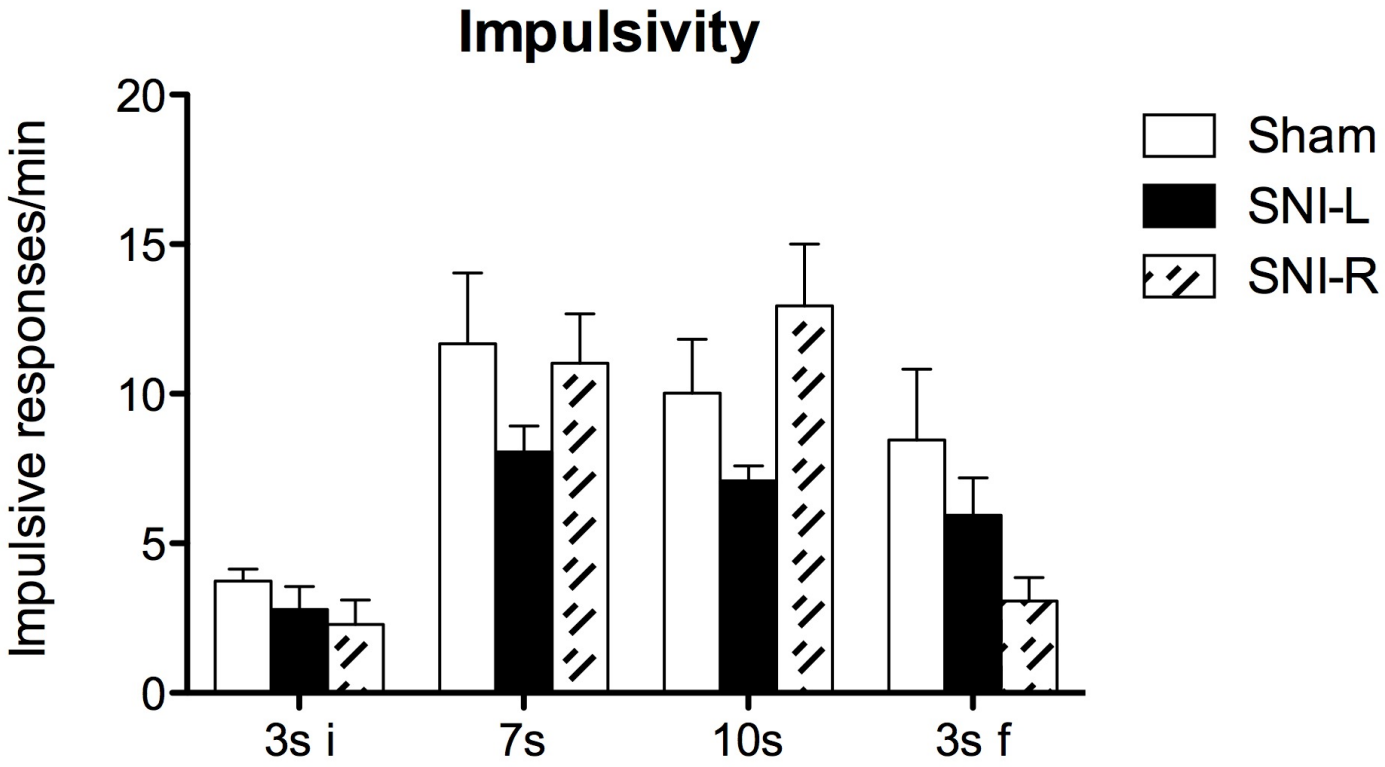
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Chapter 3

Results and discussion

3. Results and discussion

The work presented in this thesis illustrates the detrimental effect of chronic neuropathic pain on emotional and cognitive behaviour. It is in its conception a descriptive analysis, supported by a wide battery of behavioural tests. The spared nerve injury (SNI) model of chronic peripheral neuropathic pain was used in all experiments standardized to a 1 month period of undisturbed development. By keeping constant these two factors, i.e. model and neuropathy duration, the influence of two other variables, (i) the age of the animal at the time of pain onset and (ii) the laterality of the nerve lesion, were studied. The working hypotheses raised in each of these two cases shared a common ground, the neuropathy as a degenerative process that affects not only the primordial neuropathic spot but also remote areas of the nervous system either by degeneration (e.g. Wallerian), aberrant synaptic transmission, inflammation or a combination with variable degrees of contribution. In peripheral neuropathies, as is the case of the model adopted in the studies integrating this thesis, the areas being immediately affected are the spinal cord (or brainstem nuclei if a cranial nerve is affected) and the dorsal column nuclei, or to put it differently, areas where the affected nerves establish their first synapse. There, a cascade of events follows at molecular, cellular and morphological levels with immediate manifestations of abnormal sensation and hypersensitivity. Human and animal studies in the past years revealed that cortical and subcortical areas are also subject to plastic transformation as a consequence of prolonged pain and that these are temporally coincident with the emergence of altered emotional and cognitive behaviours. However, animal models have shown inconsistent results concerning the manifestation of these traits, suggesting that these models are not thoroughly characterized. The effects of age at pain onset and of lateralization of pain on such manifestations are discussed as well as the potential involvement of the prefrontal cortex (PFC).

3.1. Experimental considerations

3.1.1. Animal model and pain assessment

Over the last decades, a significant number of chronic pain animal models have been developed modelling different aspects of clinical chronic pain syndromes (reviewed in section 1.3.1.; Bennett *et al.*, 2003; Martin *et al.*, 2003; Jarvis and Boyce-Rustay, 2009; Bennett, 2010; Colleoni and Sacerdote, 2010; Jaggi *et al.*, 2011). The SNI model (Decosterd and Woolf, 2000) was adopted in the experimental work presented in this thesis. Briefly, it consists in the ligation and distal axotomy of the tibial and common peroneal branches of the sciatic nerve, preserving a third branch, the sural nerve. Characteristic signs of neuropathy emerge a few hours after the surgery, including mechanical/temperature allodynia and hyperalgesia. Importantly, the model is temporally stable in time making it particularly suited for long experiments. This, allied with its technical simplicity and reproducibility, were the main reasons for its selection.

In all experiments, the model was installed 1 month prior to the initiation of the behavioural studies in male Wistar Han rats. During this period animals were handled in a random schedule to prevent habituation while reducing bias due to novelty exposure stress. Aging studies included three groups of animals aged 2, 9 and 21 months. The SNI lesion was installed on the left side in this case. In the lateralization studies, 2 months-old animals were used. The SNI was installed either in the left or right side. Von Frey calibrated filaments were used in all cases to access the occurrence of mechanical allodynia (Chaplan *et al.*, 1994), a hallmark of ongoing neuropathy. This assessment was performed at the end of the behavioural tests to prevent any interference. Additionally, analgesia conditioned place preference (CPP) was also employed in the lateralization experiments to assess ongoing pain (King *et al.*, 2009).

3.1.2. Behavioural paradigms

3.1.2.1. Emotional behaviour

Elevated plus maze

The elevated-plus maze (EPM) test is the gold-standard paradigm used for testing anxiety-like behaviour in rodents (Handley and Mithani, 1984; Pellow *et al.*, 1985; Sousa *et al.*, 2006). It consists in an elevated plus shaped maze with opposing open and closed arms. The rationale of the test lays on the conflict between the innate drive to explore novel spaces and the aversion to open areas that occurs in rodents. Anxious behavioural profiles are correlated with shorter periods of time spent in the open arms. The validity of the test has been extensively documented (Cruz *et al.*, 1994; Rodgers and Johnson, 1995; Rodgers and Dalvi, 1997; Wall and Messier, 2001).

Forced swimming test

The forced swimming test (FST) was conceived in the mid 1970's as a screening test for putative antidepressant properties of drugs in rodents (Porsolt *et al.*, 1977a; Porsolt *et al.*, 1977b; Porsolt *et al.*, 1978). In this paradigm the animal faces an inescapable aversive scenario (a cylinder filled with water) leading to a condition of learned helplessness that is a hallmark of depressed individuals. The perseverance of the animal to escape the situation inversely correlates with depressive-like behaviour. The FST has been extensively validated and is the most common test used to assess depressive-like behaviour in rodents (Cryan *et al.*, 2005; Petit-Demouliere *et al.*, 2005; Sousa *et al.*, 2006).

3.1.2.2. Cognitive behaviour

A battery of cognitive tasks was selected to study the effect of chronic pain on cognitive performance (Sousa *et al.*, 2006). Successful performance in the majority of these tasks is known to be dependent on the PFC functional integrity (for detailed discussion see 3.4.; Dalley *et al.*, 2004; Robbins and Arnsten, 2009). This option was made based on the consistent

findings of morphofunctional maladaptative plasticity in this area during chronic pain conditions (see 1.3.2.). Morris water maze (MWM), a hippocampal dependent task, was used for comparison (Morris, 1984).

Working memory

A number of tasks have been envisaged to test for working memory (WM) in rodents (Dudchenko, 2004). In the set of studies performed, the spatial WM version of the MWM (see below for details) was used (Frick *et al.*, 1995). The task was performed in a circular pool of water where a platform was accessible but concealed slightly below the water level. Each day, for four consecutive days, the platform location was changed and the animal was given four trials to learn it. Therefore, information concerning the platform location had to be acquired (first trial) and retrieved (remaining trials). The trial-to-trial progression was analysed in terms of the amount of time (or swam distance) elapsed to reach the platform. We also analysed MWM long-term spatial reference memory for comparison. In this case the platform location was kept in the same location for four consecutive days. The daily progression in terms of the amount of time (or swam distance) elapsed to reach the platform was analysed. Water mazes are advantageous over other WM and long term memory paradigms in many aspects (Dudchenko, 2004); food or water deprivation are not required as the motivating stimulus, i.e. escaping from the water, an innate behaviour in rodents and, additionally, intramaze cues (e.g. odours) are nearly absent. The principal disadvantage relates to the fact that the animal can adopt other strategies, namely egocentric orientation, not related with the rational of WM or MWM.

Reversal learning

Reversal following MWM acquisition. The classical MWM was applied as originally described (Morris, 1984). After the fourth day of MWM, the platform location was finally moved to a new location. The percentage of time (or distance) spent in old and new quadrants were used as an index of reversal learning ability.

Attentional-set shifting task (ASST). The ASST is an analogue of the Wisconsin Card Sorting test (Berg, 1948; Milner, 1963; Monchi *et al.*, 2001) that has been adapted for primates (Dias *et*

al., 1996a, b, 1997) and rodents (Birrell and Brown, 2000). The test consists in the sequential acquisition and reversal of rules regarding information leading to a reward (Birrell and Brown, 2000). The rule indicating a food reward is constantly being shifted either within or between a perceptual dimension (odour or texture). The set of cues consists of two different odours and two different textures, being simultaneously presented to the animal (compound discrimination, CD). In the first CD, the reward was associated with one of the odours. Following a successful acquisition (six consecutive rewarded trials), the reward was then associated with the second odour - reversal (R1). Once the criterion was attained, new pairs of odours and textures were introduced. The procedure was repeated in a first instance maintaining the perceptual dimension - intradimensional shift (IDS1)/R2 – and then changing it to textures - extradimensional shift (EDS)/R3. Finally, an IDS2/R4 was performed. The ASST is advantageous over the MWM/reversal as it allows to discriminate PFC subfunctions (see below; Birrell and Brown, 2000) and limits the use of alternative strategies, e.g. egocentric orientation. Food deprivation is required, but only for a short period of time and thus it should not constitute a major drawback.

Impulsivity

The 5-choice serial reaction time task (5-csrtt) was primarily selected to test impulsive behaviour (Carli *et al.*, 1983; Robbins, 2002; Bari *et al.*, 2008). The rational behind the task follows that of Leonard's 5-csrtt developed to study sustained attention in humans (Leonard, 1959). In this task animals are trained to poke the nose in a lighted aperture out of five possible choices in order to gain a sugared reward. The attentional demand of the task is increased by successively decreasing the light signal duration whenever the animal reaches accuracy levels over 80%. We found that the amount of sessions needed to reach the last level varied substantially between animals (from 3 weeks up to 2 months), independently of their experimental condition. However, in this level we had a strong indication that right-sided SNI animals presented increased levels of impulsive responses. To validate this observation, we designed a delay-to-signal task, focusing on the impulsive response. In this task, the light signal was presented with variable delays but in a fixed location. The total number of premature responses (i.e. nose pokes during the delay period) was compared between the groups. This task is advantageous over the 5-csrtt in that animals learn it faster (up to 8 days) and more

homogeneously, implying a shorter period of alimentary restriction. Additionally, the attentional component is minimized facilitating the interpretation of the results.

3.2. Impact of neuropathic pain on emotional behaviour

The association between chronic pain and mood disorders is extensively documented in human subjects (Blackburn-Munro and Blackburn-Munro, 2001; Wiech and Tracey, 2009). It is bidirectional in the sense that both chronic pain potentiates the emergence of mood disorders (Dworkin and Gitlin, 1991), but also in that the latter can increase the risk for chronic pain (Magni *et al.*, 1994; Carroll *et al.*, 2004; Larson *et al.*, 2004). Additionally, in the past decade it was demonstrated that animal models of prolonged pain also manifest altered emotional behaviour, particularly anxiety- and depressive-like behaviour, paving the way for a new comprehension of the pathophysiology of deteriorated emotional behaviour in the context of chronic pain. However, results obtained by different researchers greatly diverge. This derives to a great extent from the experimental heterogeneity among the different studies (see table I) as factors as specie, strain, gender, age, husbandry and testing procedures are known to greatly influence the outcome measures (Mogil, 2009). These will be briefly discussed below.

Strain/genetic background. There is currently no experimental evidence that the genetic background influences the expression of emotional disorders in animal models of prolonged pain. However, this is highly probable as, when analysed individually, pain-related behaviours (LaCroix-Fralish *et al.*, 2005) and anxiety/depression manifestations (O'Neil and Moore, 2003; Hamet and Tremblay, 2005; Malkesman and Weller, 2009) greatly depend on the genetic background. Furthermore, two reports have consistently demonstrated that increased levels of basal trait anxiety are predictive of increased pain-like behaviours in neuropathy models (Vatine *et al.*, 2000; Roeska *et al.*, 2009).

Gender. All information concerning the impact of chronic pain on emotional behaviour has been obtained in male subjects (table I). Therefore gender cannot account for the diversity of published results. However it is expectable that gender plays a role, not only because emotional traits present gender-related differences in basal conditions (Palanza, 2001) but also

because sexual hormones modulate pain perception (Craft *et al.*, 2004; Aloisi and Bonifazi, 2006).

Pain duration. In chronic pain models, manifestations of abnormal emotional behaviour are delayed in relation to the emergence of pain behavioural signs. Most probably, this reflects different kinetics on the plastic adaptations taking place in the supraspinal centers and dorsal horn, respectively (see 1.3.2.). Additionally, distinct traits of emotional behaviour, like anxiety and depression, present a temporal mismatch in their manifestations, the former preceding the latter (Suzuki *et al.*, 2007; Yalcin *et al.*, 2011). Thus, the duration of the neuropathy in experimental studies is probably one of the factors mostly associated with the variability on the reported results (table I).

Pain model. A small number of studies (see table I) demonstrated that the emergence of emotional disturbances depend on the type of pain model used. Comparative analyses indicate that models associated with lower withdraw thresholds to mechanical stimulation are more anxiogenic, suggesting that pain magnitude can be a determinant factor triggering abnormal affective behaviour (Hasnie *et al.*, 2007a; Roeska *et al.*, 2008).

Experimental conditions. Factors related with the experimental organization and husbandry impact on the manifestation of behavioural traits (Sousa *et al.*, 2006; Rice *et al.*, 2008; Mogil, 2009). Although these are not always described with detail in the scientific reports, some possible sources of bias can be detected including nociceptive testing immediately prior to anxiety assessment or test repetition in paradigms that require novelty, for instance.

The impact on emotional behaviour on parameters such as age at the time of pain onset and anatomical location of the pain focus, namely its lateralization, were unexplored when the experimental work of this thesis was initiated. Both parameters were considered fundamental to the overall characterization of the phenomena in the animal model and, most of all, to guide further research on the underlying pathophysiology.

Table I. Evaluation of anxiety- and depression-like behaviour in chronic pain models

trait	paradigm	species/strain	model	age	side	overall effect	references
anxiety	EPM; DB/LB	rat; SD	SNL	y	l	No differences were detected between injured and sham controls at day 14;	Kontinen *et al.*, 1999
	EPM; DB/LB	mouse; B6	PSNL; CFA	y	r	Anxiety-like behaviour in the EPM and DB/LB at day 28; EPM tested at 7(b) and 28(a,b) day; DB/WB tested at 7(a,b), 14(a), 21(a) and 28(a,b) day;	Narita *et al.*, 2006a; Narita *et al.*, 2006b
	OF	rat; Wistar	VZV; PSNL; SNT	y	l	Positive correlation between mechanical hypersensitivity and anxiety in the VZV and SNT models at day 14;	Hasnie *et al.*, 2007a
	EPM; OF	mouse; B6	PSNL	y	l	No differences were found at days 7, 14 and 28;	Hasnie *et al.*, 2007b
	OF; DB/LB; EPM	mouse; B6	SNL	y	l	Signs of anxiety-like behaviour at post-surgery week 8 in all paradigms[1];	Suzuki *et al.*, 2007
	OF	rat; Wistar	PSNL; gp120	y	l	Signs of anxiety-like behaviour at day14; a second group was tested at day 7 but has not manifested any alteration;	Wallace *et al.*, 2007
	OF	rat; Wistar	ddC; ddC+gp120	y	syst; syst/l	Signs of anxiety-like behaviour at day 14 (ddC+gp120) and 21 (ddC);	Wallace et al., 2007b, 2008
	EPM	mouse; B6	SC	y	r	Anxiety-like behaviour at day 30;	Benbouzid *et al.*, 2008
	DB/LB; EPM	mouse; B6	PSNL	y	r	Anxiety-like behaviour at post-surgery day 27 in both paradigms;	Matsuzawa-Yanagida *et al.*, 2007
	OF; EPM	rat; Wistar	SNI	y	r	No signs of anxiety-like behaviour at day 60;	Goncalves *et al.*, 2008
	EPM	rat; Wistar	CCI; PSNL	y	l	Anxiety-like behaviour at day 28 (CCI); ≈ 40% less time in the OA PSNL compared to sham (no statistical significance);	Roeska *et al.*, 2008
	EPM	rat; Wistar (HAB and LAB)	CCI	y	n/a[2]	Anxiety-like behaviour both in HAB and LAB animals at post-surgery day 36;	Roeska *et al.*, 2009
	OF	mouse; ddY	PSNL	y	r	No signs of anxiety-like behaviour at at post-surgery days 7-9;	Kodama *et al.*, 2011
	OF; EZM; mb	mouse; Balb/c and B6	SNI; CCI; CFA	y	l	Results vary according to the paradigm, sp/st and model 30 days after pain onset. Generally, no alterations or decreased anxiety;	Urban *et al.*, 2011
	DB/LB; mb; NSF	mouse; B6	SC	n/a	r	Increased anxiety from the 4th up to the 8th post-surgery week;	Yalcin *et al.*, 2011
depression	FST	rat; SD	SNL	y	l	No differences were detected between injured and sham controls;	Kontinen *et al.*, 1999
	TST	mouse; B6	PSNL	y	l	No differences were found at post-surgery days 7, 14 and 28;	Hasnie *et al.*, 2007b
	FST	mouse; B6	SNL	y	l	Depressive-like behaviour at post-surgery week 8;	Suzuki *et al.*, 2007
	TST	mouse; B6	SC	y	r	No differences were detected between injured and sham controls;	Benbouzid *et al.*, 2008
	FST	rat; Wistar	SNI	y	r	Depressive-like behaviour at day 60;	Goncalves *et al.*, 2008
	FST	rat; Wistar	CCI	y	l	Depressive-like behaviour at post-surgery day 21-28;	Hu *et al.*, 2009
	FST	mouse; B6	SNI	y	r	Depressive-like behaviour at post-surgery day 7;	Norman *et al.*, 2009
	FST	rat; SD	SNL (L5)	y	n/a	Depressive-like behaviour at post-surgery day 29;	Hu *et al.*, 2010
	FST; SPT	mouse; Balb/c and B6	SNI; CFA	y	l	No alterations up to post-surgery day 40;	Urban *et al.*, 2011
	Splash test; FST	mouse; B6	SC	n/a	r	Depressive-like behaviour at post-surgery weeks 6-9;	Yalcin *et al.*, 2011

Table I. Summary of the literature concerning the effects of chronic pain in anxiety- and depression-like behaviour in rodent models. Male animals were used in all studies. Abbreviations: CCI – chronic constriction injury; CFA - complete Freund's adjuvant; DB/LB – dark/light box; ddC – 2'-3'-dideoxycytidine (zalcitabine; antiretroviral agent); EPM – elevated-plus maze; EZM – elevated-zero maze; FST – forced swimming test; gp120 - immunodeficiency virus type I envelope glycoprotein 120; HAB – high anxiety-like behaviour; l – left; LAB – low anxiety-like; mb – marble burying; n/a – not applicable (available); OF – open-field; NSF – novelty suppressed feeding test; PSNL – partial sciatic nerve ligation; SC sciatic nerve cuffing ; SD – Sprague-Dawley; SNI – spared nerve injury; SNL – spinal nerve ligation; SPT – sucrose preference test; syst – systemic; r – right; SNT - spinal nerve transection; TST - tail suspension test; VZV - Varicella zoster virus ; y – young. Notes: [1] in a second experiment, a group of animals was tested at post-surgery days 2, 7, 15 and 30; depressive- and anxiety-like behaviours manifested at days 15 (FST) and 30 (OF, EPM), respectively; [2] left-sided in previous reports.

3.2.1. Age effect

In chapter 2.1. of this thesis we demonstrated that young and old SNI animals had an increased anxiety-like behaviour when compared to the respective age-matched sham controls. On the contrary, mid-aged SNI and sham-operated animals presented a similar behavioural profile in the EPM. A substantial decrease in the percentage of time spent in the open-arms by the sham group (and not a recovery of SNI) accounts for this result in mid-aged animals. Additionally, there was an age-related increase in the anxiogenic profile which is in accordance with the observations made by others (Lamberty and Gower, 1990; Frussa-Filho *et al.*, 1992; Lamberty and Gower, 1992; Li *et al.*, 1995; Boguszewski and Zagrodzka, 2002; Bessa *et al.*, 2005; Pego *et al.*, 2006; Meyza *et al.*, 2011; Moretti *et al.*, 2011). Importantly, the presence of the neuropathic lesion seemed to contribute to accelerate the aging trend.

On the contrary, young and old SNI animals do not display major differences in the manifestation of depressive-like behaviour in the FST, as assessed both by the latency to immobility and the immobility parameters, when compared with the respective age-matched controls. Curiously, mid-aged controls and SNI differed significantly as a result of a decreased immobility in the sham group (when compared with young and old sham groups). This age-related pattern observed in the sham group was in accordance with studies performed by others (Miyagawa *et al.*, 1998; Zambrana *et al.*, 2007). Taking into account the data obtained in the EPM it is apparently paradoxical that in the FST, SNI did not trigger a deterioration of the emotional response. However, a recent study suggests that depressive-like behaviour in rodents with neuropathic pain is a late onset phenomena emerging around the 8th week after the neuropathy installation (Yalcin *et al.*, 2011), which may justify the absence of depressive-like manifestations in our animals (4-week neuropathy). This observation is not consensual but other studies seem to support this possibility (see table I; Suzuki *et al.*, 2007; Goncalves *et al.*, 2008).

The pathophysiology underlying altered emotional behaviour in chronic pain conditions remains largely unknown. Comparable behavioural impairments are observed in chronic stress conditions largely as a result of a sustained activation of the hypothalamic-pituitary-adrenal axis (HPA). Neuropathic pain is an inescapable stressor and in this context some groups measured

the levels of circulating adrenocorticotropic hormone (ACTH) and corticosterone (Bomholt *et al.*, 2005; Ulrich-Lai *et al.*, 2006; Norman *et al.*, 2009; Yalcin *et al.*, 2011) as well as thymus and adrenal weights (Ulrich-Lai *et al.*, 2006) to conclude that they were unaltered. However, the expression of corticotropin releasing factor (CRF) and glucocorticoid receptor (GR) were found to be increased in the Amy (and the latter also in the hippocampus) but not in the hypothalamic paraventricular nucleus (PVN), which is a central area in the HPA axis. Furthermore, CRF receptor antagonism was demonstrated to diminish pain-related (acute inflammatory condition) anxiety (Ji *et al.*, 2007). Additionally, in a chronic SNI condition, blockage of CRF binding protein (implying increased free CRF) was shown to increase the preference for the dark compartment of the dark/light box (DB/LB) paradigm (LaBuda and Fuchs, 2000) reflecting an anxiogenic effect (Bourbia *et al.*, 2010). The interpretation of this effect has however to account for the dual pro- and anti-nociceptive actions of CRF (Ji and Neugebauer, 2008; Bourbia *et al.*, 2010). Altogether, the available data support the idea that the underpinnings of emotional disturbances associated with chronic pain are mainly extra-HPA. During the ageing process, on the other hand, it is known that the regulatory mechanisms of the HPA axis are altered toward an increased level of circulatory ACTH and corticosteroids and that the inhibitory feed-back loop is less effective when a HPA response is mounted (Sapolsky, 1999; Pedersen *et al.*, 2001). It is therefore probable that both systems concur to the overall modulation of emotional behaviour, explaining the potentiation of the SNI effect observed in the aged animals.

Alternatively, the hypothesis of a neuro-immune contribution to the modulation of emotional behaviour has been gaining currency. In this context, the observation of an increased expression of interleukin 1β (IL-1β) in the brainstem, thalamus and PFC in neuropathic pain models (Apkarian *et al.*, 2006; Norman *et al.*, 2009) and, particularly, an IL-1β receptor antagonism mediated rescue of a depressive-like behaviour in the same conditions seems to support such view (Norman *et al.*, 2009). It should be noticed that the magnitude of IL-1β expression differed between the two models of neuropathic pain used by Apkarian and colleagues (2006), evidencing the singularity of each model. Additionally, alterations of opioidergic function in the Amy (Narita *et al.*, 2006a) and PFC (Narita *et al.*, 2006b) have been associated with abnormal emotional behaviour in chronic pain conditions, further highlighting the multifactorial character of the phenomenon.

3.2.2. Lateralization effect

Animals with left-sided SNI manifested increased anxiety-like behaviour when compared with both right-sided SNI and the sham control (chapter 2.2). Measurements of either evoked or ongoing pain showed no differences that could account for our observations. Only two human studies by Gagliese and colleagues tested putative differential effects of lateralized pain on emotional behaviour and both concluded similarly, i.e., left-sided pain is more anxiogenic (Schiff and Gagliese, 1994; Gagliese *et al.*, 1995). On the other hand, innumerable animal studies greatly diverge on results (see table I). These discrepancies may relate to the diversity of pain models, time points for behavioural assessment, the usage of strains that differ on basal conditions in emotional manifestations and incorrect organization of the behavioural tests (some authors report pain tests prior to EPM) hindering any comparison. This point will be further discussed in section 3.4. in the context of spinal cord ascending pathways to the PFC, a potential mediator of the lateralized effect.

3.3. Impact of neuropathic pain on cognitive performance

It has been recognised that individuals with chronic pain frequently display impaired attention and a slower ability to process information (Moriarty *et al.*, 2011). Some authors attributed such impairments to the presence of a highly salient and perseverant stimulus – pain – that, by consuming processing capacity, diminishes the efficient process of other types of information (Eccleston, 1994, 1995; Legrain *et al.*, 2009). Brain imaging studies, namely the seminal studies by Apkarian and colleagues would, however, reveal a different picture (Apkarian *et al.*, 2004). In this report, it was concluded that individuals with chronic pain presented severe structural alterations in cortical areas involved in cognitive function, namely the prefrontal cortex (PFC). To put it in the authors' own words "...the brain of a chronic pain patient is not simply a healthy brain processing pain information, but rather is altered by the persistent pain in a manner reminiscent of other neurological conditions associated with cognitive impairments." (Baliki *et al.*, 2008). Until very recently, the effect of chronic pain in cognitive performance was largely uncharacterized in the animal model. Importantly, none of the studies available was concerned with the distinction between affected and spared cognitive

domains, a fundamental aspect of validity of the model as a correlate of human pathology. This scenario prompted us to initiate a series of behavioural tests aiming to further clarify this issue. The organization rationale of the cognitive tests was similar to that followed in emotional behavioural studies (section 3.2.) both in terms of the pain model selected as well as of the parameters age and laterality of pain focus. Finally, the experimental considerations raised on section 3.2. concerning the impact of factors as strain, gender, pain model, among others, on the emotional behaviour, also apply in the context of cognitive studies. However, the scarcity of published reports hinders any detailed comparative analyses on each of these factors (table II).

3.3.1. Age effect

In chapter 2.1. we demonstrated that the increased age of the experimental subject was a determinant factor for poorer performances in the reversal learning task, marginally significant for WM task and not relevant for the performance in the MWM. Chronic pain (left-sided SNI) was detrimental for the performance only in the reversal task affecting specifically the mid-aged group, mid-aged sham operated animals performance being comparable to that of young (SNI and Sham) animals and that of mid-aged SNI comparable to old (SNI and sham) animals.

Aging seemed to be selectively detrimental in the tasks that are more dependent on the functional integrity of the PFC, i.e., reversal learning, and spared the hippocampal dependent MWM. WM task depends on the hippocampal-to-PFC communication (Cerqueira *et al.*, 2007) and in that sense it is not surprising that the performance on this task was also affected by aging though not as severely as the reversal learning task. In agreement with our observations, in a cross sectional study in humans, negative associations between volume and age were found to be stronger in the PFC than in the hippocampus in (Raz and Rodrigue, 2006). It should however be acknowledged that interindividual heterogeneity concerning the onset and the affected brain areas has been found in a longitudinal study on healthy subjects (Raz *et al.*, 2005).

Current data on the association between pain and age is controversial, some authors defending a putatively increased algesic quality of neuropathies in mid-aged individuals while others not (Gagliese and Melzack, 2000). In our experiments we showed that pain-related

behaviours evoked by stimulation of the affected dermatomes with calibrated Von frey filaments did not differ between young, mid-aged and old animals and, therefore, pain intensity should not be an accountable factor for the cognitive impairment observed.

3.3.2. Lateralization effect

Following the initial studies on aged animals (chapter 2.1.), the PFC emerged as a probable candidate mediating the observed behavioural effects considering that its dependent functions appeared particularly vulnerable in chronic pain conditions. We have therefore increased the battery of cognitive tests in the second series of experimental work in order to test for other PFC-dependent functions like impulse control and attentional-set shifting (see chapter 2.2.). We demonstrated that in all these paradigms animals with right-sided neuropathy had consistently poorer performances when compared to sham operated animals or left-sided SNI animals. Importantly, we have found no differences in the hippocampal dependent MWM reinforcing the idea that the SNI lesion, particularly when right-sided, is more detrimental for PFC-dependent functions.

In the spatial reference WM test we observed that the trial-to-trial average distance difference in the water maze was significantly smaller in rats with a right-sided neuropathy when compared to left-sided and controls, reflecting an impaired learning ability. Contrary to our observations, in a recently published study (table II; Ren *et al.*, 2011) left-sided SNI animals were found to have an impaired WM performance in the 8-arm radial maze (8-arm; Olton and Samuelson, 1976). The paradigms used in the two studies, although sharing a common rationale, differ significantly in many aspects (Sharma *et al.*, 2010), which can possibly account for the observed differences. A curious aspect of the 8-arm study is that while control animals demonstrate a clear learning curve, stabilizing on the 3rd day close to their best performance, SNI maintain a stable performance throughout the entire experimental period (9 days), i.e., these animal do not learn. This fact strongly suggests that stereotypic strategies (e.g. clockwise movements), not related with WM, might be operating (Dubreuil *et al.*, 2003).

In the ASST we observed that right-sided SNI animals required more trials to accomplish the criteria in the reversal (R1-4) steps. Curiously, in the EDS step no differences were detected between groups. EDS and reversal have been demonstrated to depend on the functional integrity of distinct areas of the PFC, the medial PFC (mPFC) and orbitofrontal cortex (OFC), respectively (Bissonette *et al.*, 2008). This suggests that the deleterious role of the neuropathic lesion is mainly restricted to the OFC (discussed in section 3.4). To the best of our knowledge, this is the first observation of impaired attentional-set shifting ever reported.

Impulse control in a delay-to-signal task was also affected in the right-sided SNI animals. The effect was particularly evident when the delay was incremented from 3 to 7 or 10 seconds. This is in agreement with a previous unpublished observation of ours in the classic 5-csrtt. Again, impulsivity was only evident when the task demand was increased. Concerning the lateralized effect of the lesion, Pais-Vieira and colleagues tested the performance of animals in left-sided inflammatory monoarthritis model in the 5-csrtt and, in agreement with our observations, failed to demonstrate alterations in impulsive behaviour (see table II; Pais-Vieira *et al.*, 2009a).

In the MWM, SNI lesion on either side was not detrimental for the animal performance. In the above mentioned study by Ren and colleagues, left-sided SNI animals though presenting an increased number of WM errors in the 8-arm, had similar long-term reference memory errors when compared to controls (Ren *et al.*, 2011). This result, and considering the construct similarities between the two paradigms, is comparable to ours, despite the ethological considerations raised before.

Table II. Evaluation of cognitive performance in chronic pain models

trait	paradigm	species; strain	model	age	side	overall effect	references
Sustained attention	Delayed nonmatching-to-position operant task	rat; Lewis	polyarthritis	y	n/a	Increased percentage of incorrect choices 1 to 2 months post pain onset;	Cain *et al.*, 1997; Lindner *et al.*, 1999
Attention/memory	NORT	rat; SD	colitis	y	n/a	Decreased attention toward a novel object up to 7 days after pain onset;	Millecamps *et al.*, 2004
Sustained attention/impulsivity	5-csrtt	rat; LH	monoarthritis	y	l	Decreased accuracy and increased omissions immediately after monoarthritis induction (sustained for 10 days); No difference in impulsive responses;	Pais-Vieira *et al.*, 2009a
Risk assessment	Gambling task	rat; SD	monoarthritis	y	l	Increased preference for risk choice 5, 21 and 56 days post pain onset;	Pais-Vieira *et al.*, 2009b
Spatial reference memory	MWM	rat; SD	SNL (L5)	y	n/a	Impaired learning performance at day 30;	Hu *et al.*, 2010
Attention/memory	NORT	mouse; ddY	PSNL	y	r	Decreased attention toward a novel object 7-9 days post PSNL;	Kodama *et al.*, 2011
Working memory	8-arm; NORT	rat; SD	SNI	y	l	Increased number of WM errors and no differences in spatial reference memory errors in the 8-arm at post-surgery days 10-20 and 30-40; Decreased short-term memory and no differences in long-term memory at post-surgery days 19 and 20, respectively, in the NORT;	Ren *et al.*, 2011

Table II. Summary of the literature concerning the effects of chronic pain in cognitive performance in rodent models. Male animals were used in all studies. Abbreviations: 5-csrtt – 5-choice serial reaction time task; 8-arm – 8-arm radial maze; l – left; LH – Lister Hooded; MWM – Morris water maze; n/a – not applicable (available); NORT – novel object recognition test; PSNL – partial sciatic nerve ligation (Seltzer *et al.*, 1990); r – right; SD – Sprague-Dawley; SNI – spared nerve injury (Decosterd and Woolf, 2000); SNL – spinal nerve ligation (Kim and Chung, 1992); y – young.

3.4. Is the PFC underlying maladaptive behaviour in chronic pain?

In an overall appreciation of our behavioural experiments, it is noticeable that a dysfunctional PFC could account for the behavioural shifts observed in chronic pain rats. Firstly, the behavioural domains affected are known to rely on important contributions from the PFC (Miller, 2000; Dalley *et al.*, 2004; Robbins, 2005; Salzman and Fusi, 2010; Diamond, 2011), namely working memory (Passingham and Sakai, 2004; Funahashi, 2006; D'Esposito, 2007; Galloway *et al.*, 2008; Khan and Muly, 2011), attentional set-shifting (Robbins, 2007), impulsivity (Dalley *et al.*, 2008; Kim and Lee, 2011) and emotional behaviour (Cardinal *et al.*, 2002; Davidson, 2002; Maier and Watkins, 2010; Etkin *et al.*, 2011). Secondly, the PFC is morphologically altered in chronic pain conditions (Apkarian *et al.*, 2004; May, 2008). Finally, the PFC is functionally lateralized (Cerqueira *et al.*, 2008) and, considering its functional load distribution, the behavioural impairments caused by left and right nerve lesions are in accordance with the organization of the ascending pathways (see 1.2.2.). Taking this into consideration it is important to appraise our data in the context of a PFC contribution.

Brain imaging studies in human subjects consistently indicate that the PFC is morphologically altered in a number of chronic pain conditions including chronic back pain (Apkarian *et al.*, 2004), fibromyalgia (Luerding *et al.*, 2008), chronic complex regional pain syndrome (Geha *et al.*, 2008) and myofascial-type temporomandibular disorders (Gerstner *et al.*, 2011). A similar observation was also made in a rat model of chronic neuropathic pain (Seminowicz *et al.*, 2009). The casual relation between pain and the plastic adaptation observed in the PFC has been established, the latter depending on the former (and not the opposite) (Rodriguez-Raecke *et al.*, 2009). Accordingly, the structural alterations, as well as the cognitive impairments associated, were shown to be reversible with an effective analgesic treatment (Seminowicz *et al.*, 2011). The reasons for an apparent increased susceptibility of the PFC when compared to other cortical areas are not currently understood. In the rat, it has been demonstrated that the ventral aspects of the mPFC and the OFC are the only neocortical areas that receive direct input from the contralateral spinal cord (Lima, 2009), which may render these areas more susceptible to the effects of the nerve lesions. In fact, other second order relay stations of the ascending pathways, such as the thalamus and the brainstem, have also been shown to

present similar morphological abnormalities (May, 2008), further supporting this hypothesis. Finally, the functional integrity of the mPFC and OFC is intimately associated with the behavioural domains affected in our experiments (Dalley *et al.*, 2004).

Concerning the lateralized effects of the neuropathic lesion, these can be explained by the functional organization of the PFC. Inhibition of the right, but not the left, mPFC (infralimbic area) was shown to suppress anxiety-like behaviour (Sullivan and Gratton, 1999, 2002; Wall *et al.*, 2004) and stress-induced corticosterone elevation (Sullivan and Gratton, 1999). It is therefore tempting to postulate that the deterioration of emotional behaviour we observed in the left SNI animals, reflect such organization. As stated before, measurements of circulating ACTH and corticosterone indicate that neuropathic animals do not differ from controls in basal conditions (Bomholt *et al.*, 2005; Ulrich-Lai *et al.*, 2006; Norman *et al.*, 2009; Yalcin *et al.*, 2011). However, when challenged with a stress paradigm, the HPA appears to be hyperresponsive in left lesioned animals suggesting an impairment of the regulatory mechanisms (Ulrich-Lai *et al.*, 2006). New studies are therefore needed concerning this hypothesis.

The selective cognitive deficits found in the right-sided animals are, on the other hand, more difficult to explain based on a simple lateralization of PFC, because both hemispheres are known to contribute to different aspects of cognitive construction/performance (Gazzaniga, 2000). For instance, when a decision paradigm was applied to patients with either right or left PFC lesions, the former show impaired performance in trials where the decision was based on incomplete information while the latter were impaired when complete information was provided (Goel *et al.*, 2007). An additional example comes from impulsivity studies in humans. Matsuo and colleagues (2009) found an inverse correlation between the left/right OFC volume gray and motor and nonplanning impulsivity, respectively. The construct of the behavioural paradigm employed is therefore determinant when evaluating lateralized frontal dysfunction. This probably explains the differences in the WM performance in our experiments and in those described by others (Ren *et al.*, 2011).

The PFC hypothesis is also coherent in the context of ageing. This area has been shown to present marked morphological alterations, namely cortical atrophy, in aged human individuals

(Raz and Rodrigue, 2006). As stated above, similar alterations have been found in chronic pain patients (Apkarian *et al.*, 2004) suggesting that a cumulatively effect would result, if the two factors co-occur. In fact, in our experiments both control and SNI aged animals presented an increased anxiety-like behaviour but this was more marked in the latter, supporting this hypothesis. Additionally, there is now available compelling evidence of functional cortical asymmetry loss during the aging process (Rossi *et al.*, 2004; Bergerbest *et al.*, 2009), explaining the apparently contradictory observation of impaired reversal performance of left-sided SNI mid-aged animals.

Taken together, these observations give us a compelling indication of the PFC involvement on the behavioural shifts occurring after SNI.

3.5. References

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Chapter 4

Conclusions and future perspectives

4. Conclusions and future perspectives

It stems from our experimental data that the age at the onset of pain and the lateralization of the neuropathic lesion affect the behavioural outcome in emotional and cognitive paradigms. In the first case (chapter 2.1.), the presence of chronic pain was shown to further aggravate an age-associated deterioration of emotional and cognitive functions while in the second (chapter 2.2.) emotional and cognitive domains were selectively affected after left- and right-sided neuropathic lesions, respectively. Although our data is suggestive of a strong PFC involvement in the observed behavioural impairments, the underlying neural substrates remain largely unknown. Future studies addressing this issue should include:

i. a chronological evaluation of the manifestations of abnormal emotional and cognitive behaviour; this point is essential to establish the temporal window of analyses and/or intervention in subsequent experimental studies;

ii. detailed morphological studies of brain areas that by their established role on emotional and cognitive behaviours can putatively mediate the behavioural shifts observed in chronic pain. For the reasons raised in the previous section, the PFC, namely its medial and ventral subdivisions, is a primary candidate area in such studies. These should include, among other techniques, volumetric analyses and Golgi stain studies of neural morphology;

iii. electrophysiological recordings of PFC neural activity while performing relevant tasks;