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Professora Filipa Pinto-Ribeiro
e da
Doutora Diana Amorim

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

RESUMO

Caracterização nociceptiva, emocional e eletrofisiológica do modelo experimental de dor neuropática por constrição crónica em fêmeas Wistar Han

A dor crónica neuropática afeta 7-10% da população e é frequentemente acompanhada por distúrbios emocionais, nomeadamente ansiedade e depressão. Esta patologia reduz significativamente a qualidade de vida dos pacientes, afetando as suas capacidades físicas, cognitivas, emocionais e sociais e, consequentemente, interferindo com o tratamento. No entanto, apesar de a dor crónica e dos distúrbios emocionais que lhe estão associados serem mais prevalentes em mulheres, poucos trabalhos são realizados em animais fêmea. Não obstante o modelo de lesão por constrição crónica (CCI) mimetizar neuropatias humanas em termos de hiperalgesia mecânica e térmica, alodinia e dor espontânea em ambos os sexos, são escassos os dados sobre distúrbios emocionais em ratos fêmea.

Neste trabalho, ratos jovens (Wistar Han) foram divididos em cinco grupos: machos com CCI (CCI_M), fêmeas gonadicamente intactas (SHAM/SHAM), fêmeas ovariectomizadas (SHAM/OVX), fêmeas gonadicamente intactas com CCI (CCI/SHAM) e fêmeas ovariectomizadas com CCI (CCI/OVX). A avaliação nocicetiva iniciou-se na semana anterior à cirurgia de CCI, perdurando 5 semanas, após as quais o comportamento anxiodepressivo foi também avaliado. No período pós-operatório, os animais com CCI apresentaram alterações na marcha, e o desenvolvimento de alodinia mecânica e térmica ao frio. Todas as fêmeas CCI desenvolveram alodinia mecânica e este comportamento instalou-se mais cedo nas CCI/OVX comparativamente às CCI/SHAM. A indução de CCI em fêmeas não levou ao desenvolvimento de comportamentos ansiosos e depressivos, embora a ovariectomia tenha induzido um comportamento tipo-anedónico, independentemente de CCI. As gravações eletrofisiológicas *single cell* na medula rostral ventromedial (RVM), área mediadora do processamento nociceptivo, sugerem um aumento da pronociceção descendente após neuropatia crónica. Estes resultados demonstram a existência de diferenças entre sexos na comorbidade entre a dor neuropática e distúrbios emocionais no modelo de CCI, modulada pelo controlo descendente da RVM.

Palavras-chave: ansiedade; depressão; dor neuropática; eletrofisiologia; histologia; lesão por constrição crónica; medula rostral ventromedial (RVM); nervo ciático.

ABSTRACT

Characterization of sex-specific nociceptive, emotional and electrophysiological impairments after the induction of the chronic constriction injury model of experimental neuropathic pain

Chronic neuropathic pain affects 7-10% of the population and is often accompanied by comorbid emotional impairments that adversely affect nociceptive symptomatology. This condition greatly reduces the quality of life of the patients, impairing physical, cognitive, emotional and social functioning which, consequently, interferes with treatment. Importantly, although chronic pain and emotional disorders are more prevalent in women, only a few publications focus on female animals. While the chronic constriction injury (CCI) model has been shown to mimic human neuropathies regarding mechanical/thermal hyperalgesia, allodynia and spontaneous pain in both sexes, data on CCI-induced emotional impairments on female rats remains scarce.

In this work, young adult rats (Wistar Han) were divided into five groups: males with CCI (CCI_M), gonadally intact females (SHAM/SHAM), ovariectomized females (SHAM/OVX), ovariectomized females with CCI (CCI/OVX) and gonadally intact females with CCI (CCI/SHAM). Nociceptive testing began before CCI surgery and was performed weekly throughout 5 weeks after which the anxiodepressive-like behaviour was also assessed. In the postoperative period, CCI animals displayed visible gait abnormalities, limping and guarding the affected hind paw, and the development of mechanical and cold allodynia from week 1 onwards. CCI developed mechanical allodynia, with CCI/OVX animals displaying symptoms prior to CCI/SHAM females. While no differences were found between CCI and SHAM animals concerning anxiety- and depressive-like behaviours, ovariectomy induced anhedonic-like behaviour, regardless of CCI surgery. Single cell electrophysiological data from the rostral ventromedial medulla (RVM), an area mediating nociceptive processing, suggest an increase in descending pronociception after chronic neuropathy. These results demonstrate the existence of sex differences on the comorbidity between neuropathic pain on the CCI model, modulated by RVM descending control.

Keywords: anxiety; chronic constriction injury; depression; electrophysiology; histology; neuropathic pain; rostral ventromedial medulla (RVM); sciatic nerve

TABLE OF CONTENTS

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
DRt – dorsal reticular nucleus
DRG – dorsal root ganglia
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
LPGi - nucleus paragigantocellularis lateralis
MB – marble burying test
NSFT - novelty-supressed feeding test
NS – nociceptive-specific neurons
OVX - ovariectomy
OF – open field test
PB – parabrachial area
PAG – periaqueductal grey
PEAP – place escape/avoidance test
PFC – prefrontal cortex
PNC – partial nociceptive convergent neurons
PWT – paw withdrawal threshold
RMg - nucleus raphe magnus
RVM – rostral ventromedial medulla
SDH – spinal dorsal horn
SEM – standard error of the mean
SNI – spared nerve ligation
SNL – spinal nerve ligation
SPT – sucrose preference test
TNC – total nociceptive convergent neurons
TST – tail suspension test
WDR – wide-dynamic range neurons

LIST OF FIGURES

LIST OF TABLES

Chapter 1. Introduction

1.1. Nociception

Pain is defined by the International Association for the Study of Pain (IASP, 2018) as *an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage.* It is thus a multidimensional experience, comprising sensorial, emotional-affective and cognitive components and whose treatment remains a challenge[1]. Pain is a fundamental physiological process that occurs continuously, protecting the organism from potential or actual injury and/or damage. In basic research, it is, however, important to distinguish between pain and nociception, as the latter refers only to the process by which noxious stimuli are encoded and processed[2].

1.2. Nociceptive transmission

Nociception is encoded and transmitted to the spinal cord by primary afferent neurons[3]. There are three major classes of primary afferents: Aβ, Aδ, and C-fibers[4], each with different properties, which allow them to respond to and transmit different types of information. Aβ-fibres are large myelinated fibres (4-8 μm diameter), fast conducting of innocuous mechanical stimuli (up to 70 $m.s^{-1}$)[5], and have low activation thresholds. Aδ-fibres are smaller thinly myelinated fibres (2-6 μm diameter), slower-conducting than Aβ-fibres (about 20 $m.s^{-1}$) and mediate the acute and well-localized "first pain"[6]. Lastly, C-fibres are thin unmyelinated fibres (0.4-1.2 μm diameter), being the slowest conducting fibres (less than 2 $m.s^{-1}$)[4,6,7] and are responsible for the "second pain"[6].

Since Aδ and C-fibres respond to noxious stimuli (mechanical, thermal or chemical) they are also designated as nociceptors[7]. The cell bodies of these fibres are located in the dorsal root ganglia (DRG) or the trigeminal ganglion and have a small axonal branch projecting to the spinal cord and a long branch extending to the periphery[8]. Primary afferents synapse in the dorsal horn of the spinal cord (SDH, Spinal Dorsal Horn) in an organized manner with most of Aδ and C-fibres terminating mainly in laminae I-II, and only a few reaching deeper laminae (**Figure 1**).

In the dorsal horn, there are two types of neurons, regarding their stimulus-response properties. In the superficial SDH (laminae I-II), we mainly find nociceptive-specific (NS) neurons, which only respond to painful stimuli and receive inputs from Aδ- and C-fibers[6]. The other type of neurons is the wide dynamic range (WDR) neurons, which can be found mainly in laminae III-VI. WDR neurons receive input from all types of peripheral fibres, responding to both innocuous and noxious stimuli[4].

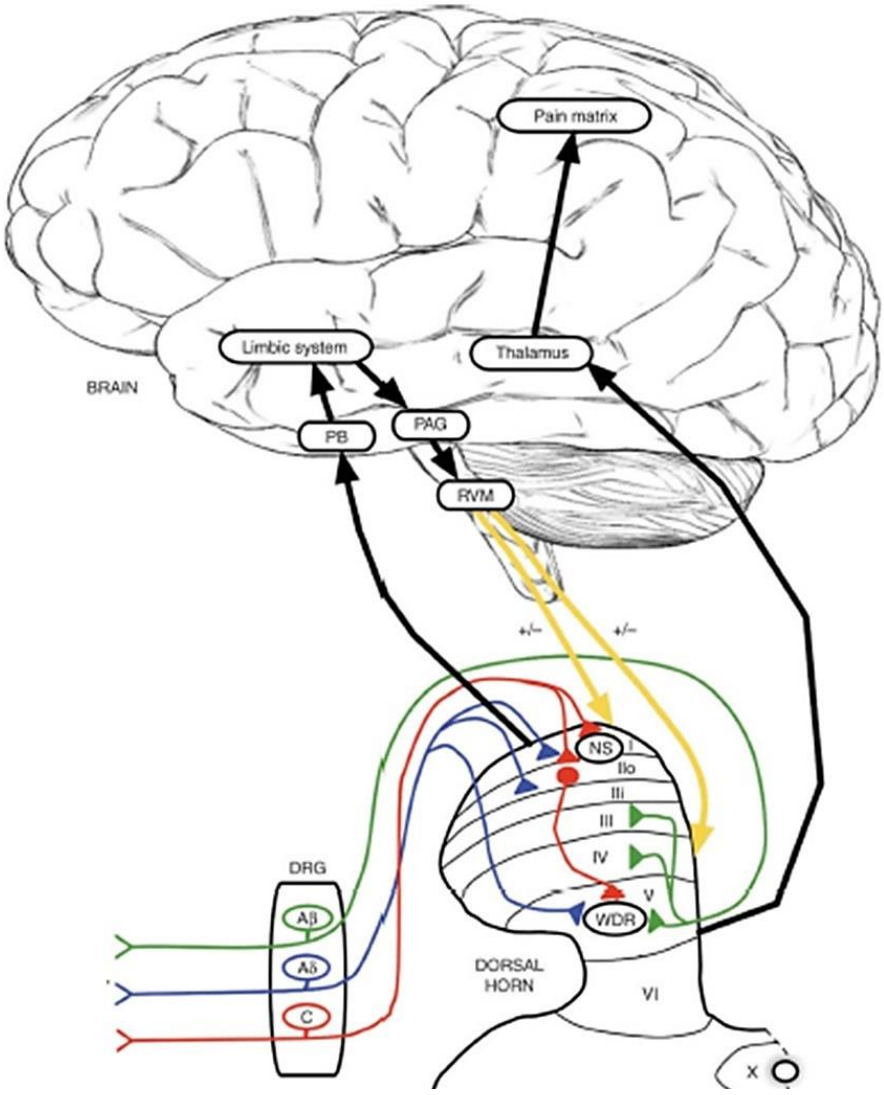

Figure 1. Summary of pain processing pathways. A peripheral noxious stimulus is signalled via specialized primary afferent fibres, called nociceptors. This nociceptive information is then conducted to the dorsal horn of the spinal cord and then transmitted via ascending nociceptive pathways, through areas such as the parabrachial area (PB), periaqueductal grey (PAG) and the thalamus. This information is processed according to the sensory-discriminative, motivational-affective and cognitive-evaluative dimensions of pain (perception). Descending pathways modulate nociceptive transmission, acting either by promoting or suppressing nociception. Limbic areas such as the limbic anterior cingulate and insular cortex, amygdala and the hypothalamus, project to the PAG, which then modulates descending pain transmission from the afferent pain system indirectly through the rostral ventromedial medulla (RVM) in the brainstem. Adapted[6].

1.3. Ascending pathways and supraspinal processing

According to their axonal projections, the SDH is comprised by two main types of neurons: (i) interneurons, with short axons, that communicate locally, and ii) projection neurons, with long axons, that communicate supraspinally, conveying nociceptive inputs for further processing[9]. Nociceptive inputs are conveyed through five major ascending pathways[10] (**Figure 2**).

The pathways responsible for the discriminative component of pain, signalling the location, intensity, and quality of the stimulus, target mainly somatosensory cortical areas. Those targeting additional cortical and brainstem pathways are related to the affective-motivational component of pain (the unpleasantness of the pain experience).

The main ascending pathways are:

(i) The spinothalamic tract transmits nociceptive information to the thalamus, which is then relayed to cortical structures, such as the somatosensory areas S1 and S2, the insular cortex, the anterior cingulate cortex, and the medial prefrontal cortex (mPFC)[11] (**Figure 2A**). Through this pathway, there is an integration of the sensory-discriminative (lateral spinothalamic tract) and motivational-affective (medial spinothalamic tract) components of the pain experience[12–15].

(ii) The spinoreticular tract projects to the brainstem reticular formation, terminating in the thalamus[13] (**Figure 2B**).

(iii) The spinomesencephalic tract targets brainstem nuclei such as the locus coeruleus and the periaqueductal grey (PAG), a major antinociceptive region[4] (**Figure 2C**). The PAG is in close interaction with limbic structures, such as the amygdala (AMY)[16–18] and the prefrontal cortex (PFC)[19].

(iv) In the cervicothalamic tract, the fibres ascend in the medial lemniscus and terminate in the ventral posterolateral nucleus of the thalamus.

(v) The spinohypothalamic tract projects mainly to the hypothalamus, but also to autonomic control centres in the thalamus and AMY[20].

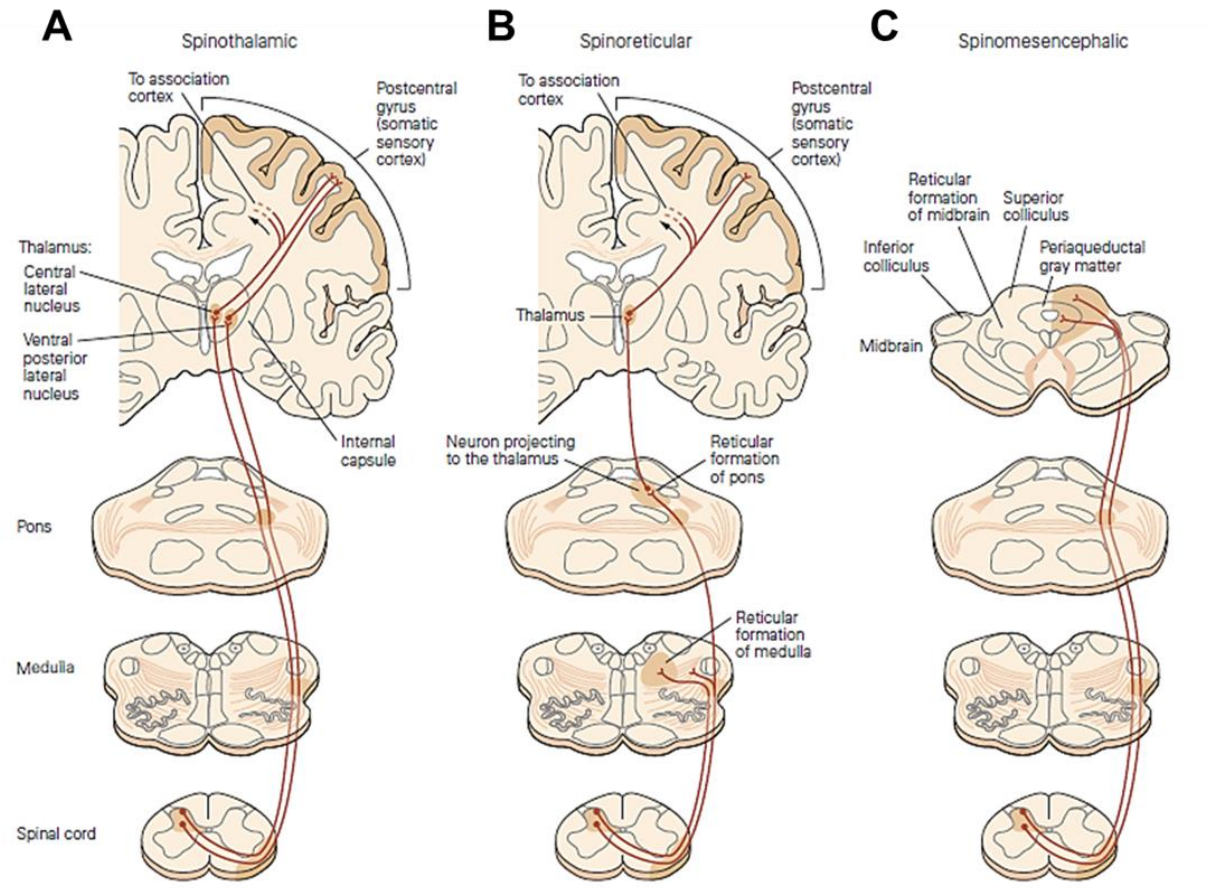

Figure 2. Schematic representation of three of the five main spinal ascending pathways, the (A) spinothalamic, (B) spinoreticular and (C) spinomesencephalic pathways[10].

1.4. Descending modulation

After supraspinal processing, nociception is modulated by descending pathways that can either facilitate (pronociception) or inhibit (antinociception) nociceptive transmission in the SDH[6,21]. Several supraspinal nuclei are recognized as involved in descending modulation, including the dorsal reticular nucleus (DRt)[22], the caudal ventrolateral medulla (CVLM)[23], the hypothalamus[24], the cortex[25] and the PAG-RVM complex[26].

The best-studied relay of descending modulation is the midline PAG-RVM complex[26], specific for noxious stimulation, which can both enhance and inhibit pain in different settings[27,28]. As mentioned previously, the PAG receives inputs from limbic forebrain areas as the anterior cingulate cortex, AMY, hypothalamus, and mPFC[29] and relays this information to the spinal cord, through the rostral ventromedial medulla (RVM)[30]. The RVM is a region of the ventral medulla that includes the midline nuclei raphe magnus (RMg), gigantocellularis reticularis (Gi), gigantocellularis pars alpha (GiA) and the paragigantocellularis lateralis

(LPGi). Projections from the RVM represent a major portion of the neurons projecting to the SDH. Electrophysiologically, the RVM is comprised of three different types of neurons, depending on their response to noxious stimulation, namely the pronociceptive On-, the antinociceptive Off- and the Neutral-cells[31].

When a noxious stimulus is applied, the activity of On-cells increases immediately before a withdrawal response while the opposite is observed for Off-cells. The third type of cells, Neutral-cells, do not alter their activity during peripheral noxious stimulation. Off-cells were first described to exert an antinociceptive action, as their activation is sufficient to produce analgesia[32–34], as well as required for the action of opioids[35,36]. On the opposite side, on-cells are considered pronociceptive, since their activation induces hyperalgesia[37,38]. The balance between the activation of on- and off-cell populations, therefore, allows a graded dual control of nociception[26].

Descending modulation occurs through three main processes: (i) a tonic effect, in which cutaneous sensory processing is inhibited, whereas pronociceptive activity is facilitated[39]; (ii) an inhibitory effect, wherein supraspinal structures inhibit the activity dorsal horn neurons, acting indirectly through interneurons[40–42] or directly by inhibiting primary afferents[43,44] and (iii) a facilitatory effect, where descending pathways enhance spinal nociceptive transmission from the SDH[45,46].

1.5. Chronic pain

Chronic pain results from an abnormal function of the nervous system, in which pain persists beyond healing time (more than 3-6 months)[47], and affects approximately 20% of the European population[48,49]. This abnormal neuronal activity includes the sensitization of the peripheral and the central nervous systems[50], leading to a heightened perception of pain[51]. According to its aetiology, chronic pain can be divided into 7 categories[47]: (i) primary pain, (ii) cancer pain, (iii) posttraumatic and postsurgical pain, (iv) neuropathic pain, (v) headache and orofacial pain, (vi) visceral pain and (vii) musculoskeletal pain (**Table 1**).

Table 1. Classification and definition of the most common clinically relevant chronic pain disorders, according to the 11th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD)[47].

Category	**Description**
Chronic primary pain	Pain in one or more anatomical regions characterized by significant emotional distress or functional disability.
Chronic cancer pain	Pain caused by the primary cancer itself or metastases, or its treatment.
Chronic post-traumatic and post-surgical pain	Pain that develops after a surgical procedure or a tissue injury and persists at least 3months after surgery or tissue trauma.

Chronic neuropathic pain	Pain caused by a lesion or disease of the somatosensory nervous system.
Chronic headache and orofacial pain	Headaches or orofacial pains that occur on at least 50% of the days during at least 3 months.
Chronic visceral pain	Persistent or recurrent pain that originates from the internal organs of the head and neck region and the thoracic, abdominal, and pelvic cavities.
Chronic musculoskeletal pain	Persistent or recurrent pain that arises as part of a disease process directly affecting bone, joint, muscle, or related soft tissues.

1.5.1. Neuropathic pain

As shown in **Table 1**, neuropathic pain is defined as *pain caused by a lesion or disease of the somatosensory system*[52] and is accompanied by the development of spontaneous pain, hyperalgesia (increased pain perception in response to a noxious stimulus) and allodynia (pain sensation in response to normally innocuous stimulus)[53]. Although the exact prevalence of chronic neuropathic pain is unknown, it is estimated that it affects 7-10% of the population[54,55]. Importantly, neuropathic pain greatly reduces the quality of life of patients, as it impairs physical, mental, emotional and social functioning[56]. Regarding treatment, many of the patients are refractory to the currently available therapies, namely nonsteroidal anti-inflammatory drugs, opioids, and antidepressants[57,58]. This leads to poorer quality of life for the patients[59], as well as higher use of healthcare resources and greater costs[60].

1.5.2. Emotional and cognitive impairments in neuropathic pain

Chronic pain goes beyond the sensory perception of pain, with sleep disturbances and emotional disorders, such as anxiety and depression, being frequently reported by patients[61]. Especially in neuropathic patients, psychiatric comorbidities have an estimated prevalence of 30%[62]. However, the mechanisms that underly the comorbidity between emotional disorders and neuropathic pain remain unclear.

Numerous studies showed negative emotions increase pain perception[63]. Pain-related anxiety can increase pain sensitivity[64–66] and worsen the subjective experience of pain[67], predisposing people to develop a chronic pain condition[68–70]. Pain modulation by anxiety results from interactions between areas involved in both emotional and sensory processing such as the activity of the thalamus[71,72], amygdala[72,73] and the PAG[73]. Regarding major depressive disorders, epidemiological studies show a prevalence of approximately 50% in chronic pain patients[74,75]. This comorbidity was associated with amplified pain perception[76], as well as

an increase in the activity of the amygdala, insula, and mPFC[77,78] – areas shown to be involved in pain modulation.

In addition to emotional impairments, cognition also affects the experience of pain[79]. Individuals with cognitive impairments display altered pain processing[80], however, pain sensitivity is variable according to the cause, which can be a consequence of ageing, neurological diseases or trauma[81]. Concerning attention, focusing on pain increases its perception decreasing the activity of areas including the thalamus and anterior cingulate cortex, and increasing the activity of others such as the PAG[82] – while a distraction can diminish it[83]. Also, a verbal cue suggesting pain relief or increase can also decrease/increase pain experience[84–86], importantly this expectancy was also shown to alter analgesic efficacy[87,88]. Fear of pain can cause persistent physical inactivity, leading to a negative spiral of worsened physical condition, frailty and distress, and subsequently more pain[59].

1.6. Animal models of neuropathic pain

The complexity of chronic pain, and especially of chronic neuropathic pain, has led to the development of a plethora of animal models, that can be categorized according to the location and method of injury (**Table 2**) into 5 gross categories[89,90]: (i) central pain models, (ii) peripheral nerve injury models, (iii) models of disease-induced, (iv) drug-induced, and (v) inherited neuropathies. In rodents, the most common experimental approach is traumatic nerve injury (full or partial) via ligation, transection, or compression with the chronic sciatic constriction injury (CCI)[91], spared nerve injury (SNI)[92] and spinal nerve ligation (SNL)[93] models being mostly used.

Table 2. Animal models of neuropathic pain.

Category	Animal models
Central pain models	Thalamic syndrome[94] Spinal cord injury: contusion[95], excitotoxic[96] and photochemical[97] Spinal hemisection[98,99]
Peripheral nerve injury models	Neuroma model[100,101] Chronic constriction injury (CCI)[91] Partial sciatic nerve injury[92] Spinal nerve ligation (SNL)[93,102,103] Spared nerve injury (SNI)[92,104,105] Brachial plexus avulsion[106,107] Photochemically-induced sciatic nerve injury[108,109] Polyethylene cuff[110,111] Partial injury of the nerve supplying the tail[112]

	Partial saphenous nerve injury[113,114] Trigeminal neuralgia[115,116] Sciatic cryoneurolysis[117,118] Caudal trunk resection[119,120] Sciatic inflammatory neuritis (SIN)[121,122] Laser-induced sciatic nerve injury[123]
Models of disease-induced neuropathic pain	Multiple sclerosis (EAE[124,125] and TMEV[126]) Postherpetic peripheral neuropathic pain model[127,128] HIV-associated sensory neuropathy[129–131] Peripheral diabetic neuropathy (PDN)[132–134] Cancer pain models[135,136]
Models of drug-induced neuropathic pain	Vincristine-induced peripheral neuropathy model[137,138] Paclitaxel-induced peripheral neuropathy model[139,140] Docetaxel-induced peripheral neuropathy[139] Cisplatin-induced peripheral neuropathy[141,142] Oxaliplatin-induced neuropathy[143–145]
Inherited neuropathies	Spontaneous inherited neuropathy[146,147] Engineered inherited neuropathies[148,149]
Others	Chronic ethanol intake/withdrawal-induced neuropathy[150,151] Pyridoxine (vitamin B6)-induced neuropathy[152,153] Orofacial pain model[154,155] Acrylamide-induced neuropathy model[156,157]

1.6.1. Chronic constriction injury model (CCI)

The CCI model mimics the symptoms of chronic nerve compression, such as nerve entrapment or compression syndromes[158,159], comprising both the inflammatory[160] and neuropathic components[161]. This model, established in 1988 by Bennet and Xie[91], results in hyperalgesia to thermal and mechanical stimuli with animals also displaying signs of spontaneous pain such as limping, limb guarding, excessive licking and avoidance of weight bearing on the injured limb[91,161,162]. It is a reliable and reproducible model, that leads to the formation of intraneural oedema, axotomizing many but not all of the sciatic nerve axons[163].

1.7. Emotional and cognitive impairments in the CCI model of neuropathic pain

In addition to the study of changes in nociception, animal models have also been used to study the anxiodepressive and cognitive components. Available behavioural evidence on the CCI model is summarized in **Tables 3-4.** Taking together, these studies show the development of anxiety- and depressive-like behaviours in the CCI model of neuropathic pain. Disregarding the general inference, results vary between studies (even between those using the same behavioural tests) concerning the time post-CCI at which they are observed and differences between tests performed on the same animals. This

variability is mostly attributable to the use of different methodological approaches, namely the animal's species and strains, the behavioural paradigms and experimental designs used, which difficult the comparison between studies. Likewise, it becomes imperative the use of female animals as experimental subjects, as these studies mainly focus on male subjects and these comorbidities develop in a sex-specific manner, with special impact in women.

1.7.1. Anxiety-like behaviour

Evaluation of anxiety-like behaviour (**Table 3**) used mostly exploratory-based tests, which play on the inner struggle of the animals between approaching or avoiding novel situations and/or stimuli[164].

In the open field (OF) test, animals are placed in a square arena with an anxiogenic illuminated centre. Overall, CCI animals displayed a reduction in the time spent in the centre of the OF arena and in the number of entries in the central area, an indicator of anxiety-like behaviour[165–169]. Some exceptions[169–171] saw no differences in time spent in the centre of the arena of the OF. Identical results were obtained in the elevated plus maze (EPM), consisting of two opposing open and brightly lit arms and two opposing closed arms, in a "plus sign" shape, elevated above floor level. Anxiety is derived from a reduction in the number of entries and/or the time spent in the open arms, which was observed in the majority of the studies[168,172–177]. In some exceptions[168,171] no differences were found between control and CCI animals. One modified version of the EPM is the elevated zero maze[178], which excludes the ambiguous central area in the traditional design[179]. Again, results were similar with CCI animals spending less time in the open quadrants, with one exception[180] which showed no differences between CCI and SHAM animals. Only one article used the light/dark box test (LDB)[181], which uses a similar exploration/avoidance conflict as the OF, by allowing the animals to freely move between a dark and a bright chamber. A decrease in time spent in the lit compartment is an indicator of an anxiety state and, accordingly, CCI spent time in the bright compartment[182].

The marble burying test (MB)[183] is based on the inherent burying behaviour of rodents on anxiogenic circumstances[183], and an increase in the numbers of marbles buried is then considered as an indicator of anxious behaviour. In the two studies that used this paradigm, anxiety-like behaviour was not observed: Wilkerson et al.[184] showed a decreased in the number of marbles buried by CCI animals, whereas Bravo and colleagues[180] showed no differences.

The place escape/avoidance paradigm (PEAP) was used to evaluate an aversion to painful experiences[185]: the animals are allowed to explore between a dark non-anxiogenic chamber, in which the injured paw

was stimulated with a noxious stimulus, and a mildly bright anxiogenic side in which there is no stimulation or of the injured paw. In all studies, CCI animals spent less time in the dark chamber, showing an aversion to painful stimuli.

1.7.2. Depressive-like behaviour

For the study of depressive-like behaviours (**Table 4**), the forced swimming (FST), tail suspension (TST), and sucrose preference (SPT) tests are the most frequently used. The FST and TST (applied only in mice) evaluate the learned helplessness component of depression. In the FST[186], the animals are placed in cylinders filled with water, in a no-escape situation. Similarly, in the TST[187] mice are suspended by the tail and similar struggling/immobility behaviours are assessed. In both tests, an increase in immobility behaviour is an indicator of behavioural despair. CCI animals displayed an increased immobility time but no differences in the time spent swimming, thus indicating the increase in immobility occurs at the expense of the decrease in the time spent climbing. The distinction between active behaviours (swimming/climbing) is important, as it is known to be differentially due to serotonergic and noradrenergic mechanisms, respectively[188,189]. Two exceptions were found[190,191], in which no differences were seen in immobility, swimming and climbing time in the FST. Similarly, in the TST, an increase in immobility was also observed.

In the sucrose preference test (SPT), the animals are allowed to choose between a sweet (sucrose) and water bottle[192]. A decrease in the intake of sucrose indicates the development of anhedonic-like behaviour. This was observed on CCI animals[165,193–196], with one exception, where saccharin was chosen and no differences were found between sham and CCI animals on its preference[168].

In the novelty-suppressed feeding test (NFST), food-deprived animals are put in a chamber with a single pellet of food and the latency to approach it is recorded[197]. This induces a conflict between the drive to eat and the fear of a novel environment. A hesitation to eat in a novel environment is described as intermix measure of anxiety and depression behavioural symptoms, observed in CCI animals[182,193].

Table 3. Summary of the literature concerning the development of anxiety-like behaviours in the chronic constriction injury model of neuropathic pain. Abbreviatures: OVX - ovariectomized; M – male; F – female; L – left; R – right; ALB - anxiety-like behaviour; OF - open field test; EPM - elevated plus-maze; EZM - elevated zero-maze; LDB - light/dark box; MB - marble burying test; PEAT - place escape/avoidance test; SI - Social interaction; N/D – unknown.

Paradigm	Species; strain	Gender	Side	Results	Ref.
OF, EPM	Rat; Wistar	M	R	No alterations observed	171
EZM, PEAP	Rat; Sprague-Dawley	M	N/D	ALB at weeks 4 and 6	198
OF	Mice; C57BL/6J	M	R	No alterations observed	199
MB	Mice; C57BL/6J	M	L	ALB at days 3 and 6	184
PEAP, EZM	Rat; Sprague-Dawley	M	L	ALB at day 30; increased place escape/avoidance	200
EPM	Mice; C57BL/6J	M	N/D	ALB at day 28	173
OF	Rat; Sprague-Dawley	M	N/D	ALB at day 14	165
EPM	Rat; Wistar	M	R	ALB at day 21	172
EPM	Rat; Wistar	M	L	ALB at days 21 and 28	174
OF, EZM	Rat; Sprague-Dawley	M	L	ALB at week 4	166
LDB	Mouse; C57BL/6J	M	R	ALB at weeks 2 to 6	182
OF, EPM	Mouse; C57BL/6J	OVX/F	R	ALB at day 26	175
EPM	Rat; Wistar	M	L	ALB at post-surgery day 25	167
EPM	Rat; Sprague-Dawley	M	R	ALB at days 7 and 21	201
OF	Mouse; C57BL/6J	M	R	No alterations observed	170
EZM, MB, PEAP	Rat; Sprague-Dawley	M	L	No alterations observed in ALB; increased place escape/avoidance at week 5	180
EZM, PEAP	Rat; Sprague-Dawley	M	L	ALB at day 28; increased place escape/avoidance at day 7	202
PEAP	Rat; Sprague-Dawley	M	L	Increased place escape/avoidance at weeks 1 and 2	191

OF, EPM	Rat; Sprague-Dawley	M	R	ALB only in OF at days 14 to 21	168
OF, EZM, MB	Mice; C57BL/6J and Balb/c	M	N/D	ALB at week 4 (only on C57BL/6J, EZM test)	169
EPM	Rat; Wistar	M	N/D	Anxiety-like behaviour both in HAB and LAB animals at day 36	176
EPM	Rat; Wistar	M	L	ALB post-surgery day 21 to 28	177

Table 4. Summary of the literature concerning the development of depressive-like behaviours in the chronic constriction injury model of neuropathic pain. Abbreviatures: M – male; F – female; L – left; R – right; DLB - Depressive-like behaviour; FST - forced swimming test; TST - tail suspension test; SPT - sucrose preference test; NSFT - novelty suppressed feeding test

Paradigm	**Species; strain**	**Gender**	**Side**	**Results**	**Ref.**
TST	Mouse; C57BL/6J	M	R	DLB at day 28	203
TST, FST	Mouse; C57BL/6J	M	R	DLB at week 8	204
FST	Rat; Sprague-Dawley	M	R	DLB at day 14	205
FST	Rat; Wistar	M	R	DLB at day 14	171
FST	Rat; Sprague-Dawley	M	N/D	DLB at week 6	198
TST	Mice; C57BL/6J	M	N/D	DLB at day 28	173
FST, NFST	Rat; Wistar	M	L	DLB at days 15 and 29	206
FST, TST	Mouse; ICR	M	R	DLB at days 7, 14, 21, 28 and 34	207
FST, SPT	Rat; Wistar	M	R	DLB at day 28	208
FST	Rat; Sprague-Dawley	M	L	DLB at days 7, 14 and 21	209
FST	Rat; Sprague-Dawley	M	L	DLB at day 14	193
FST, NFST	Mouse; C57BL/6J	M	R	DLB from week 2 to 6	182
FST	Rat; Wistar	M	L	DLB at day 32	167

FST	Mouse; C57BL/6J	M	R	DLB from week 2 to 6	210
FST	Rat; Sprague-Dawley	M	L	No alterations observed	190
FST	Rat; Sprague-Dawley	M	L	DLB at day 14	211
FST	Rat; Sprague-Dawley	M	R	DLB at days 7 and 21	201
FST	Rat; Sprague-Dawley	M	R	DLB at day 21	212
FST	Rat; Sprague-Dawley	M	L	DLB at day 21	194
SPT	Mouse; C57BL/6J	M	R	DLB from week 4 to 10	170
FST, TST	Mouse; ICR	M	R	DLB from week 2 to 6	213
FST	Rat; Sprague-Dawley	M	L	DLB at day 28	202
Anhedonia, FST	Rat; Sprague-Dawley	M	L	No alterations observed	191
FST	Rat; Sprague-Dawley	M	L	DLB at day 14	195
SPT	Rat; Sprague-Dawley	M	R	No alterations observed	168
FST	Mouse; Swiss	M	L	DLB at day 14	214
FST	Rat; Wistar	M	L	DLB from day 21 to 28	196
SPT	Rat; Wistar	M	R	DLB from day 9 to 16	215

1.8. Sex differences in pain perception and emotion

For the purpose of this thesis, the term sex will be used as it refers to biological sex, as gender is an identified sex that can be based on social or cultural values. Men and women do not experience pain in an equal manner. In chronic pain, women represent the majority of chronic pain patients, especially in chronic neuropathic pain[55,216], and have stronger and longer lasting pain symptoms[217]. Differences have been also reported in the response to treatment, mainly concerning opioid analgesia, with women having a greater antinociceptive effect[218,219]. In parallel, the prevalence of anxiety disorders and depression is also higher in women than in men and increases in the peri- and post-menopause period[60,220]. Importantly, the severity of emotional disorders is also enhanced in women[220].

In preclinical research, even though males are usually the subject of choice, sex must be taken into consideration, as previous studies showed neuropathic pain behaviours differ between males and females[221–223]. In the CCI model of neuropathic pain, sex-related differences were observed in terms of nociception, with female rats experiencing higher thermal hyperalgesic symptoms as well as mechanical allodynia in comparison with males[221,224,225]. Also, in mice, differences were observed in the development and recovery of neuropathic pain, as female mice showed higher mechanical allodynia in the first weeks post-CCI and males recovering faster from nerve injury[224].

Chapter 2. Objectives

Even though chronic pain and affective disorders are more prevalent in women, there is a male-orientated bias in the choice of the experimental subject in basic research[226] and female behaviour is presumed to be comparable to male behaviour. Consequently, a systematic review of the literature highlighted that the CCI model was never extensively characterized on female rats, especially regarding the development of mood disorders concomitant to neuropathic pain. The main goal of this work is, therefore, to characterize CCI-induced nociception and emotional impairments in female rats, comparing it with males with CCI. We will further analyse sex-specific changes in the neuronal activity of the RVM, an area involved in nociceptive and emotional processing. Taking this into consideration, this work was divided into three main goals:

(i) Characterization of CCI-induced nociceptive behaviour, in what concerns the development of mechanical and cold allodynia.

(ii) Characterization of CCI-induced emotional impairments, namely the development of anxiety- and depressive-like behaviours.

(iii) Assessment of changes in neuronal activity in the RVM, area implicated in nociceptive processing, through the analysis of single cell extracellular electrophysiology.

3.1. Animals and ethical issues

Adult Wistar Han rats (Charles Rivers, Barcelona, Spain), weighing between 200-350 g were used in all experiments, in a total of 38 animals. The animals were housed in groups of 2 in clear plastic cages with solid floors, covered with sawdust as bedding. Food and water were available *ad libitium* and animals were kept under a 12h light/dark cycle (artificially illuminated from 8h-20h), in a climate-controlled room (temperature: 20-24°C; relative humidity: 55 +/- 10%). All manipulations were carried out during the light period of animals, between 9:00 a.m. and 5:00 p.m. Each animal was considered a single unit within its experimental group. The experimental protocol followed the European Community Council Directive 86/609/ EEC and 2010/63/EU concerning the use of animals for scientific purposes and was approved by the Institutional Ethical Commission (SECVS 16/2018). This work followed the ethical guidelines of the International Association for the Study of Pain regarding the use of laboratory animals and all efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

3.2. Experimental design

Randomization for experimental group allocation was performed using a computer-generated randomization sequence (RANDOM.ORG). The animals were distributed into five groups: males with CCI (CCI_M, n=7), gonadally intact females (SHAM/SHAM, n=8), ovariectomized females (SHAM/OVX, n=7), gonadally intact females with CCI (CCI/SHAM, n=8) and ovariectomized females with CCI (CCI/OVX, n=8). The experimental timeline is illustrated in **Figure 3**. Before starting the experiments, the animals were handled daily for a minimum of 2 weeks to habituate to the experimenter. Ovariectomies were performed 3 weeks before CCI surgery. The week before CCI, a baseline evaluation of nociceptive behaviour was performed using the von Frey and acetone tests. After CCI surgery, nociception was evaluated weekly, until the end of the behavioural characterization. On week 5 post-CCI surgery, the animals were tested in the OF, EPM, SPT and FST tests. Histological and electrophysiological studies started at the end of the behavioural characterization. These experiments were performed through three independent sets to adjust the time period post-CCI surgery required for the electrophysiological recordings.

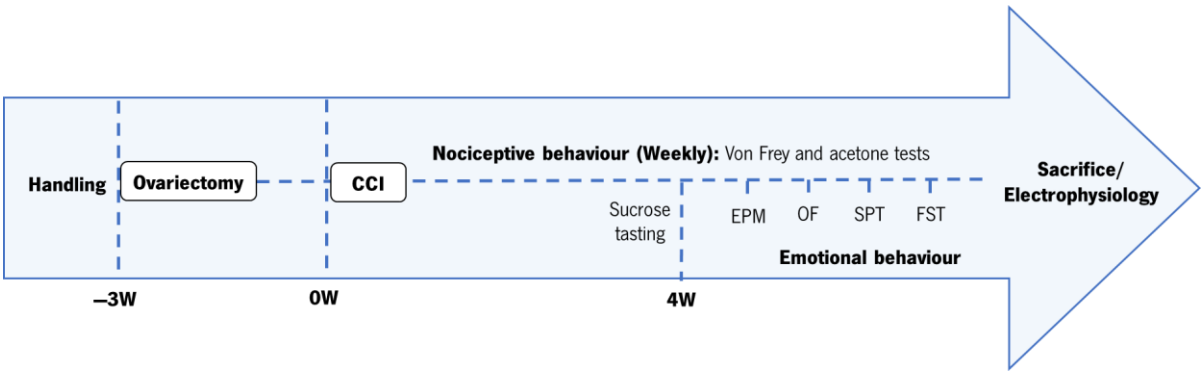

Figure 3. Schematic representation of the experimental design. Before the beginning of the experiment, all animals were handled daily by the experimenter for two weeks and, on the day of the experimental sessions, animals were left in the experimental room for an hour in order to habituate to the surroundings. After habituation, female animals underwent ovariectomy surgery and, three weeks later, chronic constriction injury induction or sham surgery, accordingly to the correspondent group. Identically, male animals underwent chronic constriction injury induction. Nociceptive behaviour was evaluated weekly, starting one week before induction. After four weeks, emotional-like behaviours were assessed, namely anxiety- and depressive-like behaviour. Following behavioural evaluations, the animals underwent electrophysiological recordings or were sacrificed, and samples were processed for histology. (CCI – chronic constriction injury; OF – open field test; EPM – elevated plus-maze; SPT – sucrose preference test; FST – forced swimming test; W - week).

3.3. Anaesthesia protocol

For both CCI and ovariectomy surgeries, the animals were anaesthetized intraperitoneally (i.p.) with a mixture of ketamine (0.75 mg/kg, Imalgene, Merial, Oeiras, Portugal) and medetomidine (0.5 mg/kg Dorbene, Esteve, Carnaxide, Portugal). At the end of the surgery, the anaesthesia was reversed with atipamezole hydrochloride (1 mg/kg, Antisedan, Pfizer, Oeiras, Portugal, i.p.). Vaseline was applied to both eyes to protect them from drying during surgery and recovery. After both surgeries, the animals were monitored until fully awake (eating and grooming) and recovered, and welfare was monitored daily (grooming, wound appearance, dehydration/weight loss and locomotor abnormalities).

During electrophysiological recordings, anaesthesia was induced (50 mg/kg, Eutasil, Ceva, Algés, Portugal) and maintained through intraperitoneal injection of pentobarbitone. The level of anaesthesia was monitored by verifying the dilation of the pupils, the general muscle tone and responses to noxious pinching every 15 min. An additional dose of pentobarbitone (20 mg/kg) was given if observed a pinch-evoked response.

3.4. Ovariectomy

Females were ovariectomized (OVX) bilaterally[227]. After a single mid-dorsal skin incision at the level of L2-L6, the cutaneous maximus and external oblique, internal oblique and transversus abdominis muscles

were transected, 1 to 2 cm in length, about 1 cm caudal to the last rib. Each ovary was exteriorized through the incision and the oviducts and ovarian vessels were ligated using 4-0 silk sutures (Mersilk, Ethicon, New Jersey, EUA) and the entire ovary removed. Surgical incisions were closed in layers and the anaesthesia reverted. The same procedure was carried out for the SHAM animals except for the removal of the ovaries.

3.5. Induction of neuropathic pain

The chronic constriction injury (CCI) model was induced as originally described[91]. Briefly, the common left sciatic nerve was exposed by blunt dissection of the biceps femoris. Proximal to the sciatic nerve's trifurcation, four 3/0 chromic gut ligatures (B. Braun Surgical, Rubi, Spain) were tied loosely with approximately 1 mm spacing, placed as to not arrest circulation. Sham animals received all the surgical procedures, but the sciatic nerve was left without ligation. Afterwards, surgical incisions were closed in layers and the anaesthesia reverted.

3.6. Behavioural assessment of nociception

3.6.1. Mechanical allodynia

The von Frey test was used for mechanical allodynia assessment, using a series of calibrated von Frey monofilaments (North Coast Medical Inc, Morgan Hill, CA). The animals were placed in transparent plastic containers on an elevated mesh floor and allowed to adjust to the environment. Von Frey filaments were applied to the plantar surface of each hindpaw in a series of ascending forces (equivalent to 0.4, 0.6, 1, 2, 4, 6, 8 and 15 g force), initiating with the 2 g filament. Positive responses included prolonged paw withdrawal, followed by licking or scratching. The 50% response threshold was calculated using the up-down method[228].

3.6.2. Cold allodynia

Cold allodynia was evaluated through the acetone test. In the same apparatus as the von Frey test, a 100 µL drop of acetone was applied with a pipette in the centre of the ventral surface of each hindpaw. The response was graded in a three-point scale: 0, no response; 1, quick withdrawal, flick or stamp of the paw; 2, prolonged withdrawal or repeated flicking of the paw; 3, repeated flicking of the paw with persistent licking directed at the ventral side of the paw. Acetone was applied alternately 3 times, at 1 min intervals, and the mean score value was calculated.

3.7. Behavioural assessment of emotional behaviour

3.7.1. Anxiety-like behaviour

3.7.1.1. Open field test (OF)

The OF test was used to evaluate anxiety-like behaviour, following a previously described protocol[229]. Briefly, the test is performed in a square arena (Med Associates Inc., St. Albans, Vermont, USA), 43.2 cm wide, with transparent acrylic walls and brightly illuminated in the centre (240 lx). The animal is placed in the centre of the arena (corresponding to a square 21.6 cm wide, equidistant from the borders) and its activity is automatically recorded for 5 min using the Activity Monitor 5 software (Med Associates Inc., St. Albans, Vermont, USA). The locomotor capability was assessed using the total distance travelled by the animals as well as average velocity, and anxiety-like behaviour through the percentage of time spent in the centre of the arena. Between each trial/animal, the arena was cleaned thoroughly with 10% alcohol.

3.7.1.2. Elevated plus maze (EPM)

Anxiety-like behaviour was also evaluated through the EPM[192]. The apparatus is composed of two open arms (50.8 cm × 10.2 cm) and two opposing closed arms (50.8 cm x 10.2 cm x 40.6 cm), elevated 72.4 cm above the ground (ENV-560; Med Associates, St. Albans, Vermont, USA). The animals were placed in the centre of the maze and allowed to explore for 5 min. Behaviour was recorded and then analysed using the EthoVision XT 13 software (Noldus Information Technology, Wageningen, Netherlands). Anxiety-like behaviour was assessed based on the time spent in the open arms, expressed as a percentage of the total testing time. The total number of arm entries was also evaluated. The maze was cleaned with 10% alcohol between trials/animals.

3.7.2. Depressive-like behaviour

3.7.2.1. Anhedonic-like behaviour

To evaluate anhedonic-like behaviour, the sucrose preference test (SPT) was performed[192], with minor modifications. One week before surgery, the animals were pre-exposed to the sucrose solution. During their active period, the animals were given free access to both water and a sucrose solution (2%) for 2h, and efforts were made to ensure that all animals tasted the solution. On test day, the animals were presented with two pre-weighted bottles (water and sucrose) for 3h. Afterwards, the bottles were weighted, and the sucrose preference calculated as follows:

$$\text{Sucrose Preference (\%)} = \frac{\text{sucrose intake}}{\text{(sucrose + water) intake}} \times 100$$

To control for potential bias associated with differences in body weight between experimental groups, sucrose preference was additionally adjusted to the animal's body weight. The total intake of sucrose and water was also assessed and likewise adjusted to the animal's body weight on testing day.

3.7.2.2. Learned helplessness

Learned helplessness was evaluated using the Forced Swimming Test (FST)[192]. The day before testing, the animals were submitted to a pre-test session, in which they were placed individually in a cylinder (diameter: 29 cm; height: 50 cm) filled with water (approximately 25°C, 30 cm in depth). After 10 min, the rats were removed from the water and towel-dried before returning to their home cages. On testing day, animals were again placed in the cylinders, for a period of 5 min, and the session recorded. Using the freeware Etholog software[230], latency to immobility, time spent immobile, swimming and climbing were quantified. Learned helplessness behaviour was defined by an increase in the time of immobility at the expense of the time spent swimming/climbing.

3.8. Electrophysiological recordings in the RVM

For the electrophysiological study, the animals were removed from the animal house randomly, one each day, and brought to the recording room. The depth of anaesthesia was assessed using the tail-pinch reflex, and recordings started when the animal was under light anaesthesia, neurones responded to noxious stimulation but no behavioural response to noxious pinching of the tail was observed. Body temperature was maintained with the help of a warming blanket (DC Temperature Controller, FHC, Bowdoin, ME, USA).

For supraspinal recordings in the RVM, the animals were initially placed in a standard stereotaxic apparatus. The skull was then exposed and an osteotomy was performed, to allow the placement of the recording electrodes according to the atlas of Paxinos and Watson[231] (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). Single neuron activity was recorded extracellularly with tungsten electrodes (tip impedance 3–10 MV at 1 kHz), the signal was amplified and filtered, and data sampling was performed through a CED Micro 1401 interface and Spike 2 software (Cambridge Electronic Design, Cambridge, UK).

The response of RVM cells to peripheral stimulation (applied to the left hindpaw) was evaluated through the following assessments, performed successively: (i) spontaneous activity; (ii) response to brushing; (iii) response to mechanical stimulation using von Frey hair, applied once (6 gf). (iv) response to pinching of the tail with a surgical clamp; (v) response to cold stimulation of the plantar skin. After finishing a recording, the animals were allowed a resting period of about 30 min starting a new recording.

To calculate the evoked response (Δ Activity) of the recorded neurons, the baseline discharge frequency was subtracted from the evoked activity using the following equation:

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

Then, RVM neurons were classified as WDR-like or NS-like according to their response to innocuous mechanical stimulation (brushing), being responsive or non-responsive, respectively. Neurons were then classified for each stimulus as (i) On-like if their activity increased during stimulation, (ii) Off-like cells if their activity decreased during the stimulation period and (iii) Neutral if none or negligible changes (<10%) were observed. This classification varied from that of Fields and colleagues[31], as the noxious-induced withdrawal reflex was not taken into consideration and only changes in neuronal activity were considered. Therefore, neurons are herein referred to as On-like and Off-like cells rather than On- or Off- cells[232,233] (**Figure 4**).

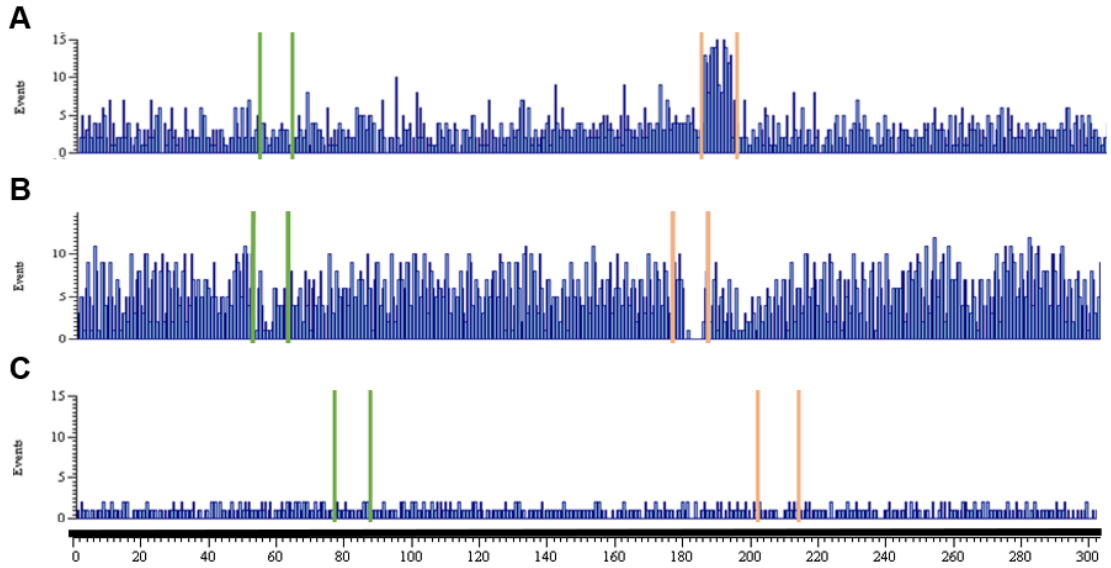

Figure 4. Example of an output from the recording software Spike 2. (A) Example of a recording of an NS On-like neuron activity. While not responding to innocuous stimulation (green interval), the cell increases its firing rate during the noxious stimulus (orange interval). (B) Sample recording of a WDR Off-like neuron. The activity of this cell is decreased not only by an innocuous stimulus (green interval) but also by noxious stimulation (orange interval). (C) Example of a recording from a neutral cell. This cell does not respond to either innocuous or noxious stimulus (Green lines – innocuous mechanical stimulation; Red lines – noxious mechanical stimulation to the tail).

After completing a recording session of approximately 4-6 hours, the animals were given a lethal dose of pentobarbitone (80 mg/kg, i.p.; Eutasil, CEVA) and were perfused with 4% paraformaldehyde (PFA).

3.9. Histology

3.9.1. Vaginal Smears

Female animals were monitored for their oestrous cycle using vaginal smears collected during habituation and on each behavioural day, after testing. In brief, for sample collection, a drop of saline is placed in a pre-labelled glass slide. A smear loop is moistened in saline and the female rat is held around the thorax, lightly gripping the tail with the same hand. The tip of the loop is then inserted carefully, with a small rotation movement, and rolled gently in the glass slide. Lastly, the animal is allowed to calm down and rest in its home cage. The samples were air-dried and fixed with 95% ethanol, followed by a Papanicolaou stain. Slides were evaluated using a brightfield microscope (Olympus Widefield Upright Microscope BX61) at the original objective magnifications of 4X, 10X and 20X (**Figure 5**).

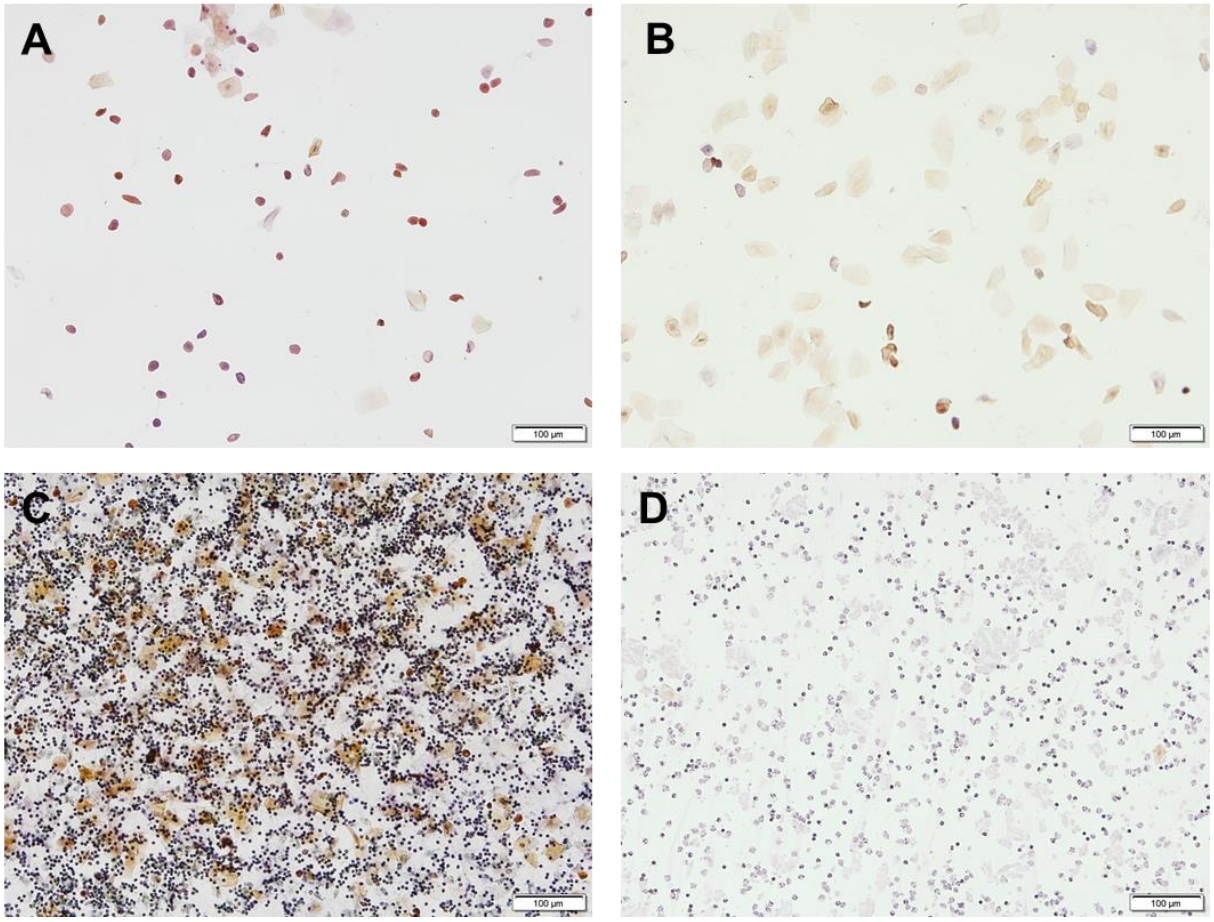

Figure 5. Sample images of the vaginal smears analysed. Represented are the **(A)** proestrus, **(B)** oestrus, **(C)** metestrus and **(D)** dioestrus phases of the oestrous cycle in female Wistar Han rats, stained with the Papanicolaou stain. Original magnification of 10X (scale: 100 µm).

The oestrous cycle was classified between 4 stages: proestrus, oestrus, metestrus and dioestrus based on the presence or absence of nucleated epithelial cells, cornified epithelial cells and leukocytes[234]. In the proestrus stage, there is a predominance of nucleated epithelial cells, while oestrous is distinctively characterized by cornified squamous epithelial cells. In metestrus, there is a mix of leucocytes and cornified squamous epithelial cells, whereas the dioestrus stage consists predominantly of leukocytes.

3.9.2. H&E staining of the sciatic nerve

Sections of the sciatic nerve were embedded in a paraffin block and sectioned into 5 μm-thick slices with a microtome. After staining with haematoxylin and eosin (H&E), slides were examined using a light microscope (Olympus Widefield Upright Microscope BX61) regarding abundance, size, and arrangement of nerve fibres.

3.10. Statistical analysis

The GraphPad Prism 6 software (GraphPad Software Inc, La Jolla, CA, USA) was used to perform the statistical analysis and graphical representations. Body weight changes and nociceptive data were analysed using a two-way repeated measures analysis of variance (ANOVA), comparing all the experimental groups. Data analysis of emotional, electrophysiological and histological data was performed using a two-way ANOVA followed by a Bonferroni test for post-hoc multiple comparisons comparing the four female groups, with ovariectomy and CCI as variables. A t-test for unpaired data with Welch's correction was used to compare CCI males with CCI females (CCI/SHAM and CCI/OVX females). Results were expressed as the mean ± standard error of the mean (SEM). In all cases, $P<0.05$ was considered statistically significant.

4.1. Animal Welfare

CCI animals walked with a definite limp, often guarding the affected hindpaw. While usually the heel is elevated, CCI animals stood with the hindpaw everted and the heel touching the floor. The toes, which are normally spread apart, were together and ventroflexed while walking or standing. Occasionally, autotomy was observed, with gnawed claw tips. These behaviours were not observed SHAM/SHAM and SHAM/OVX animals.

Weight change after CCI surgery is represented in **Figure 6**. This parameter was considered a measure of the general welfare of the animals, and no precipitous weight loss or dehydration was observed. This value was calculated in relation to the animals' body weight the week before surgery (baseline).

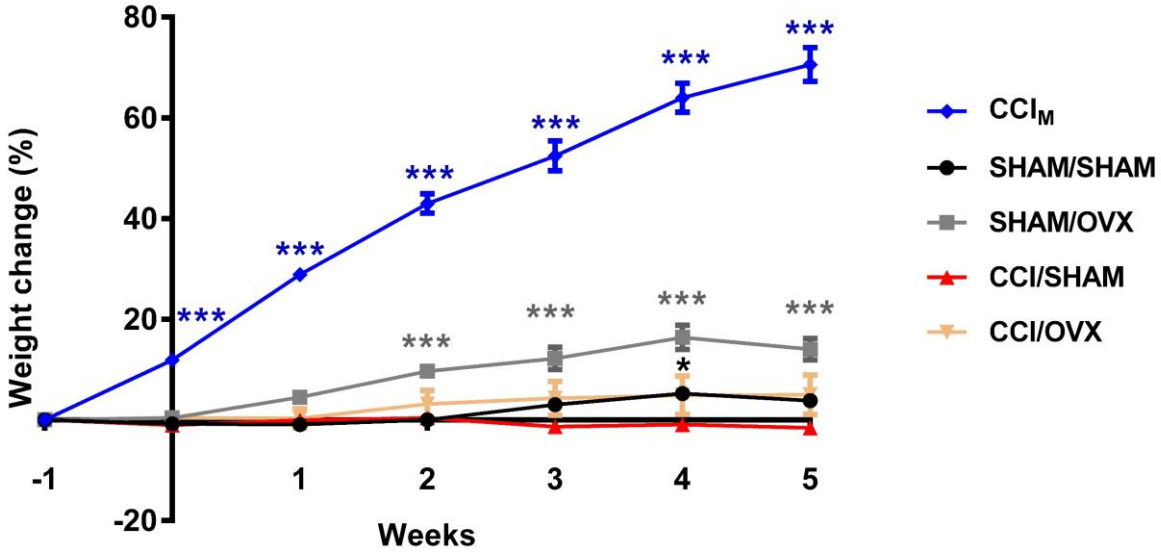

Figure 6. Variation of body weight throughout the experiments, starting one week before CCI surgery. CCI males, as well as SHAM/SHAM and SHAM/OVX females, showed an increase in body weight, while CCI females showed an arrest in weight gain. Results are presented as mean ± SEM. *P<0.05 and ***P<0.001 vs. baseline (week 0) within the respective group (t-test with a Bonferroni correction for multiple comparison t-test with a Bonferroni correction for multiple comparisons). Symbols are coloured according to their respective experimental group. ($n_{CCI/M}$=7, $n_{SHAM/SHAM}$=8, $n_{SHAM/OVX}$=7, $n_{CCI/SHAM}$=8 and $n_{CCI/OVX}$=8).

Statistical analysis showed body weight varied differently throughout time, depending on the experimental group (Interaction: $F_{21,182}$=6.34, P<0.001, η_p^2=0.12; Time: $F_{7,182}$=23.3, P<0.001, η_p^2=0.15; Group:

$F_{3,26}$=7.68, P<0.001, η_p^2=0.27). Post-hoc tests showed that, while males, SHAM/SHAM and SHAM/OVX females increased their body weight throughout the behavioural period, CCI females displayed no significant weight gain during the evaluation period, with no differences between CCI/SHAM and CCI/OVX females (P>0.99). By contrast, SHAM/OVX showed a significant increase in body weight starting at week two post-CCI surgery (P<0.001).

4.2. Behavioural assessment of nociception

To evaluate the development of nociceptive-related behaviours, the von Frey test was performed to assess mechanical allodynia and the acetone test for cold allodynia. These tests began the week before CCI surgery (baseline) until week 5 after surgery, the endpoint for behavioural assessments (**Figure 7**).

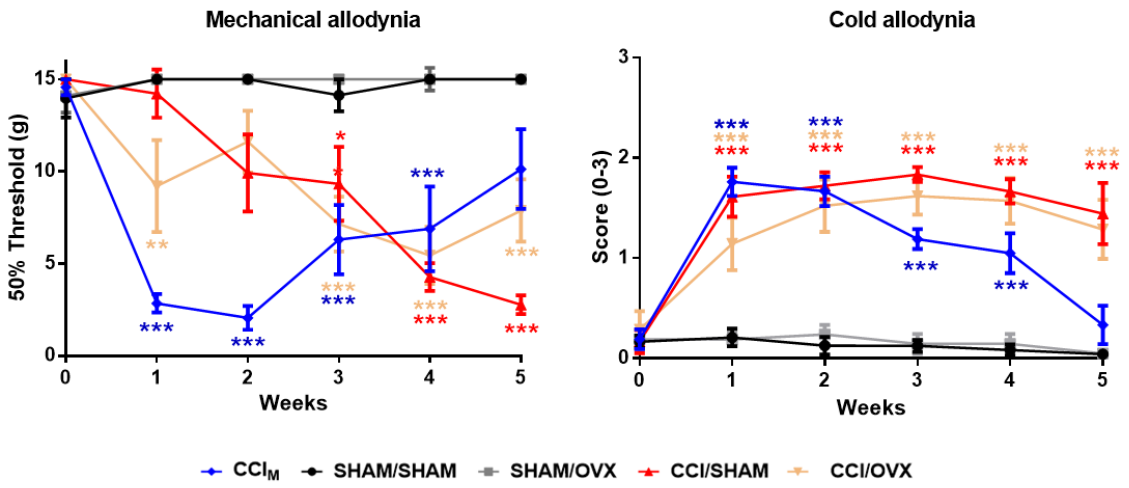

Figure 7. Assessment of nociceptive behaviour throughout the experimental period. (A) Mechanical allodynia was observed from week 1 and 2 onwards in CCI/OVX and CCI/SHAM female rats, respectively, with significant differences between the two experimental groups on weeks 1 and 5. (B) Regarding cold allodynia, CCI females displayed cold allodynia from week 1 onwards, in comparison with SHAM animals. CCI males displayed both mechanical and cold allodynia from week 1 onwards but recovering at week 5. Results are presented as mean ± SEM. *P<.05, **P<0.01 and ***P<0.001 vs. baseline (week 0) within the respective group (t-test with a Bonferroni correction for multiple comparison t-test with a Bonferroni correction for multiple comparisons). Symbols are coloured according to their respective experimental group. ($n_{CCI/M}$=7, $n_{SHAM/SHAM}$=8, $n_{SHAM/OVX}$=7, $n_{CCI/SHAM}$=6-7 and $n_{CCI/OVX}$=7).

4.2.1. Mechanical allodynia

Regarding mechanical allodynia, statistical analysis showed this parameter varied differently between experimental groups throughout time (**Figure 7A**; Interaction: $F_{20,160}$=7.23, P<0.001, η_p^2=0.24; Time: $F_{5,160}$=11.5, P<0.001, η_p^2=0.094; Group: $F_{4,32}$=39.3, P<0.001, η_p^2=0.34). Post hoc tests showed a significant decrease in withdrawal thresholds in CCI females, starting at week 1 for CCI/OVX animals and

at week 3 post-CCI surgery for CCI/SHAM females. Interestingly, while CCI/OVX females developed mechanical allodynia earlier than CCI/SHAM (P=0.041), these females appear to recover on week 5 unlike CCI/SHAM females (P=0.035).

CCI males showed a decrease in the withdrawal threshold, an indicator of the development of mechanical allodynia, starting from week 1 onwards when compared with baseline values. After week 3, recovery started to be observed in neuropathic males and, at week 5, no significant differences were observed in comparison with baseline. In comparison with neuropathic females, males were shown to have lower withdrawal thresholds at weeks 1 (CCI/SHAM: P<0.001; CCI/OVX: P=0.003) and 2 (CCI/SHAM: P<0.001; CCI/OVX: P<0.001). Oppositely, at week 5 CCI_M showed higher withdrawal thresholds than CCI/SHAM females (P<0.001) but no differences in comparison with CCI/OVX (P>0.99).

4.2.2. Cold allodynia

In the acetone test, which assesses the development of cold allodynia, ANOVA analysis showed this parameter varied differently throughout time between experimental groups (Interaction: $F_{20,150}$=7.55, P<0.001, η_p^2=0.15; Time: $F_{3,150}$=29.2, P<0.001, η_p^2=0.14; Group: $F_{4,30}$=45.7, P<0.001, η_p^2=0.49). Post hoc tests confirmed CCI_M, CCI/SHAM and CCI/OVX experimental groups cold allodynia from week 1 onwards (**Figure 7B**).

However, and contrary to what is observed for CCI females, neuropathic males steadily recovered after week 3 post-CCI induction. Cold allodynia in CCI_M was shown to be different from CCI/SHAM at weeks 1 (increased, P=0.044), 3 (decreased, P=0.045) and 5 (decreased P<0.001), as well as from CCI/OVX at week 5 (decreased, P<0.001).

4.3. Anxiety-like and locomotor behaviour

To assess the development of anxiety-like behaviour at week 5 after CCI surgery, the OF and EPM tests were performed. Basal locomotion was also evaluated through the OF test.

4.3.1. Open-field test (OF)

Anxiety-like behaviour was assessed in the OF by counting the number of *faecal boli* (**Figure 8A**) and assessing the time spent in the centre of the arena (**Figure 8B**). Two animals were excluded from the analysis due to software malfunction during the test (one from CCI/SHAM and CCI/OVX groups, respectively).

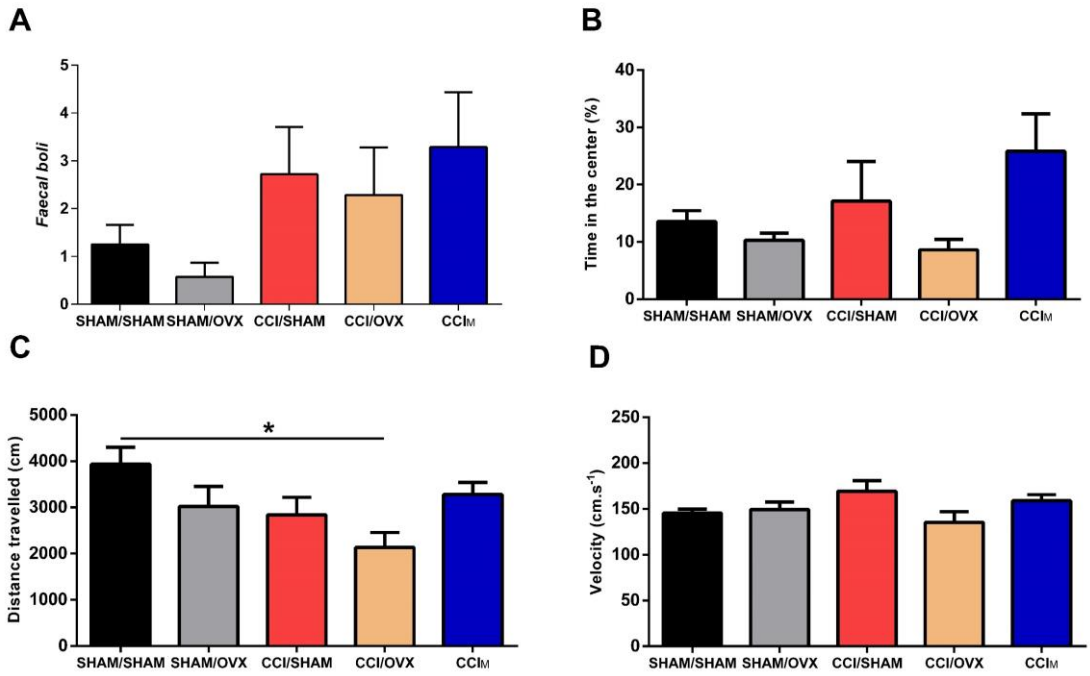

Figure 8. Evaluation of locomotion and anxiety-like behaviour in the open-field (OF) test four weeks after CCI surgery. (A) The number of faecal boli left in the arena at the end of the trial. (B) Time spent in the centre during the OF test expressed as a percentage (%) of total testing time. (C) Total distance (cm) travelled in the open field arena. (D) Average velocity during the trial. CCI/OVX animals showed a decrease in the total distance travelled, suggesting locomotor impairments Neuropathic males showed increased time spent in the centre and distance travelled in comparison with CCI/OVX females. Results are presented as mean ± SEM. *P<0.05 (t-test with a Bonferroni correction for multiple comparisons). # P<0.05 (unpaired t-test with Welch's correction). (n_{CCI}=7, $n_{SHAM/SHAM}$=8, $n_{SHAM/OVX}$=7, $n_{CCI/SHAM}$=7 and $n_{CCI/OVX}$=7).

Regarding the number of *faecal boli* left in the arena, CCI significantly increased anxiety-like behaviour in females ($F_{1,25}$=4.75, P=0.039, η_p^2=0.16), independently of ovariectomy ($F_{1,25}$=0.58, P=0.46, η_p^2=0.019) however post hoc analysis showed no differences between female experimental groups. Comparison between males and neuropathic females also showed no significant differences.

Neither CCI nor ovariectomy altered the time spent in the centre of the arena (CCI: $F_{1,25}$=0.066, P=0.80, η_p^2=0.023; OVX: $F_{1,25}$=2.57, P=0.12, η_p^2=0.092). Interestingly, CCI_M spent a higher percentage of time in the centre of the OF (t_7=2.53, P=0.039), in comparison with CCI/OVX females although no differences were found in relation to CCI/SHAM animals (t_{11}=0.96, P=0.034).

Basal locomotion was assessed in the OF test by analysing the distance travelled and average velocity of the animals. Regarding the distance travelled (**Figure 8C**), both ovariectomy and CCI independently decreased this parameter (CCI: $F_{1,25}$=6.83, P=0.015, η_p^2=0.19; OVX: $F_{1,25}$=4.55, P=0.043, η_p^2=0.12;

Interaction: $F_{1,25}$=0.076, P=0.78, η_p^2=0.002). Further comparisons showed CCI/OVX animals displayed a significant decrease in the distance travelled, compared with the SHAM/SHAM group as well as in comparison with CCI_M (t_{11}=2.76, P=0.018). Nevertheless, no differences were seen between CCI_M and CCI/SHAM females (t_{11}=0.96, P=0.36).

Regarding the average velocity (**Figure 8D**), this parameter was only decreased when animals were both OVX and CCI (Interaction: $F_{1,25}$=4.253, P=0.049, η_p^2=0.13; CCI: $F_{1,25}$=0.29, P=0.59, η_p^2=0.009; OVX: $F_{1,25}$=2.68, P=0.11, η_p^2=0.084), and no significant differences were found between experimental groups.

4.3.2. Elevated plus maze (EPM)

The EPM was also used to evaluate anxiety-like behaviour through the *faecal boli* left at the end of the trial, as well as the time spent and entries in the open arms (**Figure 9**). One CCI/SHAM animal was excluded from analysis after falling from the EPM apparatus.

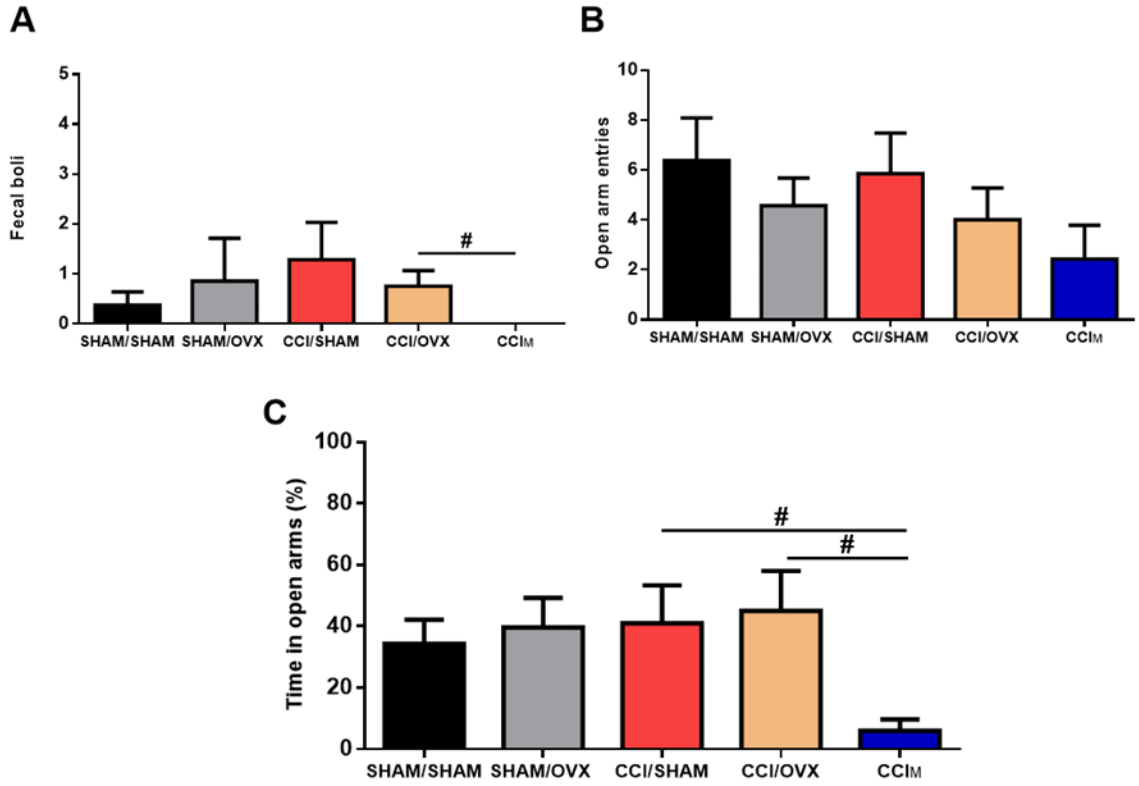

Figure 9. Behavioural evaluation of anxiety-like behaviour in the elevated plus maze (EPM) test four weeks after CCI surgery. (A) The number of *faecal boli* left in the arena at the end of the trial. (B) Time spent in the open arms during the EPM test expressed as a percentage (%) of total testing time. (C) Total of open arms entries during the trial. CCI_M left fewer faecal boli and spent less time in the open arms that CCI/OVX and CCI/SHAM females. Results are represented as mean ± SEM. # P<0.05 (unpaired t-test with Welch's correction). (n_{CCI}=7, $n_{SHAM/SHAM}$=8, $n_{SHAM/OVX}$=7, $n_{CCI/SHAM}$=7 and $n_{CCI/OVX}$=8)

Neither ovariectomy nor CCI induction altered the performance of females in the EPM regarding the number of *faecal boli* (**Figure 9A**; Interaction: $F_{1,26}$=0.79, P=0.38, η_p^2=0.003; CCI: $F_{1,26}$=0.49, P=0.49, η_p^2=0.018; OVX: $F_{1,26}$=0.002, P=0.96, η_p^2=0.008), number of open arm entries (**Figure 9B;** Interaction: $F_{1,26}$=0.0003, P=0.99, η_p^2<0.001; CCI: $F_{1,26}$=0.14, P=0.71, η_p^2=0.005; OVX: $F_{1,26}$=1.55, P=0.22, η_p^2=0.056) and time spent in open arms (**Figure 9C;** Interaction: $F_{1,26}$=0.003, P=0.96, η_p^2<0.001; CCI: $F_{1,26}$=0.29, P=0.60, η_p^2=0.011; OVX: $F_{1,26}$=0.18, P=0.59, η_p^2=0.007).

When comparing CCI_M with neuropathic females, males showed a decrease in the number of faecal boli in comparison with CCI/OVX females (t_7=2.39, P=0.047) but not with CCI/SHAM (t_6=1.72, P=0.14), as well as in time spent in the open arms in comparison with both CCI/SHAM (t_7=2.69, P=0.031) and CCI/OVX neuropathic females (t_8=2.90, P=0.020).

4.4. Depressive-like behaviour

In order to assess depressive-like behaviour, on week 5 post-CCI surgery the SPT was performed as a measure of anhedonia and the FST to evaluate learned helplessness.

4.4.1. Sucrose Preference Test (SPT)

Sucrose preference was not altered neither by CCI nor ovariectomy (**Figure 10A**; Interaction: $F_{1,27}$=3.42, P=0.075, η_p^2=0.10; CCI: $F_{1,27}$=0.011, P=0.92, η_p^2<0.001; OVX: $F_{1,27}$=3.99, P=0.056, η_p^2=0.12).

However, when adjusted for the body weight of the animals (**Figure 10B)**, ovariectomy independently decreased sucrose preference (OVX: $F_{1,27}$=28.4, P<0.001, η_p^2=0.49), indicating the development of depressive-like behaviour in ovariectomized females, while CCI did not induce anhedonic-like behaviour (CCI: $F_{1,27}$=0.024, P=0.88, η_p^2<0.001; Interaction: $F_{1,27}$=3.02, P=0.094, η_p^2=0.052). Confirming these results, multiple comparisons showed both SHAM/OVX and CCI/OVX animals decreased sucrose preference, in comparison with SHAM/SHAM animals. Males with CCI also displayed lower sucrose preference in comparison with CCI/SHAM (t_{12}=7.47, P<0.001) and CCI/OVX females (t_{12}=2.33 P=0.038).

To assess if there were differences in drinking patterns, the total intake of sucrose and water was evaluated. ANOVA analysis showed an interaction between CCI and ovariectomy (**Figure 10C;** Interaction: $F_{1,27}$=10.3, P=0.003; η_p^2=0.23; OVX: $F_{1,27}$=4.98, P=0.034; η_p^2=0.11; CCI: $F_{1,27}$=2.72, P=0.11, η_p^2=0.060). Multiple comparisons showed, in comparison with SHAM/SHAM animals, a decrease in the

total intake of SHAM/OVX and CCI/SHAM animals. When the intake was adjusted to body weight (**Figure 10D**), identical results were observed in the ANOVA analysis (Interaction: $F_{1,27}$=10.3, P=0.003, η_p^2=0.20; OVX: $F_{1,27}$=11.2, P=0.002, η_p^2=0.23; CCI: $F_{1,27}$=3.79, P=0.06, η_p^2=0.072). Multiple comparisons showed a decrease in total intake in all female experimental groups, in comparison with SHAM/SHAM animals. In both these analyses, no differences were found between CCI_M and neuropathic females.

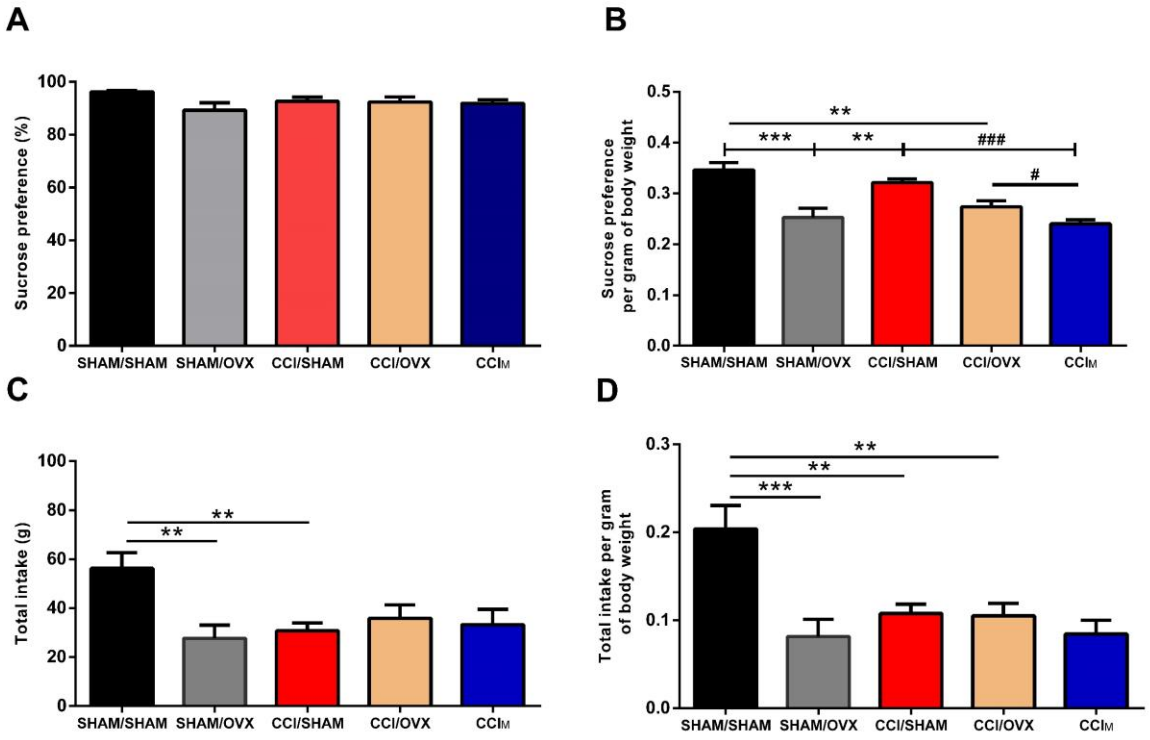

Figure 10. Behavioural evaluation of depressive-like behaviour using the sucrose preference (SPT) test four weeks after CCI surgery. **(A)** Sucrose preference in the SPT and **(B)** adjusted to body weight. **(C)** Total intake of water and sucrose solution in the SPT test and **(D)** Total intake adjusted to body weight. Ovariectomized female rats, with (CCI/OVX) and without CCI (SHAM/OVX) showed a decrease in sucrose preference, indicative of depressive-like behaviour. Results are presented as mean ± SEM. **P<0.01 and ***P<0.001 (t-test with a Bonferroni correction for multiple comparisons). # P<0.05 and ### P<0.001 (t-test for paired data with Welch's correction). (n_{CCI}=7, $n_{SHAM/SHAM}$=8, $n_{SHAM/OVX}$=7, $n_{CCI/SHAM}$=8 and $n_{CCI/OVX}$=8).

4.4.2. Forced Swimming Test (FST)

The FST was used to assess learned helplessness-like behaviour, a component of depression (**Figure 11**). Three different parameters were evaluated, namely the number of *faecal boli*, latency to immobility and immobility time. Time spent swimming and climbing is also represented in **Figure 11C**.

ANOVA analysis showed neither CCI nor ovariectomy influenced the females performance regarding (i) the number of *faecal boli* (**Figure 11A**; Interaction: $F_{1,27}$=0.20, P=0.66, η_p^2=0.097; CCI: $F_{1,27}$=0.20,

P=0.66, η_p^2=0.007; OVX: $F_{1,27}$=0.37 P=0.55, η_p^2=0.013); (ii) latency to immobility (**Figure 11B;** Interaction: $F_{1,27}$<0.001, P=0.98, η_p^2<0.001; CCI $F_{1,27}$=2.34, P=0.63, η_p^2=0.009; OVX: $F_{1,27}$=0.010, P=0.92, η_p^2<0.001) and (iii) immobility time (**Figure 11C**; Interaction: $F_{1,27}$=0.63, P=0.43, η_p^2=0.022; CCI: $F_{1,27}$=0.11, P=0.74, η_p^2=0.004; OVX: $F_{1,27}$=0.65, P=0.43, η_p^2=0.023). Further comparisons between neuropathic males and females showed no significant differences for all the parameters evaluated.

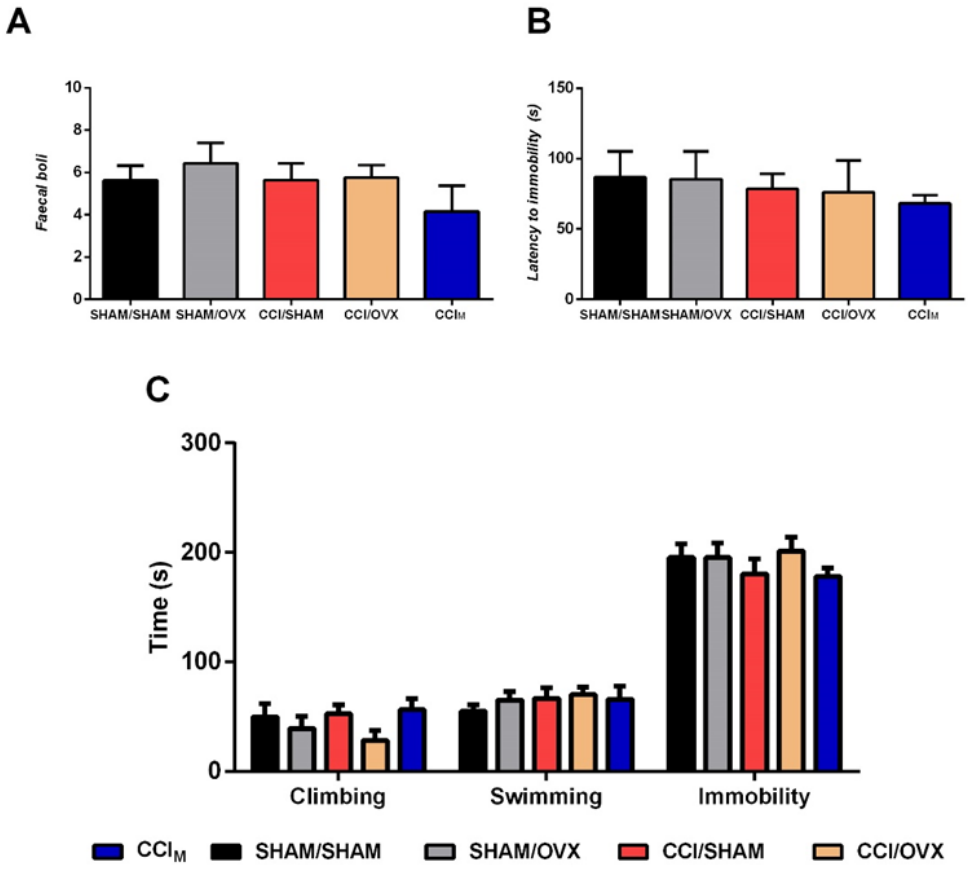

Figure 11. Behavioural evaluation of depressive-like behaviour using the forced swimming (FST) test four weeks after CCI surgery. (A) Number of faecal boli left at the end of the trial. (B) Latency to immobility. (C) Time spent climbing, swimming and in immobility measured in the FST. No significant differences were observed between experimental groups. Results are represented as mean ± SEM. (n_{CCI}=7, $n_{SHAM/SHAM}$=8, $n_{SHAM/OVX}$=7, $n_{CCI/SHAM}$=8 and $n_{CCI/OVX}$=8).

4.5. Electrophysiological recordings in the RVM

A total of 447 neurons were recorded in the RVM. As described in the methods section, neurons were initially classified as WDR-like or NS-like according to their response to innocuous mechanical stimulation, namely brushing of the animal's back (**Table 5**). For the sake of simplicity, these cells will from now on be designated as WDR and NS.

Table 5. Total number of RVM WDR- and NS-like neurons recorded in the electrophysiological study. (WDR - Wide-dynamic range; NS - nociceptive-specific; n – number of animals recorded).

	SHAM/SHAM (n=5)	SHAM/OVX (n=3)	CCI/SHAM (n=6)	CCI/OVX (n=4)	CCI_M (n=4)
WDR neurons	51	33	63	50	59
NS neurons	76	40	62	72	44
Total	127	73	125	122	103

The spontaneous activity of both WDR- and NS-like cells in the RVM is represented in **Figure 12.** ANOVA analysis showed no effects on their spontaneous activity (**Table 6**), and no differences were observed between neuropathic CCI/SHAM females and CCI_M.

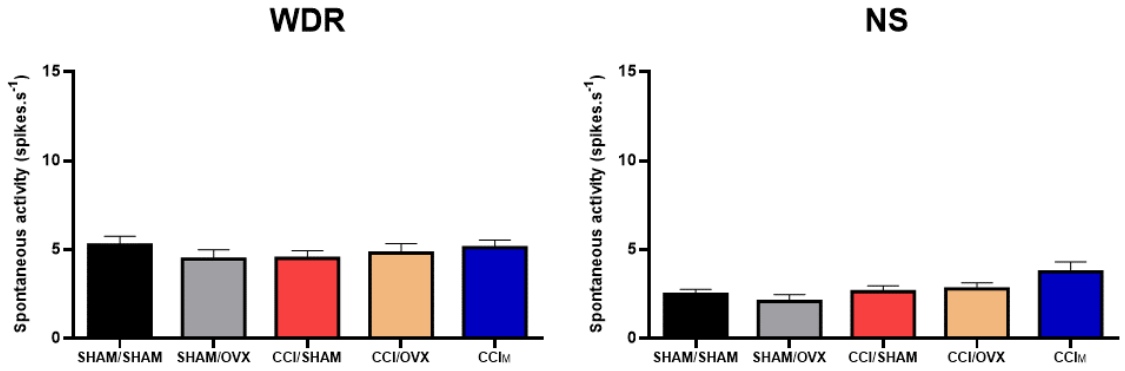

Figure 12. Spontaneous activity of WDR and NS cells recorded in the RVM. Results are presented as mean ± SEM. (WDR - Wide-dynamic range; NS - nociceptive-specific).

Table 6. Results of the statistical analysis of the differences in the spontaneous activity of RVM WDR and NS cells amongst experimental groups (WDR - Wide-dynamic range; NS - nociceptive-specific; CCI - chronic constriction injury).

	Interaction	Ovariectomy	CCI
WDR	$F_{1,193}$=1.19, P=0.28, η_p^2=0.006	$F_{1,193}$=0.24, P=0.62, η_p^2=0.001	$F_{1,193}$=0.17, P=0.68, η_p^2<0.001
NS	$F_{1,246}$=0.72, P=0.40, η_p^2=0.003	$F_{1,246}$=0.14, P=0.71, η_p^2<0.001	$F_{1,246}$=1.25, P=0.20, η_p^2=0.006

Neurons were further classified as On-, Off- or Neutral-like cells according to their response for each stimulus applied and only On- and Off-like cells were analysed in this work.

4.5.1. Mechanical stimulation: Von Frey

RVM neuronal activity was evaluated through Von Frey mechanical stimulation (6 g). Classification and the total number of neurons responsive to von Frey stimulation recorded are described in **Table 7**.

Table 7. Total number of RVM On, Off- and Neutral-like cells to mechanical stimulation with Von Frey filament. (WDR - Wide-dynamic range; NS - nociceptive-specific).

Group	On-like cells		Off-like cells		Neutral-like cells	
	WDR	NS	WDR	NS	WDR	NS
SHAM/SHAM	8	10	10	9	33	57
SHAM/OVX	7	5	5	5	21	30
CCI/SHAM	13	12	12	6	38	44
CCI/OVX	12	6	12	6	26	60
CCI_M	8	5	17	2	34	37

Spontaneous activity of RVM neurons prior to von Frey stimulation is represented in **Figure 13**. ANOVA analysis showed no differences in the spontaneous activity of all cell types between females (**Table 8**). By contrast, the spontaneous activity of NS Off-like cells was increased in males in comparison with both CCI/SHAM (t_5=3.25, P=0.020) and CCI/OVX females (t_5=3.68, P=0.012)

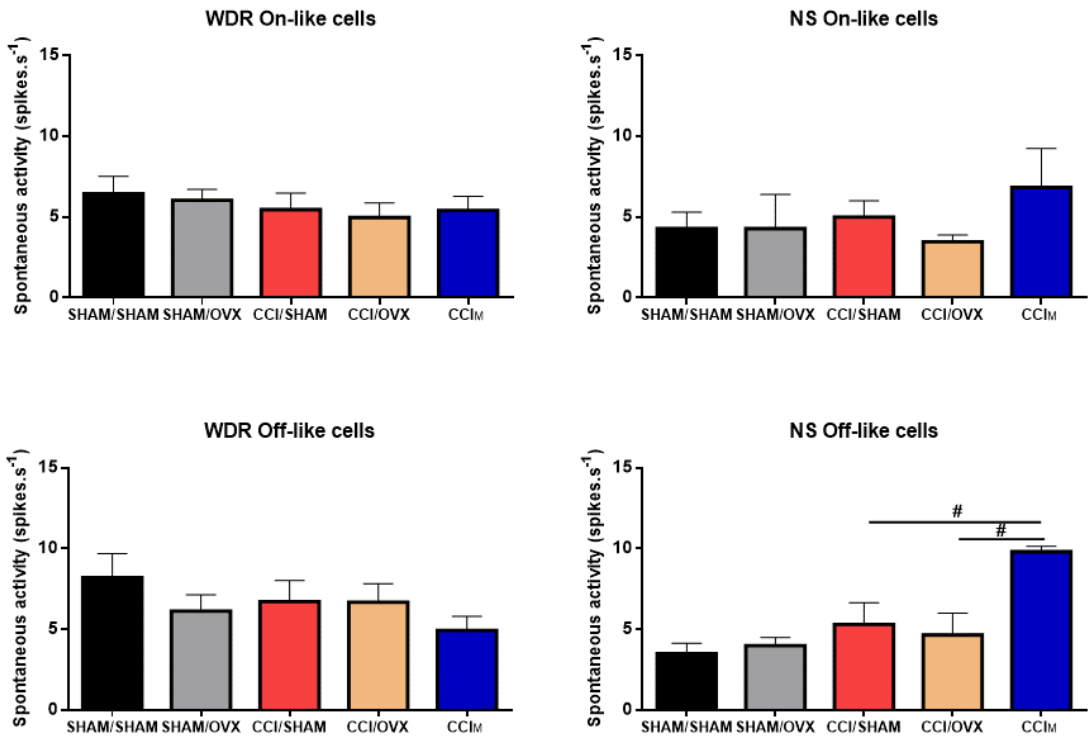

Figure 13. Spontaneous activity of RVM neurons before mechanical stimulation with von Frey hairs. No significant differences were observed between experimental groups. Results are presented as mean ± SEM. # P<0.05 (unpaired t-test with Welch's correction). (WDR - Wide-dynamic range; NS - nociceptive-specific).

Table 8. Results of the statistical analysis of the differences in the spontaneous activity of RVM neurons before mechanical stimulation with von Frey hairs between female experimental groups. (WDR - Wide-dynamic range; NS - nociceptive-specific; CCI - chronic constriction injury).

	Interaction	Ovariectomy	CCI
WDR On-like cells	$F_{1,36}$=0.001, P=0.87, η_p^2<0.001	$F_{1,36}$=0.22, P=0.64, η_p^2=0.005	$F_{1,36}$=1.10, P=0.30, η_p^2=0.029
WDR Off-like cells	$F_{1,35}$=0.56, P=0.46, η_p^2=0.015	$F_{1,35}$=0.59, P=0.45, η_p^2=0.016	$F_{1,35}$=0.11, P=0.74, η_p^2=0.003
NS On-like cells	$F_{1,29}$=0.44 P=0.51, η_p^2=0.015	$F_{1,29}$=0.42, P=0.52, η_p^2=0.014	$F_{1,29}$=0.002, P=0.96, η_p^2<0.001
NS Off-like cells	$F_{1,22}$=0.38, P=0.54, η_p^2=0.016	$F_{1,22}$=0.009, P=0.92, η_p^2<0.001	$F_{1,22}$=1.72, P=0.20, η_p^2=0.071

During Von Frey stimulation (**Figure 14**), ovariectomy independently increased the evoked response of WDR Off-like cells (**Table 9**). Multiple comparisons showed a significantly increased in WDR Off-like cells of CCI/OVX animals, in comparison with CCI/SHAM (P=0.015) and SHAM/SHAM (P=0.007) females, as well as in comparison with males (t_{11}=3.07, P=0.011). No differences were observed for NS and WDR On-like neurons.

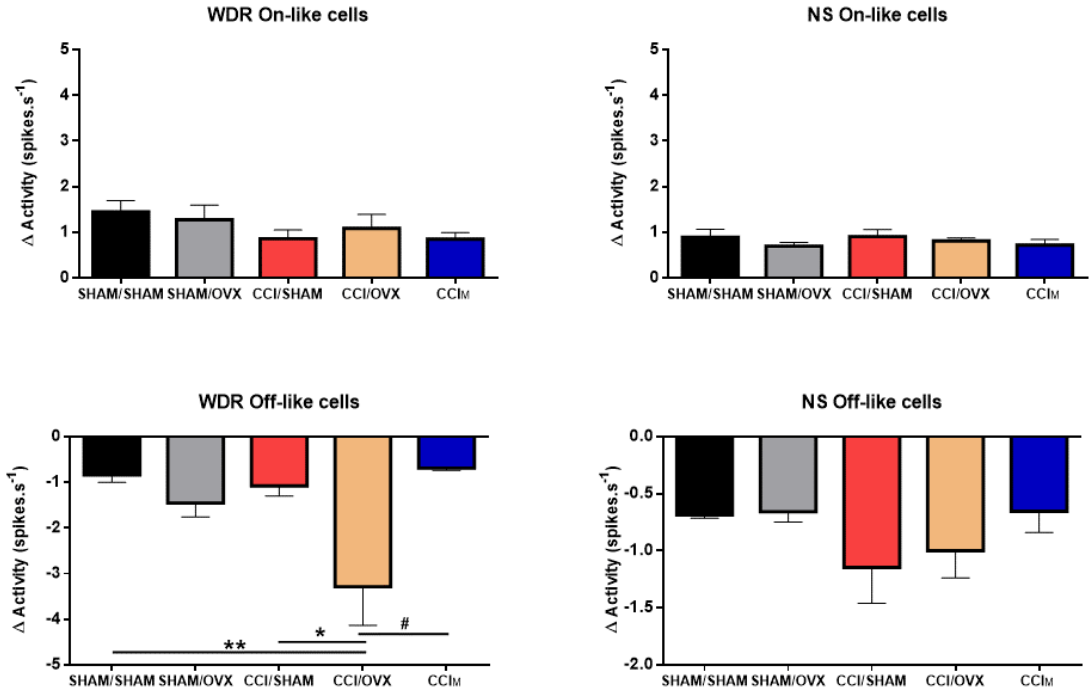

Figure 14. Evoked response of RVM neurons after mechanical stimulation with von Frey hairs. CCI/OVX showed an increased response of WDR Off-like cells in comparison with CCI/SHAM and SHAM/SHAM females. Results are presented as mean ± SEM. *P<0.05 and **P<0.01 (t-test with a Bonferroni correction for multiple comparisons). (WDR - Wide-dynamic range; NS - nociceptive-specific).

Table 9. Results of the statistical analysis of the differences in the evoked response of RVM neurons after mechanical stimulation with von Frey hairs between female experimental groups. *P<0.05. (WDR - Wide-dynamic range; NS - nociceptive-specific; CCI - chronic constriction injury).

	Interaction	Ovariectomy	CCI
WDR On-like cells	$F_{1,36}$=0.31 P=0.58, η_p^2=0.008	$F_{1,36}$=0.035, P=0.85, η_p^2<0.001	$F_{1,36}$=1.71, P=0.20, η_p^2=0.045
WDR Off-like cells	$F_{1,35}$=1.77, P=0.19, η_p^2=0.038	$F_{1,35}$=6.62, P=0.014*, η_p^2=0.14	$F_{1,35}$=3.47, P=0.071, η_p^2=0.074
NS On-like cells	$F_{1,29}$=0.098, P=0.76, η_p^2=0.003	$F_{1,29}$=0.89, P=0.35, η_p^2=0.029	$F_{1,29}$=0.098, P=0.76, η_p^2=0.003
NS Off-like cells	$F_{1,22}$=0.087, P=0.77, η_p^2=0.003	$F_{1,22}$=0.087, P=0.77, η_p^2=0.003	$F_{1,22}$=4.25, P=0.051, η_p^2=0.16

4.5.2. Mechanical stimulation: Pinch

RVM neuronal activity was also assessed after noxious mechanical stimulation, namely through pinching of the tail using a surgical clamp. Classification and the total number of neurons recorded is described in **Table 10**.

Table 10. Total number of RVM On-, On- and Neutral-like cells to noxious mechanical stimulation to the tail (pinching with a surgical clamp), recorded in the electrophysiological study. (WDR - Wide-dynamic range; NS - nociceptive-specific).

Group	On-like cells		Off-like cells		Neutral-like cells	
	WDR	NS	WDR	NS	WDR	NS
SHAM/SHAM	14	16	21	21	16	39
SHAM/OVX	13	8	10	11	10	21
CCI/SHAM	31	33	17	9	15	20
CCI/OVX	26	31	13	21	11	20
CCI_M	19	10	22	13	18	21

Spontaneous activity of the RVM neurons prior to stimulation is represented in **Figure 15**. Statistical analysis showed an effect of ovariectomy on the spontaneous activity of NS Off-like cells (**Table 11**), and further multiple comparisons showed an increase in CCI/SHAM animals when compared to CCI/OVX animals (P=0.044). No differences were observed between the remaining RVM cell types.

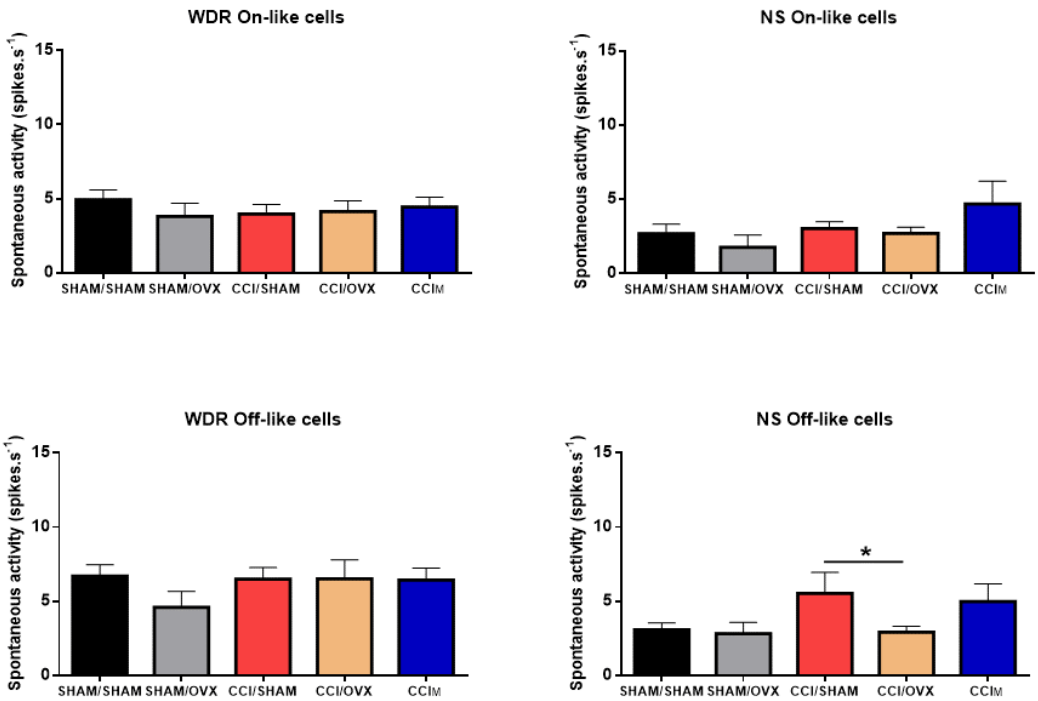

Figure 15. Spontaneous response of RVM neurons before noxious mechanical stimulation of the tail (pinching with a surgical clamp). Differences were observed on NS Off-like cells, with CCI/SHAM animals displaying an increased spotaneous activity than CCI/OVX females. Results are presented as mean ± SEM. *P<0.05 (t-test with a Bonferroni correction for multiple comparisons). (WDR - Wide-dynamic range; NS - nociceptive-specific).

Table 11. Results of the statistical analysis of the differences in the spontaneous activity of RVM neurons before noxious mechanical stimulation of the tail (pinching with a surgical clamp) between female experimental groups. *P<0.05. (WDR - Wide-dynamic range; NS - nociceptive-specific; CCI - chronic constriction injury).

	Interaction	**Ovariectomy**	**CCI**
WDR On-like cells	$F_{1,80}$=0.67, P=0.42, η_p^2=0.008	$F_{1,80}$=0.37, P=0.54, η_p^2=0.004	$F_{1,80}$=0.16, P=0.69, η_p^2=0.002
WDR Off-like cells	$F_{1,57}$=1.27, P=0.26, η_p^2=0.021	$F_{1,57}$=1.20, P=0.28, η_p^2=0.020	$F_{1,57}$=0.82, P=0.37, η_p^2=0.014
NS On-like cells	$F_{1,84}$=0.24, P=0.62, η_p^2=0.003	$F_{1,84}$=1.07, P=0.30, η_p^2=0.012	$F_{1,84}$=1.14, P=0.29, η_p^2=0.013
NS Off-like cells	$F_{1,59}$=3.31, P=0.074, η_p^2=0.047	$F_{1,59}$=5.01, P=0.029*, η_p^2=0.071	$F_{1,59}$=3.90, P=0.053, η_p^2=0.055

After noxious mechanical stimulation (**Figure 16**), statistical analysis showed CCI increased the response of WDR On-like cells, as well as an interaction between CCI and ovariectomy on the evoked response of NS Off-like cells (**Table 12**). However, multiple comparisons showed no differences between female experimental groups for all cell types. Neuropathic males showed a decreased response of WDR (CCI/SHAM: t_{46}=3.57, P<0.001; CCI/OVX: t_{42}=2.66, P=0.011) and NS On-like cells (CCI/SHAM: t_{40}=3.72, P<0.001; CCI/OVX: t_{39}=3.58, P<0.001), in comparison with both CCI/SHAM and CCI/OVX females. CCI_M also showed a decreased response of WDR Off-like cells comparing with CCI/SHAM (t_{41}=2.78, P=0.012).

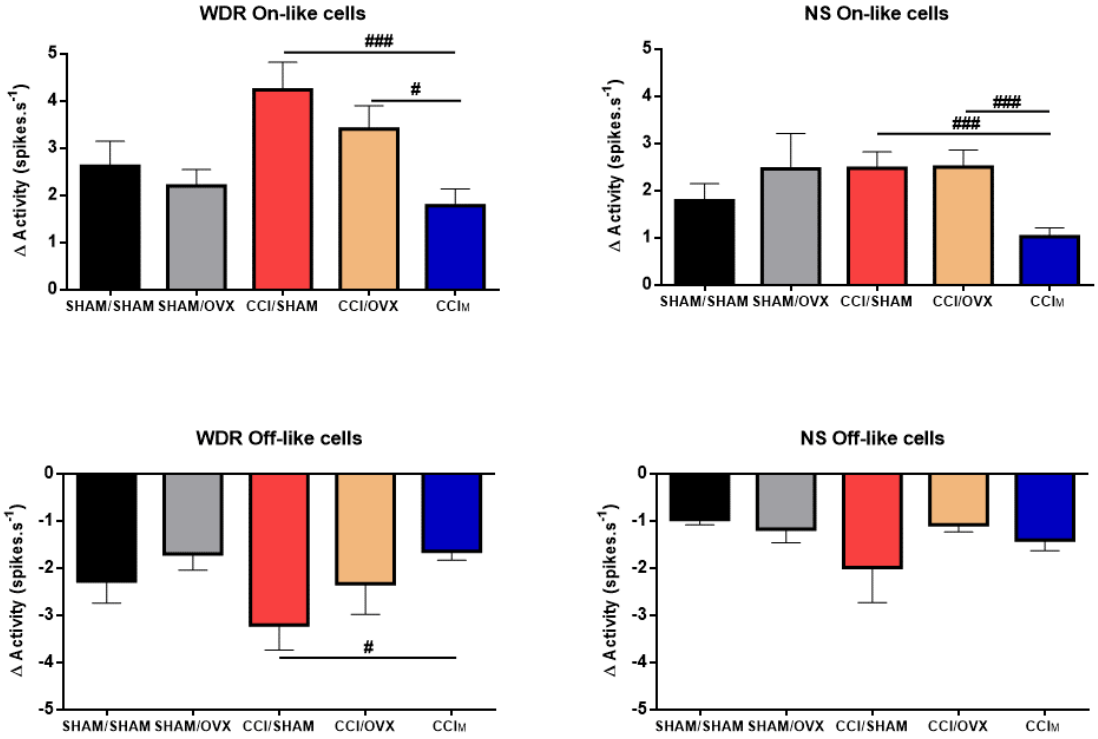

Figure 16. Evoked response of RVM neurons after noxious mechanical stimulation of the tail (pinching with a surgical clamp). No significant differences were observed between experimental groups. (WDR - Wide-dynamic range; NS - nociceptive-specific). Results are presented as mean ± SEM. # P<0.05 and ### P<0.001 (unpaired t-test with Welch's correction).

Table 12. Results of the statistical analysis of the differences in the evoked response of RVM neurons to noxious mechanical stimulation of the tail (pinching with a surgical clamp) between female experimental groups. *P<0.05. (WDR - Wide-dynamic range; NS - nociceptive-specific; CCI - chronic constriction injury).

	Interaction	Ovariectomy	CCI
WDR On-like cells	$F_{1,80}$=0.11, P=0.74, η_p^2=0.001	$F_{1,80}$=0.10, P=0.32, η_p^2=0.011	$F_{1,80}$=5.43, P=0.022*, η_p^2=0.063
WDR Off-like cells	$F_{1,57}$=0.080, P=0.78, η_p^2=0.001	$F_{1,57}$=2.00, P=0.16, η_p^2=0.33	$F_{1,57}$=2.00, P=0.16, η_p^2=0.33
NS On-like cells	$F_{1,84}$=0.56, P=0.46, η_p^2=0.007	$F_{1,84}$=0.56, P=0.46, η_p^2=0.007	$F_{1,84}$=0.56, P=0.46, η_p^2=0.007
NS Off-like cells	$F_{1,59}$=4.06, P=0.049*, η_p^2=0.061	$F_{1,59}$=1.64, P=0.21, η_p^2=0.025	$F_{1,59}$=2.72, P=0.10, η_p^2=0.041

4.5.3. Cold stimulation

Regarding innocuous thermal stimulation, RVM neuronal activity was recorded when acetone was applied to the plantar skin of the left hindpaw. The classification and the total number of neurons recorded is described in **Table 13**.

Table 13. Total number of RVM On-, Off- and Neutral-like cells to innocuous thermal stimulation (acetone applied to the plantar skin of the left hindpaw), recorded in the electrophysiological study. (WDR - Wide-dynamic range; NS - nociceptive-specific).

Group	On-like cells		Off-like cells		Neutral-like cells	
	WDR	NS	WDR	NS	WDR	NS
SHAM/SHAM	13	12	9	13	29	51
SHAM/OVX	11	6	5	2	17	32
CCI/SHAM	12	7	12	9	12	46
CCI/OVX	7	6	9	5	9	61
CCI_M	9	9	14	4	36	31

Prior to stimulation with acetone, the spontaneous activity of RVM neurons is represented in **Figure 17**.

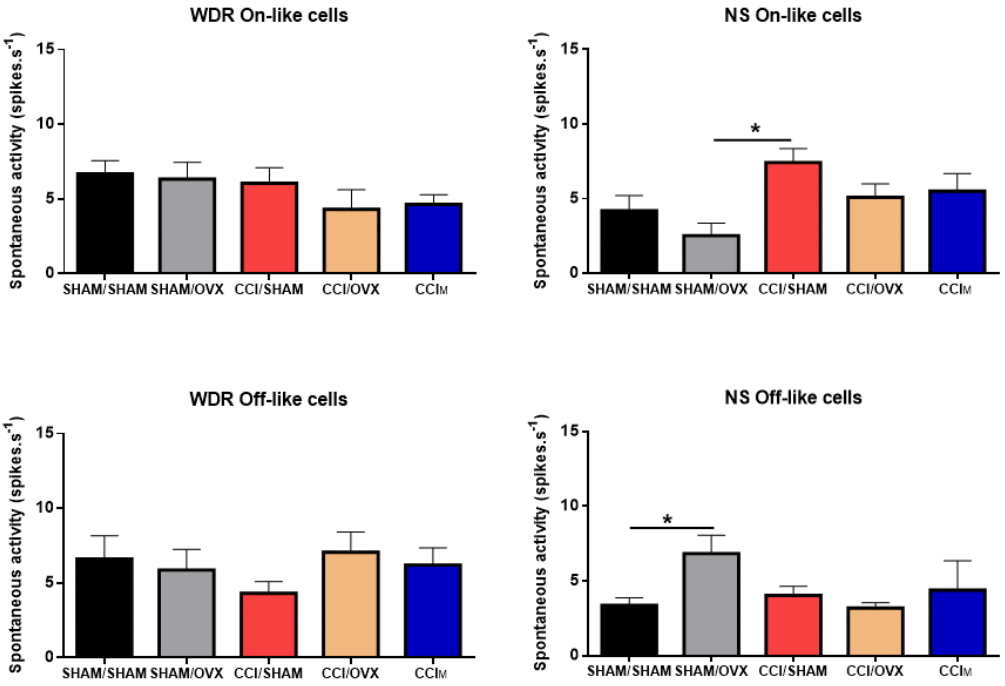

Figure 17. Spontaneous activity of RVM neurons before cold stimulation (acetone applied to the plantar skin of the left hindpaw). CCI /SHAM animals showed an increased spontaneous activity than SHAM/SHAM animals. Results are presented as mean ± SEM. *P<0.05 (t-test with a Bonferroni correction for multiple comparisons). (WDR - Wide-dynamic range; NS - nociceptive-specific).

No differences were observed in the spontaneous activity of WDR neurons. Regarding NS neurons, the spontaneous activity of NS On-like cells was increased after de induction of CCI (**Table 14**), and multiple comparisons showed increased spontaneous activity in CCI/SHAM compared with SHAM/OVX animals (P=0.018). Also, an interaction between CCI and ovariectomy was observed in NS Off-like cells, and post hoc multiple comparisons showed increased spontaneous activity in SHAM/OVX, in comparison with

SHAM/SHAM animals (P=0.048). Interestingly, no differences were found when comparing neuropathic males and females.

Table 14. Results of the statistical analysis of the differences in the spontaneous activity of RVM neurons before cold stimulation between female experimental groups. ** P<0.01. (WDR - Wide-dynamic range; NS - nociceptive-specific; CCI - chronic constriction injury).

	Interaction	Ovariectomy	CCI
WDR On-like cells	$F_{1,29}$=0.44, P=0.51, η_p^2=0.011	$F_{1,29}$=1.01, P=0.32, η_p^2=0.024	$F_{1,29}$=1.62, P=0.21, η_p^2=0.038
WDR Off-like cells	$F_{1,31}$=1.98, P=0.17, η_p^2=0.058	$F_{1,31}$=0.65, P=0.42, η_p^2=0.019	$F_{1,31}$=0.20, P=0.66, η_p^2=0.006
NS On-like cells	$F_{1,27}$=0.11, P=0.008, η_p^2=0.003	$F_{1,27}$=3.88, P=0.059, η_p^2=0.099	$F_{1,27}$=8.24, P=0.008**, η_p^2=0.21
NS Off-like cells	$F_{1,25}$=8.34, P=0.008**, η_p^2=0.21	$F_{1,25}$=3.10, P=0.091, η_p^2=0.076	$F_{1,25}$=4.06, P=0.055, η_p^2=0.10

After stimulation with acetone (**Figure 18**), CCI independently decreased the response of WDR On-like neurons (**Table 15**), and multiple comparisons showed differences between SHAM/OVX animals and both CCI/SHAM (P=0.018) and CCI/OVX neuropathic females (P=0.028). No differences were observed between experimental groups regarding NS and WDR Off-like neurons. Identically, comparisons between male and female CCI groups showed no statistically significant differences.

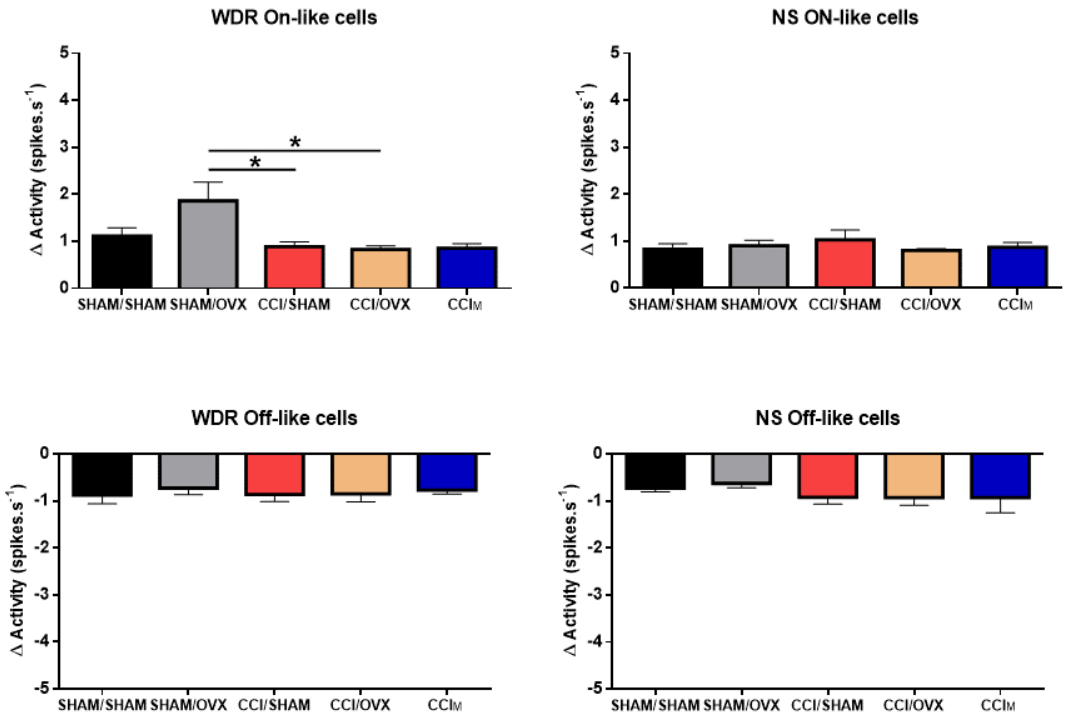

Figure 18. Evoked response of RVM neurons after cold stimulation (acetone applied to the plantar skin of the left hindpaw). CCI females (CCI/SHAM and CCI/OVX) showed a decreased response of WDR On-like cells, in comparison with SHAM/OVX. Results are presented as mean ± SEM. *P<0.05 (t-test with a Bonferroni correction for multiple comparisons. (WDR - Wide-dynamic range; NS - nociceptive-specific).

Table 15. Results of the statistical analysis of the differences in the evoked activity of RVM neurons after cold stimulation (acetone applied to the plantar skin of the hindpaw) between female experimental groups. (WDR - Wide-dynamic range; NS - nociceptive-specific; CCI - chronic constriction injury).

	Interaction	**Ovariectomy**	**CCI**
WDR On-like cells	$F_{1,29}$=3.57, P=0.066, η_p^2=0.068	$F_{1,29}$=2.16, P=0.15, η_p^2=0.041	$F_{1,29}$=7.45, P=0.009**, η_p^2=0.14
WDR Off-like cells	$F_{1,31}$=0.34, P=0.56, η_p^2=0.011	$F_{1,31}$=0.34, P=0.56, η_p^2=0.011	$F_{1,31}$=0.34, P=0.56, η_p^2=0.011
NS On-like cells	$F_{1,27}$=1.15, P=0.29, η_p^2=0.041	$F_{1,27}$=0.13, P=0.72, η_p^2=0.004	$F_{1,27}$=0.13, P=0.72, η_p^2=0.004
NS Off-like cells	$F_{1,25}$=0.10, P=0.75, η_p^2=0.004	$F_{1,25}$=0.10, P=0.75, η_p^2=0.004	$F_{1,25}$=2.58, P=0.12, η_p^2=0.001

4.6. Histopathological analysis of the sciatic nerve

At the end of the experimental period, a section of the ipsilateral sciatic nerve was used for histopathological assessment (**Figure 19**).

Control animals (SHAM/SHAM) showed a clear organization of nerve fibres and absence of infiltrating cells. Ovariectomy alone caused no histopathological alterations to the sciatic nerves, with no differences between SHAM/SHAM and SHAM/OVX females. On the other hand, differences were observed between CCI and SHAM females. Longitudinal sections of the sciatic nerve of CCI animals showed a loss of organization of nerve fibres, with the presence of degraded myelin sheath as well as several infiltrating inflammatory cells. This loss of fibre organization was also possible to observe in transversal sections, as well as, the presence of inflammatory cells. Interestingly, these infiltrates were more abundant on the sciatic nerves of CCI/OVX females.

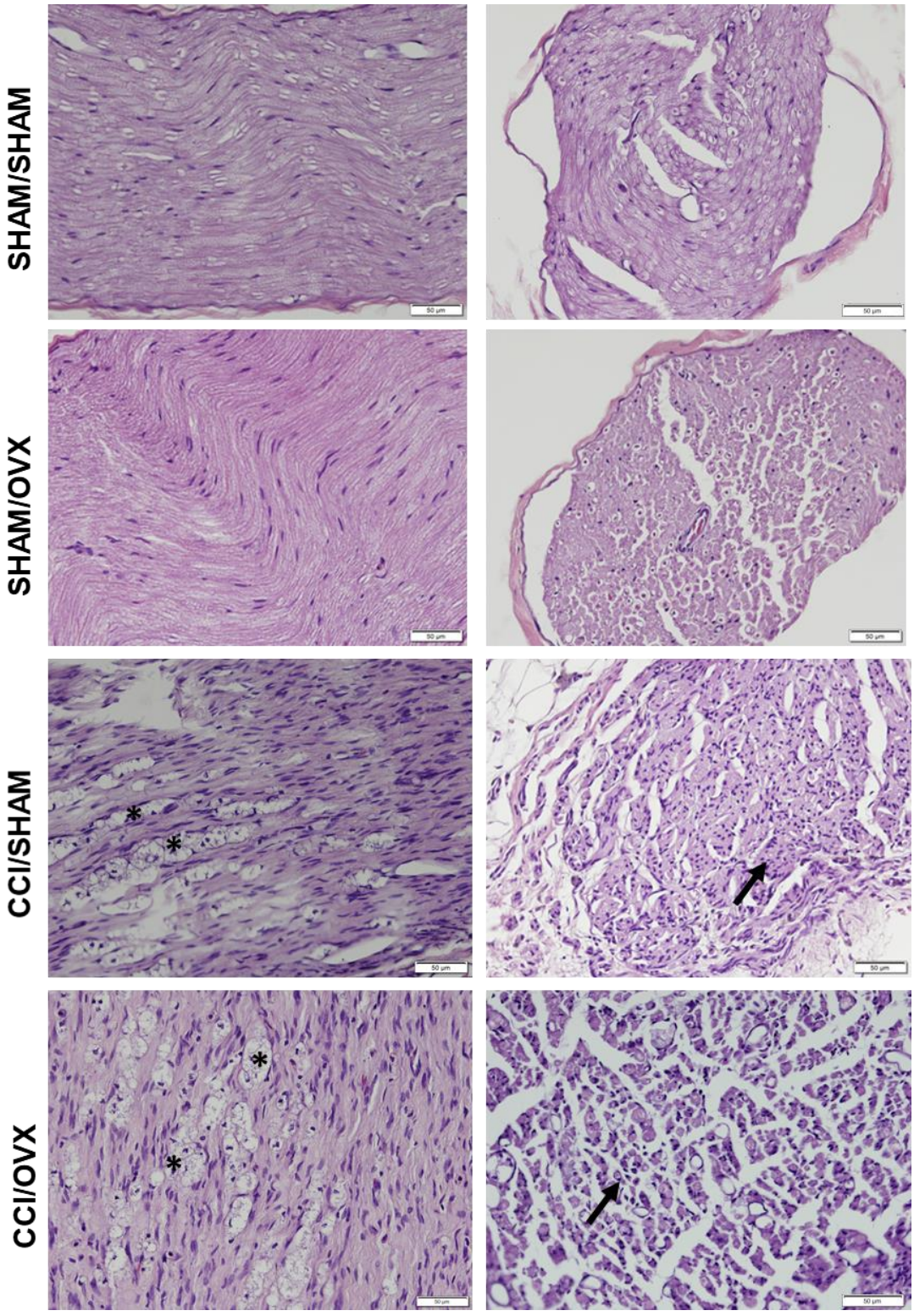

Figure 19. Histopathological analysis of the left sciatic nerve. Representative micrographs of longitudinal (Left) and transverse (Right) cuts for each female experimental group. It is possible to observe the presence of degraded myelin sheath (*) as well as infiltration of inflammatory cells (arrows) in the sciatic nerve of CCI females. Images were obtained using an Olympus BX61 brightfield microscope, coupled to an Olympus DP70 camera at the original objective magnification of 20X

Chapter 5. Discussion

In this work, the induction of CCI in female rats impaired body weight gain and lead to the development of mechanical and cold allodynia from week 1 post-CCI surgery onwards. Importantly, and contrary to what is described for males, CCI in female Wistar Han rats did not induce the development of emotional impairments, namely anxiety- or depressive-like behaviours. Interestingly, ovariectomy induced anhedonic-like behaviour with CCI/OVX females also displaying locomotor impairments. The electrophysiological results partly support our behavioural data to the extent that increased mechanical sensitivity in females and nociceptive recovery of CCI males was concomitant to changes in the neuronal activity of the RVM WDR and NS neurons. Nociceptive changes in CCI females were also accompanied by histopathological alterations of the sciatic nerve architecture.

5.1. Technical considerations

5.1.1. Animal model

The study of chronic pain depends on the use of animal models to evaluate their sensory and psychological intricacies. As mentioned previously, the CCI model of neuropathic pain[91] mimics the symptoms of chronic nerve compression or entrapment in various clinical conditions, comprising both the inflammatory and neuropathic components. This model distinguishes itself from other neuropathic pain models through the display of signs of spontaneous pain such as limping, limb guarding, excessive licking and avoidance of weight bearing on the injured limb. Notwithstanding its various advantages, the CCI model also has its demerits, such as variation in the tension of the ligatures throughout the experimental period and during induction, leading to inter-individual variability, even with the same experimenter. Another disadvantage of this model is the development of autotomy in some animals, which always progressed from the claws to the root of the toes. The root of the problem is probably the excessive trimming of the claws, as dysesthesia would damage the hindpaw randomly.

By using OVX animals, we were able to control the potential effect of hormonal depletion upon nociceptive and emotional behaviours, as well as upon neuronal activity. For the ovariectomy procedure, we opted to perform a dorsal and bilateral technique[227]. Primarily, an ovariectomy is preferred over an ovariohysterectomy as the manipulation of the uterus can lead to more surgical complications[235]. In

rodents, the dorsal approach is simple and avoids the need to manipulate and/or injury the gastrointestinal tract. In addition, as only a single incision to the skin is needed to access both ovaries, this approached is considered less invasive. It is, however, important to consider that performing a gonadectomy disrupts the normal feedback loop between gonadal hormones and the anterior pituitary and hypothalamus[236,237]. This leaves the animals in a prolonged state of altered circulating hormones causing a variety of uncontrolled consequences. The use of randomly cycling female rats did not allow to evaluate the role of gonadal hormone fluctuations on nociceptive and emotional processing. Nonetheless, we did not observe differences in variability of our results between gonadally intact and ovariectomized females.

The present work focused on the use of female experimental animals, as this model has been thoroughly characterized in male rats. However, a group of male CCI animals (CCI_M) was also included to allow comparisons between neuropathic females and males, as well as for validation of the experimental outcomes as literature on CCI males is extensive.

Regarding animal welfare, we evaluated body weight change throughout the experimental procedures. In females, ovariectomy was shown to increase the body weight of the animals, consistent with the previous reports[238]. The loss of ovarian function in rats has been known to increase daily food intake, body mass and body fat mass, which can be reversed by treatment with estradiol[239]. Interestingly, we observed this model of neuropathic pain arrested weight gain on female rats, which is not observed in males. However, our results are validated since as in other reports, CCI males continued to increase body weight despite the induction of CCI[177,196].

5.1.2. Behavioural assessment of nociception

One of the greatest difficulties in preclinical pain research is to find behavioural outcomes that properly evaluate the pain experienced by rodents. Since animals cannot self-report, the assessment of the extent and severity of pain is another major challenge in all pre-clinical models. Therefore, a battery of behavioural tests has been developed for the evaluation of nociception, with most of these tests, such as the herein used, measuring the response to a thermal or mechanical stimulus[240]. In this work, we assessed mechanical and cold allodynia using the von Frey and acetone tests, respectively. There are other behavioural measures of pain, focused on its consequences rather than behavioural responses, that may also reflect patients' symptomatology. These include the assessment of (i) adaptive postural changes, which contribute to physical disability; (ii) spontaneous behaviours, evaluating the impact of pain on daily

activities and (ii) motivational aspects, as motivation to pain relief and avoidance of painful situations[241]. These evaluations are however very time-consuming and difficult to evaluate quantitatively, while quantitative measures of allodynia/hyperalgesia are easily quantified, increase repeatability and reproducibility of the results and are present in clinical pain.

Mechanical allodynia is a high constraint symptom in chronic pain. In animal models, mechanical allodynia is often addressed using von Frey filaments applied to the plantar surface of the hindpaws[228]. The more common methodologies to determine mechanical sensitivity are the "ascending stimulus", "percent response" and "up-down" methods. While the "ascending stimulus" method avoids excessive stimulation of the hindpaw, it provides only an estimate of the withdrawal threshold. On the other way around, in the percent response" method the number of stimuli per test is significantly higher than other methodologies. In this work, the "up-down" method was used to calculate the mechanical force required to elicit a paw withdrawal response in 50% of animals (50% threshold)[228,242]. A disadvantage of this test is that the number of stimuli per animal is variable and requires repeated time-intensive measurements. This repeated stimulation can cause the animals to develop sensitization or learnt responses. Nevertheless, this test has the advantage of unrestrained assessment, being, therefore, less stress-inducing.

In rodents, a variety of tests have also been developed for the evaluation of cold allodynia such as immersion in cold water[243,244], water-alcohol bath[243], ethyl chloride spray[245], cold plate[246] and the herein used acetone test[247]. While these tests are easily performed and could provide more accurate quantification of cold sensitivity, they require specific equipment. In the acetone test, a small drop of acetone is applied to the plantar skin of the hindpaw. The great advantage of this test is the use of the same apparatus as for the von Frey test and acetone's low cost and availability. The acetone test incorporates a complex, multimodal stimulus comprising simultaneously active cold, mechanical, and chemical components. To avoid a mechanical stimulus, the acetone was applied without touching the hindpaw with the pipette. Probably, this leads to the activation of multiple types of nociceptors of the affected hindpaw[247]. Another potential disadvantage of this test is the reaction of the animals to the smell of acetone, which can lead to agitation and false responses. To avoid it, we habituated the animals to the smell of acetone prior to performing the test.

5.1.3. Behavioural paradigms of emotional behaviour

Since emotional disorders can only be evaluated indirectly, we then refer to them as anxiety-like and depressive-like behaviours. These can be assessed using a wide range of behavioural testing paradigms, which assume that rodents and humans share, to some extent, innate behaviours when faced with new challenges. However, it is necessary to take into consideration that each individual test assesses only a fraction of the emotional profile of the animal.

For the evaluation of anxiety-like behaviours, the OF and the EPM tests were performed, two of the most commonly used tests in rats[248]. The OF is an exploration test, as it is based on the animals' inner conflict between approaching or avoiding novel environments[249]. One of the greatest advantages of this tests is that it allows the analysis of both locomotion and anxiety: locomotion is evaluated through the total distance travelled by the animals, and the time spent in the centre of the arena is a measure of anxiety-like behaviour. Also, through an infrared beam system, it is largely automated and with no inter-experimenter variability on its analysis. Despite its advantages, it has been shown that OF activity can be influenced by several factors such as the species, strain and sex of the animals as well as the equipment used[249]. Similar to the OF test, the EPM is a very simple method to assess anxiety-like behaviour in rodents. This test is easy to quantify and does not comprise the presentation of noxious stimuli, relying only on the rodent's avoidance of open spaces and heights. It is important to take into consideration that the results on both these tests are influenced by habituation, resulting in the reduction of activity, so this test was only performed once. Also, while the OF test is more exploratory-based and suitable for locomotor measurements, the EPM is more suitable for anxiety testing. As some behavioural tests depend on the locomotor ability of the animals, the basal locomotor ability (without effort) of our animals was assessed in the OF test by evaluating the velocity and total distance travelled.

To evaluate depressive-like behaviours, we chose to assess two components of depression: anhedonia (SPT) and learned helplessness (FST). Regarding the SPT[250], this is an "effortless" test as it does not imply intense physical activity in order to gain access to the sweet solution. Also, our protocol does not require food and/or water deprivation prior to the test, reduces its duration and consequently the anxiety induced by isolation from the respective cage mates. Nonetheless, as this test lasts only a few hours, factors such as circadian rhythm and interindividual differences in the pattern and amount of liquid intake can influence the results. The analysis of anhedonia in rodents should be approached with caution, a reduced sucrose preference is considered to share some analogy with anhedonia and depressive behaviour in humans[251] and thus, SPT is widely used in chronic stress studies. Regarding the FST[192], a great advantage

of this test is that, through filling the tank in such depth that the animals cannot reach the ground, it allows the distinction between active behaviours (swimming/climbing), which is known to be differentially due to different serotonergic and noradrenergic mechanisms[188,189]. However, repeated testing and consequent learning processes can influence the results, as the animals recognize there is no escape from that situation and, for that reason, we opted to perform the test only once. Also, as this test is sensible to variation between observers, video recordings were analysed by two independent researchers.

It is important to point out all our emotional behavioural tests were performed on week 5 post-CCI surgery, while most studies perform anxiodepressive testing on week 4. Our choice was first based on the fact that emotional impairments, namely depressive-like behaviour is a slow-setting condition, taking several weeks to fully develop. Secondly, the development of nociceptive impairments was delayed and less extensive in females when compared to male animals. Thirdly, while the chromic cat gut used to constrict the sciatic nerve is known to deteriorate from day 28 onwards, several reports showed the presence of emotional impairments in male animals at later dates.

5.1.4. Electrophysiological recordings

Some consideration must be given to the advantages and disadvantages of the type of electrophysiological recordings used, namely a single cell recording technique. This technique allows the recording of a few cells simultaneously in every session, allowing the differentiation of functionally heterogeneous populations of neurons in the same area. This is of interest when recording the RVM, as it is comprised of functionally different cell types. As well, this allows the comparison between basal and neuronal activity evoked by several innocuous and noxious stimuli. After categorizing the recorded cells between NS and WDR, based on their response to noxious and innocuous stimuli, these cells were subdivided based on changes in their discharge rate (10% more or less in relation to spontaneous discharge rate[232]) after peripheral stimulation. A disadvantage of this classification is that, in cells with low spontaneous firing rates, a very small change is enough to classify the cell as responsive. For this reason, we considered a minimum variation of 0.45 spikes.s^{-1} to classify a cell as responsive.

Despite the advantages above mentioned, the results obtained must be analysed carefully. It is important to note that the experimental procedures were performed in animals under anaesthesia, which may interfere with brain function and bias the results due to possible effects of the sedation. Also, in large and heterogeneous areas, the small population of neurons analysed may not be a full representative of the entire area of interest. To partially overcome this constraint, we recorded as many neurons as possible

per session and at different depths. Concerning the experimental procedure, maintaining an anaesthetized state using sodium pentobarbital depended on a regular intraperitoneal injection, compromising the stability of the anaesthesia level and, consequently, the firing activity of the recorded cells. Thus, in addition to verifying the dilation of the pupils, the general muscle tone and responses to noxious pinching every 15 min, we also used spontaneous activity to control for variation in anaesthesia level. Finally, pentobarbitone has a narrow safety margin for anaesthesia, and mortality can be high – leading to a smaller sample size and fewer neurons being recorded in some animals.

5.2. Development of neuropathic pain

The hallmarks of neuropathic pain comprise the development of spontaneous pain - pain arising without stimulus -, allodynia - abnormal responses to non-painful stimuli – and hyperalgesia – exacerbated response to painful stimuli[253].

Spontaneous pain is particularly difficult to measure in rodents but since it was not within the scope of this work, no attempt at performing direct measures was made. Nevertheless, CCI animals displayed signs of limping and guarding the affected hindpaw, behaviours which are in line with those described by Bennet and Xie[91], thus supporting the CCI model was effectively induced in our animals. Also, as described earlier[91], autotomy was sometimes observed, with gnawed claw tips, leading to the exclusion of some CCI animals from the further nociceptive evaluation. This behaviour is present on conditions that cause anaesthesia dolorosa and was proposed to be triggered by abnormal afferent signaling generated in the ligation site[254]. Importantly, no differences were observed between neuropathic males and females regarding this parameter.

Neuropathy caused by CCI surgery led to the development of mechanical and cold allodynia, in both CCI/SHAM and CCI/OVX females, starting from week 1. While ovariectomy solely did not lead to detectable nociceptive abnormalities, gonadally intact and ovariectomized females with CCI showed differences in the development of mechanical allodynia. Whereas CCI/OVX females developed mechanical allodynia prior to CCI/SHAM, gonadally intact females showed increasingly lower withdrawal thresholds throughout the weeks while ovariectomy stagnated the development of mechanical allodynia. Some studies have shown alterations in sensitivity to nociceptive stimuli following OVX. A recent study by Li and colleagues[238] showed the development of mechanical allodynia 5 weeks after OVX, using the von Frey test, however, in this same study, no differences were seen between intact and ovariectomized females one-week post-CCI, which we observed in this work. As this assessment was performed only once

after CCI induction, it may not reflect the time-dependent development of mechanical allodynia observed herein. Altogether, these results point to a role of gonadal hormones not only on the early development of CCI-induced allodynia but also impacting the further maintenance of nociceptive symptoms.

As expected, CCI-induced neuropathy led to the development of mechanical and cold allodynia, in both CCI/OVX and CCI/SHAM females. While ovariectomy by itself did not lead to nociceptive abnormalities, CCI/SHAM and CCI/OVX females displayed differences in the development of mechanical allodynia, with CCI/OVX females showing symptoms two weeks prior to CCI/SHAM. In addition, while mechanical allodynia is gradually worsened in CCI/SHAM females, CCI/OVX partially recovered in the last experimental week. Some studies have shown alterations in sensitivity to nociceptive stimuli following OVX. A recent study by Li and colleagues[238] showed the development of mechanical allodynia 5 weeks after OVX, using the von Frey test, however, in this same study and contrary to our results, no differences were seen between intact and ovariectomized females one-week post-CCI. As Li's assessment was performed only once after CCI induction, it may not reflect the time-dependent development of mechanical allodynia observed herein. These results point to a role of gonadal hormones not only on the early development of CCI-induced allodynia but also in the maintenance of nociceptive symptoms.

The establishment of CCI in our females is further validated by results from the CCI_M group. The development of mechanical and cold allodynia in CCI males followed a similar pattern to that of females. However, despite displaying lower withdrawal thresholds earlier when compared to CCI females at weeks 1 and 2, recovery signs started at week 3 and full recovery was observed at week 5. These results are in clear contrast to those of CCI females as these did not recover to baseline values, displaying nociceptive responses until week 5. This sex-specific difference is in accordance with what was observed by Tall and colleagues[221], where neither ovariectomized nor intact female rats recovered after the induction of neuropathic pain, contrary to what's observed in males by day 35 post-CCI surgery. Another work from Vacca *et al.* [224] also observed in mice that, while males showed a gradual decrease of allodynic responses, females showed no recovery throughout 121 days of CCI. This data supports slower regenerative processes resulting from the injury in female animals when compared with males.

Altogether, our results highlight the existence of sex-differences in the CCI model, not only on the development and maintenance of neuropathic pain but also on general welfare measures such as body weight, as discussed in the technical considerations section. Importantly, we show an impact of ovarian hormones on body weight as well as on the development of mechanical allodynia on CCI females which should be further investigated to verify if it interferes with the efficiency of pain management therapies.

While CCI by itself did not affect the locomotor ability of females, when associated with OVX it highlighted the development of locomotor impairments in CCI/OVX females translated in a significant decrease in the total distance travelled in the OF, probably due to decreased velocity. Accordingly, while the locomotor performance of CCI/SHAM females was identical to that of males, the total distance travelled of CCI/OVX females was significantly lower than that of CCI males. Except for CCI/OVX females, our data is in accordance with literature showing no motor dysfunctions in neuropathic animals[169]. However, a work by Li and colleagues[238] showed ovariectomy reduced the distance travelled in the OF thus support our CCI/OVX data. These results call to the attention that concomitant CCI and ovariectomy impair the locomotor abilities of female animals and these must be considered when performing locomotion-dependent behavioural tests. Additionally, it is important to note that the performance of CCI males and CCI/OVX females cannot be considered comparable.

5.3. Neuropathic pain and the development of mood disorders

In male animals, the development of emotional impairments in CCI has been thoroughly described, both regarding anxiety- and depressive-like behaviour[61], as shown in **Section 1.7**. The opposite is true for female rats thus being of great interest to uncover sex differences on the comorbidity between experimental neuropathic pain and emotional impairments.

In terms of anxiety-like behaviour, our results were not consistent among tests since the increased defecation of CCI females in the OF suggested the development of anxiety-like behaviour, but this observation was not supported by the remaining OF and EPM data. Although negative, our results were not completely unexpected as only one study[175] (**Section 1.7.1**) reported the development of anxiety-like behaviour, using the OF and EPM tests, on right operated intact and ovariectomized female mice on day 26. On the other hand, CCI_M performance indicates the development of anxiety-like behaviour in these animals. The number of *faecal boli* left in the OF arena by CCI_M was similar to that of CCI females, and these animals spent considerably less time in the EPM open arms than their female counterparts. The measurement of *faecal boli* is not validated across laboratories and is usually only used to support other anxiogenic measures. So, while it is interesting that results between CCI females and males were identical, it is not enough to establish an anxious-like phenotype.

One possible reason for CCI females not displaying an anxious-like phenotype is the time point chosen to evaluate. In fact, it has been demonstrated in the cuff model of chronic neuropathic pain[255] that anxiety-like behaviour is present in earlier stages, while depressive-like behaviour develops later on. Accordingly,

a study using the sciatic nerve ligation (SNL) of experimental neuropathic pain[256] showed a decrease in the percentage of time spent in the open arms of the EPM on day 20 post neuropathic pain induction.

Concerning ovariectomy, a recent study by Puga-Olguín et al.[257] showed the development of anxiety-like behaviour, using the EPM, three weeks after ovariectomy through a decrease in the time spent in open arms. Thus, since we tested our animals in week 5, it is possible we lost this window of opportunity. On the other hand, the intensity and timeline of mechanical allodynia settlement in females, especially in CCI/SHAM females, was slower when compared with CCI_M, and, if so, it is possible that more time was needed for the development of anxiety-like behaviour in our CCI females. This assumption is also partly supported by our OVX females data as literature shows only long-term ovariectomy leads to an anxiogenic state in the OF test in female rats and mice[258–260].

In what regards depressive-like behaviours, two different components were assessed: anhedonia through the SPT and learned helplessness in the FST. Identically to what was observed for anxiety, the induction of CCI did not lead to the development of depressive-like behaviour in females.

Considering CCI females still displayed mechanical and cold allodynia at the end of the experimental period, SPT results in CCI females were rather unexpected. In a study using chemically induced experimental gastritis[261], female, but not male, Sprague-Dawley rats displayed decreased sucrose preference. However, there is a strong inflammatory component associated with gastritis which might account for the behavioural differences in females. Our data are nonetheless in accordance with a study using the SNL[262] where females also did not decrease sucrose preference. Comparability between works is however limited since not only the neuropathic pain model used are different, but one evaluation was performed on day 15 post SNL induction and ours much later. As argued previously in relation to anxiety, it is also possible we missed the time window to detect it, thus both an earlier (3-4 weeks) and a later (8-12 weeks) timepoints should be investigated in future studies. Nonetheless, the SPT results in our OVX females are in accordance with previous reports showing the development of anhedonic-like behaviour on day 28[263], 5 weeks[264] and 4 months post-OVX induction[265].

Interestingly, CCI_M displayed anhedonic-like behaviour but not learned helplessness. Of the four works using SPT (**Section 1.7.2**), one in mice showed anhedonic-like behaviour on right side operated animals between weeks 4 and 10 while in rats[170], one study using right-operated Sprague-Dawley rats showed no anhedonia[168] and two using right side operated Wistar Han rats showed anhedonic-like behaviour on days 9, 16 and 28[208,215]. Interestingly, all these studies were performed on right side operated animals, while in

this work CCI was induced on the left. Again, data from CCI_M adds to the literature, showing that while mechanical and cold allodynia was apparently resolved in males, these animals still display anhedonic-like behaviour.

Lack of significant differences between experimental groups in the FST was surprising, especially for the CCI_M group since these animals displayed anhedonic-like behaviour. Concerning CCI females, our results contrast with data from the study using SNL[262] where neuropathic females displayed increased immobility and decreased time spent swimming in the FST. In addition, another work[256] showed similar results but on day 20. Yet, these differences might again not only be related to the use of different experimental models of neuropathic pain but also different testing time points, 15 and 20 days vs. 5 weeks.

Contrary to what was observed for anhedonic-like behaviour, ovariectomy did not induce the development of behavioural despair. The literature on this subject is contradictory, with different results being reported depending on the strain and the testing timepoints chosen. In female Wistar rats, ovariectomy was shown to increase immobility at week 1, but not at weeks 3 and 12[238] or after 15 months[266] post-surgery, which is in accordance with our results. However, increased immobility was observed on recent studies, namely 6 weeks after ovariectomy[257] in Wistar Han rats and in Sprague-Dawley rats at week 5[238]. Thus, and as proposed before, for a better understanding of the effect of ovariectomy on depressive-like behaviours, further studies should include different post-ovariectomy periods to assess the possibility of a time-dependent effect of this behaviour.

As male animals were shown to develop learned helplessness following CCI (**Section 1.7.2**), our CCI_M results come as unexpected. Data from previous works on males show learned helplessness was observed up to week 6 on Sprague-Dawley rats[198] and week 5 on Wistar Han rats[167]. It is possible the amelioration of mechanical and cold allodynia from week 3 onwards prevented the development of learned helplessness in these animals. In fact, a study[208] using right side operated Wistar Han rats showed increased immobility and decreased time spent climbing in the FST on day 28 post CCI-induction but paw-withdrawal threshold remained low. Identically, in another two studies left side operated Wistar Han rats also display increased immobility time in the FST[167,196] and decreased time spent climbing[196] on day 28 but again paw-withdrawal threshold in these animals remained low.

Altogether, our results highlight the existence of sex differences in the development of anxiety- and depressive-like behaviours after CCI-induced neuropathy, as well as an impact of hormonal withdrawal on anhedonic behaviour of ovariectomized female rats.

5.4. CCI-induced changes in RVM neuronal activity

In this work, we assessed whether RVM neuronal activity was affected by CCI-induced neuropathic pain or ovariectomy. The RVM is an area involved in descending modulation of pain, through a balance between the activity of RVM pronociceptive On- and antinociceptive Off-cells. As clinical studies show hypersensitivity to cold and mechanical stimulation are hallmarks of neuropathic injuries[267], when assessing the response of RVM cells to peripheral stimulation we focused on these stimuli.

While the spontaneous activity of RVM WDR and NS cells was not increased prior to VF stimulation in females, we observed in CCI males an increase in the spontaneous activity of NS Off-like cells. This increase in the tonic activity of NS RVM OFF-like neurons can, at least partly, be correlated to the recovery observed in behavioural mechanical allodynia due to the antinociceptive role of these cells. Interestingly, during VF stimulation, the response of WDR Off-like cells in the CCI/OVX group was significantly increased which, considering their antinociceptive role, supports the increased in behavioural mechanical allodynia threshold in comparison with CCI/SHAM females on week 5. CCI-induction *per se* did not alter RVM cells response to von Frey stimulation, which is in accordance with a study by Heinricher *et al.*[268] where, in the SNL model, no differences were observed between Sham and ligated animals during von Frey stimulation.

Regarding tail pinch stimulation, results are more difficult to interpret because the activity of both RVM On- and Off-like cells is enhanced. Nonetheless, our results suggest the heightened response of pronociceptive WDR RVM On-like cells to noxious stimulation is matched by an attempt to counteract it by WDR RVM Off-like cells in CCI/OVX females. Again, this falls in line with the increase in behavioural mechanical allodynia threshold in comparison with CCI/SHAM females on week 5. The increased response of pronociceptive female On-like cells when compared to that of neuropathic males, suggests increased descending pronociception and could partly explain the differences in behavioural mechanical allodynia experienced between female and male CCI animals at the end of the experimental period.

While our results suggest a profound alteration of RVM noxious stimuli processing in CCI females, with increased descending pronociception when compared with CCI males, it is important to remember this noxious stimulation was applied to the tail and not the affected hindpaw.

Despite the fact that CCI did not alter the spontaneous activity of pinch-responsive neurons, previous studies using other neuropathic[269] and inflammatory[270] chronic pain models showed altered spontaneous activity of RVM On/Off-cells. In a diabetic neuropathy model[269], an increase in the spontaneous activity of On-cells, as well as a decrease in Off-cells, was demonstrated. However, in an arthritis model[270], both RVM

On- and Off-cells increased spontaneous activity, similarly to our results. These findings indicate the recruitment of RVM On- and Off-cells differs significantly between neuropathic and inflammatory chronic pain models.

Regarding cold stimulation, the increase in the spontaneous activity of NS RVM On-like neurons in CCI animals suggests enhanced activation of pronociceptive descending pathways with a potential counteracting effect of NS RVM Off-cells in CCI/OVX females. Surprisingly, especially bearing in mind the behavioural cold allodynia data, during stimulation with acetone the response of WDR RVM On-like cells is decreased in CCI females. Our results also in opposition to those reported by a previous study using the SNI model of neuropathic pain[269] and showing an increase in cold-evoked responses of RVM On-like cells at week 8.

Overall the activity of RVM pain modulatory neurones partially supports our behavioural observations, except in what concerns cold allodynia. Differences in mechanical allodynia between CCI/SHAM and CCI/OVX animals are concomitant with subtle changes in both the spontaneous and evoked activity of mainly RVM WDR cells. Differences between the activity of RVM neurones of CCI females and males suggest enhanced activity of descending pronociceptive pathways in the former. Nociceptive recovery of CCI males appears to be associated with an overall concomitant increased in the spontaneous activity of RVM NS antinociceptive neurones and a decrease in the evoked activity of RVM WDR and NS pronociceptive cells.

Additionally, studies point to a possible role of Neutral cells in nociceptive modulation[271] despite their lack of response to acute peripheral noxious stimulation. The latter were not assessed in this work however they emerge as a potential target of future studies.

In sum, these results suggest an increase in descending pronociception in female rats by both CCI and ovariectomy, modulated by the RVM. This pronociceptive shift is known to facilitate nociceptive signaling and could explain the exacerbated mechanical allodynia seen in females, in comparison with males.

5.5. Histological evaluation of the sciatic nerve

We evaluated whether CCI induced histopathological changes in the structure of the sciatic nerve. During sampling of the nerve, we observed the ligated region was opaque in a dull yellow color, and through H&E staining, we observed that nerve constriction led to degraded myelin sheets and inflammation. These are common consequences of peripheral nerve injury, confirming the proper induction of the CCI model, as well as the inflammatory response induced by the chromic gut suture. This same CCI-induced pathology

was also observed in previous studies, from nerve derangement to inflammation on the affected site[272,273]. Interestingly, we observed an increase in inflammatory infiltrates in CCI/OVX females which could be explained by the more pro-inflammatory state induced by ovariectomy[274].

Nonetheless, our analysis had its setbacks as some of the sciatic nerves were damaged throughout processing and prevented a clear visualization of the nerve structure. As well, the absence of data from males due to time-constraints hinders comparisons between neuropathic males and females. It would be of interest in the future for these samples to be assessed by a senior neuropathologist to confirm our results. Future studies could also include the evaluation of demyelination in the sciatic nerve of neuropathic animals through more thorough histological and techniques.

Chapter 6. Concluding remarks and future perspectives

In the present thesis, using a behavioural and electrophysiological approach, we demonstrated: (i) CCI lead to the development of both mechanical and cold allodynia in female rats, that, contrary to what is observed in males, do not show signs of recovery at 5 weeks post-CCI surgery; (ii) CCI did not induce the development of anxiety or depressive-like behaviours in females; (iii) ovariectomy induced anhedonic-like behaviour, and locomotor impairments in CCI females; (iv) neuropathic males perform differently from females on anxiety- and depressive-like behavioural paradigms and (v) CCI and ovariectomy induced alterations in the neuronal activity of the RVM, with increased descending pronociception on week 6 post-CCI surgery.

Our results highlight the existence of sex differences in the development and maintenance of CCI-induced neuropathic pain as well as emotional comorbidities. Thus, our data challenge the assumption the use of male subjects on basic research is equivalent to that of females and straightforwardly translatable to women. Future studies should try to elucidate the mechanisms underlying these sex differences, and how this impacts the efficiency of chronic pain management therapies.

The observed differences between gonadally intact and ovariectomized females further confirm the role of gonadal hormones in this neuropathic pain-emotions comorbidity. Further work should investigate hormonal replacement therapy to determine which and how gonadal hormones are involved in these alterations. In addition, it would be of interest to assess if ovariectomy would differentially affect the development of emotional impairments if performed after the induction of peripheral neuropathy.

It remains to be studied how CCI-induced changes in the response properties of RVM neurons observed could be altering supraspinal processing or the response of spinal dorsal horn neurons after peripheral neuropathy, as well as if these can be modulated through pharmacological intervention. Also, possible cellular and structural changes could be associated with the development of chronic neuropathic pain and further studies could include the stereological analysis of the RVM as well as other areas involved in pain and emotional modulation.

Chapter 7. References

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