Universidade do Minho
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STATEMENT OF INTEGRITY

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RESUMO

Caracterização temporal das diferenças entre sexos no modelo experimental de dor neuropática por constrição crónica (CCI)

A dor neuropática crónica afeta 7-10% da população e é frequentemente acompanhada por distúrbios emocionais (ansiedade/depressão), que reduzem significativamente a qualidade de vida dos doentes. Recentemente, mostrámos que no modelo experimental de dor neuropática por constrição crónica do nervo ciático (CCI), ratos fêmea mostraram um desenvolvimento retardado, mas progressivo de alodinia mecânica e térmica que, ao contrário do que se observa nos machos, não melhorou ao longo do tempo. Além disso, as fêmeas não demonstraram comportamentos ansiosos - nem depressivos - 4 semanas após a indução. Neste trabalho, o objetivo foi então caracterizar os distúrbios nociceptivos e emocionais num período inicial (2 semanas) e mais tardio (8 semanas) após a indução do modelo de CCI, tanto em ratos machos como em fêmeas.

Os ratos Wistar Han foram divididos em um de quatro grupos: fêmeas controlo ($SHAM_F$), fêmeas neuropáticas (CCI_F), machos controlo ($SHAM_M$) e machos neuropáticos (CCI_M). A presença de dor foi avaliada durante 2 ou 8 semanas, após os quais a ansiedade e o comportamento depressivo foram avaliados, seguido de gravações eletrofisiológicas *single cell* na medula rostral ventromedial (RVM). Tanto os machos neuropáticos como as fêmeas desenvolveram alodinia mecânica a partir da semana 1, no entanto, verificou-se que os machos recuperavam mais cedo na semana 3, enquanto as fêmeas só recuperavam a partir da semana 6. Importantemente, tanto os machos como as fêmeas mostraram alodinia térmica durante todo o período experimental (8 semanas). Em relação aos distúrbios emocionais, os machos mostraram um fenótipo mais ansioso e depressivo do que as fêmeas, independentemente do CCI. As gravações eletrofisiológicas sugerem que a RVM está apenas parcialmente envolvida na modulação descendente da dor neuropática induzida por este modelo. No seu conjunto, os nossos resultados mostram a existência de diferenças específicas entre machos e fêmeas no desenvolvimento de distúrbios nociceptivos e emocionais após a indução do modelo de CCI, corroboradas por dados eletrofisiológicos, destacando a necessidade de incluir indivíduos do sexo feminino no estudo dos mecanismos e tratamentos da dor neuropática crónica.

Palavras-chave: ansiedade/depressão; dor neuropática crónica; medula rostral ventromedial (RVM); histopatologia

ABSTRACT

Time-dependent characterization of sex-differences on the chronic constriction injury (CCI) model of neuropathic pain

Chronic neuropathic pain affects 7-10% of the population and is often accompanied by emotional impairments (anxiety/depression), greatly reducing the quality of life of patients. Recently, we showed that in the Chronic Constriction Injury (CCI) model of experimental neuropathic pain, female rats showed a delayed but progressive development of mechanical and cold allodynia that, contrary to what is observed for males, did not improve over time. Also, female rats did not display anxious- nor depressive-like behaviour 4 weeks post- induction. In this work, we aim to further characterize the CCI-induced nociceptive and emotional impairments at earlier (2 weeks) and later (8 weeks) time-points in both male and female rats.

Wistar Han rats were assigned to one of four groups: sham-operated females ($SHAM_F$), neuropathic females (CCI_F), sham-operated males ($SHAM_M$) and neuropathic males (CCI_M). Animals were allowed to develop neuropathic pain for 2 or 8 weeks, after which anxiety and depressive-like behaviour were assessed and followed by single-cell electrophysiological recordings of the rostral ventromedial medulla (RVM) cells. Both neuropathic males and females developed mechanical allodynia from week 1 onwards, however, males were shown to recover earlier at week 3, while females only recovered from week 6 onwards. Importantly, both males and females displayed cold allodynia throughout the entire experimental period (8 weeks). Regarding emotional impairments, males showed a more anxious and depressive phenotype than females, independently of CCI. Electrophysiological recordings suggest the RVM is only partially involved in descending modulation of CCI-induced neuropathic pain. Altogether, our results show the existence of sex-specific differences in the development of CCI-induced nociceptive and emotional impairments, corroborated by electrophysiological data, highlighting the need to include female subjects in the study of neuropathic pain mechanisms and treatments.

Keywords: anxiety/depression; chronic neuropathic pain; rostral ventromedial medulla (RVM); histopathology

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ABBREVIATIONS

ACC - anterior cingulate cortex
AMY - amygdala
ANOVA – analysis of variance
CCI – chronic constriction injury
CVLM - caudal ventrolateral medulla
DMH - dorsomedial nucleus of the hypothalamus
DRG – dorsal root ganglia
DRt – dorsal reticular nucleus
EPM – elevated plus-maze test
FST – forced swimming test
Gi - gigantocellularis reticularis
GiA - gigantocellularis pars alpha
IASP – International Association for the Study of Pain
LDB – light/dark box test
LFB - luxol fast blue technique
LPGi - paragigantocellularis lateralis
mPFC – medial prefrontal cortex
NSFT - novelty-suppressed feeding test
OF – open field test
PAG – periaqueductal grey matter
PFA – paraformaldehyde
PFC – prefrontal cortex
RMg - nuclei raphe magnus
RVM – rostral ventromedial medulla
SDH – spinal dorsal horn
SEM – standard error of the mean
SHAM – control group
SNI – spared nerve injury
SNL – spinal nerve ligation
SNT – spinal nerve transection
SPT – sucrose preference test
TST – tail suspension test

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Chapter 1. Introduction

1.1. Nociception

The International Association for the Study of Pain (IASP, 2020) defines pain as *an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage* (Raja et al., 2020). In this context, it is also important to acknowledge nociception and pain are distinct concepts. Pain is a subjective experience with sensory, social, emotional, and cognitive elements (Williams & Craig, 2016), while nociception is the physiological process involved in the encoding and processing of noxious stimuli, which protects the organism from tissue injury (Loeser & Treede, 2008; Baliki & Apkarian, 2015).

1.1.1. Nociceptive Transmission

Noxious stimuli (thermal, mechanical or chemical) are encoded by peripheral nerve fibres, known as nociceptors (Dubin & Patapoutian, 2010). There are three main classes of primary afferent fibres involved in nociception, namely Aβ-, Aδ-, and C-fibres, based on their anatomical features (conduction velocity, diameter, amount of myelination) and the different types of stimuli they respond to (Julius & Basbaum, 2001; D'Mello & Dickenson, 2008; Basbaum et al., 2009).

Firstly, Aβ-fibres are myelinated with a large diameter (4-8 μm diameter), which conduct innocuous mechanical stimuli (e.g. light touch) rapidly (as fast as 70 $m.s^{-1}$) and display low activation thresholds (Djouhri & Lawson, 2004). Aδ-fibres have a smaller diameter (2-6 μm diameter), are thinly myelinated, slower than Aβ-fibres (up to 20 $m.s^{-1}$) and have higher activation thresholds (D'Mello & Dickenson, 2008). This type of fibres mediates acute "first" pain (Julius & Basbaum, 2001; Basbaum et al., 2009). Finally, C-fibres are unmyelinated and present the smallest diameter (0.4-1.2 μm diameter), so they conduct impulses at the slowest rate (0.6–2 $m.s^{-1}$) and have the highest activation thresholds (D'Mello & Dickenson, 2008; Basbaum et al., 2009; Dubin & Patapoutian, 2010). Most C-fibres are polymodal, meaning they respond to both noxious thermal and mechanical stimuli, and mediate the "second" pain. Only Aδ and C are considered nociceptors since both respond to noxious stimuli (mechanical, thermal, or chemical) (Julius & Basbaum, 2001; D'Mello & Dickenson, 2008; Basbaum et al., 2009).

The cell bodies of nociceptors are found in the dorsal root ganglia (DRG) and the trigeminal ganglion. Primary afferent nerve fibres project to the dorsal horn of the spinal cord (SDH), which is divided into different laminae (**Figure 1**). Nociceptors, Aδ- and C-fibres, terminate in lamina I and II (superficial dorsal horn), with fewer projecting to lamina V (deeper dorsal horn). On the other hand, Aβ-fibres reach mostly deep laminae (III, IV, and V). Two categories of neurons that form synapses with sensory fibres were identified in the dorsal horn. Nociceptive-specific (NS) cells, mainly superficial (lamina I), only respond to noxious stimuli, through Aδ- and C-fibres, whereas wide dynamic range (WDR) neurons, located in lamina V, receive input from all three types of peripheral sensory fibres and respond to both innocuous (e.g. light touch) and noxious (e.g. pinch, heat, and chemicals) stimuli (Calvino & Grilo, 2006; D'Mello & Dickenson, 2008; Basbaum et al., 2009; Comitato & Bardoni, 2021).

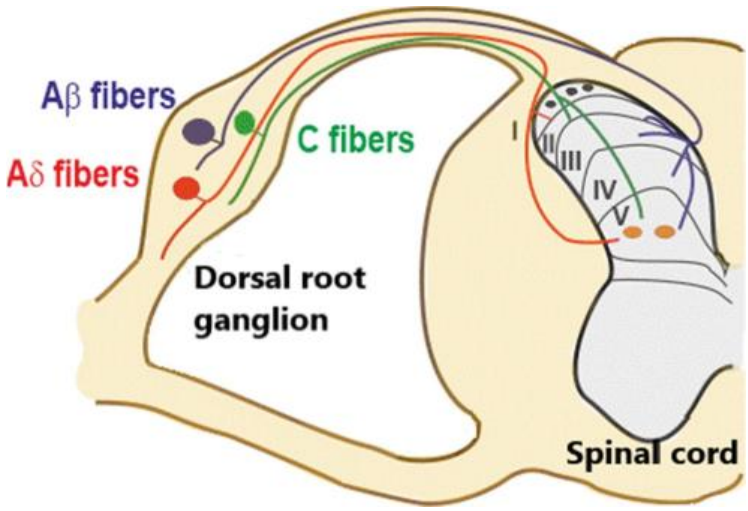

Figure 1. Schematic representation of the primary afferent sensory fibres reaching different dorsal horn laminae. Aδ- and C-fibres project to lamina I and II, as Aβ-fibres terminate in laminae III, IV, and V. NS-cells respond only to noxious stimuli and are found in the superficial dorsal horn, while WDR neurons (laminae V) receive input from Aδ-, Aβ- and C-fibres and respond to both innocuous and noxious stimuli. (From Comitato & Bardoni, 2021)

1.1.2. Ascending Pain Pathways

Nociceptive inputs are further transmitted to supraspinal structures via several ascending pathways such as the (i) spinothalamic, (ii) spinoreticular, (iii) spinomesencephalic, (iv) spinohypothalamic and (v) cervicothalamic tracts (Kandel et al., 2000).

The spinothalamic tract is critical in the transmission of nociceptive information. The lateral spinothalamic tract projects information directly to the ventral posterior nucleus (the ventral posterolateral and ventral posteromedial nuclei) of the thalamus and is implicated in the sensory-discriminative aspects of pain, whereas the medial spinothalamic tract is involved in the motivational – affective components of pain and projects to the intralaminar nuclei and posterior medial complex (Treede, 2002; Almeida et al., 2004; Renn & Dorsey, 2005; Bourne et al., 2014). After reaching the thalamus, the information is relayed to several cortical structures, including the somatosensory areas S1 and S2, the insular cortex, anterior cingulate cortex (ACC) and medial prefrontal cortex (mPFC) (Renn & Dorsey, 2005).

The spinoreticular tract terminates in various nuclei of the reticular formation of the brainstem and the medial thalamus (Willis & Westlund, 1997; Kandel et al., 2000). The lateral reticular formation is implicated in motor control, while the medial reticular formation is involved in the nociceptive transmission. This tract plays an important role in descending modulation, leading to the activation of brainstem structures involved in descending inhibition of pain (Almeida et al., 2004).

The spinomesencephalic tract projects to the periaqueductal gray (PAG) matter and the mesencephalic reticular formation (Kandel et al., 2000). The periaqueductal gray is an important area in descending modulation (Heinricher et al., 2009).

The spinohypothalamic tract terminates in the hypothalamic nuclei, thalamus, and amygdala (autonomic control centres). This tract has a role in the activation of neuroendocrine and cardiovascular responses (Kandel et al., 2000; Lemke, 2004).

Lastly, the cervicothalamic tract projects to the midbrain nuclei and the ventroposterior lateral and posteromedial nuclei of the thalamus (Kandel et al., 2000).

1.1.3. Descending Pain Modulation

Nociceptive transmission in the SDH is either facilitated (pronociceptive) or inhibited (antinociceptive) by descending pain modulatory pathways (Millan, 2002; Mendell, 2011) (**Figure 2**). There are key supraspinal structures involved in descending modulation such as the periaqueductal gray matter (PAG) (Heinricher & Ingram, 2008; Heinricher et al., 2009), rostral ventromedial medulla (RVM) (Silva et al., 2013), caudal ventrolateral medulla (CVLM) (Pinto-Ribeiro et al., 2011), the dorsomedial nucleus of the hypothalamus (DMH) (Pinto-Ribeiro et al., 2013), amygdala (AMY) (Neugebauer et al., 2004; Li & Sheets, 2018), prefrontal cortex (PFC) (Ong et al., 2019), anterior cingulate cortex (ACC) (Zhang et al., 2005) and dorsal reticular nucleus (DRt) (Lima & Almeida, 2002).

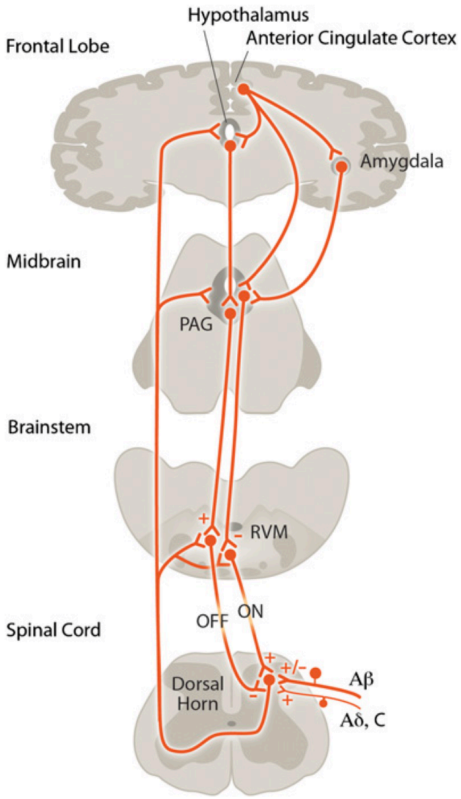

Figure 2. Schematic representation of pain modulatory pathways. A peripheral noxious stimulus is encoded by nociceptors, specifically by Aδ- and C-fibres, which then transmit the nociceptive information to the SDH. This information is processed by several supraspinal regions via ascending pathways. After the supraspinal processing, nociceptive inputs are modulated through the PAG-RVM system. These are key structures in descending modulation that can either facilitate (pronociception) or inhibit (antinociception) nociception. Moreover, in the RVM, there are three types of neurons, according to their response to thermal, mechanical, or chemical stimuli, which are On-, Off- and Neutral-cells. PAG - periaqueductal gray; RVM - rostral ventromedial medulla; SDH - spinal dorsal horn. (From Mendell, 2011)

The PAG and RVM are usually referred to as the PAG-RVM complex (Heinricher & Ingram, 2008; Heinricher et al., 2009) and remains the most well-studied circuit in descending pain modulation (Chen & Heinricher, 2019). The PAG receives information from limbic structures such as the medial prefrontal cortex (mPFC) (Ong et al., 2019), dorsomedial nucleus of the hypothalamus (DMH) (Pinto-Ribeiro et al., 2013), the anterior cingulate cortex (ACC) (Zhang et al., 2005) and the AMY (Neugebauer et al., 2004). This midbrain area transmits nociceptive information to the RVM, which is then relayed to the SDH (Millan, 2002; Heinricher & Ingram, 2008; Heinricher et al., 2009).

The RVM includes the midline nuclei raphe magnus (RMg), gigantocellularis reticularis (Gi), gigantocellularis pars alpha (GiA) and the paragigantocellularis lateralis (LPGi) and plays an important role in the nociceptive modulation and can either facilitate (pronociceptive) or inhibit (antinociceptive) nociceptive inputs (Heinricher et al., 1989; Heinricher & Ingram, 2008; Heinricher et al., 2009; Ossipov et al., 2010; Ossipov et al., 2014).

Fields et al. identified three types of neurons on the RVM, based on their response to noxious stimulation: (i) On- (pronociceptive), (ii) Off- (antinociceptive) and (iii) Neutral-cells. On-cells increase their neuronal activity in response to noxious stimuli immediately before the withdrawal reflex, whereas the neuronal activity of Off-cells decreases (Fields et al., 1983). Neutral cells do not display changes in their neuronal activity during noxious stimulation. Importantly, On-cells are important mediators of hyperalgesia, as their activation is thought to contribute to increased sensitivity to noxious stimulus or decreased sensitivity to pain medication (Neubert et al., 2004). This type of RVM cells is inhibited by opioids (e.g., morphine), whereas Off-cells are activated, which is crucial to produce analgesia (Millan, 2002; Ossipov et al., 2014). These neurons allow both a positive and negative descending modulation from the PAG-RVM circuit in a balanced manner (Ossipov et al., 2010; Ossipov et al., 2014).

1.2. Chronic Pain

Chronic pain is defined as pain that persists beyond the time of healing, normally between three and six months (Treede et al., 2015). Taking into account its aetiology, chronic pain is classified into seven types (Treede et al., 2015; Treede et al., 2019), namely: (i) chronic primary pain (pain in one or more anatomic regions and is accompanied by emotional distress or functional disability); (ii) chronic cancer pain (pain as a debilitating side effect of cancer caused by the primary tumour or metastases, or by the treatment itself); (iii) chronic post-traumatic and post-surgical pain (pain that lasts for at least three

months after a surgical procedure or a tissue injury); (iv) chronic neuropathic pain (caused by a somatosensory nervous system lesion or disease); (v) chronic secondary headache and orofacial pain (pain that lasts more than 2 hours and arise at least 50% of the days for at least three months); (vi) chronic secondary visceral pain (pain that originates in the internal organs of the head and neck region, as well as the thoracic, abdominal, and pelvic cavities), and (vii) chronic secondary musculoskeletal pain (caused by a disease process that directly affects bones, joints, muscles, or related soft tissues).

1.3. Neuropathic Pain

Neuropathic pain is defined as *pain caused by a somatosensory nervous system lesion or disease* (Treede et al., 2019) and can be classified as either peripheral or central, depending on where the lesion occurs within the nervous system. This disorder has various aetiologies, which include infections, trauma, metabolic abnormalities, chemotherapy, surgery, radiation, neurotoxins, nerve compression, inflammation, and tumour infiltration (Dworkin, 2002).

Approximated 7-10% of the population worldwide (Bouhassira et al., 2008; Colloca et al., 2017) suffer from neuropathic pain, developing symptoms that include spontaneous pain (pain present without any stimulation that can be continuous or intermittent), hyperalgesia (increased pain perception caused by a painful stimulus), allodynia (pain caused by an innocuous stimulus), dysesthesias (evoked or spontaneous abnormal unpleasant sensation), and paraesthesia (non-unpleasant abnormal sensation) (Baron, 2006; Baron et al., 2010). Most neuropathic pain therapies focus on the management of peripheral and central neuronal hyperexcitability and include opioids, anticonvulsants, and antidepressants, among others (Jensen et al., 2009; Finnerup et al., 2010). Antidepressants, on the other hand, may be the first drug of choice in chronic pain patients suffering from depression because they help to ameliorate pain symptoms (Magni, 1991).

Neuropathic pain is a manifestation of both peripheral and central sensitization mechanisms. Under normal physiological conditions, C- and Aδ- fibres are only triggered by a noxious stimulus, not responding in the absence of any stimulation. Nevertheless, after peripheral nerve injury, these neurons become unusually responsive and display spontaneous activity due to the molecular and cellular changes caused by nerve lesions (Baron, 2006). Unusual spontaneous discharges named ectopic discharges are produced by injured primary afferent sensory fibres, which also are thought to cause central sensitization and continuous pain (Yoon et al., 1996; Devor, 2009). This phenomenon may be important in the early stages of neuropathic pain, but its significance diminishes over time (Sun et al., 2005).

In chronic stages, allodynia is thought to be caused by central sensitization and disinhibition, which occur when inhibitory control mechanisms are suppressed, in combination with changes in the periphery such as nociceptor sensitization due to changes in ion channels (Bridges et al., 2001; Pasero, 2004; Latremoliere & Woolf, 2009). Indeed, in some neuropathic pain patients, Aβ fibre stimulation can cause extreme pain, when they are usually involved in the transmission of innocuous tactile sensation (Pasero, 2004).

Neuropathic pain is more common in women and middle-aged patients (Bouhassira et al., 2008; Dieleman et al., 2008; Colloca et al., 2017; Zghoul et al., 2017). Importantly, in terms of clinical pain, women experience more severe pain, more frequently, and for a longer period than men (Unruh, 1996; Mogil & Bailey, 2010). In addition, women have a higher pain perception during the menstrual and premenstrual stages than during the mid-menstrual and ovulatory stages (Hellström & Anderberg, 2003).

1.4. Comorbidity between pain and emotional/cognitive impairments

Anxiety and depression, as well as sleep deprivation, are common comorbidities of chronic pain conditions (Magni et al., 1990; Nicholson & Verma, 2004). A recent study found that around 30% of patients with neuropathic pain report anxiety and/or depression symptoms (Radat et al., 2013).

These mood disorders can alter pain perception (Klossika et al., 2006; Peters, 2015), as pain intensity was noted to be increased in depressed pain patients (Haythornthwaite et al., 1991). Epidemiological studies indicate a prevalence of depression of around 50% in patients with chronic pain (Bair et al., 2003). The presence of both pain and depressive symptoms has a negative impact on patients' quality of life. (Elliott et al., 2003). Thus, to improve treatment response, it is critical to diagnose and address this comorbidity, implementing therapies that simultaneously treat mood disorders and ameliorate neuropathic pain.

Similarly to what was described for chronic pain conditions, female patients also present a higher prevalence of anxiety and depression, as well as chronic pain disorders, than males (Munce & Stewart, 2007). Due to this duality, it becomes imperative for female subjects to be included in pain research not only in clinical trials but also in preclinical studies. Nonetheless, at this moment, male animals are almost exclusively used to study chronic pain conditions in general (Mogil & Chanda, 2005) and neuropathic pain specifically, as shown by a recent review on the chronic constriction injury model

(Fonseca-Rodrigues et al., 2021). The term sex will be used in this thesis, as gender refers to social and cultural factors related to being a man or a woman in a particular historical and cultural context, while we aim to study the physiological differences between males and females.

1.5. Animal Models of Neuropathic Pain

Several animal models of neuropathic pain have been developed, based on the site and technique of injury, to study its mechanistic and pathophysiological characteristics and develop novel therapeutic approaches (Colleoni & Sacerdote, 2010; Jaggi et al., 2011). Models can be classified into five main categories: (i) central pain models, (ii) peripheral nerve injury models, (iii) disease-induced and (iv) drug-induced models, and (v) inherited neuropathies. The chronic sciatic constriction injury (CCI) (Bennett & Xie, 1988), spared nerve injury (SNI) (Decosterd & Woolf, 2000) and spinal nerve ligation (SNL) (Ho Kim & Mo Chung, 1992) are the most used models in rodents.

1.5.1. Chronic Constriction Injury Model (CCI)

The chronic constriction injury (CCI) of the sciatic nerve is the most used model of peripheral nerve injury (Bennett & Xie, 1988). This animal model has been used on both the left and right hind paws of mice and rats, and mimics nerve constriction symptoms, with both inflammatory (Maves et al., 1993) and neuropathic characteristics, resulting in hyperalgesia to thermal stimulation up to seven weeks and allodynia to mechanical stimuli until week 11 after induction (Dellarole et al., 2014). Animals also display symptoms of spontaneous pain, including limping, limb guarding, extreme licking, and avoidance of weight-bearing on the injured limb (Bennett & Xie, 1988). Additionally, recent studies showed animals with CCI display anxiodepressive-like behaviour (Yalcin et al., 2014; Fonseca-Rodrigues et al., 2021), being a reliable tool to study the comorbidity between mood impairments and chronic neuropathic pain.

In terms of nociception, preclinical studies revealed CCI induces thermal hyperalgesia, and mechanical and cold allodynia in both rats and mice. Thermal hyperalgesia has been observed in CCI animals starting 14 days after surgery up to day 35 (Tall et al., 2001). On the other hand, CCI induces mechanical allodynia beginning week 1 until week 8 (Barcelon et al., 2019) whereas cold allodynia was present from week 1 to week 12 post-surgery (Gopalsamy et al., 2019).

Regarding the emotional component, as reviewed by Fonseca-Rodrigues and colleagues, CCI induces anxious- and depressive-like behaviour, starting hours after induction, in several strains and species as

assessed using different paradigms (Fonseca-Rodrigues et al., 2021). Anxiety-like behaviour in the CCI model has been demonstrated in earlier stages, up to 6 weeks post-induction (Alba-Delgado et al., 2018), while depressive-like behaviours could be present up to 10 weeks after surgery (Dellarole et al., 2014). Accordingly, this model has been validated for modelling the development of emotional impairments in neuropathic pain conditions.

Although the CCI model has been characterized in both males and females in terms of nociceptive behaviour (Tall et al., 2001; Vacca et al., 2014; Fonseca-Rodrigues et al., 2021) very little is known on the development of mood disorders associated with chronic neuropathic pain in females.

1.5.2. Sex differences in animal models of neuropathic pain

In preclinical research, some studies have showed sex differences in nociceptive, emotional, and cognitive impairments in different animal models of neuropathic pain, as well as on the response to pharmacological treatment. Neuropathic females were shown to develop an increased mechanical and thermal hypersensitivity, and recovering later, than males in the CCI model (Vacca et al., 2014; Fonseca-Rodrigues et al., 2021), the SNI model (Boullon et al., 2021), after injury to the infraorbital and sciatic nerves (Dominguez et al., 2009), in a chemotherapy-induced neuropathic pain model (Naji-Esfahani et al., 2015), and in a HIV-induced model of neuropathic pain (Guindon et al., 2019). Additionally, in the spinal nerve transection (SNT) model of neuropathic pain, female mice do not develop cognitive impairments as males (Won et al., 2020), and, in the SNI model, are not responsive to metformin treatment (Inyang et al., 2019).

Chapter 2. Objectives

Neuropathic pain (Bouhassira et al., 2008; Zghoul et al., 2017) and mood disorders (depression/anxiety) (Munce & Stewart, 2007; Abate, 2013) are more prevalent in women. Given all the information previously mentioned and considering male animals are primarily used in basic studies (Mogil & Chanda, 2005), it grows in importance the inclusion of female subjects in studies regarding the comorbidity between chronic pain and emotional impairments. However, in the CCI animal model, the literature on emotional comorbidities in females remains scarce. Previous works by our group (Fonseca-Rodrigues et al., 2021) showed differences in the development of nociceptive impairments, with neuropathic males recovering four weeks post-induction while CCI females did not. Importantly, female rats did not display anxious- nor depressive-like behaviour five weeks after CCI induction, unlike their counterpart males. However, questions arose as to whether the differences observed in emotional behaviour and RVM neuronal activity between sexes could be due to the time frame at which these were evaluated. Thereby, this project aims to characterize sex differences in CCI-induced nociceptive and emotional impairments at earlier (2 weeks) and later (8 weeks) timepoints post-induction. Additionally, we aim to assess CCI-induced changes in the RVM neuronal activity as well as on the sciatic nerve's histopathology. For that purpose, this project will be divided into four main tasks:

(i) Characterization of CCI-induced nociceptive behaviour. Nociceptive behaviour will be evaluated weekly through the von Frey test (mechanical allodynia) and acetone test (cold allodynia).

(ii) Characterization of CCI-induced emotional impairments. Two and eight weeks after CCI induction, emotional impairments will be evaluated. Anxiety-like behaviour will be assessed through the open-field and elevated plus-maze test, and depressive-like behaviour using the sucrose preference (anhedonia) and forced swimming (learned helplessness) tests.

(iii) Electrophysiological characterization of CCI-induced changes in RVM neuronal activity. After behavioural assessments, changes in basal and evoked response in neuronal activity of the RVM, an important area in descending pain modulation, will be measured through single-cell electrophysiological recordings.

(iv) Histopathological analysis of CCI-induced changes to the sciatic nerve. Haematoxylin and eosin-stained sections of the sciatic nerve will be evaluated in what regards structural and cellular changes, as well as fibres density.

Chapter 3. Materials and Methods

3.1. Animals

Experiments were performed in 78 adult Wistar Han rats (Charles Rivers Laboratories, Barcelona, Spain), weighing between 200–400 g at the beginning of the experiments. The animals were housed in pairs (22°C and relative humidity of 55%) with food and water available *ad libitium*, and under a 12h light cycle (starting at 08.00 a.m.). All animals were manipulated between 9:00 a.m. and 5:00 p.m. (light period) except for the sucrose preference test (section 3.4.3), which was performed in the dark phase. All procedures followed the European Community Council Directive 86/609/EEC and 2010/63/EU regarding the use of animals for scientific purposes and were approved by the ICVS Ethical Commission (SECVS 016/2018). Protocols were designed to minimize animal suffering and to use the number of animals necessary to produce reliable scientific data, and each animal was considered a single unit within its experimental group.

3.2. Induction of neuropathic pain

Induction of neuropathic pain was performed as described previously (Bennett & Xie, 1988). Animals were anaesthetized with a mixture of ketamine (0.75 mg/kg; Imalgene®, Merial, Lisbon, Portugal) and medetomidine (0.5 mg/kg; Dorbene®, ESTEVE, Carnaxide, Portugal) administered intraperitoneally (i.p.). Vaseline was applied to both eyes of the animal to prevent dehydration of the cornea. After a skin incision, the common branch of the left sciatic nerve was exposed through a blunt dissection of the biceps femoris. The constriction of the sciatic nerve was performed proximal to the sciatic trifurcation, using three loose 3/0 chromic gut ligatures around the sciatic nerve (B. Braun Surgical, Rubi, Spain), separated by approximately 1 mm (with care not to arrest the sciatic blood flow). Control animals (SHAM) underwent the same surgical procedure without nerve ligation. Then, the incisions were closed by layers with adsorbable sutures, and the anaesthesia was reversed with atipamezole hydrochloride (1 mg/kg, Antisedan, Pfizer, Oeiras, Portugal, i.p.). Until completely recovered, animals were placed in a separate cage on top of a paper bed and monitored to prevent unconscious choking. In the following seven days, animal welfare (wound healing, grooming, locomotion, and dehydration/weight loss) was assessed daily.

3.3. Nociceptive behaviour

3.3.1. von Frey test

Mechanical allodynia was assessed through the von Frey test, using the "up-down" method (Chaplan et al., 1994). The animals were placed in upside down transparent plastic containers on an elevated mesh floor and allowed to adjust to the environment. Calibrated von Frey monofilaments (North Coast Medical Inc, Morgan Hill, CA) with sequentially increasing forces (0.4, 0.6, 1, 2, 4, 6, 8 and 15 g force) were applied on the plantar surface of each hind paw. In the "up-down" method, the test starts with the 2 g filament and an absence of a response to a filament requires the use of the next higher filament, whereas a positive response requires the use of the next lower filament. After the first positive response, a maximum of four von Frey stimulations are performed or when reached the 15 g force filament. Positive responses include extended paw withdrawal, followed by licking or shaking. The pattern of response is then used to calculate the 50% withdrawal response threshold (force required to elicit a withdrawal response in 50% of animals) (Dixon, 1980).

3.3.2. Acetone test

The acetone test (Yoon et al., 1994) was performed to evaluate cold allodynia. In the same apparatus as the von Frey test, a drop of acetone (100 μL) was applied to the plantar surface of each hind paw of the animal, three times with a 1 min interval. The animal's response was observed for 30 seconds and then classified (0: no response; 1: rapid withdrawal, flick, or stamp of the paw; 2: continued withdrawal/flicking of the paw; 3: constant flicking of the paw with licking) and the mean of the responses of each animal calculated.

3.4. Emotional Behaviour

3.4.1. Open-Field test (OF) test

The OF test (Leite-Almeida et al., 2009) was performed to assess locomotor activity and anxious-like behaviour. This test was carried out in a square arena (43.2 cm x 43.2 cm x 30.5 cm) with transparent acrylic walls (Med Associates Inc., St. Albans, Vermont, USA) in a brightly illuminated centre (240 lx in the centre of the arena). The animals were placed at the centre of the arena and their exploratory activity was automatically recorded (Activity Monitor 5 software, Med Associates Inc., St. Albans, Vermont, USA) for 5 min. The arena was cleaned with ethanol (10%) between each trial. Locomotor activity was evaluated using the total distance travelled by the animal and the average velocity during

the trial. The time spent in the centre of the arena, as well as the number of faeces (*faecal boli)* left in the arena at the end of each trial, were considered a measure of anxiety-like behaviour.

3.4.2. Elevated-Plus Maze (EPM) test

Anxiety-like behaviour was also evaluated using the EPM test (Bessa et al., 2009). The EPM apparatus (ENV-560; Med Associates, St. Albans, Vermont, USA) is composed of two opposite open arms (50.8 cm × 10.2 cm) and two opposite closed arms (50.8 cm × 10.2 cm × 40.6 cm), elevated 72.4 cm above the floor. The animals were placed in the centre of the maze and allowed to explore for 5 minutes. The trial was recorded and then analysed using the EthoVision XT 13 software (Noldus Information Technology, Wageningen, Netherlands). Between each trial, the maze was cleaned with 10% ethanol. Anxiety-like behaviour was assessed using the number of *faecal boli* left in the arena, the time spent in open arms and the open arms entries.

3.4.3. Sucrose Preference Test (SPT)

The SPT (Bessa et al., 2009) was performed to assess anhedonic-like behaviour, the inability to experience pleasure from rewarding or enjoyable activities, a component of depression. One week before the test, the animals were pre-exposed to the sucrose solution (3%) in their home cage, having free access to both water and a sucrose solution for two hours. On the test day, and without any previous water and food restriction, the animals were placed in individual cages and presented with two previously weighted bottles of water and sucrose solution (3%) for three hours during their active period (8:00–11:00 p.m.). Afterwards, the bottles were re-weighted, and sucrose preference was determined using the following equation:

$$\text{Sucrose preference} = [\text{sucrose intake} / (\text{sucrose intake} + \text{water intake})] \times 100$$

To control for the effect of body weight differences between males and females in the SPT outcomes, the sucrose preference and the total intake were adjusted to the animals' body weight on the day of testing.

3.4.4. Forced Swimming Test (FST)

To measure learned helplessness, a component of depression, the FST (Bessa et al., 2009) was performed. In the first session, each animal, individually, was placed in a cylinder filled with water (25ªC; 30 cm in depth) for 10 minutes. Afterwards, on the test day (24h later), the animals were placed again in cylinders filled with water for 5 minutes. Then, the animal was removed from the water and towel-dried before returning to their home cage. The recordings of the sessions were analysed using the Etholog software by a blind observer, in which latency to immobility, time spent immobile, swimming, and climbing were measured. A reduction in the latency to immobility, as well as an increase in time of immobility at the expense of a decrease in swimming/struggling time, were considered a measure of depressive-like behaviour.

3.5. Electrophysiological recordings

Basal and noxious-evoked neuronal activity in the RVM was evaluated through single-cell electrophysiological recordings (Pinto-Ribeiro et al., 2013). Each animal was removed from the animal house and anaesthetized with pentobarbitone (50 mg/kg, Eutasil®, CEVA, Portugal, i.p.). The level of anaesthesia was evaluated by observing the dilation of the pupils, the general muscle tone, as well as responses to noxious pinching of the tail, and the body temperature was controlled through a warming blanket (DC Temperature Controller, FHC, Bowdoin, ME, USA). Anaesthesia was reinforced using pentobarbitone (20 mg/kg) when a pinch-evoked response was observed. Vaseline was applied to prevent dehydration of the cornea and lidocaine as a local anaesthetic before supraspinal access.

The animals were placed in standard stereotaxic equipment and an osteotomy was performed according to the atlas of Paxinos and Watson (Paxinos & Watson, 2006) (AP: 10.92 mm caudal to the bregma; ML: 0.00 mm from the midline; DV: 10.0 mm below the surface of the skull) to allow the placement of the recording electrode. Single-cell extracellular recordings were performed with tungsten electrodes (tip impedance 3–10 MV at 1 kHz) and a CED Micro 1401 interface coupled to Spike 2 software (Cambridge Electronic Design, Cambridge, UK) to amplify and filter the signal and for data sampling.

The neuronal activity of the RVM was quantified during different assessments performed sequentially: (i) spontaneous activity; (ii) response to brushing; (iii) response to mechanical stimulation with von Frey hair (6g); (iv) response to cold stimulation using acetone and (v) response to pinching of the tail with a surgical clamp. After finishing a recording, the animals were allowed a resting period of 30 minutes

before the start of a new recording. The evoked response (Δ Activity) of the recorded neurons was determined using the following equation:

$$\Delta \text{ Activity} = (\text{cell activity during stimulation}) - (\text{basal cell activity before stimulation})$$

Afterwards, for every peripheral stimulation assessment applied on the left hind paw, RVM neurons were classified as (i) On-like cells if their activity is amplified throughout the stimulation period, (ii) Off-like cells if their activity decreased during evaluation, and (iii) Neutral if no alterations are noticed or negligible changes (<10%) in the discharge rates were found. This categorization was adapted from Fields and colleagues (Fields et al., 1983) since only neuronal activity changes were considered.

At the end of the electrophysiological recordings, the animals were injected with a lethal dose of pentobarbitone intraperitoneally (50 mg/kg, Eutasil®, CEVA, Portugal). Subsequently, the sciatic nerve and brain were collected and preserved in 4% paraformaldehyde (PFA) for future analysis.

3.6. Histology

3.6.1. Vaginal Smears

The oestrous cycle in female rats was monitored throughout the experimental period, after each behavioural test, starting from the baseline. Firstly, in a pre-labelled glass slide, a drop of saline representative of each animal was placed. Then, a moistened tip of the smear loop was placed cautiously on the female animal, held by the thorax with the tail slightly gripped, and a minor rotation movement was performed. The smear loop was gently rolled in the glass slide and left to air-dry. The vaginal samples were fixed with 96% ethanol before a Papanicolaou staining. A brightfield microscope (Olympus Widefield Upright Microscope BX61) was used to assess the slides with the original objective magnifications of 4X, 10X and 20X (**Figure 3**).

The stages of the oestrous cycle are defined by the ratio (presence or absence) and abundance of four different cell types - leukocytes, cornified epithelial, small, and large nucleated epithelial cells (Cora et al., 2015). The cycle is divided into proestrus, oestrus, metestrus and dioestrus. Proestrus is characterized by a predominant presence of small nucleated epithelial cells, whereas in the oestrus stage, the cornified epithelial cells are predominant. The metestrus stage is characterized by a mixture of cornified squamous epithelial cells and leucocytes while dioestrus is characterized by the presence of leukocytes and a decrease in the number of epithelial cells.

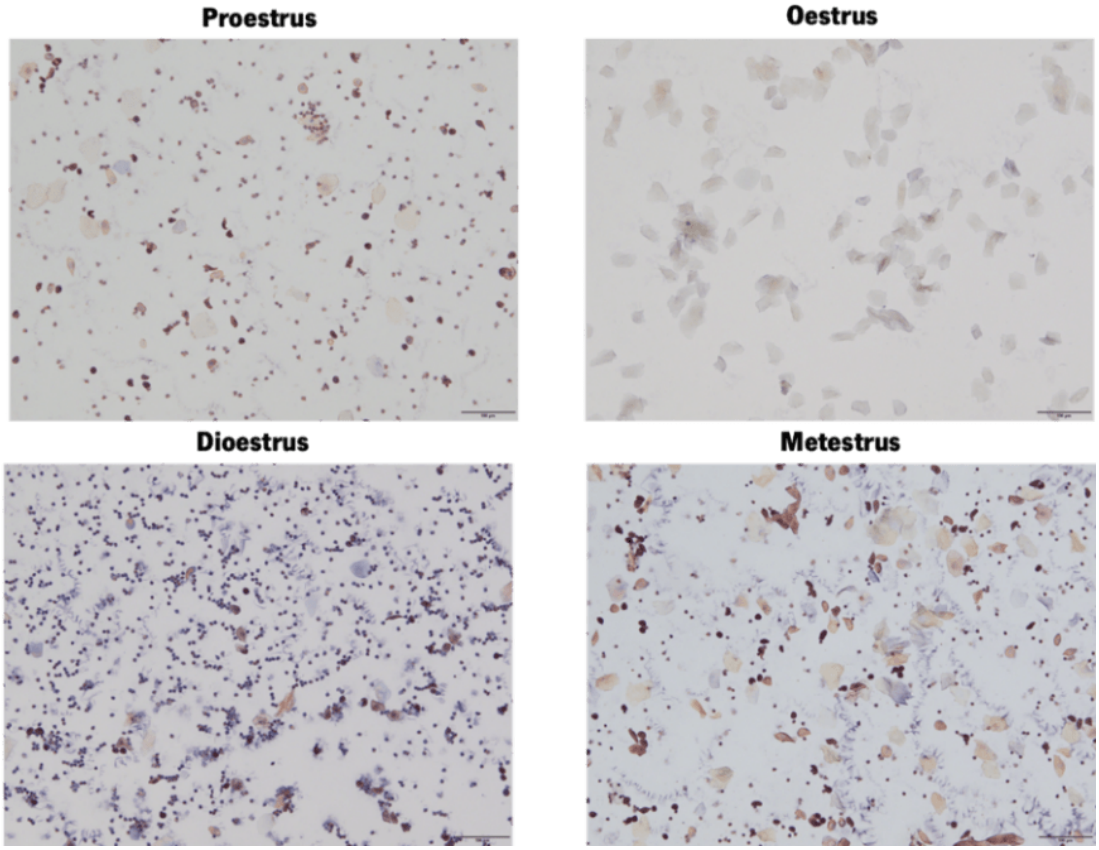

Figure 3. Sample images of vaginal smears obtained from female Wistar Han rats, representing the four different phases of the oestrous cycle with the Papanicolaou stain. Original amplification of 10X (scale: 100 μm).

3.6.2. Haematoxylin & Eosin staining of the sciatic nerve

The sciatic nerve was fixed in paraffin, cut into 5 μm sections and stained with haematoxylin and eosin (H&E) for posterior histological analysis (longitudinal and transversal sections).

For the analysis of the general morphology of the sciatic nerve, two transverse and two longitudinal slides per animal were analysed using a light microscope (Olympus Widefield Upright Microscope BX61) attached to an Olympus DP70 camera. The number, size, and densities of myelinated fibres were also assessed using the ImageJ software, through the analysis of 4-5 distinct microscopic fields of vision at an original amplification of 60X (two transverse slices per animal). The optical fractionator method was used to estimate the total number of myelinated fibres using the Visiopharm Integrator System software (Hoersholm, Denmark) and a motorized microscope (Olympus BX51) coupled to a digital camera (Olympus U-TV1X-2).

Square probes were placed over the previously drawn sciatic nerve area, evenly spaced, covering 15% of the total defined area and fibres numbers were quantified and normalized for the total area. The random positioning of this grid by the software ensured an unbiased and effective sampling, and the experimenter was blind to the experimental conditions of the animals.

3.7. Experimental Design

The experimental timeline is described in **Figure 4**. At the beginning of the experimental period, all animals were handled daily by the experimenter for two weeks. Before any procedure, animals were left in the experimental room for an hour to habituate to the surroundings. Animals were randomly divided into four experimental groups: control females ($SHAM_F$, n=21), neuropathic females (CCI_F, n=19) control males ($SHAM_M$, n=19) and neuropathic males (CCI_M, n=19). Two independent experiments were performed, where animals will develop the CCI model throughout a two- or eight-week period. One week preceding the CCI induction, mechanical and cold allodynia were assessed through von Frey and acetone test, respectively (baseline, week 0). Mechanical allodynia and cold allodynia were evaluated weekly. Anxiety- and depressive-like behaviours were assessed either at two or eight weeks after CCI induction using the OF, EPM, SPT, and FST tests. Single-cell electrophysiological recordings and histological analysis were carried out at the end of the behavioural assessments. Three independent sets were performed during this project to adjust the period post-CCI surgery necessary for the electrophysiological recordings.

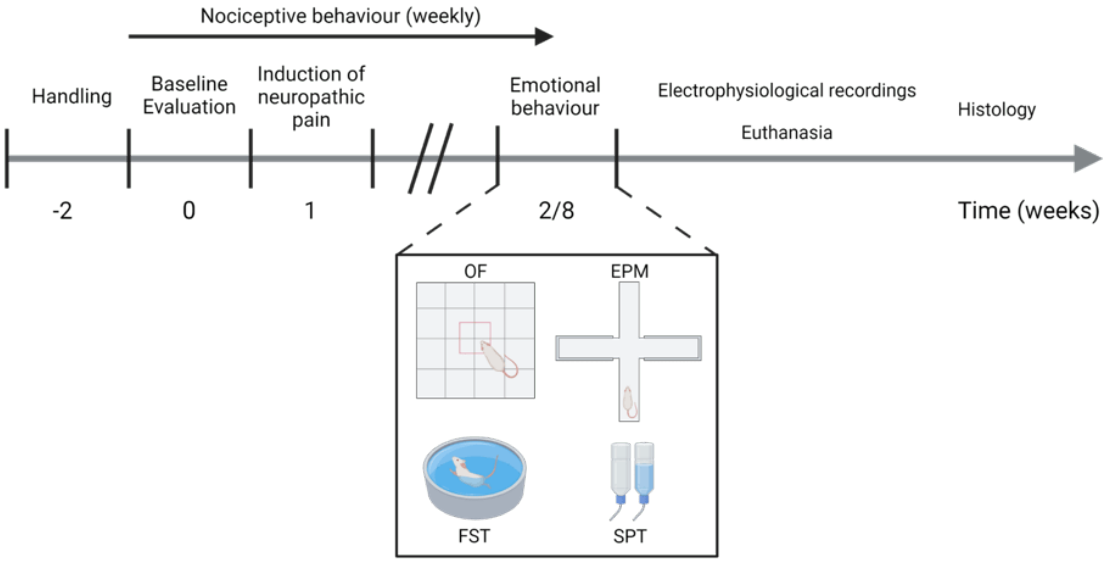

Figure 4. Schematic representation of the experimental design. One week preceding the CCI induction, animals were submitted to a baseline evaluation of the von Frey test and acetone test. Nociception was evaluated weekly throughout two or eight weeks using the von Frey test (mechanical allodynia) and the acetone test (cold allodynia). Two or eight weeks after CCI induction, according to the animals' experimental group, anxiety- and depressive-like behaviours were evaluated using the open field (OF), elevated plus-maze (EPM), sucrose preference (SPT), and forced swimming (FST) tests. At the end of the behavioural evaluations, single-cell electrophysiological recordings were performed, and biological samples (brain and sciatic nerve) were collected for further histological processing and analysis.

3.8. Statistical Analysis

The GPower 3.1 software was used to calculate the sample size required to produce reliable scientific data. For each experimental group, RANDOM.ORG (online random number generator) was used to assign the animals to their respective experimental group. After determining normality (Shapiro–Wilk test), differences in body weight, nociceptive behaviour, emotional behaviour, electrophysiological and histological data between all experimental groups were analysed using a two-way analysis of variance (ANOVA), with the sex of the animals and CCI as independent variables, followed by a Bonferroni's multiple comparisons test. Comparisons were performed between control and neuropathic animals, as well as between males and their counterpart females. For all comparisons, a significance level of $P<0.05$ were considered. Results were presented as mean ± standard error of the mean (SEM).

Chapter 4. Results

4.1. Animal Welfare

CCI animals walked with a distinguishable limp, frequently guarding the left hind paw, and stood with the hind paw everted and the heel touching the floor. The toes, which are generally spread apart, were brought together and ventroflexed while walking or standing. Autotomy was sporadically observed, with gnawed claw tips. SHAM animals did not display these behaviours.

Body weight change was evaluated as a measure of animal welfare (**Figure 5**). ANOVA analysis showed body weight varied differently between experimental groups throughout time (Interaction: $F(24,432)=1.11$, $P=0.32$, $\eta_p^2=0.041$; Time: $F(8,432)=19.6$, $P<0.0001$, $\eta_p^2=0.24$; Group: $F(3,432)=14.2$, $P<0.0001$, $\eta_p^2=0.065$). Post hoc tests showed no significant differences between neuropathic animals and their counterpart controls. In comparison with baseline values, neuropathic males (CCI_M) gained weight from weeks 6 to 8, while females (CCI_F) showed an increase in body weight from week 5 onwards. In control animals, while males showed no significant weight gain during the experimental periods, females gained weight on week 2 and from weeks 4 to 8.

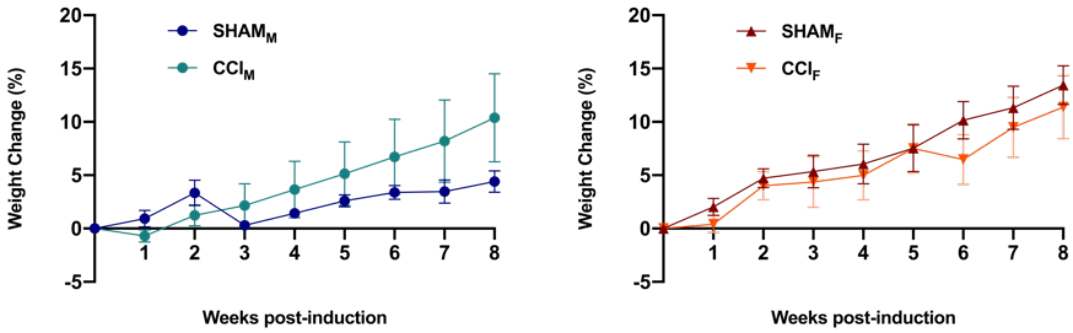

Figure 5. Body weight variation (%) throughout the experimental period, starting the day of the CCI surgery. No differences were found between the experimental groups. Data presented as mean ± SEM [$n(SHAM_F)=21$, $n(CCI_F)=19$, $n(SHAM_M)=19$, $n(CCI_M)=19$). SHAM vs CCI. Symbols are coloured in accordance with the experimental group (blue-males, orange-females).

4.2. Nociceptive Behaviour

4.2.1. von Frey test

The von Frey test was used to assess the development of mechanical allodynia. Concerning the ipsilateral side (**Figure 6**, **Table 1**), mechanical allodynia varied differently between experimental groups throughout time. Multiple comparisons showed neuropathic females displayed a significant decrease in withdrawal threshold between weeks 1 and 5 in comparison with control females and baseline values. Interestingly, CCI males recovered earlier, at week 3. There were no significant differences found on the contralateral limb.

Table 1. Statistical analysis of mechanical and cold allodynia assessments on the ipsilateral and contralateral limb throughout the experimental period, through a two-way analysis of variance (ANOVA).

		Interaction	Time	Group
Von Frey	**Ipsilateral**	$F(24,432)=8.06$, $P<0.0001$, $\eta_p^2=0.18$	$F(8,432)=22.7$, $P<0.0001$, $\eta_p^2=0.17$	$F(3,432)=73.5$, $P<0.0001$, $\eta_p^2=0.20$
	Contralateral	$F(24,432)=0.82$, $P=0.72$, $\eta_p^2=0.042$	$F(8,432)=1.36$, $P=0.21$, $\eta_p^2=0.024$	$F(3,432)=0.17$, $P=0.92$, $\eta_p^2=0.001$
Acetone Test	**Ipsilateral**	$F(24,431)=7.95$, $P<0.0001$, $\eta_p^2=0.14$	$F(8,431)=25.4$, $P<0.0001$, $\eta_p^2=0.15$	$F(3,431)=148$, $P<0.0001$, $\eta_p^2=0.33$
	Contralateral	$F(24,431)=1.98$, $P=0.004$, $\eta_p^2=0.091$	$F(8,431)=6.37$, $P<0.0001$, $\eta_p^2=0.097$	$F(3,431)=1.93$, $P=0.13$, $\eta_p^2=0.011$

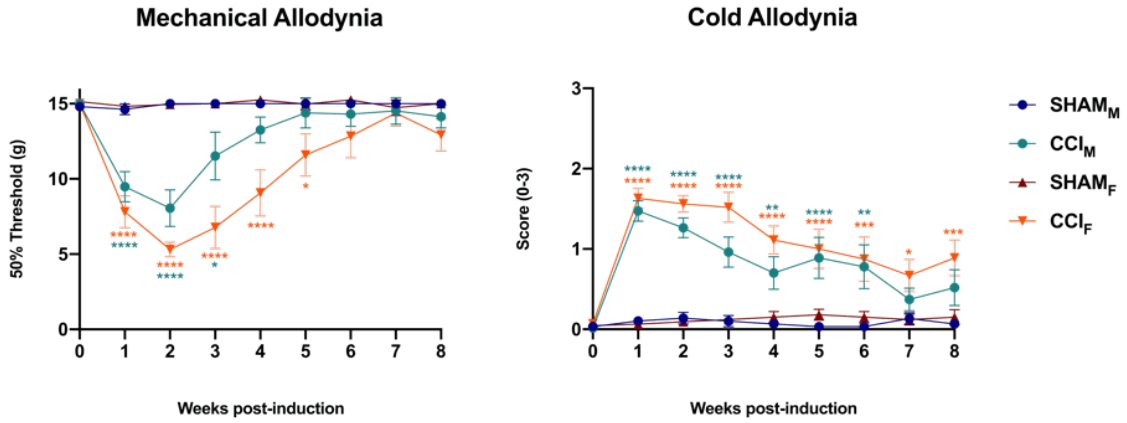

Figure 6. Assessment of mechanical and cold allodynia on the ipsilateral limb throughout the experimental period. Mechanical allodynia was observed from weeks 1 to 5 on neuropathic females, recovering afterwards, while CCI male rats recovered earlier at week 3. Both neuropathic males and females displayed cold allodynia from week 1 onwards, but neuropathic males recovered at week 6. Data presented as mean ± SEM [$n(SHAM_F)=21$, $n(CCI_F)=19$, $n(SHAM_M)=19$, $n(CCI_M)=19$]. *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$. SHAM vs CCI. Symbols are coloured in accordance with their experimental group (blue-males, orange-females).

4.2.2. Acetone test

To evaluate the progression of cold allodynia, the acetone test was performed. In the ipsilateral limb, CCI animals displayed cold allodynia throughout time (**Figure 6**, **Table 1**). Multiple comparisons showed neuropathic females developed cold allodynia from week 1 onwards, whereas males did not display cold allodynia on weeks 7 and 8 compared to their counterpart controls. Similarly, in comparison with baseline values, neuropathic animals, both males and females, displayed cold allodynia up to week 8. Regarding the contralateral side, ANOVA analysis showed cold allodynia varied both with time and experimental group.

4.3. Emotional Behaviour

4.3.1. Open-Field test

The OF test was used to assess both locomotor activities, through the total distance travelled and average velocity of the animals, and anxious-like behaviour, through the number of *faecal boli* and time spent in the centre of the arena, two and eight weeks post-CCI induction. One animal (CCI_M) was identified as an outlier for the time in the centre (%) using the ROUT method (Q=0.1%) and removed from the analysis (2 weeks).

Table 2. Statistical analysis of the results obtained in the open field (OF) test, two and eight weeks after chronic constriction injury (CCI) induction, through a two-way analysis of variance (ANOVA).

		Interaction	Sex	CCI
Faecal boli	**2 weeks**	F(1,34)=0.003, P=0.96, η_p^2<0.0001	F(1,34)=1.93, P=0.17, η_p^2=0.054	F(1,34)=0.034, P=0.86, η_p^2=0.0009
	8 weeks	F(1,35)=3.74, P=0.061, η_p^2=0.096	F(1,35)=0.27, P=0.61, η_p^2=0.007	F(1,35)=0.028, P=0.87, η_p^2=0.0007
Time in the centre	**2 weeks**	F(1,34)=0.42, P=0.52, η_p^2=0.010	F(1,34)=4.90, P=0.034, η_p^2=0.11	F(1,34)=4.38, P=0.044, η_p^2=0.10
	8 weeks	F(1,35)=3.33, P=0.077, η_p^2=0.081	F(1,35)=1.94, P=0.17, η_p^2=0.047	F(1,35)=1.28, P=0.27, η_p^2=0.031
Velocity	**2 weeks**	F(1,34) =2.07, P=0.16, η_p^2=0.051	F(1,34)=4.51, P=0.041, η_p^2=0.11	F(1,34)=0.007, P=0.94, η_p^2=0.0002
	8 weeks	F(1,35)=0.56, P=0.46, η_p^2=0.016	F(1,35)=0.13, P=0.72, η_p^2=0.004	F(1,35)=0.17, P=0.68, η_p^2=0.005
Distance travelled	**2 weeks**	F(1,34)=0.59, P=0.45, η_p^2=0.014	F(1,34)=4.50, P=0.041, η_p^2=0.10	F(1,34)=4.15, P=0.049, η_p^2=0.096
	8 weeks	F(1,35)=0.023, P=0.88, η_p^2=0.0005	F(1,35)=7.22, P=0.011, η_p^2=0.17	F(1,35)=0.17, P=0.68, η_p^2=0.004

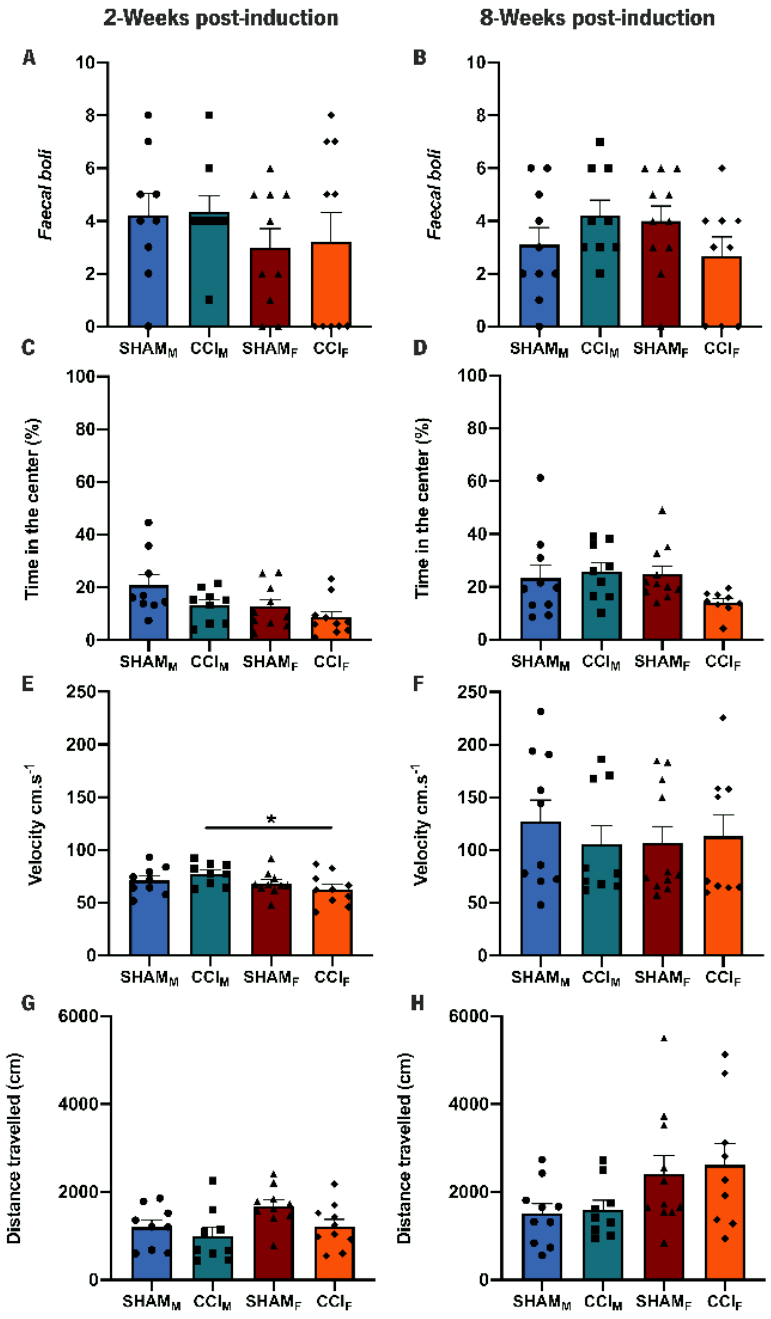

Figure 7. Anxiety-like behaviour assessment using the open field test (OF) two and eight weeks post-CCI induction. **(A, B)** No differences were found between experimental groups regarding the number of *faecal boli* and **(C, D)** the time spent in the centre of the arena at both two and eight weeks after CCI induction. **(E)** Neuropathic females present a decreased velocity, compared to males, however **(F)** no differences were found between experimental groups at week 8. **(G, H)** Regarding the distance travelled, no significant differences were found between experimental groups at both weeks 2 and 8 post-CCI. Data presented as mean ± SEM [$n(SHAM_F)_{2W}$=10, $n(CCI_F)_{2W}$=10, $n(SHAM_M)_{2W}$=9, $n(CCI_M)_{2W}$=9]); [$n(SHAM_F)_{8W}$=11, $n(CCI_F)_{8W}$=9, $n(SHAM_M)_{8W}$=10, $n(CCI_M)_{8W}$=9]. *P<0.05

Regarding the number of *faecal boli* left in the arena, there were no statistically significant differences between the experimental groups at week 2 (**Table 2**, **Figure 7A**) and week 8 (**Table 2**, **Figure 7B**).

Two weeks post-induction, the time spent in the centre of the arena varied according to both sex and CCI independently (**Table 2**, **Figure 7C**), although multiple comparisons showed no significant differences between experimental groups. No differences were found between experimental groups at week 8 (**Table 2**, **Figure 7D**).

The average velocity was significantly different between male and female animals at week 2, independently of CCI (**Table 2**, **Figure 7E**). Multiple comparisons showed a decrease in the velocity in neuropathic females, as compared to males (P=0.033). On week 8, there were no statistically significant differences between experimental groups (**Figure 7F**).

Concerning the distance travelled by the animals during the OF test, both sex and CCI independently impacted this variable two weeks post-induction (**Table 2**, **Figure 7G**), but no significant differences were found between experimental groups. On week 8 after CCI induction, distance travelled was significantly different between males and females, independently of CCI (**Table 2**, **Figure 7H**), but multiple comparisons showed no significant differences between experimental groups.

4.3.2. Elevated-Plus Maze

Anxiety-like behaviour was evaluated two and eight weeks post-CCI induction using the EPM test. After falling from the apparatus during the test, one CCI_M animal was removed from the analysis (2 weeks). Two animals (one $SHAM_F$ and one CCI_F) were identified as outliers (ROUT method, Q=0.1%) and removed from the statistical analysis for *faecal boli* in week 2, and one $SHAM_F$ for open arms entries in week 8.

On week 2, the number of *faecal boli* left in the arena was significantly different between male and female animals, independently of CCI (**Table 3**, **Figure 8A**). Multiple comparisons showed male animals (CCI and SHAM) left a greater number of *faecal boli* in the arena in comparison with their female counterparts. However, at week 8, no differences were found between experimental groups (**Table 3**, **Figure 8B**).

Table 3. Statistical analysis of the results obtained in the elevated plus-maze (EPM) test, two and eight weeks after chronic constriction injury (CCI) induction, through a two-way analysis of variance (ANOVA).

		Interaction	Sex	CCI
Faecal boli	**2 weeks**	F(1,32)=2.12, P=0.16, η_p^2=0.033	F(1,32)=28.5, P<0.0001, η_p^2=0.44	F(1,32)=2.12, P=0.16, η_p^2=0.033
	8 weeks	F(1,34)=0.60, P=0.45, η_p^2=0.017	F(1,34)=0.83, P=0.37, η_p^2=0.023	F(1,34)=0.51, P=0.48, η_p^2=0.014
Time in open arms	**2 weeks**	F(1,32)=0.81, P=0.37, η_p^2=0.024	F(1,32)=0.043, P=0.84, η_p^2=0.001	F(1,32)=0.67, P=0.42, η_p^2=0.020
	8 weeks	F(1,34)=1.44, P=0.24, η_p^2=0.030	F(1,34)=10.4, P=0.003, η_p^2=0.21	F(1,34)=2.38, P=0.13, η_p^2=0.049
Open arms entries	**2 weeks**	F(1,32)=2.55, P=0.12, η_p^2=0.065	F(1,32)=4.39, P=0.044, η_p^2=0.11	F(1,32)=0.50, P=0.48, η_p^2=0.013
	8 weeks	F(1,34)=8.54, P=0.006, η_p^2=0.20	F(1,34)=0.26, P=0.61, η_p^2=0.006	F(1,34)=0.29, P=0.60, η_p^2=0.007

Regarding the time spent in the open arms, no significant differences were found between experimental groups two weeks post-induction (**Table 3**, **Figure 8C**). However, on week 8 the time in the open arms was different between males and females, independently of CCI (**Table 3**, **Figure 8D**). Multiple comparisons showed $SHAM_M$ spent less time in the open arms, in comparison with $SHAM_F$ (P=0.006), however with no differences between control and neuropathic animals.

The number of open arms entries was also significantly different between male and female animals, independently of CCI, at week 2 (**Table 3**, **Figure 8E**). Post hoc tests showed $SHAM_M$ displayed a significant decrease in the number of open arms entries when compared to $SHAM_F$ (P= 0.027). On week 8, ANOVA analysis showed an interaction between sex and CCI (**Table 3**, **Figure 8F**). Comparisons between experimental groups showed a significant decrease in open arms entries of $SHAM_M$ when compared to both CCI_M (P=0.040) and $SHAM_F$ (P=0.035).

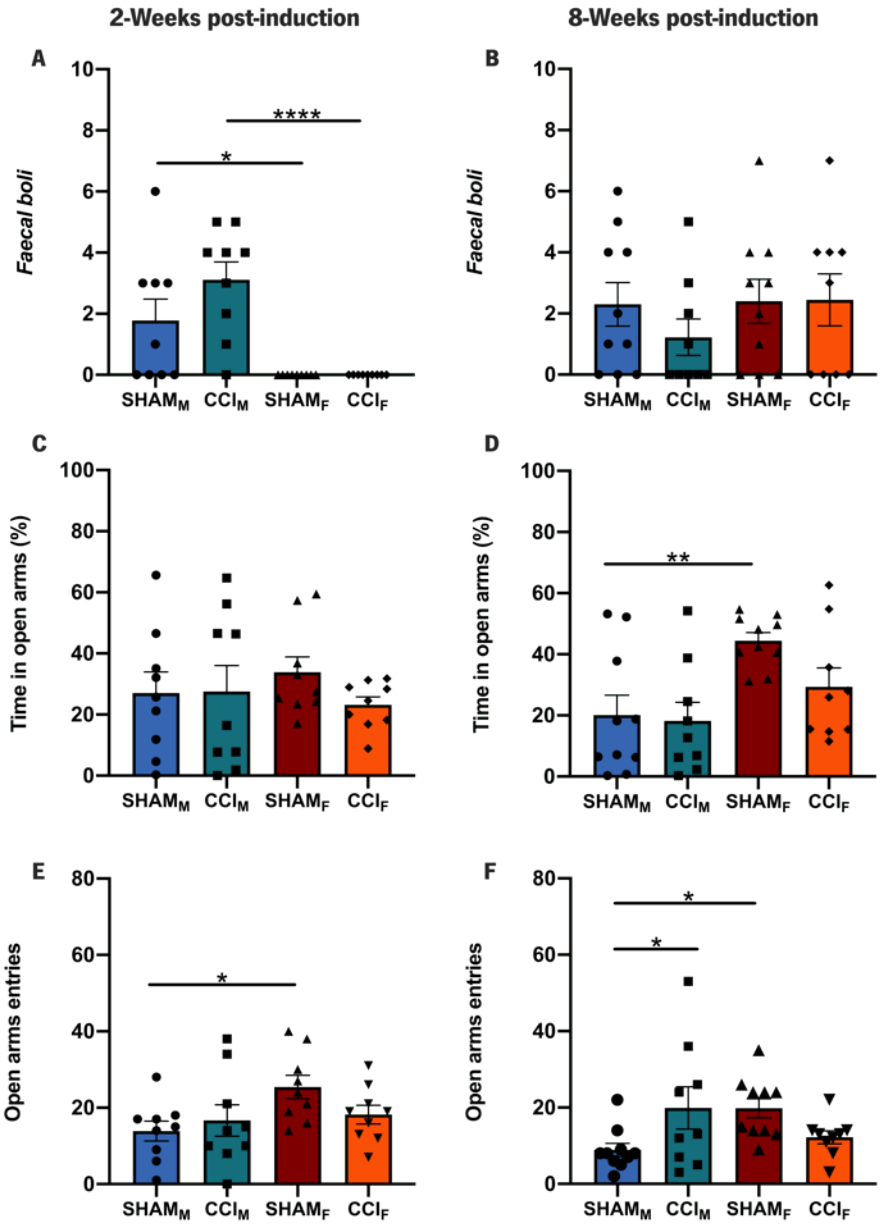

Figure 8. Anxiety-like behaviour assessment through the elevated-plus maze test (EPM) two and eight weeks post-CCI induction. **(A, B)** Female rats left a lower number of *faecal boli* on the arena on week 2 post-CCI induction, however, no differences were found between experimental groups at week 8. **(C, D)** Regarding the time spent in the open arms, no differences were found between experimental groups on week 2, but eight weeks post-CCI induction control males ($SHAM_M$) spent less time in the open arms when compared to females ($SHAM_F$). **(E, F)** At both earlier and later time-points, control males ($SHAM_M$) displayed a decrease in the number of open arms entries when compared to females ($SHAM_F$), as well as neuropathic males (CCI_M) at week 8. Data presented as mean ± SEM [$n(SHAM_F)_{2W}$=9, $n(CCI_F)_{2W}$=9, $n(SHAM_M)_{2W}$=9, $n(CCI_M)_{2W}$=9]); [$n(SHAM_F)_{8W}$=10, $n(CCI_F)_{8W}$=9, $n(SHAM_M)_{8W}$=10, $n(CCI_M)_{8W}$=9]. *P<0.05, **P<0.01, and ****P<0.0001

4.3.3. Sucrose Preference Test

The SPT test was used to evaluate anhedonic-like behaviour two and eight weeks post-CCI induction. Two animals (CCI_F and CCI_M) were removed from the analysis (2 weeks) due to a bottle malfunction throughout the test. After testing for the presence of outliers for the sucrose preference (%) using the ROUT method, one animal (CCI_F) was removed from the two weeks analysis and one $SHAM_M$ and two $SHAM_F$ were removed in week 8.

Sucrose preference was significantly different between males and females, independently of CCI, at week 2 (**Table 4**, **Figure 9A**). Post hoc tests showed a decrease in sucrose preference in neuropathic males when compared to neuropathic females (P=0.006). In contrast, on week 8, ANOVA analysis showed an interaction between sex and CCI (**Table 4**, **Figure 9B**). Further comparisons showed a significant decrease in control males ($SHAM_M$) as compared to females (P=0.002).

Table 4. Statistical analysis of the results obtained in the sucrose preference test (SPT), two and eight weeks after chronic constriction injury (CCI) induction, through a two-way analysis of variance (ANOVA).

		Interaction	Sex	CCI
Sucrose Preference	**2 weeks**	F(1,32)=2.60, P=0.12, η_p^2=0.055	F(1,32)=9.44, P=0.004, η_p^2=0.20	F(1,32)=2.77, P=0.11, η_p^2=0.059
	8 weeks	F(1,32)=4.54, P=0.041, η_p^2=0.10	F(1,32)=8.43, P=0.007, η_p^2=0.19	F(1,32)=0.44, P=0.51, η_p^2=0.010
Sucrose Preference per gram of body weight	**2 weeks**	F(1,32)=0.40, P=0.53, η_p^2=0.003	F(1,32)=111, P<0.0001, η_p^2=0.76	F(1,32)=1.59, P=0.22, η_p^2=0.011
	8 weeks	F(1,32)=3.47, P=0.072, η_p^2=0.009	F(1,32)=359, P<0.0001, η_p^2=0.91	F(1,32)=0.27, P=0.61, η_p^2=0.0007
Total Intake	**2 weeks**	F(1,32)=3.12, P=0.087, η_p^2=0.081	F(1,32)=3.27, P=0.080, η_p^2=0.084	F(1,32)=0.006, P=0.94, η_p^2=0.0002
	8 weeks	F(1,32)=5.48, P=0.026, η_p^2=0.11	F(1,32)=7.82, P=0.009, η_p^2=0.16	F(1,32)=3.89, P=0.057, η_p^2=0.079
Total Intake per gram of body weight	**2 weeks**	F(1,32)=2.18, P=0.15, η_p^2=0.037	F(1,32)=23.1, P<0.0001, η_p^2=0.39	F(1,32)=0.51, P=0.48, η_p^2=0.009
	8 weeks	F(1,32)=6.49, P=0.016, η_p^2=0.065	F(1,32)=57.2, P<0.0001, η_p^2=0.57	F(1,32)=4.32, P=0.046, η_p^2=0.043

Similarly, when adjusted to the body weight of the animals, sucrose preference was again significantly different between males and females, independently of CCI at both an earlier (**Table 4**, **Figure 9C**) and later time-points (**Table 4**, **Figure 9D**). Multiple comparisons showed male animals (SHAM and CCI) displayed lower sucrose preference, as compared to female animals (SHAM and CCI), respectively at both 2 and 8 weeks after CCI induction.

Regarding the total intake of water and sucrose, no significant differences were found between experimental groups two weeks post-induction (**Table 4**, **Figure 9E**). At week 8, ANOVA analysis showed an interaction between sex and CCI in the total intake (**Table 4**, **Figure 9F**). Comparisons showed SHAM males (P=0.002) and neuropathic females (P=0.009) displayed a lower intake when compared to SHAM females.

When adjusted to the animal's body weight, the total intake was significantly different between male and female animals, independently of CCI on week 2 (**Table 4**, **Figure 9G**). Comparisons between experimental groups showed $SHAM_F$ present a higher total intake when compared to $SHAM_M$ (P=0.0001). However, eight weeks post-CCI induction, ANOVA analysis showed an interaction between sex and CCI on the total intake (**Table 4**, **Figure 9H**). Post hoc tests showed a decrease in male rats (SHAM and CCI) in comparison with female rats (SHAM and CCI), respectively. Also, $SHAM_F$ displayed a higher intake when compared to CCI_F (P=0.005).

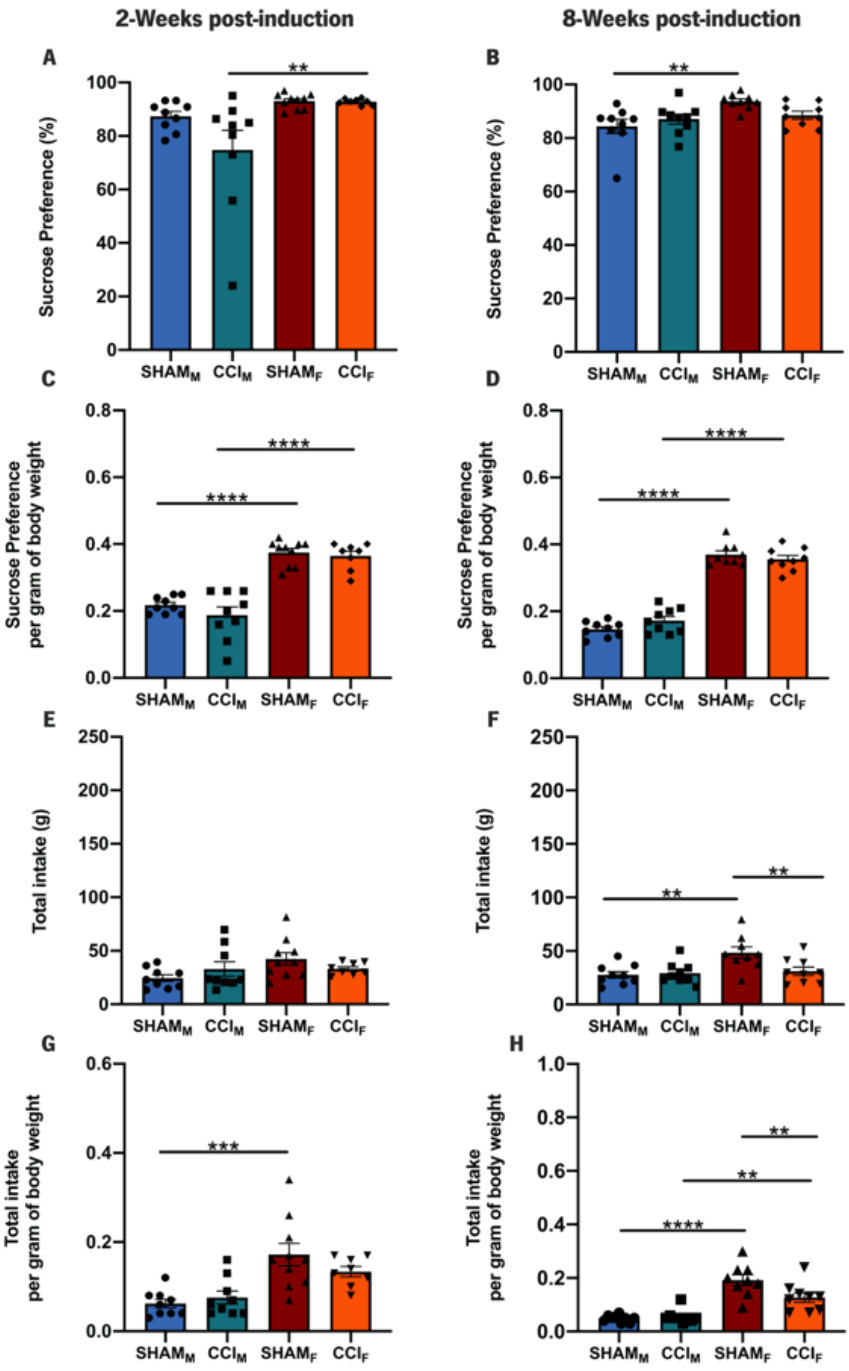

Figure 9. Depressive–like behaviour assessment using the sucrose preference test (SPT) two and eight weeks post-CCI induction. **(A, B)** Neuropathic males presented a decrease in the sucrose preference at week 2 as $SHAM_M$ displayed a lower percentage at week 8. **(C, D)** When adjusted to body weight, male rats (CCI and SHAM) displayed a lower sucrose preference compared to their female counterparts. **(E, F)**. Regarding the total intake, at week 2 no differences were found between experimental groups while on week 8 $SHAM_F$ displayed a higher intake when compared to $SHAM_M$ and CCI_F. **(G, H)** SHAM females displayed a higher total intake in comparison with both CCI females and SHAM males, which was also observed when adjusted to the animal's body weight at week 8. Furthermore, CCI females were shown to drink less than controls. Data presented as mean ± SEM [$n(SHAM_F)_{2W}$=10, $n(CCI_F)_{2W}$=8, $n(SHAM_M)_{2W}$=9, $n(CCI_M)_{2W}$=9]); [$n(SHAM_F)_{8W}$=9, $n(CCI_F)_{8W}$=9, $n(SHAM_M)_{8W}$=9, $n(CCI_M)_{8W}$=9]. ** P<0.01, *** P<0.001 and **** P<0.0001.

4.3.4. Forced Swimming Test

Learned helplessness-like behaviour was evaluated two and eight weeks post-CCI induction using the FST. Due to a software malfunction during the recordings of the test, four animals (one CCI_M, two $SHAM_F$ and one CCI_F) were removed from the analysis (2 weeks). Three animals (one $SHAM_F$ and two CCI_F) were identified as outliers through the ROUT method for the latency to immobility and removed from the statistical analysis at week 2 and one animal ($SHAM_F$) was removed from eight weeks analysis.

Concerning the number of *faecal boli;* ANOVA analysis showed an interaction between sex and CCI, two weeks after CCI induction (**Table 5**, **Figure 10A**). Multiple comparisons showed neuropathic females (CCI_F) displayed fewer *faecal boli*, in comparison with controls ($SHAM_F$) (P=0.049). At week 8, no differences were found between experimental groups (**Table 5**, **Figure 10B**).

Table 5. Statistical analysis of the results obtained in the forced swimming test (FST), two and eight weeks after chronic constriction injury (CCI) induction, through a two-way analysis of variance (ANOVA).

		Interaction	Sex	CCI
Faecal boli	**2 weeks**	F(1,28)=4.38, P=0.046, η_p^2=0.13	F(1,28)=0.70, P=0.41, η_p^2=0.020	F(1,28)=2.18, P=0.15, η_p^2=0.063
	8 weeks	F(1,34)=0.51, P=0.48, η_p^2=0.015	F(1,34)=0.075, P=0.79, η_p^2=0.002	F(1,34)=0.20, P=0.66, η_p^2=0.006
Climbing duration	**2 weeks**	F(1,28)=3.81, P=0.061, η_p^2=0.11	F(1,28)=3.75, P=0.063, η_p^2=0.11	F(1,28)=0.0005, P=0.98, η_p^2<0.0001
	8 weeks	F(1,34)=0.018, P=0.89, η_p^2=0.0005	F(1,34)=0.007, P=0.94, η_p^2=0.0002	F(1,34)=4.88, P=0.034, η_p^2=0.13
Swimming duration	**2 weeks**	F(1,28)=0.78, P=0.38, η_p^2=0.023	F(1,28)=2.57, P=0.12, η_p^2=0.077	F(1,28)=1.71, P=0.20, η_p^2=0.051
	8 weeks	F(1,34)=1.10, P=0.30, η_p^2=0.029	F(1,34)=2.56, P=0.12, η_p^2=0.067	F(1,34)=0.27, P=0.61, η_p^2=0.007
Floating duration	**2 weeks**	F(1,28)=0.79, P=0.38, η_p^2=0.023	F(1,28)=5.63, P=0.025, η_p^2=0.16	F(1,28)=0.65, P=0.43, η_p^2=0.019
	8 weeks	F(1,34)=0.30, P=0.59, η_p^2=0.008	F(1,34)=1.08, P=0.31, η_p^2=0.030	F(1,34)=1.15, P=0.29, η_p^2=0.031
Latency to immobility	**2 weeks**	F(1,28)=1.97, P=0.17, η_p^2=0.051	F(1,28)=8.75, P=0.006, η_p^2=0.23	F(1,28)=0.32, P=0.58, η_p^2=0.008
	8 weeks	F(1,34)=0.53, P=0.47, η_p^2=0.013	F(1,34)=3.58, P=0.067, η_p^2=0.091	F(1,34)=0.97, P=0.33, η_p^2=0.025

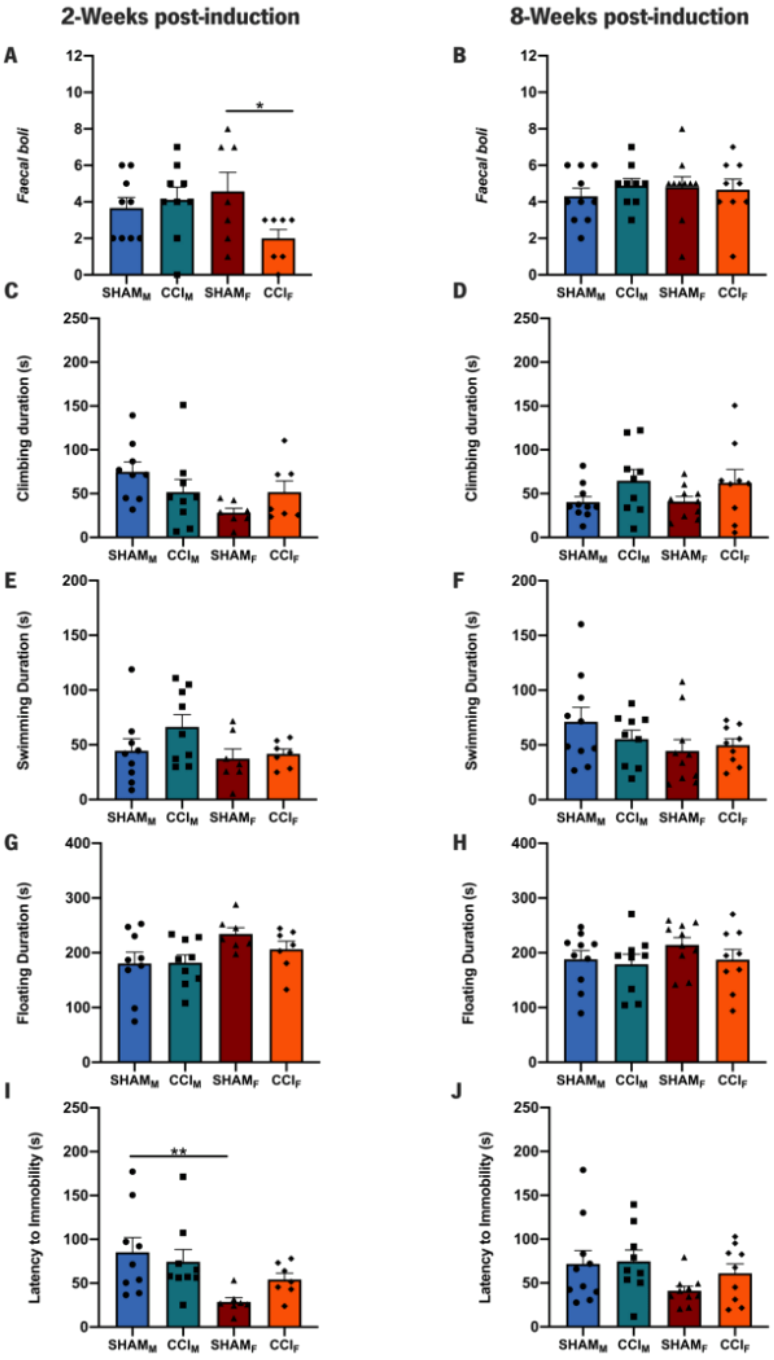

Figure 10. Depressive-like behaviour assessment through the forced swimming test (FST) two and eight weeks post-CCI induction. **(A, B)** Neuropathic females (CCI_F) displayed fewer *faecal boli*, in comparison with controls, however, no differences were found between experimental groups at week 8. **(C, D**) No differences were found between experimental groups two and eight weeks post-CCI induction, however, CCI independently increased the climbing duration on week 8. **(E, F)** Concerning the swimming duration, no differences were found between experimental groups at both earlier and later time-points. **(G, H)** No significant differences were found between experimental groups regarding the time spent immobile (floating duration), both on weeks 2 and 8. **(I, J)** Control females ($SHAM_F$) displayed a decreased latency to immobility on week 2, in comparison with males, however, no differences were found between control and neuropathic animals at both time-points. Data presented as mean ± SEM [$n(SHAM_F)_{2W}$=7, $n(CCI_F)_{2W}$=7, $n(SHAM_M)_{2W}$=9, $n(CCI_M)_{2W}$=9]); [$n(SHAM_F)_{8W}$=10, $n(CCI_F)_{8W}$=9, $n(SHAM_M)_{8W}$=10, $n(CCI_M)_{8W}$=9]. *P<0.05, **P<0.01

While no differences were found between experimental groups at week 2 (**Table 5**, **Figure 10C**), CCI independently increased the time spent climbing at week 8, (**Table 5**, **Figure 10D**), but multiple comparisons showed no significant differences between experimental groups. Neither sex nor CCI varied the swimming duration, both two (**Table 5**, **Figure 10E**) and eight weeks post-CCI induction (**Table 5**, **Figure 10F**).

On week 2, the time spent floating (immobility) was significantly different between male and female animals, independently of CCI (**Table 5**, **Figure 10G**), although post hoc showed no significant differences between experimental groups. In contrast, on week 8 after induction, no statistically significant differences were found between experimental groups (**Table 5**, **Figure 10H**).

Regarding the latency to immobility, ANOVA analysis showed differences between males and females, independently of CCI two weeks after CCI induction (**Table 5**, **Figure 10I**). Post hoc tests showed a decrease in the latency to immobility in control females ($SHAM_F$) when compared to males ($SHAM_M$) (P=0.009). At 8 weeks after induction, there were no statistically significant differences between experimental groups (**Table 5**, **Figure 10J**).

4.4. Electrophysiological recordings on RVM

In this work, a total of 806 RVM neurons were recorded two weeks post-induction and 668 RVM neurons at eight weeks post-induction (**Table 6**).

Table 6. Total number of RVM neurons recorded in the electrophysiological study, two and eight weeks after CCI induction.

	$SHAM_M$ (n=16)	**CCI_M (n=16)**	**$SHAM_F$ (n=11)**	**CCI_F (n=19)**
RVM neurons (2 weeks)	226	203	109	268
RVM neurons (8 weeks)	192	225	133	118

4.4.1. Mechanical stimulation (von Frey)

Neuronal activity in the RVM was recorded before and after mechanical stimulation using von Frey hairs (6g), and the total number of neurons recorded following the stimulation, at weeks 2 and 8, is described in **Table 7**. After identifying outliers for the evoked response, through the ROUT method, 42 neurons were removed from the analysis.

Table 7. Total number of RVM On-, Off- and Neutral-like cells recorded in the electrophysiological study, after Von Frey stimulation, two and eight weeks post-CCI induction. (-) outliers removed.

	Group	On-like cells	Off-like cells	Neutral-like cells
2 weeks	**$SHAM_M$**	37 (-2)	43 (-6)	148
	CCI_M	26 (-7)	42 (-3)	140
	$SHAM_F$	15 (-1)	19 (-1)	73
	CCI_F	37(-1)	46 (-1)	177
8 weeks	**$SHAM_M$**	20 (-1)	28 (-2)	141
	CCI_M	38 (-9)	47 (-6)	141
	$SHAM_F$	16	21 (-2)	58
	CCI_F	17	15	88

Regarding the spontaneous activity of RVM neurons (**Table 8**, **Figure 11**), sex independently altered the activity of On-like cells two weeks post-induction, but no differences were found between experimental groups on week 8. ANOVA analysis showed no differences in the spontaneous activity of Off-like cells, at both earlier and later time-points.

Table 8. Statistical analysis of the spontaneous activity of RVM neurons before Von Frey stimulation, two and eight weeks after CCI induction.

		Interaction	Sex	CCI
2 weeks	**On-like cells**	$F(1,100)=0.72$, $P=0.40$, $\eta_p^2=0.007$	$F(1,100)=4.71$, $P=0.032$, $\eta_p^2=0.043$	$F(1,100)=0.63$, $P=0.43$, $\eta_p^2=0.006$
	Off-like cells	$F(1,135)=0.64$, $P=0.43$, $\eta_p^2=0.005$	$F(1,135)=0.067$, $P=0.80$, $\eta_p^2=0.0005$	$F(1,135)=3.00$, $P=0.086$, $\eta_p^2=0.022$
8 weeks	**On-like cells**	$F(1,77)=0.21$, $P=0.65$, $\eta_p^2=0.003$	$F(1,77)=0.040$, $P=0.84$, $\eta_p^2=0.0005$	$F(1,77)=0.085$, $P=0.77$, $\eta_p^2=0.001$
	Off-like cells	$F(1,97)=1.01$, $P=0.32$, $\eta_p^2=0.010$	$F(1,97)=0.008$, $P=0.93$, $\eta_p^2<0.0001$	$F(1,97)=0.25$, $P=0.62$, $\eta_p^2=0.003$

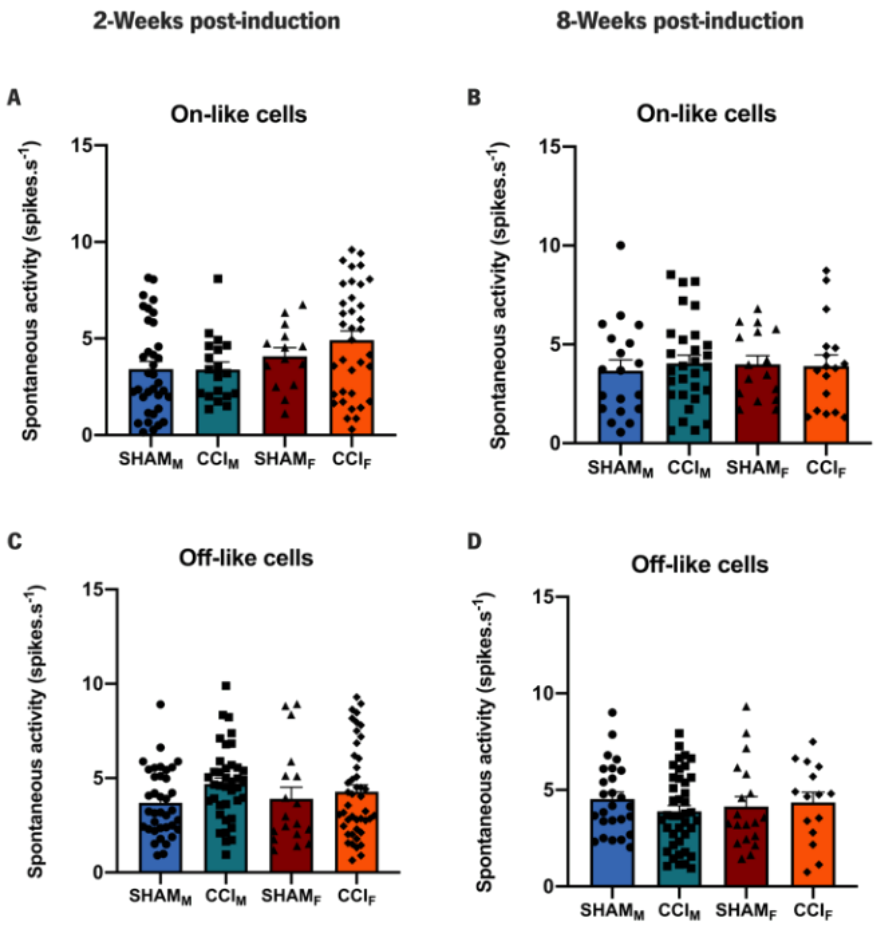

Figure 11. Spontaneous activity of RVM neurons before the von Frey stimulation. No differences were found between the experimental groups. Data presented as mean ± SEM.

Regarding the evoked response of RVM neurons to von Frey stimulation (**Table 9**, **Figure 12**), ANOVA analysis showed an interaction between sex and CCI in the response of On-like cells on week 2. Further comparisons showed an increase in the response of On-like cells of neuropathic females (CCI_F) when compared to both control females ($SHAM_F$, P=0.022) and neuropathic males (CCI_M, P= 0.009). On the other hand, eight weeks post-induction, CCI significantly decreased the evoked response of On-like cells, particularly in males. Regarding the evoked response of the Off-like cells, neither sex nor CCI altered this variable on week 2. However, ANOVA analysis showed an interaction between sex and CCI on week 8, and multiple comparisons showed neuropathic females (CCI_F) displayed a decrease in the response of Off-like cells when compared to control females ($SHAM_F$, P=0.011).

Table 9. Statistical analysis of the evoked response of RVM neurons after the Von Frey stimulation, two and eight weeks after CCI induction.

		Interaction	Sex	CCI
2 weeks	**On-like cells**	F(1,100)=10.4, P=0.002, η_p^2=0.093	F(1,100)=0.46, P=0.50, η_p^2=0.004	F(1,100)=0.39, P=0.53, η_p^2=0.004
	Off-like cells	F(1,135)=0.61, P=0.44, η_p^2=0.004	F(1,135)=0.13, P=0.72, η_p^2=0.001	F(1,135)=2.20, P=0.14, η_p^2=0.016
8 weeks	**On-like cells**	F(1,77)=0.16, P=0.69, η_p^2=0.002	F(1,77)=0.40, P=0.53, η_p^2=0.005	F(1,77)=7.36, P=0.008, η_p^2=0.086
	Off-like cells	F(1,97)=6.32, P=0.014, η_p^2=0.060	F(1,97)=0.34, P=0.56, η_p^2=0.003	F(1,97)=4.27, P=0.042, η_p^2=0.041

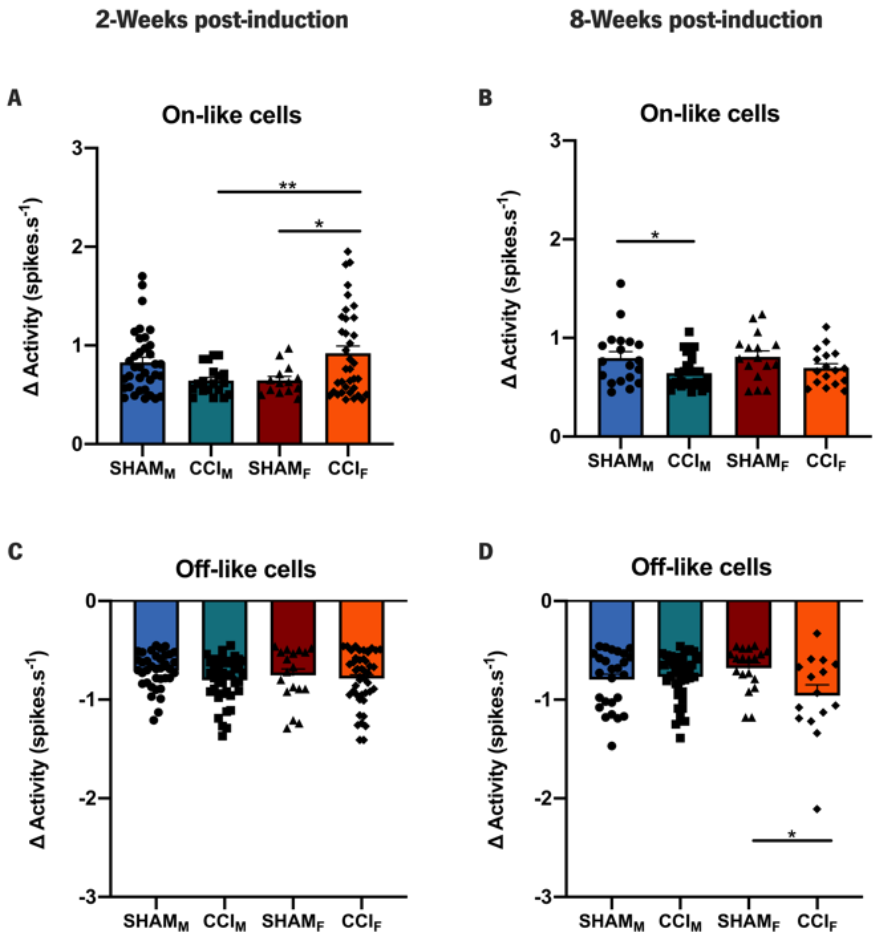

Figure 12. Evoked response (Δ Activity) of RVM neurons after mechanical stimulation with von Frey hairs. Neuropathic females (CCI_F) displayed an increased response of pronociceptive On-like two weeks post-induction, and a decreased activation of antinociceptive Off-like cells at week 8. Neuropathic males (CCI_M) showed a decrease in the evoked response of pronociceptive On-like cells, however only at week 8 after CCI induction. Data presented as mean ± SEM. *P<0.05, **P<0.01.

4.4.2. Cold stimulation (Acetone)

RVM neuronal activity was assessed using innocuous cold stimulation, namely, acetone applied to the plantar skin of the left hind paw. **Table 10** describes the total number of neurons recorded following stimulation at weeks 2 and 8 after CCI induction. Thirty-two neurons were identified as outliers for evoked response (ROUT method) and removed from the statistical analysis.

Table 10. Total number of RVM On-, Off- and Neutral-like cells recorded in the electrophysiological study, after acetone stimulation, two and eight weeks post-CCI induction. (-) outliers removed.

	Group	On-like cells	Off-like cells	Neutral-like cells
2 weeks	**$SHAM_M$**	39 (-1)	39	149
	CCI_M	27 (-4)	32 (-2)	144
	$SHAM_F$	13 (-1)	16 (-1)	79
	CCI_F	41 (-3)	33 (-11)	189
8 weeks	**$SHAM_M$**	22 (-3)	20	150
	CCI_M	36 (-2)	32 (-4)	160
	$SHAM_F$	18	19	92
	CCI_F	8	23	97

Regarding the spontaneous activity of RVM neurons (**Table 11**, **Figure 13**), no significant differences were found in the spontaneous activity of On-like cells at both earlier and later time-points. ANOVA analysis showed an interaction between sex and CCI regarding the spontaneous activity of Off-like cells at week 2, and post hoc tests showed an increase in the spontaneous activity in control females ($SHAM_F$) in comparison with neuropathic females (CCI_F, P=0.004) and control males ($SHAM_M$, P=0.019). No statistically significant differences were found between experimental groups in the spontaneous activity of OFF-like cells at week 8.

Table 11. Statistical analysis of the spontaneous activity of RVM neurons before acetone stimulation, two and eight weeks after CCI induction.

		Interaction	Sex	CCI
2 weeks	**On-like cells**	$F(1,107)=0.69$, $P=0.41$, $\eta_p^2=0.006$	$F(1,107)=0.068$, $P=0.80$, $\eta_p^2=0.0006$	$F(1,107)=0.047$, $P=0.83$, $\eta_p^2=0.0004$
	Off-like cells	$F(1,102)=8.99$, $P=0.003$, $\eta_p^2=0.080$	$F(1,102)=0.81$, $P=0.37$, $\eta_p^2=0.007$	$F(1,102)=4.39$, $P=0.039$, $\eta_p^2=0.039$
8 weeks	**On-like cells**	$F(1,75)=0.17$, $P=0.68$, $\eta_p^2=0.002$	$F(1,75)=0.12$, $P=0.73$, $\eta_p^2=0.002$	$F(1,75)=0.19$, $P=0.66$, $\eta_p^2=0.003$
	Off-like cells	$F(1,86)=0.004$, $P=0.95$, $\eta_p^2<0.0001$	$F(1,86)=0.55$, $P=0.46$, $\eta_p^2=0.006$	$F(1,86)=0.35$, $P=0.55$, $\eta_p^2=0.004$

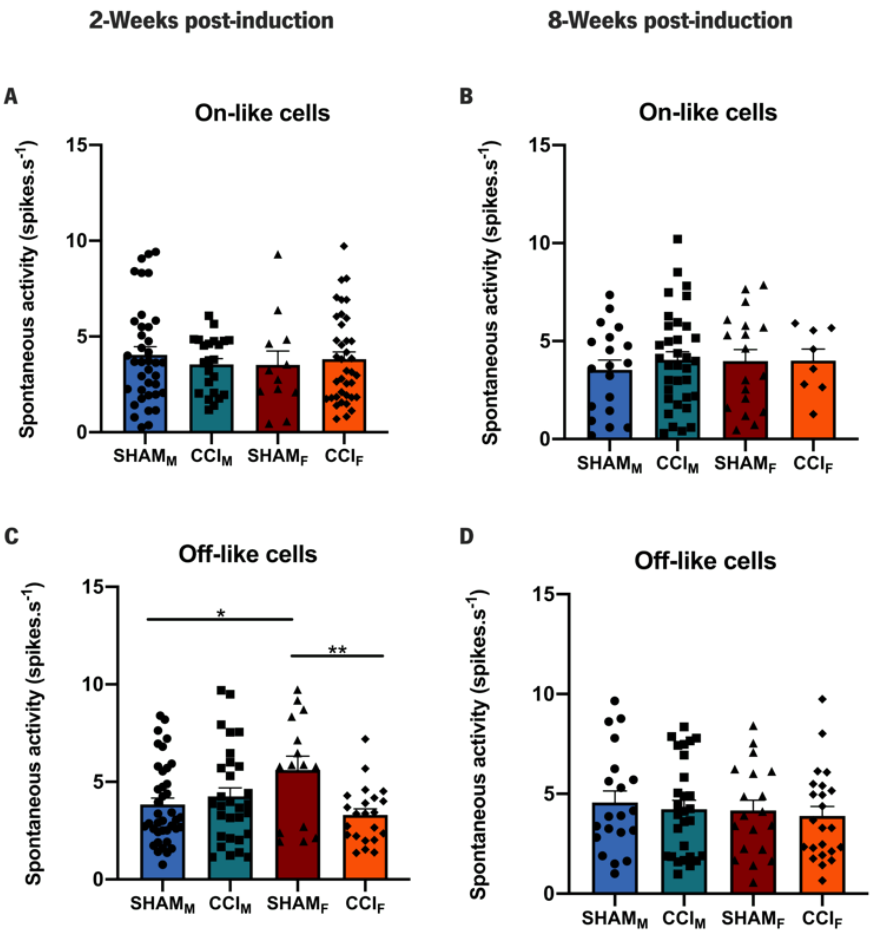

Figure 13. Spontaneous activity of RVM neurons before cold stimulation with acetone. Control females displayed an increase in the spontaneous activity of Off-like cells in comparison with males, and neuropathic females displayed a decrease in the response of these neurons. No differences were found between experimental groups regarding the remaining cells. Data presented as mean ± SEM. *P<0.05, **P<0.01.

Regarding the neuronal response of RVM cells to cold stimulation (**Table 12**, **Figure 14**), two weeks post-induction, CCI independently decreased the neuronal response of pronociceptive On-like cells, but no significant differences were found between experimental groups on weeks 2 and 8. CCI significantly increased the evoked response of antinociceptive Off-like cells at week 2, and multiple comparisons showed an increase in neuropathic animals, both males (P=0.022) and females (P=0.010) when compared to their counterpart controls. Neither CCI nor sex altered the response of Off-like cells on week 8.

Table 12. Statistical analysis of the evoked response of RVM neurons after the acetone stimulation, two and eight weeks after CCI induction.

		Interaction	Sex	CCI
2 weeks	**On-like cells**	F(1,107)=0.040, P=0.84, η_p^2=0.0003	F(1,107)=1.24, P=0.27, η_p^2=0.011	F(1,107)=4.96, P=0.028, η_p^2=0.042
	Off-like cells	F(1,102)=0.63, P=0.43, η_p^2=0.005	F(1,102)=1.64, P=0.20, η_p^2=0.014	F(1,102)=14.7, P=0.0002, η_p^2=0.12
8 weeks	**On-like cells**	F(1,74)=0.001, P=0.97, η_p^2<0.0001	F(1,74)=1.62, P=0.21, η_p^2=0.021	F(1,74)=0.027, P=0.87, η_p^2=0.0004
	Off-like cells	F(1,86)=2.53, P=0.12, η_p^2=0.027	F(1,86)=2.87, P=0.094, η_p^2=0.030	F(1,86)=1.62, P=0.21, η_p^2=0.017

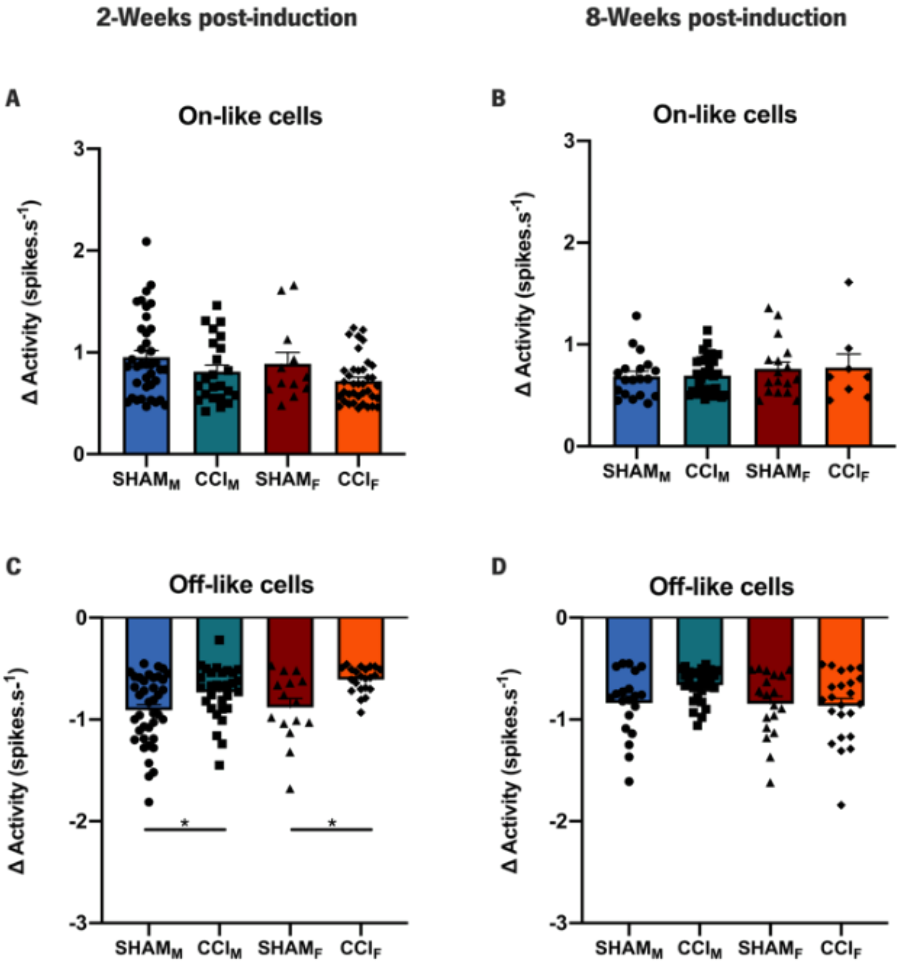

Figure 14. Evoked response (Δ Activity) of the RVM neurons after cold stimulation with acetone. Neuropathic animals, both males and females, displayed an increased activation of antinociceptive Off-like cells at week 2 post-CCI induction. No significant differences were found between experimental groups for the remaining cells. Data presented as mean ± SEM. *P<0.05

4.4.3. Mechanical stimulation (Pinch)

RVM neuronal activity was also evaluated using noxious stimulation by the pinch of the animals' tails. The total number of neurons recorded at weeks 2 and 8 are described in **Table 13**. After identifying outliers for spontaneous activity and evoked response with the ROUT method, 78 neurons were removed from the statistical analysis.

Table 13. Total number of RVM On-, Off- and Neutral-like cells recorded in the electrophysiological study, after noxious stimulation of the tail, two and eight weeks post-CCI induction. (-) outliers removed.

	Group	On-like cells	Off-like cells	Neutral-like cells
2 weeks	**$SHAM_M$**	108 (-8)	45 (-1)	77
	CCI_M	64 (-7)	48 (-10)	92
	$SHAM_F$	35 (-5)	22 (-8)	51
	CCI_F	96 (-6)	37 (-2)	130
8 weeks	**$SHAM_M$**	81 (-9)	26 (-3)	77
	CCI_M	82 (-6)	52 (-1)	103
	$SHAM_F$	51 (-3)	32 (-3)	47
	CCI_F	36 (-5)	21 (-1)	61

Regarding the spontaneous activity of RVM cells (**Table 14**, **Figure 15**), neither CCI nor sex altered the activity of pronociceptive On-like cells at both earlier (2 weeks) and later (8 weeks) time-points. On the other hand, ANOVA analysis showed an interaction between CCI and sex in the spontaneous activity of antinociceptive Off-like cells two weeks post-induction, and further comparisons showed a decrease in neuronal activity in control females ($SHAM_F$) in comparison with males ($SHAM_M$, P=0.012) and neuropathic females (CCI_F, P=0.001). No statistically significant differences were found between experimental groups in the spontaneous activity of Off-like cells on week 8.

Table 14. Statistical analysis of the spontaneous activity of RVM neurons before pinch stimulation, two and eight weeks after CCI induction.

		Interaction	Sex	CCI
2 weeks	**On-like cells**	$F(1,273)=0.45$, $P=0.50$, $\eta_p^2=0.002$	$F(1,273)=1.05$, $P=0.31$, $\eta_p^2=0.004$	$F(1,273)=1.09$, $P=0.30$, $\eta_p^2=0.004$
	Off-like cells	$F(1,129)=11.4$, $P=0.001$, $\eta_p^2=0.080$	$F(1,129)=1.18$, $P=0.28$, $\eta_p^2=0.008$	$F(1,129)=5.90$, $P=0.017$, $\eta_p^2=0.041$
8 weeks	**On-like cells**	$F(1,221)=1.11$, $P=0.29$, $\eta_p^2=0.005$	$F(1,221)=0.24$, $P=0.62$, $\eta_p^2=0.001$	$F(1,221)=1.79$, $P=0.18$, $\eta_p^2=0.008$
	Off-like cells	$F(1,119)=0.20$, $P=0.65$, $\eta_p^2=0.002$	$F(1,119)=2.26$, $P=0.14$, $\eta_p^2=0.018$	$F(1,119)=1.39$, $P=0.24$, $\eta_p^2=0.011$

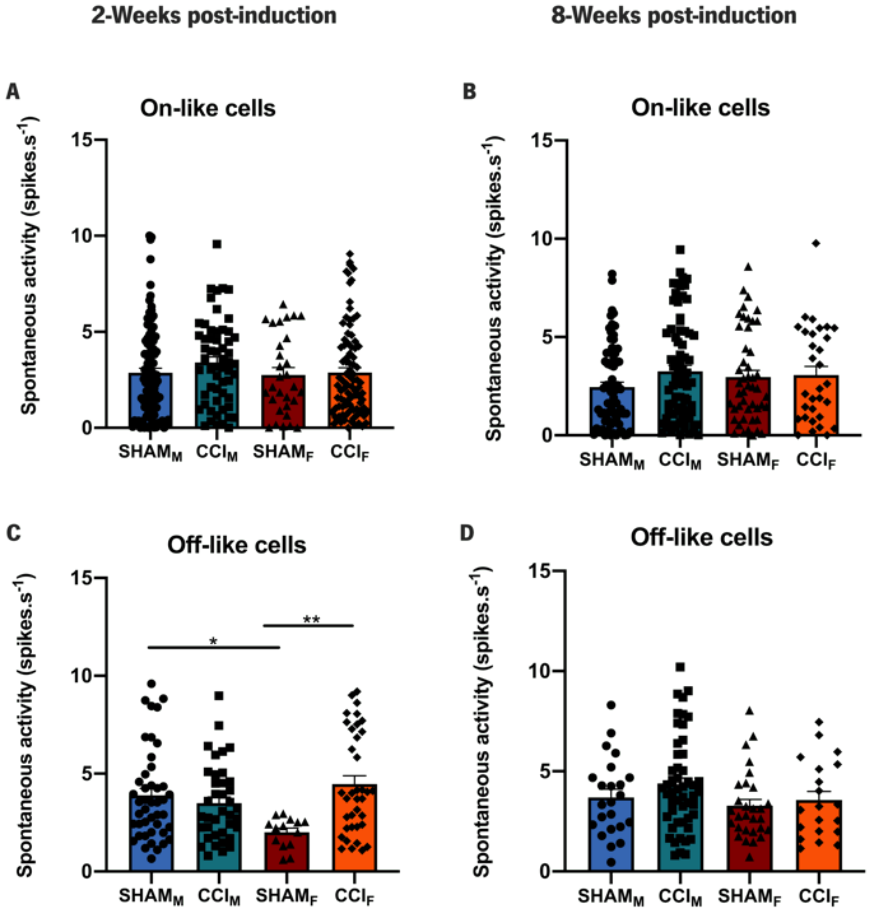

Figure 15. Spontaneous activity of RVM neurons before noxious mechanical stimulation of the animal's tails. Control females ($SHAM_F$) displayed a decrease in the spontaneous activity of Off-like cells when compared to both control males ($SHAM_M$) and neuropathic females (CCI_F). No significant differences were found between experimental groups for the remaining cells. Data presented as mean ± SEM. *P<0.05, **P<0.01.

Regarding the response of RVM neurons evoked by mechanical stimulation (**Table 15**, **Figure 16**), the response of pronociceptive On-like cells was altered neither by CCI nor sex at weeks 2 and 8. On the other hand, ANOVA analysis showed an interaction between sex and CCI at week 2 on the response of antinociceptive Off-like cells (**Table 15**), with no significant differences between experimental groups. Eight weeks post-induction, CCI independently decreased the evoked response of Off-like cells, and multiple comparisons showed a decreased in neuropathic females (CCI_F) compared to their counterpart controls ($SHAM_F$, P=0.035).

Table 15. Statistical analysis of the evoked response of RVM neurons after the pinch stimulation, two and eight weeks after CCI induction.

		Interaction	Sex	CCI
2 weeks	**On-like cells**	F(1,273)=2.17, P=0.14, η_p^2=0.008	F(1,273)=0.68, P=0.41, η_p^2 =0.003	F(1,273)=0.026, P=0.87, η_p^2=0.0001
	Off-like cells	F(1,127)=6.41, P=0.013, η_p^2=0.048	F(1,127)=0.13, P=0.72, η_p^2=0.001	F(1,127)=0.41, P=0.52, η_p^2=0.003
8 weeks	**On-like cells**	F(1,222)=1.70, P=0.19, η_p^2=0.008	F(1,222)=0.16, P=0.69, η_p^2=0.0007	F(1,222)=0.96, P=0.33, η_p^2=0.004
	Off-like cells	F(1,119)=1.43, P=0.24, η_p^2=0.011	F(1,119)=1.33, P=0.25, η_p^2=0.011	F(1,119)=6.02, P=0.016,η_p^2=0.048

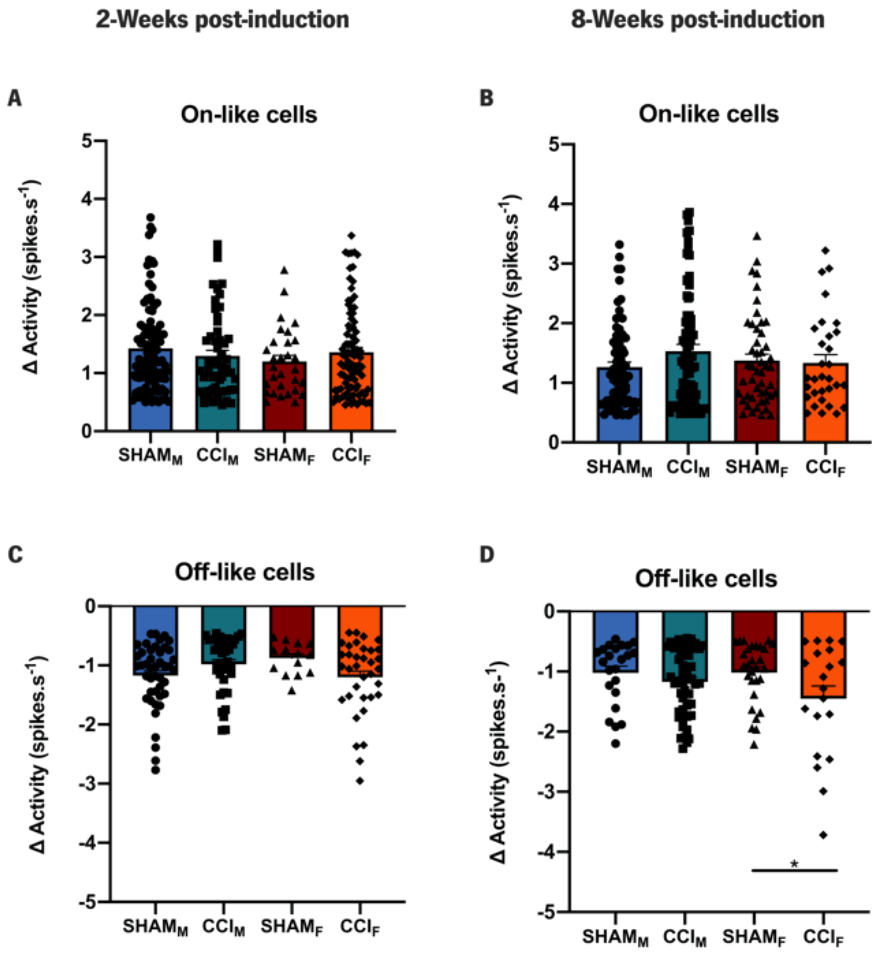

Figure 16. Evoked response (Δ Activity) of the RVM neurons after noxious mechanical stimulation of the animal's tails. No significant differences were found between experimental groups regarding the response of On-like cells. Neuropathic females (CCI_F) displayed a decreased activation of antinociceptive Off-like cells eight weeks after CCI induction, in comparison with controls ($SHAM_F$). Data presented as mean ± SEM. *P<0.05

4.2. Histopathological analysis of the sciatic nerve

After the behavioural and electrophysiological study, samples of the ipsilateral sciatic nerve were collected for histopathological evaluation. Control animals, both males and females, presented a well-organized structure of nerve fibres and an absence of inflammatory cells at both weeks 2 and week 8. On both weeks 2 and 8, longitudinal and transversal sections of the sciatic nerve of neuropathic animals showed an inflammatory reaction, with the presence of infiltrating inflammatory cells, as well as the degradation of the myelin sheath (**Figure 17**).

To assess the impact of CCI on nerve fibres, we assessed the total nerve area as well as the density of myelinated fibres through a stereological approach (**Figure 18**, **Table 16**).

Table 16. Statistical analysis of the area and density of myelinated fibres of the left sciatic nerve, two and eight weeks after CCI induction.

		Interaction	Sex	CCI
2 Weeks	**Area (mm^2)**	$F(1,28) = 0.15$, $P = 0.70$, $\eta_p^2 = 0.004$	$F(1,28) = 1.55$, $P = 0.22$, $\eta_p^2 = 0.043$	$F(1,28) = 5.84$ $P = 0.023$, $\eta_p^2 = 0.16$
	Myelinated fibres/ mm^2	$F(1,28) = 1.65$, $P = 0.21$, $\eta_p^2 = 0.045$	$F(1,28) = 0.18$, $P = 0.68$, $\eta_p^2 = 0.005$	$F(1,28) = 6.80$, $P = 0.014$, $\eta_p^2 = 0.19$
8 weeks	**Area (mm^2)**	$F(1,31) = 0.095$, $P = 0.76$, $\eta_p^2 = 0.003$	$F(1,31) = 1.12$, $P = 0.30$, $\eta_p^2 = 0.034$	$F(1,31) = 0.22$, $P = 0.64$, $\eta_p^2 = 0.007$
	Myelinated fibres/ mm^2	$F(1,31) = 4.89$, $P = 0.034$, $\eta_p^2 = 0.12$	$F(1,31) = 0.59$, $P = 0.45$, $\eta_p^2 = 0.015$	$F(1,31) = 3.38$, $P = 0.076$, $\eta_p^2 = 0.083$

CCI independently increased the total nerve area two weeks post-CCI induction, however, multiple comparisons showed no significant differences between experimental groups. No significant differences were found between experimental groups at week 8.

CCI significantly decreased the total density of myelinated fibres at week 2, particularly in males ($P = 0.026$). At week 8 after CCI induction, ANOVA analysis showed an interaction between sex and CCI on the total density of myelinated fibres, and further comparisons showed a decrease in fibre density in neuropathic females (CCI_F), in comparison with controls ($SHAM_F$, $P = 0.010$).

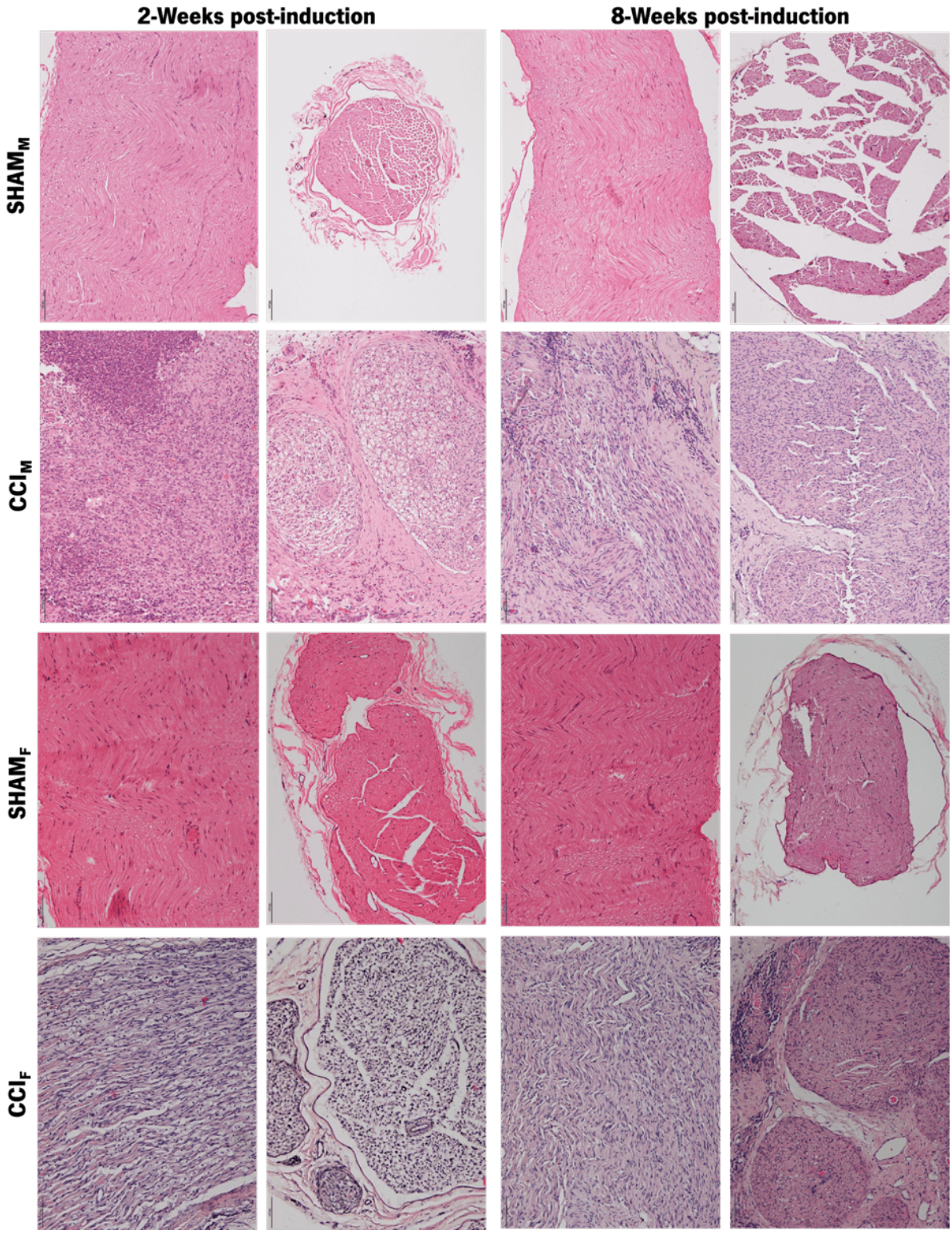

Figure 17. Sample micrographs of longitudinal (left) and transverse (right) sections of the ipsilateral sciatic nerve from each experimental group, stained with haematoxylin and eosin. CCI animals (male and females) display a lack of nerve fibre structure, inflammatory reaction, as well as degraded myelin sheath, on weeks 2 and 8. Images were collected using an Olympus BX61 brightfield microscope, attached to an Olympus DP70 camera at the original objective magnification of 10×. (Scale bar = 100 μm).

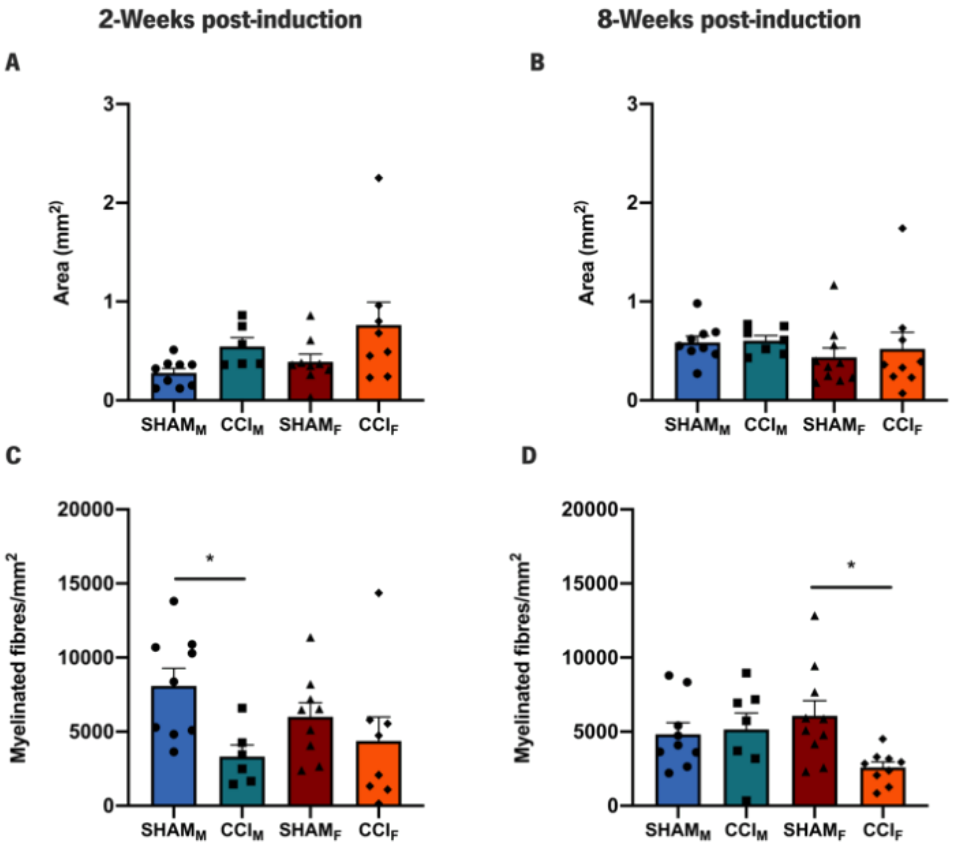

Figure 18. Nerve area and myelinated nerve fibre density of the ipsilateral sciatic nerve. **(A, B)** No significant differences were found between experimental groups, however CCI increased the total nerve area at week 2. **(C)** CCI_M displayed a decrease in the total density of myelinated fibres compared to $SHAM_M$. **(D)** Neuropathic females showed a decrease in the total density of myelinated fibres in comparison with control females. Data presented as mean ± SEM. *P<0.05.

Morphometric analysis was performed, assessing the different distributions of myelinated fibres' diameters, to address the extent of myelinated fibre breakdown and degeneration (**Figure 19**, **Table 17**). ANOVA analysis also revealed that the distribution of the diameter of myelinated fibres in both males and females differed between control and neuropathic animals, two weeks post-CCI induction. Accordingly, multiple comparisons showed an increase in the number of myelinated fibres with 2-4 μm of diameter (P<0.0001) and a decrease in myelinated fibres with 6-8 μm (P<0.0001) in neuropathic males (CCI_M) in comparison with controls ($SHAM_M$) males. Regarding females, CCI induced an increase in the number of myelinated fibres under 2 μm (P=0.030) compared controls ($SHAM_F$). At week 8 after CCI induction, ANOVA analysis showed the distribution of the diameter of myelinated fibres in both males and females varied between control and neuropathic animals.

Multiple comparisons showed CCI increased the number of myelinated fibres between 2-4 μm in males (P=0.031). Neuropathic females displayed an increase in the number of myelinated fibres under 2 μm (P=0.019) and between 2-4 μm (P=0.023), as well as a decrease in myelinated fibres with 6-8 μm of diameter (P=0.022).

Table 17. Statistical analysis of the diameter distribution of the left sciatic nerve between male and female experimental groups, two and eight weeks after CCI induction.

		Interaction	Diameter	CCI
2 Weeks	**Males**	F(6,98)=13.1, P<0.0001, η_p^2=0.23	F(6,98)=27.4, P<0.0001,η_p^2=0.48	F(1,98)=0.002, P=0.96, η_p^2<0.0001
	Females	F(6,119)=2.81, P=0.014, η_p^2=0.071	F(6,119)=16.4, P<0.0001, η_p^2=0.42	F(1,119)=0.003, P=0.96, η_p^2<0.0001
8 weeks	**Males**	F(6,105)=3.60, P=0.003, η_p^2=0.064	F(6,105)=33.1, P<0.0001,η_p^2=0.59	F(1,105)<0.0001, P=0.99, η_p^2<0.0001
	Females	F(6,119)=5.22, P<0.0001, η_p^2=0.13	F(6,119)=14.1 P<0.0001, η_p^2=0.36	F(1,119)=0.002, P=0.96, η_p^2<0.0001

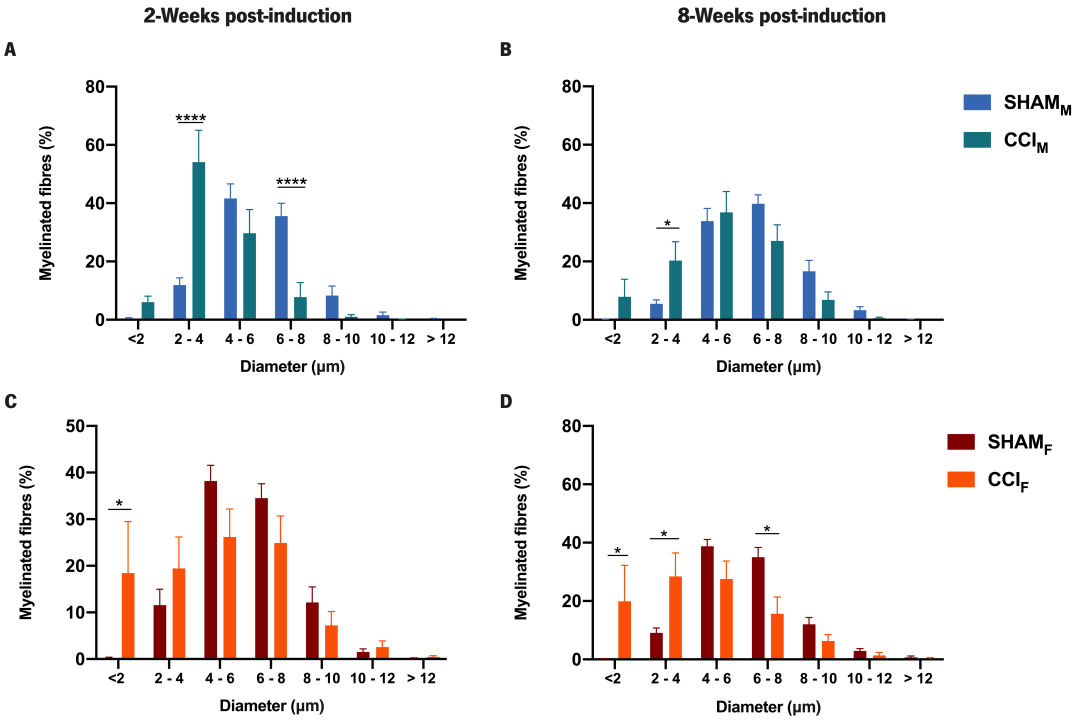

Figure 19. Diameter distribution of myelinated nerve fibres of the ipsilateral sciatic nerve. **(A, B)** Neuropathic males displayed an increase in the number of myelinated fibres with 2-4 μm, concomitant with a decrease in myelinated fibres with 6-8 μm, at week 2 but not at week 8. **(C, D)** Neuropathic females displayed an increased percentage of myelinated fibres under 2 μm at week 2 post-induction. At week 8, females showed an increased percentage of myelinated fibres <2 and 2-4 μm, concomitant to a decrease in the number of myelinated fibres between 6-8 μm. Data presented as mean ± SEM. *P<0.05 and ****P<0.0001.

Chapter 5. Discussion

In this experimental work, CCI induction resulted in the development of mechanical and cold allodynia starting the first week after surgery in both males and females, without impacting body weight gain. Interestingly, CCI animals, both male and female, did not display emotional impairments at weeks 2 and 8 after its induction. The electrophysiological data partially corroborated our behavioural findings, as nociceptive changes observed in male and female animals were associated with changes in the neuronal activity of the RVM neurons. Additionally, our histopathological analysis showed CCI caused significant nerve damage, myelin sheath degradation, and inflammation of the sciatic nerve, observed as early as two weeks after induction.

5.1. Technical considerations on the animal model

Several animal models have been developed to study the mechanisms and novel treatments for chronic neuropathic pain. The CCI model (Bennett & Xie, 1988) mimics the symptoms of nerve constriction and comprises both inflammatory and neuropathic characteristics. Additionally, CCI animals present signs of spontaneous pain, namely limping, guarding behaviour, extreme licking, and avoidance of weight-bearing on the injured limb. However, the CCI model presents some disadvantages such as the possible variation in ligature tension throughout the experimental period and during induction, which might lead to inter-individual variability even when induced by the same experimenter. Another limitation of this approach is the development of autonomy in some animals, which progresses from the claws to the toes' roots, which, in some cases, requires the animals' euthanasia. The CCI model was successfully induced in our work, and CCI animals exhibited limping and guarding of the injured limb, as well as autotomy.

According to the work of Maves and colleagues, it is not only the constriction of the sciatic nerve that leads to the development of thermal hyperalgesia and limb guarding but also the use of chromic gut suture (Maves et al., 1993). This suture is absorbable in 90 to 120 days, and its tensile strength begins to deteriorate between days 14 to 21 days. Therefore, the nerve compression is resolved, allowing us to investigate the mechanisms underlying the development and maintenance of chronic pain, as well as sex differences in these mechanisms.

In this work, we used three 3/0 chromic gut ligatures around the sciatic nerve, however, Gopalsamy and colleagues showed in mice, that one ligation with 4/0 silk suture can establish the CCI-induced

animal model (Gopalsamy et al., 2019). The use of one ligation can lead to a decreased variability between animals as well as a decrease in surgical time, and silk suture is non-absorbable, which means the animals will not be able to lose nerve compression. However, using the chromic gut suture induces an inflammatory response, a strength of the CCI model. We also decide to perform the CCI surgery in the left hind paw, as previous research conducted by Leite-Almeida and colleagues, using the SNI model of neuropathic pain, found the side of injury has a different impact on the development of emotional and cognitive impairments – when neuropathic pain was induced in the left side was associated with emotional impairments, while on the right side with cognitive deficits (Leite-Almeida et al., 2012).

Among the alternatives to this model are the spinal nerve ligation (SNL), in which L_5 and L_6 spinal nerves are tightly ligated with 6–0 silk suture (Ho Kim & Mo Chung, 1992), and the spared nerve injury model (SNI) model, where the common peroneal and tibial nerves are tightly ligated using 5–0 silk and axotomized (2 mm of the distal nerve) (Decosterd & Woolf, 2000). As previously stated, unlike the CCI model, these neuropathic pain models do not allow the animal to recover, as the latter involves the irreversible axotomy of the tibial and common peroneal nerves.

To assess the impact of CCI on animal welfare, we monitored body weight changes throughout the experimental period. Interestingly, CCI induction did not arrest weight gain, and both male and female neuropathic animals gained weight during the experimental time. Our findings are supported by other studies that showed CCI males continued to gain weight after neuropathy (De Vry et al., 2004; Roeska et al., 2008; Hu et al., 2009; Caspani et al., 2014). Therefore, this animal model has the great advantage of accurately mimicking human peripheral nerve injuries without jeopardizing animal welfare.

5.2. Assessment of mechanical and cold allodynia after CCI induction

An array of behavioural assessments have been developed for the evaluation of pain responses in animal models, namely the response to a thermal or mechanical stimulus (Deuis et al., 2017). In this work, we used the von Frey and acetone tests to evaluate mechanical and cold allodynia, respectively.

Mechanical allodynia is frequently measured in animal models by applying von Frey filaments to the plantar surface of the hind paws, and there are three different methods to calculate the limb withdrawal threshold: the "ascending stimulus," "percent response," and the herein used "up-down". The "ascending stimulus" method avoids overstimulating the hind paw; however, it only gives an estimate of the withdrawal threshold, and the number of stimuli per test in the "percent response" procedure is

greater than in other methods. The "up-down" method was used in this work to calculate the 50% withdrawal response threshold (force required to elicit a withdrawal response in 50% of animals) (Dixon, 1980; Chaplan et al., 1994; Bonin et al., 2014). This method has several advantages such as allowing for unrestricted evaluation, reducing the stress induced by the test. However, the number of stimuli presented to each animal varies and necessitates repeated time-consuming measurements, which can result in learned responses or sensitization development.

The assessment of mechanical and cold allodynia, common symptoms of neuropathic pain, showed neuropathic females displayed mechanical allodynia up to week 5, while neuropathic males recovered earlier at week 3.

Other reports on the CCI model showed male CD1 mice and Sprague Dawley rats developed mechanical allodynia up to 3 weeks post-CCI surgery (Li et al., 2014; Medeiros et al., 2020), in agreement with our results. Nevertheless, previous studies demonstrated male Wistar Han rats developed mechanical allodynia from week 1 until week 7 post-induction (De Vry et al., 2004), and male mice (C57BL/6) up to 8 weeks after CCI induction (Barcelon et al., 2019) without a recovery. In other peripheral nerve injury models, for example, spinal nerve ligation, male Sprague-Dawley rats developed mechanical allodynia for ten weeks minimum (Ho Kim & Mo Chung, 1992).

The recovery observed in neuropathic males was expected since the suture used herein is absorbable and starts losing its tensile strength on week 2. However, females displayed mechanical hypersensitivity for longer, showing a sex difference in the maintenance of neuropathic pain. In line with our results, previous studies have also demonstrated sex differences in mechanical sensitivity in both rats and mice, with female animals displaying a higher mechanical threshold and recovering later than males (Vacca et al., 2014; Fonseca-Rodrigues et al., 2021). Moreover, when compared to SNI males, neuropathic female rats developed mechanical and cold sensitivity earlier and with greater intensity in the SNI model (Boullon et al., 2021).

Besides the acetone test (Yoon et al., 1994), immersion in cold water (Kayser et al., 1995; Kayser & Christensen, 2000), water-alcohol bath (Kayser et al., 1995), ethyl chloride spray (Hao et al., 1996) and cold plate (Jasmin et al., 1998) are used to assess cold allodynia. These tests are simple to perform and provide an accurate measurement of thermal sensitivity. The herein used acetone test presents some advantages: it does not require the use of additional specialized equipment as we use the same apparatus used for the von Frey test thus reducing the habituation period; and we apply the

stimulus, a drop of acetone, directly to the plantar surface of each hind paw, which is inexpensive and widely available. Additionally, the acetone test is performed with animals unrestrained, decreasing potential acute stress. However, the main disadvantage of this test is the smell of acetone, which induces stress and agitation in the animals, and may induce false responses. To tackle this limitation, we habituated the animals to the acetone odour before the test.

Concerning cold allodynia, while CCI females displayed cold allodynia during the eight weeks post-induction, males recovered at week 6. Previous reports showed the presence of cold allodynia two weeks post-CCI induction in Wistar rats, male and female, thus supporting our results (Jaggi & Singh, 2011; Khangura et al., 2017). On the other hand, while our males showed a recovery after six weeks, male ICR mice were shown to present mechanical and cold allodynia for up to 12 weeks (Gopalsamy et al., 2019). However, it is important to take into consideration that different species may present different mechanisms of chronic pain development. In the SNI model, Sprague-Dawley male rats were shown to display cold allodynia for no less than 16 weeks (Yoon et al., 1994). Additionally, as most studies are conducted on male animals, we have a limited number of studies on the CCI model in females to compare our findings to. Our results, nonetheless, are consistent with previous research, confirming the development and maintenance of neuropathic pain after CCI induction, and emphasizing the presence of sex differences in this model.

Neuropathic animals further showed no locomotor impairments in the OF. However, this is not in line with the literature, as CCI induction was shown to cause locomotor impairments in male Wistar, assessed using the OF and rotarod test (Bagriyanik et al., 2014) and Sprague Dawley rats assessed through the OF (Gregoire et al., 2012). Additionally, neuropathic females further displayed a decreased velocity in comparison with neuropathic males in the OF, suggesting a sex difference in the development of locomotor impairments after CCI induction.

While we assessed the oestrous cycle of the animals, due to our small sample size we were not able to correlate these with nociceptive behaviour on female animals. However, it is important to note female animals did not show increased variability in nociceptive responses, and the oestrus cycle did not hamper the comparison between males and females.

To sum up, our results showed sex differences in the development and maintenance of CCI-induced neuropathic pain. Importantly, the differences between male and female animals may be due to gonadal hormones, as previous research has shown these have an impact on nociceptive perception, and ovariectomized females display reduced hypersensitivity (Guindon et al., 2019; Fonseca-Rodrigues

et al., 2021). However, additional research is still needed to elucidate the mechanisms underlying these sex differences.

5.3. Assessment of anxiety- and depressive-like behaviours after CCI induction

We evaluated the development of comorbid mood disorders in Wistar Han rats, namely anxiety and depressive-like behaviours two and eight weeks post-CCI induction.

Anxiety-like behaviour was assessed through the OF and EPM tests, frequently used in pre-clinical research. The OF test (Walsh & Cummins, 1976) can also be used to assess basal locomotor impairments, an advantage when compared to other behavioural tests. On the contrary, while it is considered a standardized test, it is influenced by a variety of external factors such as the size and shape of the apparatus, as well as the room illumination and duration of testing. The EPM test (Pellow et al., 1985) is a well-validated behavioural assay for assessing anxiety-like behaviour in rodents. The main advantages of this test are its simplicity and the fact that the animals do not need to be trained before the test. Nevertheless, as with the OF, results may be inaccurate because anxious behaviour can be influenced by both genetic and environmental factors such as housing conditions, circadian rhythm/light cycle, apparatus construction, and illumination (Carobrez & Bertoglio, 2005). To control for these variables, these tests were carried out at the same time of day, in the same room, and with low lighting. Alternative options to these tests include the light/dark box test (LDB) (Crawley & Goodwin, 1980) which assesses the animal's preference for a light/open or dark/closed environment and, like the OF and EPM, is based on the animal's natural aversion of bright spaces and disposition to explore (Sousa et al., 2006).

Regarding anxiety-like behaviour, both male and female neuropathic did not display anxious behaviour in either OF or EPM tests. These results are not in accordance with previous studies, showing male Sprague-Dawley rats displayed anxiety-like behaviour in the OF test, two weeks post-CCI surgery (Missig et al., 2017). Moreover, other research conducted in neuropathic male Sprague-Dawley rats showed a decrease in the time spent in the centre using the OF test, between two and three weeks after CCI induction (Gregoire et al., 2012). In later time-points, namely after week 8 post-CCI induction only one study evaluated anxiety-like behaviour and showed no differences between control and neuropathic males (Dellarole et al., 2014), in line with our results. Most studies, however, have shown the development of anxiety-like behaviour between weeks 3 and 5 after CCI induction in both male rats (Roeska et al., 2008; Alba-Delgado et al., 2016; Fonseca-Rodrigues et al., 2021) and mice (Yalcin et al.,

2011; Ferreira-Chamorro et al., 2018). Overall, our findings suggest that the onset of anxiety-like behaviour might occur earlier after CCI induction, between weeks two and four, and is not observed at later time-points.

Interestingly, in the EPM test, control females spent more time in the open arms and had a larger number of open arms entries than their male counterparts, in week 8. Indeed, our data is corroborated by prior studies which have shown female animals spent more time in the open arms and displayed an increase in the number of open-arms entries in comparison with males, highlighting the existence of sex differences in behavioural testing (Scholl et al., 2019).

The SPT and FST tests were chosen to assess depressive-like behaviour. The SPT (Liu et al., 2018) assesses anhedonic-like behaviour, or a reduced ability to experience pleasure, using a two-bottle choice paradigm between water and a sweet likeable solution, and is a simple and easy test to perform. Nonetheless, because different protocols are used by different laboratories, the SPT schedule and parameters differ. The FST (Porsolt et al., 1977) assesses learned helplessness by placing animals in a short-term stressful situation with no escape. This test is a simple procedure that produces fast and robust results. However, its results are dependent on the animals' swimming ability and cannot be performed when they are unable to float. In addition to the previously mentioned tests, the novelty-suppressed feeding test (NSFT) (Shephard & Broadhurst, 1982) is an alternative to assess depressive-like behaviour, measuring the time until the rodent manifests feeding behaviour to a new factor and also the amount of food consumed.

Similarly, to what was observed with anxiety, inducing CCI did not result in the development of anhedonic-like behaviour in the SPT at weeks 2 or 8. Additionally, neuropathic females showed a significant decrease in total intake (sucrose intake + water intake) when compared to control females, nonetheless, however, this reduction did not affect the sucrose preference. Also, neuropathic males displayed a significant decrease in the total intake compared to neuropathic females. This could be due to a general decrease in fluid consumption rather than a direct manifestation of anhedonia, as previous research found female rats consumed more sucrose than males in the SPT (Dalla et al., 2005). Two weeks after induction, our results are in line with the literature, showing that neuropathic Sprague-Dawley animals do not display anhedonic behaviour in the SPT (Gregoire et al., 2012). However, at later time-points, reports on the right common sciatic nerve showed anhedonia from week 4 to 10 in male C57BL/6 mice (Dellarole et al., 2014), which contrasts with what was observed in our work.

Anhedonic behaviour was only observed four weeks after CCI induction in male Wistar rats (Fonseca-Rodrigues et al., 2021).

Neuropathic animals did not develop learned helplessness behaviour at week 2 or week 8. Neuropathic females presented a reduced number of *faecal boli* in comparison with controls, yet *faecal boli* measurement has not been validated across laboratories and is typically used to supplement other measures. However, our data is not in line with the literature as previous studies showed CCI animals, namely male Sprague–Dawley rats, display an increase in immobility time in FST, two weeks after CCI surgery (Li et al., 2019). Another study in male Swiss mice further found an increase in immobility time in neuropathic animals on day 14 upon induction (Jesse et al., 2010). Regarding the later time-point at which we evaluate our animals (eight weeks), Barcelon and colleagues observed an increase in immobility time in both tail suspension test (TST) and FST in male C57BL/6 mice with nerve constriction performed in the right sciatic nerve at week 8 post-induction. Furthermore, in other animal models of neuropathic pain, male Wistar rats displayed depressive-like behaviour seven weeks after SNI induction (Gonçalves et al., 2008), and male C57BL/6 mice exhibited anxious and depressive-like behaviour eight weeks after SNL injury (Suzuki et al., 2007).

Most studies, however, show the development of depressive-like behaviours in the CCI model between weeks 4 and 6 after induction, in male Wistar rats (Hu et al., 2009; Li et al., 2017), Sprague-Dawley rats (Alba-Delgado et al., 2013), ICR mice (Zhao et al., 2014) and C57BL/6J mice (Yalcin et al., 2011). It is important to note in most studies constriction of the sciatic nerve was performed on the right side, while in our work we induced the CCI model in the left hind paw.

To summarize, our findings emphasize a probable time-dependent development of emotional behaviour. As a result, anxiety-like behaviour is mostly present between two and four weeks after CCI induction, whereas depressive-like behaviour is mostly observed after week four. This conclusion in accordance with a previous study conducted by Yalcin and colleagues, which found emotional impairments develop in a time-dependent manner, with neuropathic mice displaying anxiety-like behaviour four weeks after CCI induction and depressive-like behaviour later, after six weeks (Yalcin et al., 2011). Nonetheless, it is critical to continue researching these sex differences in the development of mood comorbidities, so that they can be translated to the clinics. Future work could include other emotional tests to corroborate our findings, as well as studying the existence of sex differences in the CCI model in different species or strains.

5.4. Electrophysiological evaluation of CCI-induced changes in RVM neuronal activity

Basal and noxious-evoked neuronal activity in the RVM was evaluated through single-cell electrophysiological recordings. This method enables the simultaneous recording of several cells in every session and allows for the comparison of basal and neuronal activity evoked by innocuous and noxious stimuli. Importantly, we recorded as many neurons as possible per session and at various depths, to obtain a complete representation of the entire area of interest. After a peripheral stimulus, noxious and innocuous, the recorded cells were classified based on changes in discharge rate <10% in regards to the rate of spontaneous discharge (Pinto-Ribeiro et al., 2013), as responsive or not to a specific stimulus. However, in cells with low spontaneous firing rates, a minor change is enough to label the cell as responsive. As a result, a lower limit difference of 0.45 spikes/s was used to categorize a cell as responsive. Another limitation of this technique was the anaesthesia (sodium pentobarbital), as it might alter the cells' firing activity due to sedation's effects. During the recordings, we used the spontaneous activity to control for changes in anaesthesia level, in addition to checking pupil dilation, general muscle tone, and responses to noxious pinching every 15 minutes (standard procedure). This anaesthesia has indeed such a narrow anaesthesia safety margin (higher mortality rate), resulting in reduced sample size and very few neurons recorded. Unfortunately, there are very few drugs that can be used to record in vivo neuronal activity as most will either suppress neuronal discharge, as is the case of inhaled anaesthesia, or are too toxic to be used such as urethane.

Thenceforth, we evaluated changes in the neuronal activity of the RVM, a central player in descending modulation of pain, induced by CCI at an earlier (two weeks) and later (eight weeks) after surgery.

Before VF stimulation, we did not observe any change in the basal activity of On and Off-like cells at week 2 and week 8. However, after stimulation, at two weeks post-induction, we observed an increase in the activation of On-like cells in neuropathic females, in comparison with controls and neuropathic males. This increase in descending pronociception supports our mechanical allodynia results in females, where CCI_F showed a lower mechanical threshold, implying greater sensitivity to these stimuli. Furthermore, at week 8, neuropathic males displayed a decrease in the neuronal activity of pronociceptive On-like cells, which partially supports our behaviour data, as neuropathic males recovered earlier (week 3). Surprisingly, the activation Off-like cells in neuropathic females was significantly reduced eight weeks post-induction, which suggests a decrease in descending antinociception.

In terms of cold stimulation, there was a decrease in the spontaneous activity of Off-like neurons in neuropathic females, two-weeks post-induction, suggesting an enhanced activation of pronociceptive descending pathways at a basal state. Indeed, we have previously shown a decrease in spontaneous activity in Off-like cells at both time-points in the SNI model of neuropathic pain (Gonçalves et al., 2007). On the other hand, after stimulation, both neuropathic females and males displayed an increased activation of Off-like cells, which seems to indicate an increase in descending antinociception at earlier stages of CCI. These results are not in accordance with our behavioural data, as neuropathic animals still displayed cold allodynia at week 2. Additionally, previous studies have shown, using the SNI model, an increase in the response of On-like cells concomitant to a decrease in the response of Off-like cells at weeks 1 and 8 post-surgery in male rats after cold stimulation (Gonçalves et al., 2007). Nonetheless, eight weeks after CCI induction, this activation of Off-like cells was not observed, and this loss of antinociceptive response could partially explain why neuropathic animals do not shown a recovery in cold allodynia at this time point.

Regarding tail pinch stimulation, at week 2, Off-like cells showed an increase in the spontaneous activity in neuropathic females, which suggests a tonic antinociceptive effect. Yet, at week 8, the activation of Off-like cells was reduced in neuropathic females, suggesting a decrease in descending antinociceptive pathways. This could partly explain the mechanical allodynia experienced by neuropathic females at an earlier time point. Additionally, we showed no changes in the response of RVM neurons after CCI induction in males, in accordance with Gonçalves and colleagues, who, using the SNI model of neuropathic pain, showed neuronal activity of Off-like cells was not altered after pinch stimulation in both earlier and later time-points (Gonçalves et al., 2007).

Overall, our results partially support our behavioural data, as changes in the neuronal activity of the RVM neurons were concomitant with nociceptive behaviour observed in male and female animals. However, as only minor changes were observed, our results confirm our previous statement (Fonseca-Rodrigues et al., 2021) that probably the RVM is not a key player in neuropathic pain modulation in the CCI model. Future work could include cellular evaluation such as RVM neuronal death, as well as research into other structures involved in pain modulation both faciliatory and inhibitory such as anterior cingulate cortex (ACC) (Zhang et al., 2005), amygdala (AMY) (Neugebauer et al., 2004), dorsomedial nucleus of the hypothalamus (DMH) (Pinto-Ribeiro et al., 2013), the dorsal reticular nucleus (DRt) (Lima & Almeida, 2002) and caudal ventrolateral medulla (CVLM) (Tavares & Lima, 2002).

5.5. Histopathological analysis of the sciatic nerve

The haematoxylin and eosin (H&E) stain is widely used in most laboratories for microscopic observation. Haematoxylin is used to stain nucleic acids (dark blue) and eosin stain proteins (red to pink or orange). The tissues of interest should be embedded in paraffin and sectioned to a thickness of 3–5 μm. A few processing errors can happen, resulting in an overly processed or dehydrated tissue. Also, the reagents must be changed regularly to avoid contamination and disrupt efficient processing. The intensity of the staining can also vary depending on the experimenter's training and experience. Additionally, haematoxylin and eosin solutions should always be stored properly as, for instance, when the haematoxylin solution becomes over oxidized, the nuclei lose staining (Cardiff et al., 2014). Alternatives to this staining procedure include the Toluidine blue stain, which also stains nucleic acids.

Longitudinal and transversal sections of the ipsilateral sciatic nerve of neuropathic animals revealed an inflammatory response, with infiltrating inflammatory cells and myelin sheath degradation on both weeks 2 and 8 after CCI induction, as expected due to the type of suture used in this work, as discussed previously.

Morphometric analysis of the sciatic nerve showed, a decrease in the diameter of myelinated fibres after CCI induction, with a shift to the left in the neuropathic animals' histogram in comparison with controls (Gopalsamy et al., 2019; Fonseca-Rodrigues et al., 2021). These findings show that degeneration of the sciatic nerve is correlated with an increased number of smaller width myelinated fibres, which could be due to demyelination of largely myelinated fibres (Aα and Aβ), and far less effect on small myelinated (Aδ) or unmyelinated axons (C-fibres). However, eight weeks after CCI surgery, neuropathic males did not display a significant decrease in the density of myelinated fibres and did not present a decrease in 6-8 μm myelinated fibres, suggesting nerve regeneration in comparison with females. Our results are in accordance with data presented by Vacca and colleagues, showing nerve regeneration was indeed accelerated in CCI males (Vacca et al., 2014). This data supports our behavioural data, showing neuropathic males recovered faster than neuropathic females from mechanical and thermal allodynia.

Further work should include a more precise assessment of demyelination in the sciatic nerve of neuropathic animals, including myelin staining, for example, through the luxol fast blue (LFB) method, that dyes myelin blue and an axon staining using the Palmgren's silver impregnation method.

Chapter 6. Conclusion

In the present thesis, using a behavioural, electrophysiological, and histopathological approach, we showed that, in the CCI model: (i) mechanical and cold allodynia is observed in both female and male animals, with the latter recovering faster; (ii) neuropathic animals do not display anxiety or depressive-like behaviours neither at earlier nor at later stages; (iii) the RVM does not appear to play a critical role in neuropathic pain modulation; and (iv) chronic constriction induces neuroinflammation and degradation of the myelin sheath of the sciatic nerve, more severe in females, with males showing amelioration of these changes eight weeks after induction.

In conclusion, our findings suggest sex differences in the development and maintenance of CCI-induced nociceptive and emotional impairments, which are partially supported by electrophysiological data. Importantly, our results further highlight the need to include female subjects in studies on neuropathic pain mechanisms and treatments, as their behaviour cannot be assumed to match that of their male counterparts.

Chapter 7. References

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