The effect of postnatal morphine exposure on spinal processing of sensory inputs

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Amy Platten

20161058

Supervisors: Professor Gareth Hathway & Professor Victoria Chapman

School of Life Sciences, Faculty of Medicine and Health Sciences

University of Nottingham

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Contents

Abstract

Neonates and children differ from adults in pain processing, particularly in descending pain pathways, which mature from facilitatory to inhibitory during a critical period in postnatal development. Opioid exposure has been shown to accelerate this maturation process. Therefore, this study aims to further understand the effect of postnatal morphine exposure using immunohistochemistry to analyse markers in rat spinal cord dorsal horns. We examined primary afferent termination patterns, mu opioid receptor (MOR) expression, markers parvalbumin (PV), and protein kinase C gamma (PKCγ), and explored potential sex-specific interactions. We hypothesised that, following morphine exposure there would be an increase in PV and MOR intensity, but no significant changes in primary afferent termination. Our findings showed a significant elevation of IB4 intensity in male rats following morphine exposure, but no significant differences in MOR intensity. Morphine exposure revealed an increased neuronal cell count in PV labelling area for females, and an increased PKCγ total intensity in male rats. Overall, this study demonstrates that exposure to opioids during critical periods of postnatal development can influence nociceptive markers later in life, with sex differences, highlighting the importance in the inclusion of both sexes in pain and opioid research.

Introduction

1.1 What is Pain?

Simply put, pain is a subjective unpleasant experience, however it is far from simple. The multidimensional nature of pain means defining the concept is complex, particularly in appreciating both the sensory and emotional aspects.

The International Association for the study of Pain (IASP) updated the definition as "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" (Raja et al. 2020). The key change from the 1979 version is in the wording, regarding the ability to describe one's experience of pain, whereby the previous definition included "or described in terms of such damage" (IASP). The replacement of this language was to enable the definition to be inclusive of infants, elderly and those who cannot verbalise their painful experience (IASP).

IASP defines the term 'nociception' as the "neural process of encoding noxious stimuli". This definition refers specifically to the physiological processes underlying the detection and transmission of harmful stimuli, distinct to the perceptual and subjective experience involved in pain. The difference in terminology is essential, particularly in animal studies where there is an inability to receive feedback or any description of pain, and therefore focus on observable nociceptive responses (National Research Council, US. 2009)

1.2 Epidemiology of pain

Though pain is a universal experience, the Global Burden of Disease study in 2016 confirmed that worldwide, the prominence of pain and pain-related diseases is the leading cause of disability and disease burden (GBD, 2016). Pain that is persistent beyond three months is considered to be chronic pain, and it is believed to affect between 35% to 51.3% of the UK population, and interestingly found to be more common in females than males.

To make sense of this figure, it translates to almost 28 million people in total (Fayaz et al. 2016). Although there is a trend of increased prevalence with increasing age, the occurrence of chronic pain in people aged 18-39 may still be as high as 30% (Fayaz et al. 2016). Pain can have devastating societal impacts on an individual's livelihood and quality of life. Furthermore, it is estimated that in Europe alone, the financial burden of pain is suggested to be between 1.5-3% of GDP, accounting for significant expenditure in healthcare sectors (Phillips, 2006). This indicates the importance of furthering our understanding of pain to find long-lasting, effective solutions in the alleviation of pain.

1.3 Peripheral mechanisms of pain

The complexity of the peripheral nervous system (PNS) that stems beyond the brain and spinal cord, is a sophisticated and extremely intricate sensory network, structurally consisting of twelve pairs of cranial and thirty-one pairs of spinal nerves (Akinrodoye & Lui, 2022). It can be broadly categorised into two domains: the autonomic and somatic subsystems. The former is made up of the parasympathetic, sympathetic and enteric systems that are responsible for the automatic bodily functions referring to those such as heart rate, breathing, blood pressure, digestion (Hall, 2023). The latter is responsible for functions we manage, and consciously influence, including the voluntary movement via skeletal muscles in our body. It includes both sensory (afferent) and motor (efferent) nerves that connect our central nervous system (CNS) to our organs and striated muscle to allow us to perform daily functions (Akinrodoye & Lui, 2022). It also involves the reflex arc: the automatic response between sensory neuronal input to create a specific motor neuronal output. The simplest is known as the monosynaptic reflex that comprises of one synapse between the sensory and motor neurons (Akinrodoye & Lui, 2022).

Of particular interest is the somatosensory system, that conveys information from the skin, viscera, bone and muscles regarding sensorial stimulation, such as pain

temperature, touch (Murtazina & Adameyko, 2023). To identify and process a noxious stimulus, e.g. burning your hand on a hot surface, will firstly involve the activation of nociceptors in peripheral tissue (Osterweis et al. 1987). There are various cutaneous receptors that are selectively sensitive to certain stimuli. For example, there are low threshold mechanoreceptors that will encode information regarding texture and shape. A specialised subset of cutaneous receptors, of notable focus, are the relatively high threshold receptors that respond preferentially to a noxious stimulus, known as 'nociceptors' (McMahon, 2013). Nociceptors have free nerve endings in the cutaneous skin, viscera and muscles and are polymodal, in that they respond to tissue-damaging stimuli activated by major modalities such as; mechanical (i.e. pressure), thermal and chemical stimulation (Purves et al. 2001). These sensory receptors can also be classified into rapidly adapting or slowly adapting (tonic); the former displaying a maximal but brief response to stimuli, decreasing if the stimulus is sustained, and the latter involving receptors that continuously transduce signals as long as the stimulus is maintained and present (Purves et al. 2001).

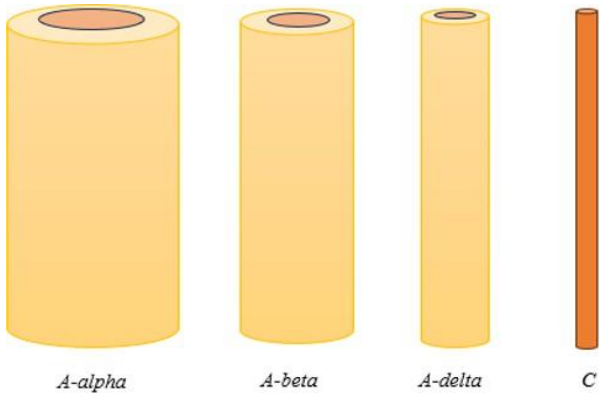
1.3.1 Primary afferent fibres

The nociceptors relay the signal and detection of noxious stimuli onto specialised neurons of the peripheral nervous system called primary afferent fibres (PAF). They are the integral in conveying sensory information from the periphery to the CNS (Hunt & Mantyh. 2001). The somata of these nociceptive primary afferent fibres that innervate the head are situated in the trigeminal ganglia, whereas the cell bodies of those that innervate the body are located in the Dorsal root ganglion (DRG). With a bilateral structure that spans the periphery to the spinal cord (SC) and vertebral column, the DRG is involved in

sensory transmission, including the transduction of pain (Ahimsadasan et al. 2022, Julius & Basbaum, 2001).

These sensory fibres can be classified into different groups based on anatomy, the modalities that are evoking responses, neurochemical markers, as well as conduction velocities (Todd, 2010, see figure 1.1). Electrophysiology studies established a correlation of afferent fibre activity with certain sensory modalities using a cathode-ray oscilloscope (Erlanger et al. 1924, Perl. 2007). Erlanger et al. (1924) discovered that brief electrical stimuli to peripheral nerves evokes a sequence of electrical waves. Thus, subsequent studies on the compound action potential showed correlation of differing primary afferent fibre activity, with the sensory stimuli invoked (Perl. 2007). There are three anatomically distinct groups of myelinated A-fibres (conduction velocity >2 m/sec), and unmyelinated, smaller diameter C-fibres (conduction velocity <2 m/sec) (Hunt & Mantyh, 2001, McMahon, 2013). The largest diameter Aα fibres are responsible for proprioceptive information, such as transmitting signals from muscle spindles and golgi tendon organs. The Aβ are low-threshold fibres that convey sensory information from mechanoreceptors relating to touch, vibration and pressure. However, it is important to note that there have been in vivo studies that demonstrate the presence of Aβ nociceptors using intracellular recordings in DRG neurons, showing a significant proportion of A-fibre nociceptors do in fact conduct within the Aβ-fibre conduction velocity in cats (Koerber et al. 1988) as well as in the rats (Ritter & Mendell, 1992). The Aδ-fibres carry information about temperature and nociceptive signalling, and slower conducting C-fibres that are also responsible in nociception signalling, activated by various modalities

such as mechanical, thermal and chemical stimuli. This is not an exhaustive list of the functions of c-fibres with evidence of tactile c-fibres role in affective touch sensations (Yam et al. 2018, Liljencrantz & Olausson, 2014). It is generally recognised that Aδ respond to the 'first' acute, sharp pain and C-fibres mediate the poorly localised, dull, delayed pain (Basbaum et al. 2009).



Nerve fibre	A-alpha	A-beta	A-delta	C
Size	Large, myelinated (13-20um)	Medium, myelinated (6-12um)	Small, myelinated (1-5um)	Small, unmyelinated (0.2-1.5um)
Conduction velocity	80-120 m/s	33-75 m/s	3-30 m/s	0.5-2 m/s

Figure 1.1 An adapted illustration and description of the properties of primary afferent fibres regarding their size, conduction velocities (Yam et al. 2018, McMahon, 2013).

The heterogeneity of these nociceptive fibres is demonstrated by the further classification based on the biochemical markers they express or release. They can be categorised between 'peptidergic' neurons, those that contain peptides (e.g. Substance P or calcitonin gene-related peptide- CGRP) and 'non-peptidergic', those that do not (McMahon. 2013). They express different receptors and project to distal dorsal horn (DH) laminae, with peptidergic fibres predominantly projecting to lamina I and II outer, whereas non-peptidergic fibres tend to project to lamina II inner. Therefore, these different fibres may

show different sensitivity to a given stimulus (McMahon. 2013, Woolf & Fitzgerald. 1986).

1.4 Central mechanisms of pain

Primary afferent fibres innervate almost exclusively in the DH of the SC, the first synapse in the pathway of ascending sensory information to perceive pain. The laminae of the of spinal cord were first anatomically organised by Rexed (1952) studying a cat (see figure 1.2). The six parallel laminae in the DH were divided based on anatomical features of neurons such as the size and packing density and differences between them. It has since been applied to other species including rat, monkey and human (McMahon. 2013, Rexed. 1952).

1.4.1 Organisation of the spinal cord

The interior anatomy of the SC is formed by grey matter that is divided into sections referred to as: dorsal (posterior) and ventral (anterior) horns (Purves et al. 2001). The white matter, surrounding the gray matter in the spinal cord, is also divided into dorsal, lateral and ventral columns that each contain axon tracts with specific functions. For example, the dorsal column carries ascending sensory signals from somatic mechanoreceptors, whereas the ventral columns carry both descending motor information, as well as ascending pain, and temperature information to higher brain centres (Purves et al. 2001).

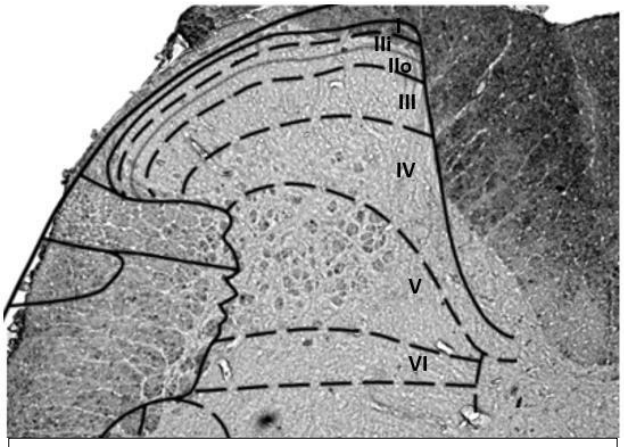


Figure 1.2. An illustration of the laminae portraying the anatomical layers of the rat spinal cord dorsal horn (adapted from Rexed 1952)

Lamina I, also referred to as the marginal layer, is the most superficial dorsal layer of the DH. It contains both projection neurons and interneurons of diverse sizes and shapes, with most dendrites of the cells remaining within the lamina. Lamina II, the substantia gelatinosa, has a translucent appearance due to the lack of myelinated fibres within the layer. The majority of neurons here are interneurons, and the most outer layer of lamina II are where they are densely packed. These layers of the DH receive input from the small diameter Aδ- and C-fibres that innervate all tissues of the body and therefore is essential to the processing of noxious stimuli (McMahon, 2013). Lamina III also displays a high density of interneurons but are somewhat larger than those visible in lamina II. A distinct feature that separates lamina II and III is the myelination present in lamina III (McMahon, 2013). In the deep DH, lamina IV-VI become more difficult to distinguish with definitive borders due to the heterogenous nature and various sizes seen across the laminae, here primarily receives Aα and Aβ-fibre input (McMahon, 2013). In the deeper DH are wide dynamic range (WDR) neurons, that are distributed at a depth of 500-1200 μm beneath the surface of the DH, most concentrated in laminae V-VI (Zhang et al. 2024). The 'WDR' term is based on the polymodal properties of these neurons. Laminae IV and VI are predominantly inhibitory interneurons, whereas lamina V contains a greater proportion of excitatory interneurons. They respond to both noxious and innocuous stimuli and are important in the modulation and integration of sensory inputs. WDR neurons exhibit the 'wind-up' phenomenon that essentially describes the increase in excitability of the spinal cord neurons from repetitive stimulation (Herrero et al. 2000). It has been implicated in the development of chronic pain as an explanation of the amplification and maintenance of transmitting pain signals (Herrero et al. 2000, Zhang et al. 2024)

1.4.2 Sensory integration

Primary afferent fibres, upon stimulation, release various chemical mediators into the synaptic cleft between the fibre terminal and postsynaptic neurons in the DH, where excitatory neurotransmitter glutamate is predominantly released. These neurons that form excitatory synapses to PAFs in the DH include projection neurons (PNs) (McMahon, 2013). The axons of these PNs transmit information to supraspinal targets including the thalamus, the midbrain periaqueductal gray (PAG), as well as various areas of the medulla (McMahon, 2013). However, the majority of neurons in the DH have axons and dendrites that remain within the spinal cord, contributing to the local neuronal circuit, and thus described as locally projecting interneurons. These can be categorised into two major groups of inhibitory or excitatory, based on the neurotransmitter expression profile (Abraira & Ginty, 2013).

During the early 1960s, the first revolutionary model that defined sensory processing, at the level of the spinal cord, was developed and is known as the Gate control theory (GCT) of Pain (Melzack & Wall, 1965). The theory accepts the notion of nociceptors and innocuous touch fibres, and postulates that there are essentially ‘gates’ in the SC. These gates can modulate the transmission of sensory information from the primary afferent fibres to ‘transmission cells’, with control large fibres closing the gate, or essentially inhibiting the sensory output. If a stimulus reaches a threshold of noxious activity detected by small fibres, the gate opens, facilitating the transmission and ultimately experience of feeling pain (Moayedi & Davis, 2013, Melzack & Wall, 1965). The GCT remains a foundational model to understand pain due to its oversimplification and exclusion of numerous aspects, such as the implications of neuroimmune interactions. Nonetheless, it

was pivotal in advancing our understanding that sensory signals can be modulated at the SC (Moayedi & Davis, 2013).

1.5 Central mechanisms of pain processing

The interaction between spinal and supraspinal sites is required for the transmission of nociception as studies have indicated that activity of peripheral nociceptors and fibres within the DH, does not always correlate to the severity of pain measured at behavioural level. Activity beyond the SC also contributes in the sensitivity of pain (Kwok et al. 2014).

1.5.1 Ascending pathway

Conveying pain and sensory information to higher brain centres from noxious evoking stimuli will activate projecting neurons in the ascending pathway. The main ascending pathways in processing pain are components of the anterolateral system in the SC, and play a role in perception, motivation and the emotional response to pain (Wang et al. 2022). The spinothalamic tract (SST) terminates in the thalamus and is primarily involved in the sensory-discriminative aspect of somatosensation, such as the location, intensity and duration of pain, temperature, crude touch and pressure (McMahon, 2013, Wang et al. 2022). The Spinoreticular tract (SRT) projects to the reticular formation in the brainstem and thus, has a role in the autonomic responses to pain, as well as the arousal and attention of pain (Willis & Westlund, 1997, McMahon, 2013). The spinomesencephalic tract (SMT) projects from the DH to the periaqueductal gray (PAG) in the midbrain, responsible largely in the modulation of pain and reflexive responses (Willis & Westlund, 1997, McMahon, 2013). The spinoparabrachial tract (SPBT) contributes largely to the emotional and autonomic responses to pain, with projections

from the DH to the parabrachial nucleus in the pons, and relaying signals to limbic structures such as the amygdala (Wang et al. 2022).

The SST, is comprised of two pathways: anterior (specifically carrying input regarding touch) and lateral (regarding pain and temperature), is responsible to convey nociceptive information from our peripheral to the somatosensory region of the thalamus in the brain (see figure 1.3). It is part of the anterolateral system in the SC (Al-Chalabi et al. 2023, McMahon, 2013). The axons of SST neurons arise in DRG and cross over the SC to the opposite side via the anterior white commissure, to then ascend through the length of the SC in the ventrolateral region of spinal white matter. The spinothalamic tract second order neurons terminate in the ventral posterolateral nucleus (VPL) of the thalamus. The third order neurons then terminate in the primary somatosensory cortex (Al-Chalabi et al. 2023).

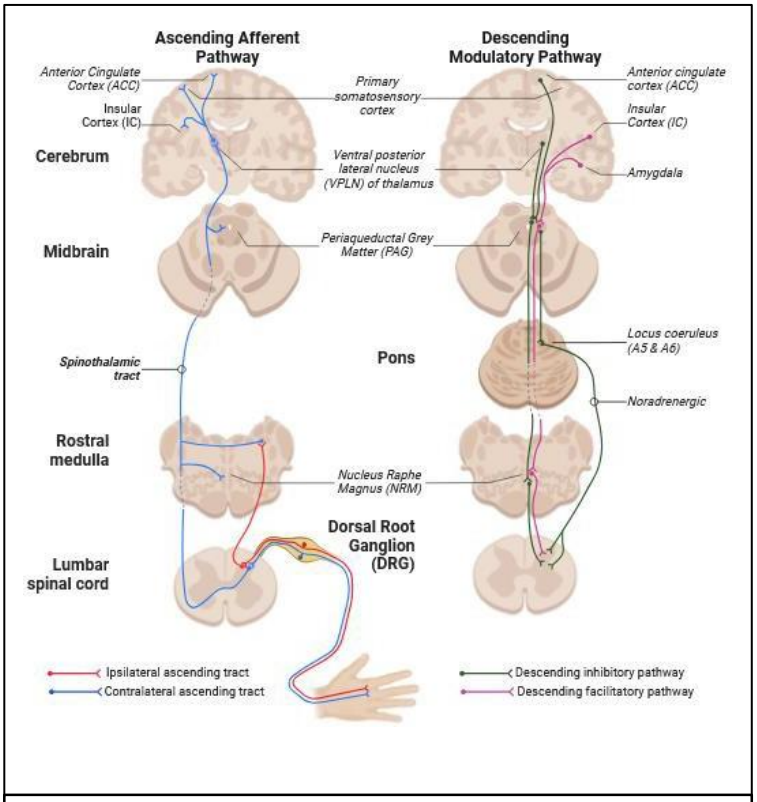


Figure 1.3. A diagram of the ascending and descending pain pathways to illustrate the conveying of pain from the periphery to higher brain regions, and the modulatory processing from the brain to the spinal dorsal horn (adapted from Biorender.com)

It is not one specific brain region wholly responsible for processing pain, but rather an activation of various structures. However, there are regions more responsible for certain components than others. For example, the thalamus has considerable connections to the

somatosensory cortex, integrating information amongst cortical regions (Yao et al. 2023). The somatosensory cortex is significantly involved in discriminating the location and intensity of pain (Kenshalo & Isensee, 1983). Other regions more involved in the affective, emotional aspects of pain processing are regions such as the parabrachial nucleus, that receives nociceptive input from the SC and projects to numerous regions, such as the amygdala. Projections to the amygdala, anterior cingulate cortex and other limbic system regions are widely recognised as areas responsible for processing the emotional, fear-related aspects of pain and lesions in these areas have altered emotional responses to pain (Berthier et al. 1988, Bushnell et al. 2013). Though this is not an exhaustive list of higher brain centres involved, it reveals the 'pain matrix' of an interconnected network that contribute to our experience and perception of pain (Yao et al. 2023).

1.5.2 Descending pathway

Decades of research has led to the recognition that the descending pathway (see figure 1.3) has the powerful ability to control the transmission of pain signaling at the level of the SC, as it can have a bidirectional influence on pain (Basbaum et al. 2009). Originating in different higher regions (prefrontal cortex, anterior cingulate cortex, amygdala, hypothalamus and periaqueductal gray), the descending pathway modulates nociceptive signaling by transmitting signals via the brainstem nuclei in the PAG and medulla that project down to the SC (Millan, 2002, Yao et al. 2023).

The PAG is the gray matter around the cerebral aqueduct located within the tegmentum of the midbrain. It was in 1969 that Reynolds et al, whilst completing abdominal surgery on rats without chemical anaesthesia, discovered that stimulating the midbrain PAG had a strong analgesic effect (Ossipov et al. 2014). It is thought that the PAG primarily exerts

its descending influence via its reciprocal connections with the rostroventromedial medulla (RVM). There is evidence of PAG neuron excitation also exciting RVM neurons, resulting in inhibition reflexes to nociceptive input in rats. The spinal termination pattern of RVM descending axons is the densest in the substantia gelatinosa, as well as lamina V (Ossipov et al. 2014, McMahon et al. 2013, Behbehani et al. 1979).

The RVM also includes the serotonergic nucleus raphe magnus (NRM), the nucleus reticularis gigantocellularis-pars alpha as well as the nucleus paragigantoceulluaris lateralis (Vanegas & Schaible, 2004). This structure is known to be integral in opioid analgesia. It is thought that there are three physiologically defined classes of cells in the RVM; 'neutral' that display no change in activity in response to noxious stimuli or administration of opioids, 'ON cells', and finally 'OFF cells', that do have roles in modulating nociception. 'On cells' are characterised by sudden, excitatory amino acid-mediated (i.e. glutamate) burst of firing, that occurs just prior to the reflex of noxious input i.e. tail flick response from noxious heating of a rodent tail. Hence, the activity of these particular cells indicates a facilitatory response of neurons on nociception (Vanegas & Schaible, 2004, Heinricher & Roychowdhury, 1997). Electrode recordings have shown that 'off-cells' exhibit an abrupt pause in firing at the time of nociceptive reflex, mediated by gamma-aminobutyric acid (GABA). These neurons therefore exert an inhibitory response on nociception (Vanegas & Schaible, 2004, Heinricher & Roychowdhury, 1997). In fact, Urban et al. (1996) found mustard oil (a chemical irritant which selectively activates C-fibers) induced facilitation of the tail-flick reflex, which was then significantly inhibited in rats with electrolytic lesions in the RVM. With the descending projections of the RVM to the DH, it provides a neuronal context for both the positive and negative inflection on pain from the PAG/RVM network (Ossipov et al. 2014).

Though the PAG-RVM is the most studied descending pathway, it is not the only

mechanism that modulates pain. The locus coeruleus (LC) is an important structure in the brainstem that projects descending fibres to the DH, and releases noradrenaline that act on α2-adrenergic receptors to supress nociceptive signalling at the level of the SC (Pertovaara, 2006). Understanding the holistic role of this pathway and the implication of the LC is ongoing research. Particularly in understanding its potential affiliation to chronic pain states and the development of allodynic symptoms, that indicates a bilateral effect in pain processing, in certain pathological instances (Llorca-Torralba et al. 2016).

1.5.3 Sex-dependent effects in descending control

Population-based studies have indicated that women are more likely to experience chronic pain syndromes, and more frequently report a higher intensity of pain than men (Andersson et al. 1993, Lee & Ho, 2013). It is widely recognised that are sex differences in pain, but what remains less clear are the brain mechanisms for these differences (Failla et al. 2024). For example, a study measured descending control nociception by an increase in the percentage of response in hind-paw withdrawal (longer latency to withdraw indicating analgesia) after administration of capsaicin to the forepaw. Results indicated that naïve male rats may possess a stronger descending control of nociception response in comparison to naïve female rats (Fiatcoski et al. 2024).

Analysis of all quantitative rodent research regarding sex differences in pain showed that 85.4% of female rodents were reported to exhibit greater sensitivity to pain than male rodents. Furthermore, it was also reported that across the research, male rodents (77.4%) were more sensitive to analgesics (majority being opioids) in comparison to female rodents (Mogil, 2020). Studies have shown that females have approximately 33% more RVM-projecting neurons in the descending circuit than males, and females need 30%

more morphine in order to achieve a similar level of analgesia compared to male counterparts (Lloyd & Murphy, 2014, Cepeda & Carr, 2003). This research supports the notion that there is clear sexual dimorphism in the descending modulatory pathway.

1.5.4 Central Sensitisation

Central sensitisation details the amplified response and function of nociceptors and neurons in the CNS. This heightened response is developed from persistent noxious input, leading to increased membrane excitability, synaptic efficacy and the decrease of inhibition that results in hypersensitivity to stimuli, including innocuous sensations causing allodynic symptoms (Latremoliere & Woolf, 2009). Changes in the properties of CNS neurons subsequently changes the sensory response. Central sensitisation involves the recruitment, of what was formerly subthreshold synaptic inputs, to nociceptive neurons (Latremoliere & Woolf, 2009). This causes profound alterations to receptive field thresholds, as well as the temporal and spatial properties, indicating the high malleability and plasticity of somatosensory neurons. It demonstrates a major functional shift from high to low-threshold pain sensitivity that ultimately increases sustained excitation at level of the DH and is fundamental in the development of chronic pain conditions (Latremoliere & Woolf, 2009).

1.6 Neurotransmitters in pain modulation

As previously mentioned, the PAG/RVM system is an established pain-modulating network and supports the analgesic effects of opioids. There are three classic opioid receptors: μ-mu, δ-delta, and κ-kappa, as well as the later discovered fourth receptor ORL1- opioid receptor-like receptors. These are all present in both the PAG, RVM and DH respectively (McMahon, 2013). Distribution of opioid receptors (OR) vary in the different structures they are expressed. They are primarily expressed in the cortex, limbic

system and the brain stem, and though the binding sites overlap between the three in most structures, there are certain regions that will contain greater expression of one receptor over others (Le Merrer et al. 2009). For example, Mu opioid receptor (MOR) is most abundantly expressed in the amygdala, thalamus and certain brain stem nuclei, whereas delta is the most expressed OR in the olfactory bulb (Le Merrer et al, 2009). Expression of MOR in the DH is located pre-synaptically on terminals of primary afferent fibres, and in laminae I and II, expression is seen post-synaptically of neurons in the DH (Moriwaki et al. 1996).

The pharmacology, in particular of in the PAG-RVM system, has various neurotransmitters and neuropeptides that have various actions both anti and pro-nociceptive effects (McMahon et al. 2013).

1.6.1 Endogenous & Exogenous opioids

The action of endogenous opioids is regulated by the specific OR to which they bind as agonists. The three distinct endogenous opioid peptides: Endorphins, enkephalins and dynorphin are all neurotransmitters that induce effects by changing the electrical properties of the respective target neurons (Shenoy & Lui, 2023, Winters et al. 2017). Each of the opioid peptides have both pre- and pro-forms that are cleaved sequentially. They are modified by post-translational actions such as acetylation, methylation and phosphorylation that can alter potencies, receptor affinity and selectivity and therefore pose critical steps in the regulation of the endogenous opioidergic system (Shenoy & Lui, 2023, Le Merrer et al. 2009). The endogenous opioids are derived from precursors prodynophin (PDYN), pro-enkephalin (PENK) and pro-opiomelanocortin (POMC) (Le Merrer et al. 2009).

It is widely accepted that through binding and activation of MOR, endogenous opioids can produce pain relief. Analgesia from electrical stimulation of the PAG was reversed when naloxone, a non-specific opioid antagonist, was administered in 41 Sprague-Dawley male rats (Akil et al. 1976). This effect was also seen in a clinical case with a male, where the study wanted to investigate these effects in humans (Adams et al. 1976). The research carried out suggested that stimulation-induced analgesia released, what we now know as, endogenous opioids. A study by Yaksh et al. (1977) demonstrated that intrathecal administration of the endogenous opioid enkephalin produced analgesia in groups of four to eight male albino rats per condition. This effect is seemingly mediated via the activation of the same opioid receptors that bind to morphine, as the analgesic response was also antagonised by naloxone.

Likewise, exogenous opioids such as morphine, heroin and fentanyl are all substances that act and bind to the same opioid receptors, both peripherally and centrally. Morphine is acknowledged as the gold standard analgesic drug; it was first isolated in the year 1804 by German pharmacist Freidrich Willhelm Adam Serturner (Trescot et al. 2008).

Each OR is G-protein coupled and contains an extracellular N-terminus, seven transmembrane helical twists and three extracellular and intracellular loops, and finally an intracellular C-terminus. Upon activation from either endogenous or exogenous opioids, a cascade of actions occurs. Part of the G protein is released and diffused within the membrane to reach its target, that being an enzyme or an ion channel (Trescot et al. 2008). The activated targets alter phosphorylation via inhibition of cyclic adenosine monophosphate (cAMP). This molecule acts as an intracellular secondary messenger that then activates protein kinases, and longer-term effects could incur through triggered gene transcription proteins or gene transcription (Trescot et al. 2008). When the OR located on the presynaptic terminals of C and Aδ-fibres are activated, it will result in the indirect

inhibition of voltage-gated calcium channels and consequently decrease levels of cAMP (Trescot et al. 2008). Ultimately blocking the release of neurotransmitters from nociceptive fibres (for example: glutamate; substance P and calcitonin gene-related peptide). These series of actions result in analgesia (McCleane & Smith, 2007, Trescot et al. 2008).

1.6.2 Other major pain-mediating neurotransmitters

Glutamate, Substance P (SP), GABA and Calcitonin Gene-Related Peptide (CGRP) and Adenosine triphosphate (ATP) are all neurotransmitters that mediate the process of nociception, though this is not an exhaustive list of all that are involved.

Of the excitatory neurotransmitters, glutamate is most abundant. It is active in contributing to over 50% of synaptic connections in the brain, as well as being present at sites of peripheral inflammation (Yam et al. 2018). Glutamate acts at two types of receptors: ionotropic and metabotropic (e.g mGluR1). Alpha amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) and N-methyl-d-aspartate receptors (NMDAR) are examples of ionotropic receptors found on sensory neurons and when activated, cause an influx of Na^+ ions, depolarising the cell and thus making it more likely to fire an action potential (Yam et al. 2018). Repeated stimulation also leads to the co-release of other neuropeptides such as SP that will bind to their respective receptor. These neuropeptides are abundant in the DH causing excitation, that removes the magnesium ($Mg2^+$) block found at resting membrane potential at NMDA receptors. At this receptor, glutamate and co-agonist glycine, a major inhibitory neurotransmitter, will bind to the NMDA receptor, causing an influx of calcium ions and thus resulting in hyperexcitability in the postsynaptic neuron, sending the pain signals across the neuronal pathway (Yam et al. 2018)

Substance P is a neuromodulator that plays a role in mediating both the inflammatory response in mediating the production of cytokines, but also has a facilitatory role in nociception and chronic pain models (Sahbaie et a. 2009). SP is predominantly found in the DH of the SC and in C-fibres of the PNS. The molecule binds to Gq-coupled receptor neurokinin type 1 receptor (NK_1) and via the phospholipase C (PLC) signalling pathway will trigger an increase of intracellular Ca^{2+} ions (Yam et al. 2018). A study showed that by using the neurotoxin [Sar^9,$Met(O_2)$]-substance P-saporin (SSP-SAP) to selectively ablate NK1 receptors, the pronociceptive effects decreased, and was found to prevent the mechanical hyperalgesia induced by joint injury models (Weisshaar et al. 2014).

CGRP is also related to the excitatory effects of SP that cause the release of Ca^{2+} ions, as its receptors are G_s-protein coupled metabotropic receptors. It is produced both in the CNS and PNS, but studies using both electrophysiological and immunohistochemical approaches have demonstrated that CGRP-expressing neurons are either C or Aδ peripheral afferent fibres (Iyengar et al. 2017, Lawson et al. 2002)

GABA is a major inhibitory neurotransmitter that is most widely distributed in a mammalian CNS. It is widely found in interneurons of the SC, neocortex, as well as the cerebellum, and is thought to contribute to approximately 40% of synapses in the brain (Watanabe et al. 2002). GABA binds to ionotropic $GABA_A$ receptors, or metabotropic $GABA_B$ receptors, with the former permeable to an influx of extracellular Cl^- ions into the neuron; causing a reduction in membrane potential, decreasing the likelihood of an action potential and thus an inhibitory effect. The latter metabotropic receptors are concentrated at presynaptic nerve terminals. When bound by GABA, as they are G_i-protein coupled, causes inhibition through downstream signalling, that results in an increase in cyclic adenosine monophosphate (cAMP) (Yam et al. 2018, Hyland & Cryan,

2010). GABA-dominant inhibitory interneurons in the DH are mainly found in lamina I-IIo. Whereas glycine, another major inhibitory neurotransmitter, is important in the regulation of inhibitory tone in neurons of lamina II-III (Bardoni et al. 2013)

1.6.3 The role of Parvalbumin and Protein Kinase C in pain

Parvalbumin (PV) is a small, acidic, calcium-binding protein that a population of spinal interneurons express. Within the DH, parvalbumin-expressing interneurons are found largely in lamina IIi and III. Immunohistochemical studies have demonstrated that approximately 75% of PV-positive cells in laminae I-III express both inhibitory neurotransmitters GABA and glycine; the remaining population of PV-positive cells are thought to be glutamatergic excitatory interneurons found to populate primarily lamina I and IIo, though these proportions do vary slightly within the literature (Laing et al. 1994, Gradwell et al. 2022). Presynaptic inhibition of inhibitory parvalbumin expressing interneurons (iPVIN) is mediated by the transmission of GABA, and postsynaptic inhibition was found to be mediated by both GABA and glycine, the latter signalling more prominent (Gradwell et al. 2022).

Interestingly, many have identified that there are a population of these inhibitory PV-interneurons (iPVIN) that have a ‘gate-keeping’ like role in controlling the transmission of nociceptive information to the higher brain regions by essentially preventing peripheral touch inputs from activating the nociceptive pathways previously mentioned (Qiu et al. 2024, Petitjean et al. 2015). In fact, recent approaches using pharmacogenetic activation in mice demonstrated its contribution as selective ablation of these iPVINs produced neuropathic pain-like mechanical allodynia. This hypersensitivity was attenuated when PV neurons were activated in nerve-injured mice. Thus, indicates that following nerve injury, an increase in PV-interneuron activity has the ability to reduce the mechanical hypersensitivity (Petitjean et al. 2015).

Qiu et al. (2024) showed that this decreased inhibition that occurs from iPVIN could be due to the change in firing pattern in these neurons. Parvalbumin may be sustaining the high frequency tonic firing patterns exhibited at normal conditions. However, with nerve injury, Qiu et al. (2024) discovered a transition to adaptive firing patterns from PVNs and there was a decrease in the expression of PV. This change in firing pattern was a result of the recruitment of calcium-activated potassium channels. Additionally, blocking these channels in chronic pain attenuated symptoms and restored the tonic firing pattern (Qiu et al. 2024).

Furthermore, with the use of electron microscopy, there have been synaptic contacts identified between the terminals of parvalbumin positive interneurons, and protein kinase C gamma (PKCγ) positive excitatory interneurons (Petitjean et al. 2015). Moreover, ablation of the PV neurons or following nerve injury, the number of these synaptic contacts is significantly reduced, meaning there is no disinhibition of these PKCγ interneurons, and as a result, lead to mechanical allodynia. In both conditions of nerve injury and ablated PV neurons, inhibiting these PKCγ interneurons reduced mechanical allodynia (Petitjean et al. 2015).

PKCγ is part of the classical PKC family of serine- and threonine-specific protein kinases, that functionally have been identified by similar enzymatic properties (Site & Parker, 1991). The PKCγ mRNA is solely found in the spinal cord and brain, and neuronal distribution of the γ isoform of PKC is concentrated in interneurons in lamina IIi of the DH and as previously mentioned, has local circuits that can reach nociceptive output neurons (Saito & Shirai, 2002, Peirs et al. 2014).

1.7 Pain in development

A big challenge in treating children with pain is their ability, or lack thereof, to verbalise and communicate the pain for effective management and treatment. Processing pain in

early life, contrary to previous beliefs, is not just simply on a smaller scale than that seen in adults. There was a misconception that children either did not feel pain, or neonates are not born with mature pain processing pathways and undergo crucial postnatal changes from behavioural to neurophysiological processes that impact how they respond to both noxious stimuli and analgesia (Pancekauskaitė & Jankauskaitė, 2018). Due to ethics and accessibility to subjects, the majority of research in early life pain in the literature is more comprehensive in rats than in humans so it is important to note the translation of data between the ages. In comparison to humans, rats are born prematurely and are quick to develop, comparing an average gestational period of 21.5 days to a humans 38.5 weeks (Mittendorf, 1990, Kwok et al. 2014). In Rats, up to postnatal day 7 (P7) rats are considered neonatal, at P21 rats are weaned (separated from birth mother) and from P35 rats are considered sexually mature though this number can vary. This age is considered the beginning of adolescence. At approximately P63, rats are considered to be in the adult phase of their life (Sengupta, 2013).

1.7.1 Maturation of the pain pathway

Peripheral nociceptive neurons are established early in development, but unlike adults, synaptic connections with SC neurons are not yet formed (Pancekauskaitė & Jankauskaitė, 2018). Sensory neurons in the DRG are determined in the embryonic period, between E12-E16, and require neuronal determination genes: neurogenin 1 or 2 (NGN1 or NGN2). These genes encode basic helix-loop-helix transcription factors, NGN1 predominantly for the establishment of smaller diameter tyrosine kinase receptor A (TrkA) expressing neurons, with NGN2 for larger diameter tyrosine kinase receptor B and C (TrkB/ TrkC) positive neurons (Coggeshall et al. 1994, Kitao et al. 1996, Fitzgerald, 2005). The small diameter sensory neurons develop to form C-fibres, and it is thought that the peptidergic subtype of these fibres are produced before IB4 expressing

non-peptidergic fibres (Kitao et al. 1996). Primary afferent fibres from the DRG eventually enter the grey matter of the SC at approximately E15-17 for A-fibres, and E18-20 for C-fibres (Mirnics & Koerber,1995, Fitzgerald, 2005). However, despite $IB4^+$ C-fibres are in the DRG from E18, the detection of these fibres are not found in the DH until postnatal day 5 (P5). The relatively delayed generation of central C-fibre synaptic connections means that central processing of nociception is immature in the postnatal period (Benn et al. 2001, Fitzgerald, 2005).

1.7.2 Postnatal processing of pain

Neonates tend to display larger cutaneous receptive fields of DH cells relative to those in adults, and in the first two postnatal weeks quickly decrease in size (Fitzgerald, 1985, Fitzgerald & Jennings, 1999). Neonates have also been shown to exhibit lower pain behaviour thresholds and respond inappropriately to noxious stimuli, at times moving towards the stimulus. Noxious stimuli to neonates displayed prolonged excitability in that after the stimulus was applied, action potential activity continued for a further 30-90 seconds that with age decreased in both the amplitude and duration of the response (Andrews & Fitzgerald et al. 1994, Waldenström et al. 2003).

The characteristics of pain in early life suggests a lack of synaptic inhibition, which is in part due to a difference in supraspinal control. This matures with postnatal development and is a process dependent upon the action of endogenous opioids during a critical period (Hathway et al. 2012). This is supported by research that showed blocking tonic opioidergic activity during the 4^{th} postnatal week (P21-P28) in rats, prevented the normal development of RVM descending inhibition in the SC to nociceptive stimuli. This did not occur outside of this critical window (Hathway et al. 2012). Furthermore, the application of chronic morphine between P7 to P14 to enhance opioidergic activity, accelerated the maturation of descending RVM inhibition, with thresholds being significantly higher to

evoke descending inhibitory activity (Hathway et al. 2012). This maturation is typically observed in rats a lot later, at P25 or older (Hathway et al. 2009).

1.8 The opioid epidemic

Opioids have long been used both recreationally and medically for centuries. Though they are regarded the most effective in treating pain, the adverse effects of addiction and abuse mean that the appropriate use of opioid therapy is of great importance (Rosenblum et al. 2008). Data has shown that between 1999 to 2019, nearly 500,000 people have died from an opioid overdose, inclusive of prescription opioids (Bushak, 2016). This has led to a controversial concern upon the effectiveness and safety of long-term opioid use in treating chronic pain. The potential development of the opioid-induced hyperalgesia (OIH) has caused some patients to experience worsening pain with escalating doses, the reverse effects opioids intend to have (Ballantyne, 2006). Though the molecular mechanism of OIH is not entirely understood and its theories varying in the literature, it is most commonly thought that the central glutaminergic system is associated with the neuroplastic alterations that occur in the pronociceptive pathways (Lee et al. 2011).

Prescription opioids were increasing throughout the 1980s and early 1990s, but it was in the mid-1990s when OxyContin was released from Purdue Pharma, offering a new slow-release form of oxycodone. It caused a controversial surge in the national prescriptions for opioids in the US and arguably was the pivotal drug that, due to many factors including FDA label approval and unprecedented marketing, caused an outbreak in addiction (DeWeerdt, 2019, Van Zee, 2009). In 2009, deaths from prescription opioids exceeded 15,000, this figure being three times the number from the previous decade; it was the first time that overdoses outnumbered car accident fatalities (McGreal. 2018). The rise in opioid use, both as a result of this epidemic, but also with general medicinal use, has sparked further research into the long-term effects of opiate exposure, particularly during

adolescence and perinatal exposure to opioids (Testa et al. 2022).

Infants who have been exposed to opioids can have a higher risk of being born prematurely (before 37 weeks), as well as at a lower birth weight, less than the normal range of 2500-4000g, as well as other cognitive deficits (Hunt et al. 2008, Harder et al. 2019). They are also at risk of developing neonatal opioid withdrawal syndrome (NOWS) when born, as approximately 60-80% of in utero opioid exposed infants are diagnosed with NOWS (Patrick et al. 2012). Both in premature births and in many cases of NOWS, infants are admitted to the neonatal intensive care unit (NICU) where they undergo a number of painful and stressful procedures, many of which are without analgesia (Carbajal et al. 2008). In rats, cutaneous tissue inflammation in the first postnatal week can affect the long-term maturation of nociceptive circuits in the spinal cord, and alter pain behavioural responses in adulthood (Zhang et al. 2011). These long-term changes may be as a result of epigenetic effects that have led to alterations in gene expression that effect noxious responses at the level of the spinal cord (Ren et al. 2005). This illustrates the effect opioid exposure may directly or indirectly have on infants in early life, especially during the critical period of the maturation of pain pathways during development.

1.9 Aims

The aim of this thesis is to further understand the effects of morphine exposure during the critical period of postnatal development. This will entail using immunohistochemistry to:

1. Quantify a potential difference in neuronal cell count between morphine-exposed and saline tissue.
2. Localise the expression of μ-opioid receptor (MOR), parvalbumin and PKCγ in the DH of the rat, and quantify the intensity of expression
3. Identify any sex differences that may be apparent in markers of pain

processing

1.9.1 Hypothesis

Research has shown the postnatal refinement of nociceptive pathways and endogenous opioid signalling, and a critical period in which exposure to opioids can have significant effects (Hathway et al. 2012). I hypothesise that following morphine exposure between P21-28, there will be an increase in PV intensity and neuronal cell count, increased MOR intensity of labelling in the DH, and that there will be significant interaction of sex and morphine exposure on these markers. I do not predict any significant changes in primary afferent termination due to maturation occurring at P21, prior to any morphine exposure.

2.0 Methods

2.1 Animals

All animal procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986/2012 and licensed by the UK Home Office. Animals (Sprague-Dawley rats) obtained from Charles River (UK) and delivered at (P) 17, weaned off their mothers from (P) 21 prior to dosing. Rat pups (12♂, 12♀) were treated with either morphine hydrochloride (BioTechne, catalogue no. 5158) (s.c., 3mg/kg, twice daily, n=12) or saline (1mL/kg, n=12) for 7 days from postnatal day (P) 21 until (P) 28. Behavioural tests to measure nociceptive thresholds (application of Von Frey filaments), anxiety scores (elevated plus maze) and locomotor activity were performed and recorded between P29 to P40, however data from these experiments do not form part of this thesis. The number of animals per experimental group (n=6) was determined based on previous data from the research group in a model of inflammatory pain: Pain = 5.436, Control = 3.451, shared sigma = 1.028, 80% power, alpha = 0.05 n = 4. To allow for potentially smaller effect sizes in this study and correlation of anatomical and behavioural or

physiological data from the same animal, n = 6.

2.2 Perfusion and tissue preparation

At P42, rats received an overdose of pentobarbitone (Biosupport Unit, University of Nottingham) [100 mg/mL, i.p.] and were transcardially perfused with saline, followed by 4% paraformaldehyde (Sigma Aldrich). The spinal cord was removed, post-fixed (4% paraformaldehyde solution) and stored overnight. After 24 hours, tissues were kept in 30% sucrose in 0.1 M phosphate buffer/0.05% sodium azide solution for long term storage at 4 °C. Spinal cords were embedded in OCT medium (optimal cutting temperature, VMR Chemicals, catalogue no. 361603E) and Lumbar region L4 were cryo-sectioned at 20um using a cryostat (Thermofisher, catalogue no. HM550), as well as at 10um for use of RNAscope testing for PhD student Neave Smith.

2.3 Method optimisation

To determine the optimum concentration of antibodies used, a series of experiments were conducted for both Guinea pig anti-parvalbumin (Synaptic systems, catalogue no. 195 308, Germany) and rabbit anti-PKCg (Thermofisher, catalogue no. #14364-1-AP, UK), as well two trial triple staining experiments to ensure each antibody used was at a dilution that provided the most accurate signal to noise ratio. Guinea-pig anti-PV was tested firstly with a 1:5 serial dilution (1:200, 1:1000 and 1:5000) whereby 1:5000 showed the best images. However, upon repeated testing of the trial triple staining experiment, the signalling looked sub-optimal and thus a 1:2000 repeat dilution was performed and decided as the best concentration for this antibody. The rabbit anti-PKCg antibody was similarly tested with a 1:5 serial dilution (1:200, 1:1000 and 1:5000) and 1:1000 dilution was selected for the quantifying experiment (see figure 2.1). Concentrations for antibodies IB4, CGRP and MOR used in experiment one had previously been optimised by the research group Pain Centre vs Arthritis (University of Nottingham). Sections were

originally cut at 40um and used for multiple optimisation and trial experiments. However, to maximise use of tissue, sections were ultimately cut and retested at 20um so that the same spinal tissue could also be used for RNAscope experiments at 10um, and for other labelling immunohistochemistry experiments. A comparison of the labelling was made between 40um and 20um prior. To ensure the longevity of the antigen retrieval did not cause any irreversible damage to the 20um thinner sections, or potentially an impact of the signal to background ratio, the use of two different antigen retrieval times of either 10 or 20 minutes was tested. It was confirmed that 20um tissue for the immunohistochemistry was still successful at 20 minutes antigen retrieval (see figure 2.2).

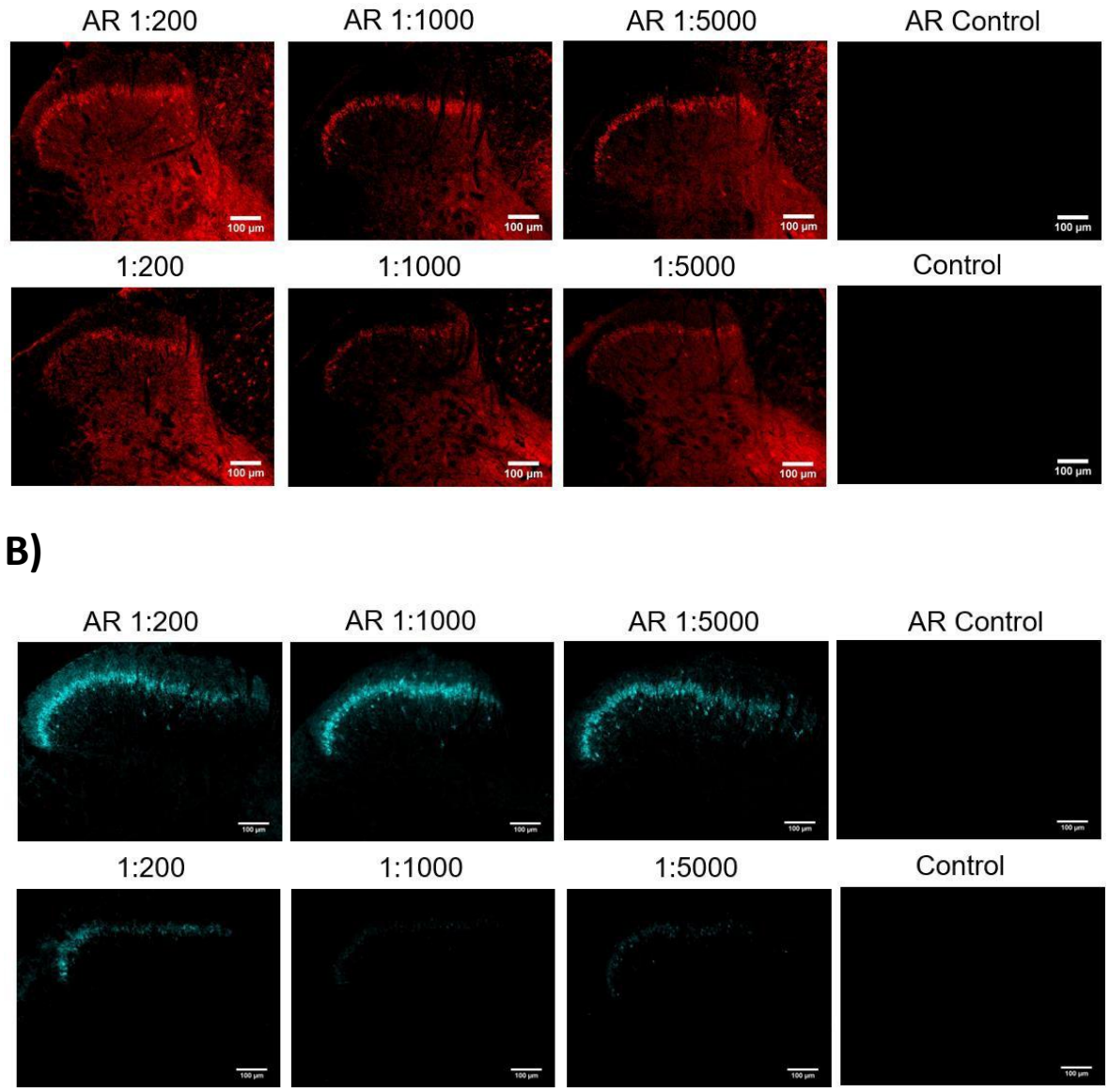


Figure 2.1: Early lumbar sections (20um) of the dorsal horn in the rat spinal cord with a series of 1.5 serial dilutions of tested antibodies. A) Optimisation of guinea-pig anti-parvalbumin antibody (Synaptic systems, catalogue no. 195 308, Germany). Images show sections that have been labelled (displayed in red) at dilutions of 1:200, 1:1000 and 1:5000, and those with and without antigen retrieval (AR). Strong and specific labelling was displayed at both 1:1000 (AR) and 1:5000 (AR). Ultimately, following a trial experiment an intermediate of 1:2000 was decided as the final concentration. Images for parvalbumin antibody were taken in TRITC channel B) Optimisation of rabbit anti-PKCg antibody (Thermofisher, catalogue no. #14364-1-AP, UK). Specific labelling (cyan) was clearest and strongest at 1:1000 dilution with antigen retrieval. The PKCg antibody was taken in Cy5 channel. Scale bar 100um. Images for each antibody in their respective channels were taken at the same exposure time to ensure consistency. Scale bar= 100um.

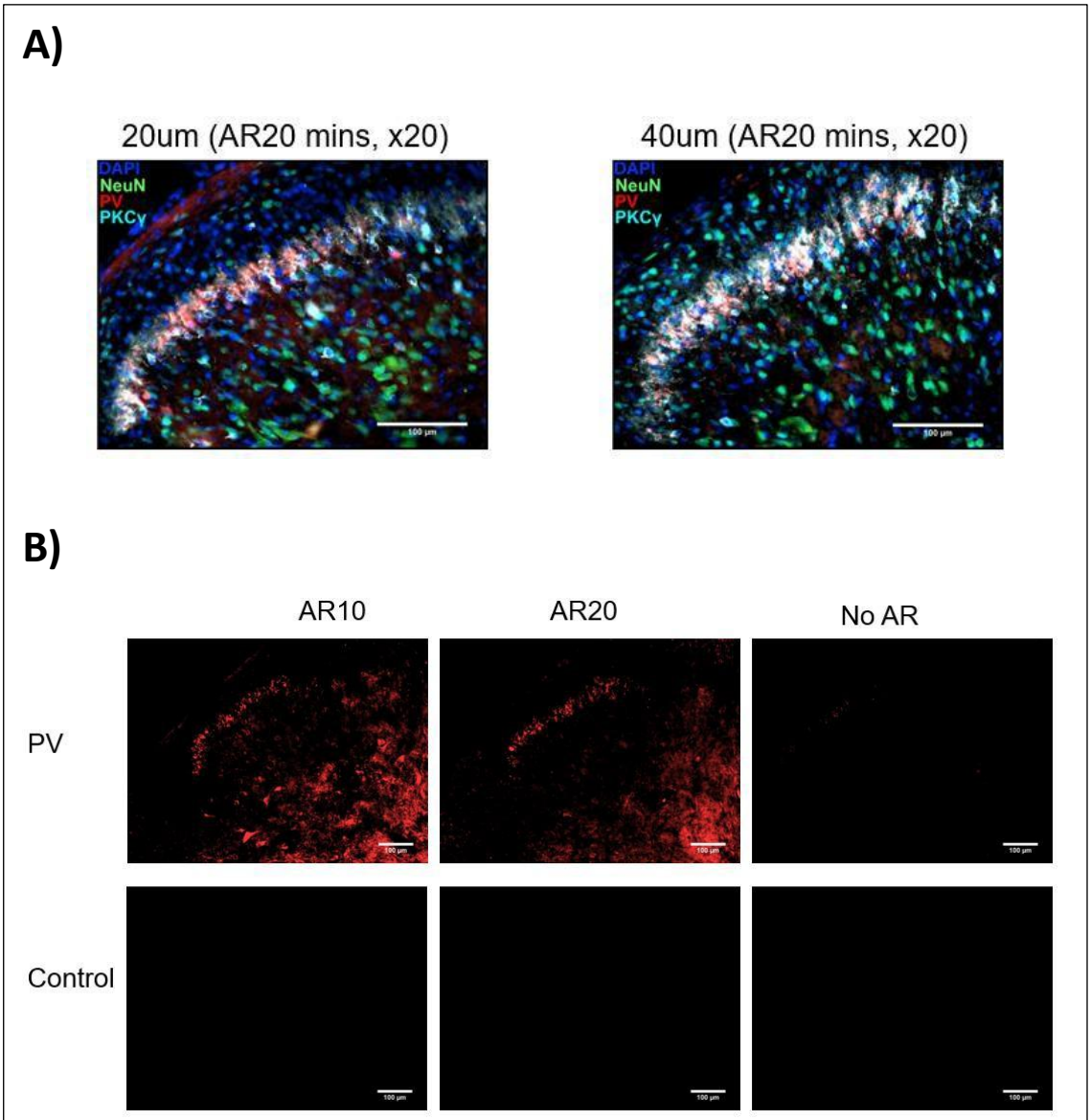


Figure 2.2: Trial experiments using different thicknesses to maximise experimental use of tissue. A) A comparison of composite images (x20 maginifaction) of 20um sections and 40um sections (both with an antigen retrieval time of 20 minutes) labelled with DAPI (blue), NeuN (green), parvalbumin (red) and PKC gamma (cyan). Images show integrity was upheld in 20um sections. B) A comparison of the parvalbumin labelling (red) in 20um sections with antigen retrieval (AR) times of 10 minutes, 20 minutes and lastly no AR at all. Bottom row shows secondary only controls. Scale bar = 100um.

2.4 Immunohistochemistry

In the first experiment, sections were triple labelled through free floating immunohistochemistry (see table 1 for antibody details) in a 24-well plate for markers of two major populations of nociceptive primary afferent fibres using IB4-488 (1:1000, Invitrogen, mp21410); sheep anti-CGRP (1:5000, Enzo, BML-CA1137-0100); and expression of the mu-opioid receptor using rabbit anti-MOR (1:200, Abcam, ab134054). The second experiment also triple labelled sections with free floating immunohistochemistry using: mouse anti-NeuN (1:1000, Millipore, MAB377); guinea pig anti-parvalbumin (1:2000, Synaptic systems, 195 308); and rabbit anti-PKCγ (1:1000, Thermofisher, #14364-1-AP).

Primary antibody	**Supplier/ Catalogue no.**	**Secondary antibody**	**Supplier/ Catalogue no.**
IB4-AF488	Invitrogen, mp21410	-	-
Sheep anti-CGRP	Enzo, BML-CA1137-0100	Donkey anti-sheep 568	Invitrogen, A21099
Rabbit anti-MOR	Abcam, ab134054	Donkey anti-rabbit 647	Invitrogen, A31573
Mouse anti-NeuN	Millipore, MAB377	Donkey anti-mouse 488	Invitrogen, A11001
Guinea pig anti-parvalbumin	Synaptic systems, 195 308	Goat anti-guinea pig 568	Invitrogen, A11075
Rabbit anti-PKCγ	Thermofisher, #14364-1-AP	Donkey anti-rabbit 647	Invitrogen, A31573

Table 1: Details and suppliers of the primary antibodies used and their respective secondary antibody for the immunohistochemistry experiments.

Sections were first placed into Eppendorf's of citrate buffer and incubated for 20 minutes in a 90^{o}C water bath for antigen retrieval. Sections were then washed in 0.1M phosphate buffer saline (PBS) three times (5 minutes, RT). Nonspecific binding was then blocked and tissue permeabilised by incubation with of normal donkey serum (Scientific Laboratory Supplies, cat. No. D9663), 0.3% Triton X100 (Sigma), in PBS (1 hour, RT). Sections were subsequently incubated with primary antibodies diluted in PBS (overnight, 4°C). Following this, sections were thoroughly washed in PBS and appropriate secondary bodies (see table 1) were added (all 1:500 dilution, 2 hours, in the dark, RT). Sections

were repeatedly washed in PBS and incubated with DAPI (20 minutes, dark, RT). Negative control sections were incubated with PBS only during primary antibody incubations and then incubated with the same secondary antibodies as experimental sections to determine any non-specific signal from secondary binding. Lastly, all sections were washed in PBS, mounted onto labelled gelatinised glass slides, and cover slipped with fluoromount. Slides were stored at 4°C in the dark for imaging the following day.

2.5 Imaging analysis

Sections were imaged using the Zeiss Exciter Wide Field Microscope (Zeiss, Oberkochen, Germany) Approximately three to four DH's were imaged from sections of each animal using FIJI software and the same exposure time for the image acquisition was used for each staining of the different antibodies across all sections and animals to ensure consistent brightness in each image. To ensure the unbiased analysis of the various spinal cords, I was blinded to experimental conditions.

For experiment one analysis, a macro was devised (see appendix 1) to threshold images and measure the area and mean gray intensities (MGI) of IB4, CGRP and MOR labelling. The average area and MGI were multiplied by each other to calculate the total intensity of each label. Manual DAPI-positive cell count in the MOR labelled region of the DH was completed. The macro also included measuring MOR labelling within CGRP, and MOR within IB4, for any colocalisation of the Mu Opioid receptor and the primary afferent termination to draw comparisons between morphine treated and saline treated experimental groups. For the second experiment, a second macro was created (see appendix 2) to also threshold images and measure both area and MGI's of PKCγ and parvalbumin labelling and again multiply these values to calculate the total intensity. Thresholds used were consistent across experimental groups for fluorescence markers, and measurements were taken from these thresholded images of the DH rather than

predefined anatomical regions of interest (ROIs). This was to assess both spatial distribution and total area of staining, to ensure total intensity reflected marker expression without imposing anatomical boundaries within the DH.

Manual cell-count of both DAPI positive cells and NeuN+ cells were completed in PKC labelled regions, as well as PV positive regions of the DH. A further measure of PV within PKCγ labelling for colocalisation was included. Lastly, an additional manual cell count for both DAPI+ve cells and NeuN+ve cells were collected (see appendix 3) in three regions: superficial, intermediate and deeper DH. These cells were counted in rectangles (width= 200um, height= 100um) placed in each of the regions of interest across the DH. All data was calculated in three different sections per animal, and a mean average was subsequently calculated and plotted to graph.

2.6 Statistical analysis

All statistical analysis was performed using GraphPad Prism 9 Software (San Diego, CA, USA). The mean ± standard deviation (SD) was calculated for all data where applicable. To compare experimental groups, Student's unpaired t-tests was used and for statistical comparisons between the saline and morphine exposed groups and sexes were made using 2-way analysis of variance (ANOVA), as well as the Fisher's least significant difference (LSD) post hoc test. Data are presented as mean ± SD. *$P < 0.05$; **$P < 0.01$.

3.0 Results

3.1 Morphine exposure during postnatal day (P) 21 and (P) 28 has no significant effect on the area of the spinal Dorsal Horn in rats.

A macro was devised to measure the area (um^2) of the dorsal horn in the spinal cords of rats to assess if, between the adolescent growth of P21 and P28, exposure to morphine has any impact on the total size of the structure (figure 3.1A). As can be seen in Figure

3.1B, there were no significant differences between the groups.

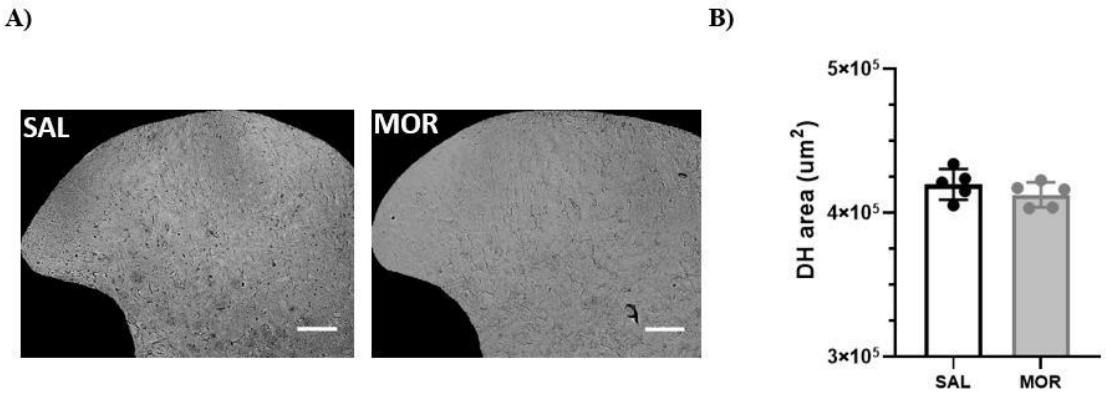


Figure 3.1. Comparison of DH area of rat spinal cords between saline and morphine treatment groups. A) Low power representative images (X10, brightfield) of saline exposed (left) and morphine exposed (right) rat DH. B) Histogram showing no statistically significant difference in area (um^2) of the DH in rat spinal cord sections from saline and morphine exposed groups (t(8)=1.173, P=0.2745). Scale bar= 100um. N=10 (5=saline, 5= morphine). Data are presented as mean ± SD.

3.2 Morphine exposure from P21 to P28 has no significant effect on total number of cells and total neuronal number in the dorsal horn.

Exposure to morphine in adolescence may have impacted the number of cells in the mature DH especially neurons. Manual cell counts of DAPI+ve cells and NeuN+ve cells were completed in three different regions of the DH, from three sections per spinal cord and any significant differences between rats that had been exposed to either saline or morphine between postnatal day 21 and 28 (figure 3.2A, figure 3.2C). Data shows that there was no significant difference between experimental groups for DAPI+ve total cell count in all three regions of the superficial (P=0.7476), intermediate (P=0.9667) and deep (P=0.3826) dorsal horn (figure 3.2B). There were also no significant differences in NeuN+ve cells between saline and morphine treatment groups in the superficial (P=0.9984), intermediate (P=0.8568) and deep (P=0.9362) DH (figure 3.2D).

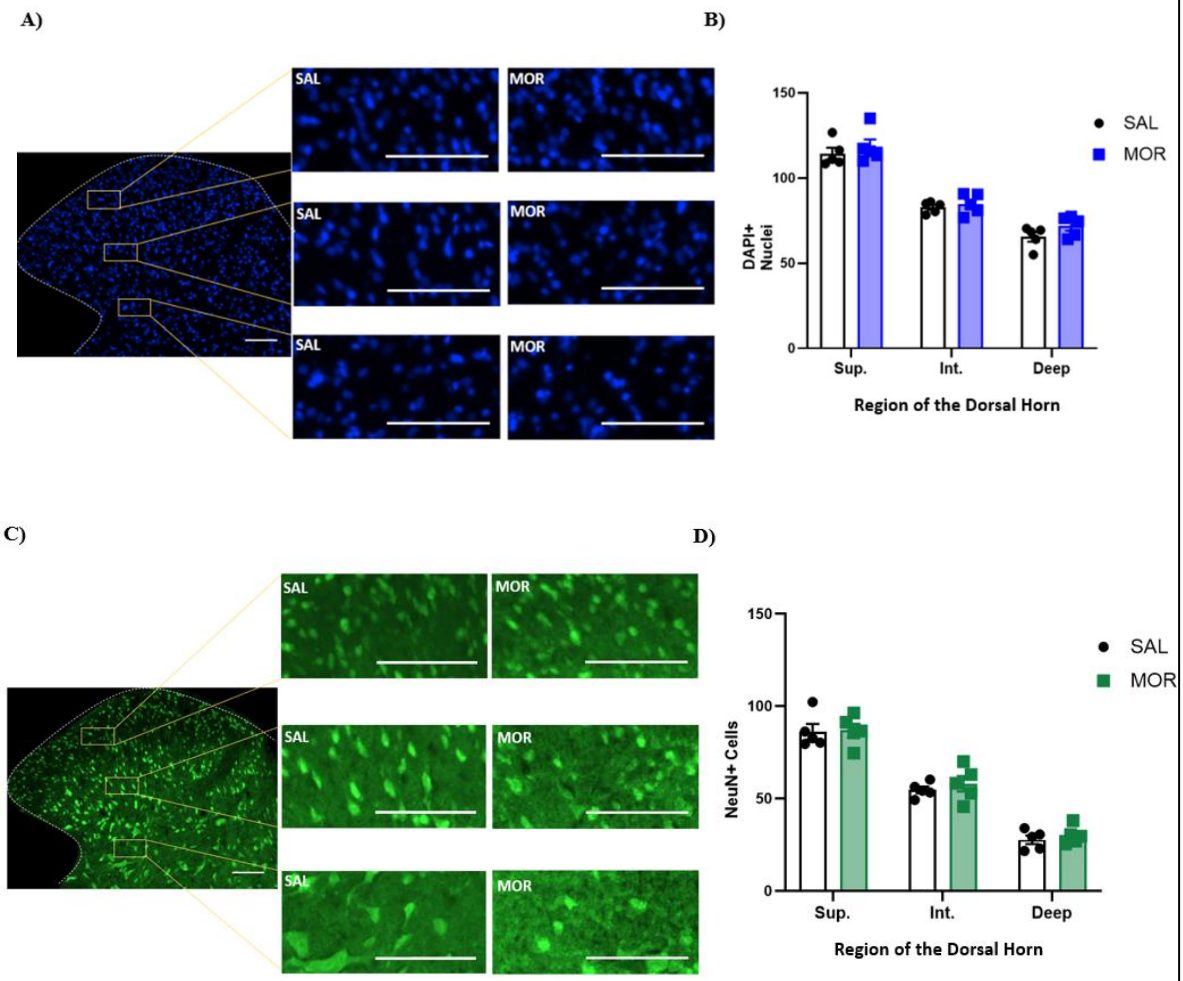


Figure 3.2: Immunohistochemical labelling of DAPI positive nuclei and NeuN positive cell bodies in the dorsal horn of the rat spinal cord. A) DAPI labelling of nuclei in the DH with saline and morphine images (rectangles of width= 200um, height= 100um) in the respective order of the superficial, intermediate and deeper DH. B) Histogram displaying no significant difference between saline and morphine DAPI positive nuclei in the superficial (P=0.7476), intermediate (P=0.9667) and deep (P=0.3826) regions of the DH.

C) NeuN labelling of neurons in the DH with saline and morphine images (rectangles of width= 200um, height= 100um) in the respective order of the superficial, intermediate, and deep DH. D) Histogram showing no significance between saline and morphine groups for the NeuN positive cell labelling in the superficial (P=0.9984), intermediate (P=0.8568) and deep (P=0.9362) regions of the DH. Images taken at X10 magnification. Scale bar= 100um, N=10 (5= saline, 5= morphine, analysis of three sections per spinal cord). Statistical comparisons between the treatment groups across the three regions were made using 2- way analysis of variance (ANOVA) and the Fisher's least significant difference (LSD) post hoc test. Data are presented as mean ± SD.

3.3 A significant interaction of sex and treatment group on the area and DAPI+ve cells for MOR labelling

Immunohistochemical labelling for MOR was performed to assess the regions in which the receptor is expressed in the DH as well as the intensity of labelling between treatment groups (figure 3.3A). Signal intensity measurements were based on thresholded images isolating signal-specific regions, instead of fixed ROIs to ensure quantification reflected distribution as well as intensity of labelling. Although no significant difference was found between treatment groups regarding the total intensity of MOR labelling in the DH (figure 3.3C), there was a significant interaction of sex and treatment group on the area of MOR labelling (um^2) within the DH ($f_{(1,20)}$=7.454, P=0.0129; 2-way ANOVA). Post hoc analysis revealed that female morphine exposed rats displayed significantly greater area (um^2) of MOR labelling in comparison to male rats exposed to morphine (P=0.0338), even with no overall change in DH area, as seen previously (figure 3.3B, figure 3.1). It is also worth noting that there is a visible trend that shows in male rats, morphine exposure led to a decrease in MOR area, whereas in female rats, exposure to morphine resulted in an increase of MOR area. Furthermore, there was a significant interaction between sex and treatment group for DAPI+ve cell count within MOR labelling in the DH ($f_{(1,20)}$=10.54, P=0.004; 2-way ANOVA) (figure 3.3D). Post hoc analysis revealed that male morphine exposed rats exhibited a greater DAPI+ve cell count in the MOR region than female morphine exposed rats (P=0.0133). It also revealed that between treatment groups for female rats, morphine exposed female rats had significantly less DAPI+ve cells in the MOR labelled region compared to female saline controls (P=0.0029). Though not significant, morphine exposure showed a trend that in male rats it led to an increase in DAPI+ve cells in MOR labelling, and opposingly in female rats, morphine exposure led to a decrease in DAPI+ve cells. These results indicate a significant interaction of sex and

morphine exposure that effect MOR area and affect the number of DAPI+ve cells within the labelling of MOR (figure 3.3).

3.4 An increase of IB4 area and total intensity in morphine exposed male rats.

Triple staining immunohistochemistry of both IB4, CGRP and MOR was conducted to analyse the primary afferent termination pattern in the spinal DH of to show if morphine exposure affects the area and intensity of primary afferent fibres, as well as measuring the expression of MOR in the IB4 positive labelling, and MOR in the CGRP labelling (figure 3.4 figure 3.5). Data showed that there were no significant differences found in CGRP area and total intensity between sexes and experimental groups (figure 3.5A, figure 3.5B). However, there was a significant effect of morphine on male rats on the area (um^2) of IB4 labelling ($F_{(1,20)}$=8.827, P=0.0076; 2-way ANOVA). Post hoc analysis showed that the IB4 labelled area (um^2) was significantly greater in morphine exposed male rats compared to saline exposed male controls (P=0.0041), this was not found in female rats (figure 3.5C). There was a significant interaction of sex and treatment group on IB4 total intensity in the DH ($F_{(1,20)}$=4.471, P=0.0472; 2-way ANOVA), as well as a significant effect of treatment group on IB4 total intensity ($F_{(1,20)}$=9.241, P=0.0065; 2-way ANOVA). Post hoc analysis showed that male rats exposed to morphine had significantly greater IB4 total intensity compared to male saline exposed control rats (P=0.0016). Furthermore, between controls, male rats exhibited a significantly lower total intensity of IB4 compared to female rats (P=0.0159) (figure 3.5D). There were no significant interactions for MOR intensity in CGRP labelling (figure 3.5E), however there was a significant interaction between sex and treatment group on MOR intensity in IB4 labelling ($f_{(1,20)}$=4.985, P=0.0372; 2-way ANOVA) (figure 3.5F). Post hoc analysis indicated that MOR intensity in IB4 labelling was found to be significantly greater in morphine exposed male rats compared to male saline controls (P=0.0083). This is in line with an increased area for

IB4, as previously mentioned, and therefore likely due to an increase in IB4+ve c-fibres. Interestingly, there was a sex difference between the saline control cohort of rats; female rats had significantly greater MOR intensity in IB4 staining in contrast to the male saline exposed rats (P=0.0396) (figure 3.5F).

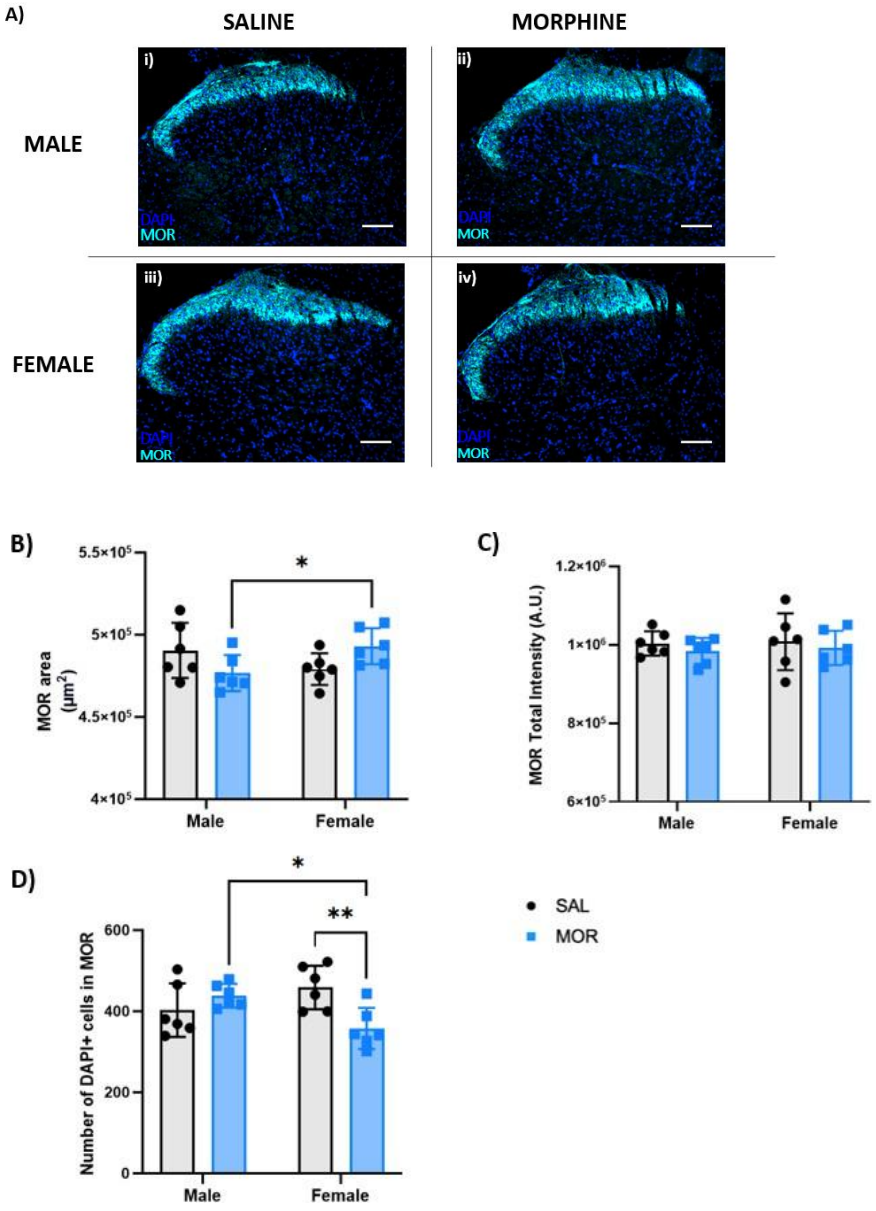


Figure. 3.3: Mu Opioid receptor intensity in the rat DH in male and female rats exposed to either saline or morphine during P21-P28. A) Representative images of immunohistochemistry labelling of the MOR and DAPI (i) male saline exposed; (ii) male morphine exposed; (iii) female saline exposed; and female morphine exposed (iv) rat DH. Scale bar= 100um. B) Histogram of the area (um^2) of MOR labelling showing significantly greater area in female morphine group compared to male morphine group ($f_{(1,20)}$=7.454, P=0.0129). C) Histogram of the total intensity of MOR labelling between sexes and experimental groups of morphine and saline. D) Histogram of the DAPI+ve cell count within MOR labelling, male morphine group showing significantly greater than female morphine group (P=0.0133), and female saline controls showing significant increase in number (P=0.0029). N=24, 12♂, 12♀ (12= morphine, 12= saline). Student's unpaired t-tests was used and for statistical comparisons between the treatment groups and sexes were made using 2-way ANOVA, as well as the Fisher's least significant difference (LSD) post hoc test. Data are presented as mean ± SD. *P < 0.05; **P < 0.01.

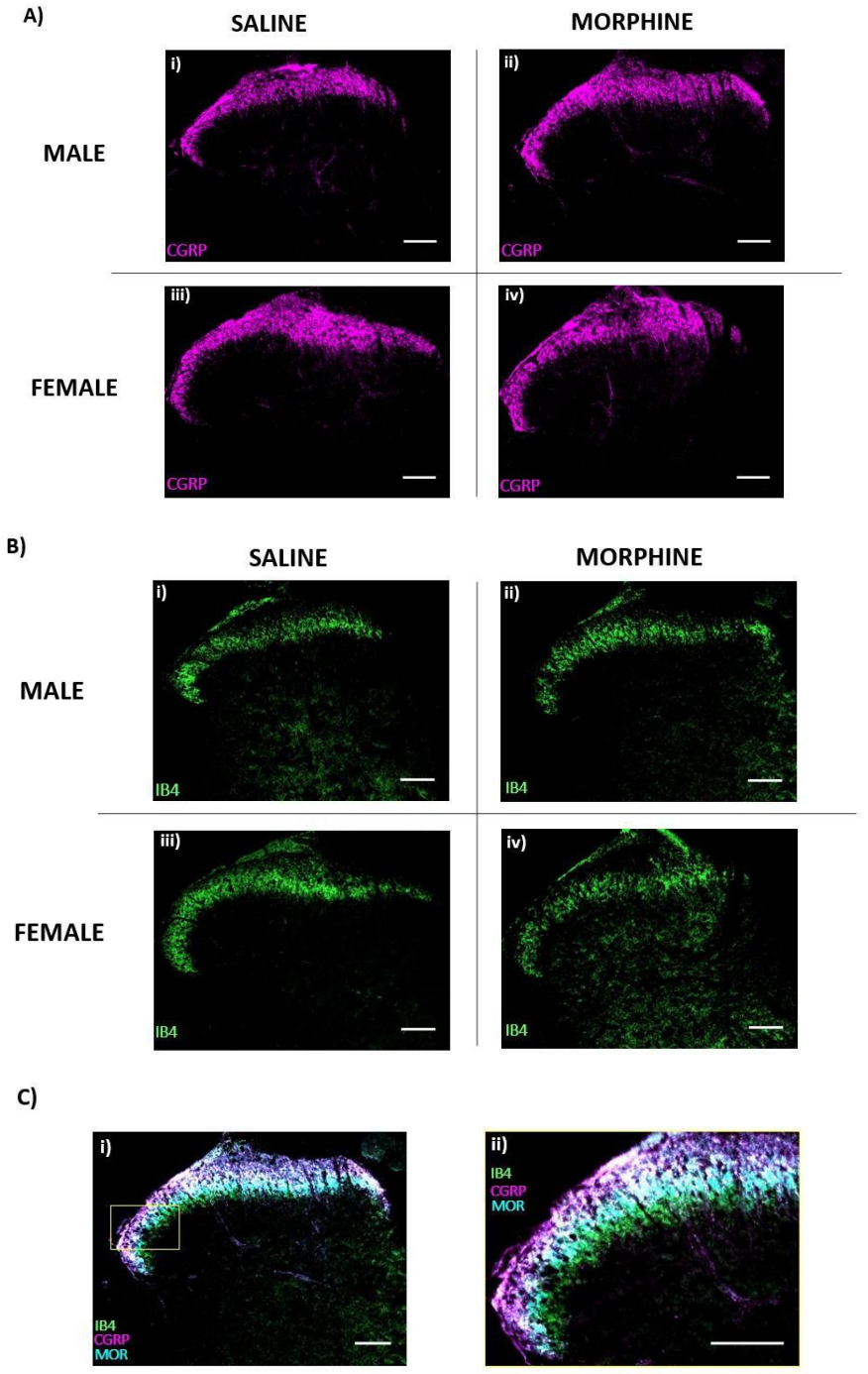


Figure 3.4 Immunohistochemistry triple labelling primary afferent termination patters of IB4, CGRP and MOR in the spinal DH in male and female rats exposed to saline or morphine. A) Representative images (x10) of calcitonin gene-related peptide labelling in the DH of (i) male control saline exposed, (ii) male morphine exposed, (iii) female control saline exposed, (iv) female morphine exposed rats. B) representative images (x10) of IB4 labelling in the DH of (i) male control saline exposed, (ii) male morphine exposed, (iii) female control saline exposed, (iv) female morphine exposed rats. C) Representative triple labelling image of IB4 (green), CGRP (pink) and MOR (cyan) in the DH of the rat spinal cord at (i) X10 and (ii) X20 magnification. Scale bar= 100um. N=24, 12♂, 12♀ (12= morphine, 12= saline).

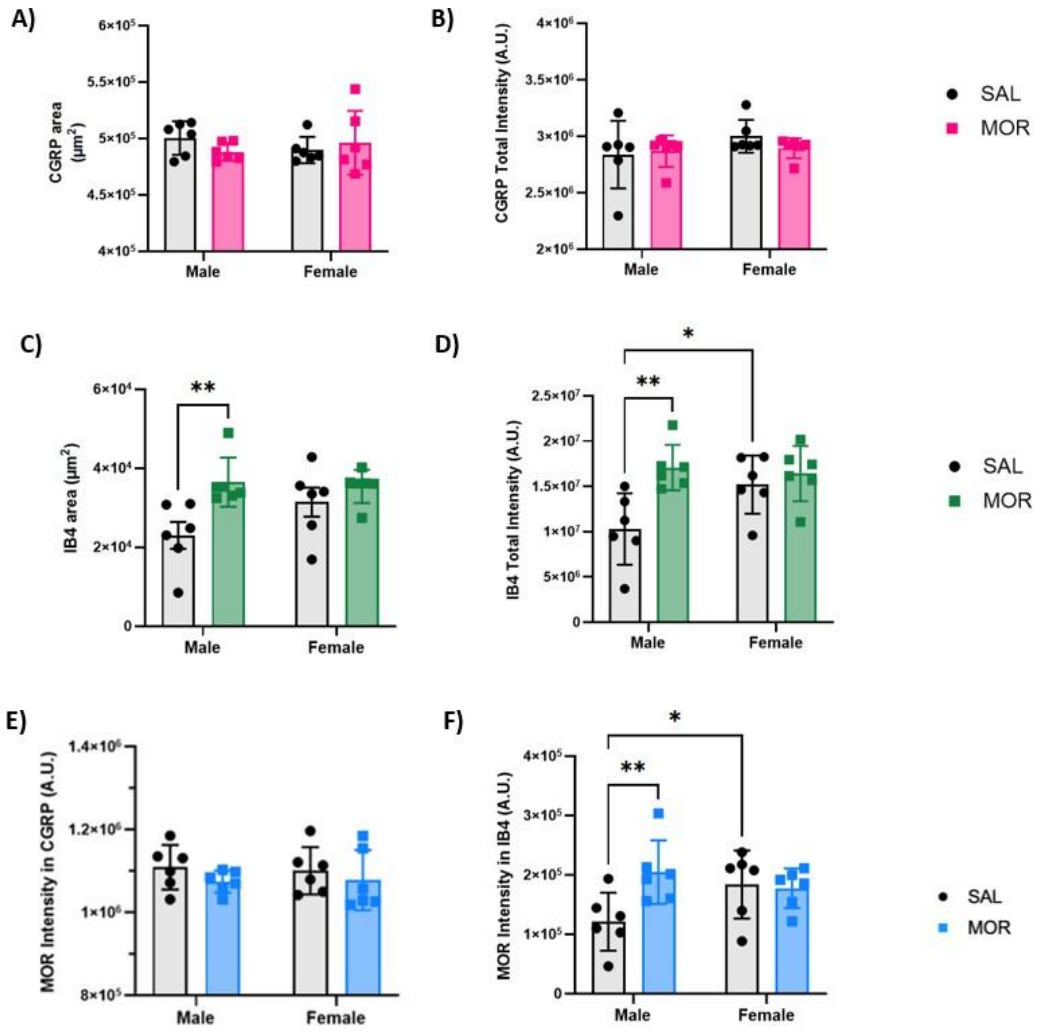


Figure 3.5. Histograms of CGRP, IB4 and MOR intensity within CGRP and IB4 comparing male and female rats DH exposed to morphine or saline during P21-P28. A) Histogram comparing the CGRP labelled area (um^2) in the rat DH between sexes and experimental groups with no significance. B) Histogram showing no significance between groups of CGRP total intensity. C) Histogram of the area of IB4 labelling in rat DH showing significance between male morphine and saline groups (P=0.0041). D) Histogram showing significantly greater IB4 total intensity in male morphine exposed rats compared to male controls (P=0.0016), and significantly greater between sexes control groups (P=0.0159). E) Histogram showing no significant differences between groups for MOR intensity in CGRP labelling. F) Histogram of MOR intensity in IB4 labelling displaying significant data between male and female saline groups (P=0.0396), and between treatment groups in male rats (P=0.0083). N=24, 12♂, 12♀ (12= morphine, 12= saline). Student's unpaired t-tests was used and for statistical comparisons between the treatment groups and sexes were made using 2-way ANOVA, as well as the Fisher's least significant difference (LSD) post hoc test. Data are presented as mean ± SD. *P < 0.05; **P < 0.01.

3.5. Morphine exposure between P21-P28 significantly increases NeuN+ve parvalbumin cells in females

Immunohistochemical labelling of parvalbumin was also performed in male and female rats from the morphine and saline treatment groups (figure 3.6A). Data showed no significant differences between sexes and experimental groups for parvalbumin labelling area and total intensity (figure 3.6B, figure 3.6C, figure 3.6D). However, there was a significant interaction of sex and treatment group for the number of PV+ neurons within the DH ($F_{(1,20)}$= 5, P=0.0356; 2-way ANOVA). Post hoc analysis of the data revealed that there was a greater number of PV neurons in females exposed to morphine during preadolescence, compared to saline-exposed female controls (P= 0.0160) (figure 3.6E). As overall NeuN+ve cell count was previously completed and found no significant differences, there is an increase in neurons expressing NeuN and PV in females exposed to morphine. This pattern was not observed in male rat counterparts.

3.6 A significant increase of Protein Kinase C gamma total intensity observed in male rats exposed to morphine

Immuno-labelling of PKCγ allowed us to localise and measure both the area and total intensity in morphine exposed rat spinal DH, and compare these datasets against saline controls, as well as compare between the sexes for any sex-dependent differences (figure 3.7A). Whilst there is no evident difference in area of PKCγ between sexes and treatment groups (figure 3.7B), there was a significant interaction between sex and treatment groups on PKCγ total intensity ($F_{(1,20)}$=4.359, P=0.0498; 2-way ANOVA) (figure 3.7C). Post hoc analysis showed that male morphine exposed rats had significantly greater PKCγ total intensity in the DH than male saline exposed controls (P=0.0426) (figure 3.7C). Between saline control rats, females had a significantly greater PKCγ total intensity relative to male saline controls (P=0.0109) (figure 3.7C). Manual cell- count of DAPI+ve and

NeuN+ve cells in PKCγ labelling displayed no significant differences between sexes and treatment groups (figure 3.7D, figure 3.7E).

3.7 Sex differences displayed in the coexpression of Parvalbumin and Protein Kinase C gamma

The triple labelling of NeuN, PV and PKCγ enabled us to investigate the coexpression of PV and PKCγ across the different treatment groups and assess any apparent sex differences (figure 3.8A). There was a significant effect of morphine in female rats on parvalbumin intensity in PKCγ labelling, female morphine exposed rats displayed a significant increase in PV intensity in PKCγ labelling relative to female saline controls ($F_{(1,20)}$=3.329, P=0.0460) (figure 3.8B). Male morphine and saline exposed groups had no significant differences between them for PV intensity in PKCγ so this pattern seems to be exclusive to female rats.

It was also found that there is a significant interaction of sex and treatment group on PKCγ intensity in PV labelling ($F_{(1,20)}$=6.847, P=0.0165) and a significant effect sex has on PKCγ intensity in PV area ($F_{(1,20)}$=6.315, P=0.0207) (figure 3.8C). Post hoc analysis revealed that male rats exposed to morphine had significantly greater PKCγ intensity in PV labelling, compared to male saline exposed controls (P=0.0099). It also showed that between the sexes of the control group, female saline exposed rats had significantly greater PKCγ intensity in PV area than male saline exposed rats (P=0.0017). Taken together, this data indicates sex and morphine exposure has an effect on the coexpression of PKCγ in PV labelling.

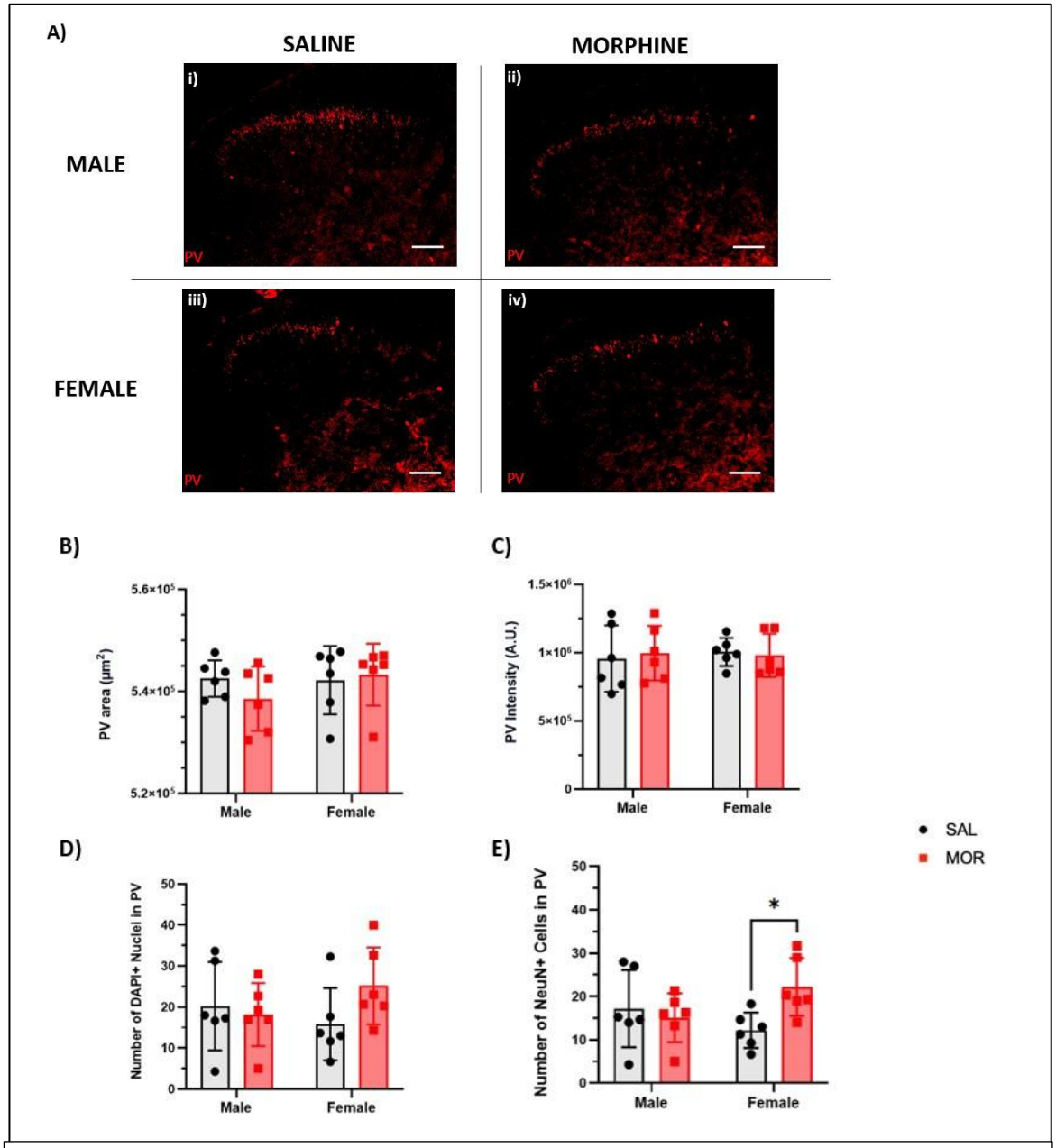


Figure 3.6: Localisation and intensity of parvalbumin labelling in the rat spinal DH. A) representative images (X10) of parvalbumin labelling in (i) male control saline exposed rats; (ii) male morphine exposed rats; (iii) female control saline exposed rats; (iv) female morphine exposed rats. Scale bar=100um. B) Histogram of PV area (um^2) showing no significance between sexes and treatment groups. C) Histogram of PV total intensity in the DH of male and female rats of both treatment groups showing no significant differences. D) Histogram showing DAPI+ve nuclei in PV labelling with an increase in female morphine exposed rats compared to saline female rats but no statistical significance. E) Histogram of NeuN+ve cells in PV region of the DH showing significant increase of PV neurons in female morphine exposed rats DH relative to female control groups (saline) (P= 0.0160). N=24, 12♂, 12♀ (12= morphine, 12= saline). Student's unpaired t-tests was used and for statistical comparisons between the treatment groups and sexes were made using 2-way ANOVA, as well as the Fisher's least significant difference (LSD) post hoc test. Data are presented as mean ± SD. *P < 0.05.

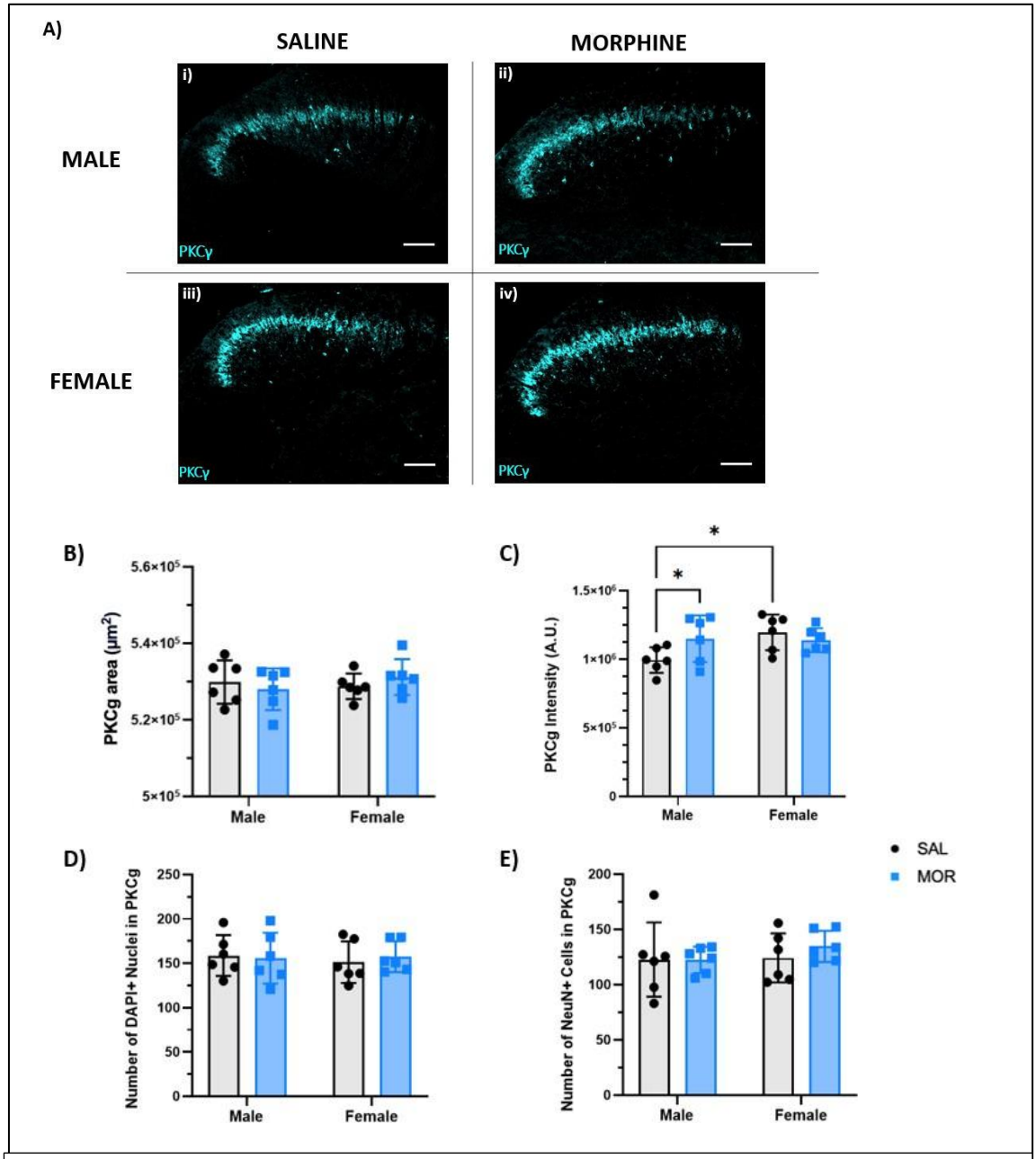


Figure 3.7: Localisation and intensity of PKCγ labelling in the rat DH. A) representative images (X10) of PKCγ labelling in (i) male control saline exposed rats; (ii) male morphine exposed rats; (iii) female control saline exposed rats; (iv) female morphine exposed rats. Scale bar=100um. B) Histogram of PKCγ area (um^2) with no significant disparities between sexes and treatment groups. C) Histogram of PKCγ total intensity displaying a significant increase of intensity in male morphine exposed rats compared to controls (saline) (P=0.0426), and between sexes of control groups an increase in female PKCγ intensity (P=0.0109). D) Histogram showing no differences of DAPI+ve cell counts between sexes and treatment groups. E) Histogram showing no significant changes of NeuN+ve cell counts between sexes and treatment groups. N=24, 12♂, 12♀ (12= morphine, 12= saline). Student's unpaired t-tests was used and for statistical comparisons between the treatment groups and sexes were made using 2-way ANOVA, as well as the Fisher's least significant difference (LSD) post hoc test. Data are presented as mean ± SD. *P < 0.05.

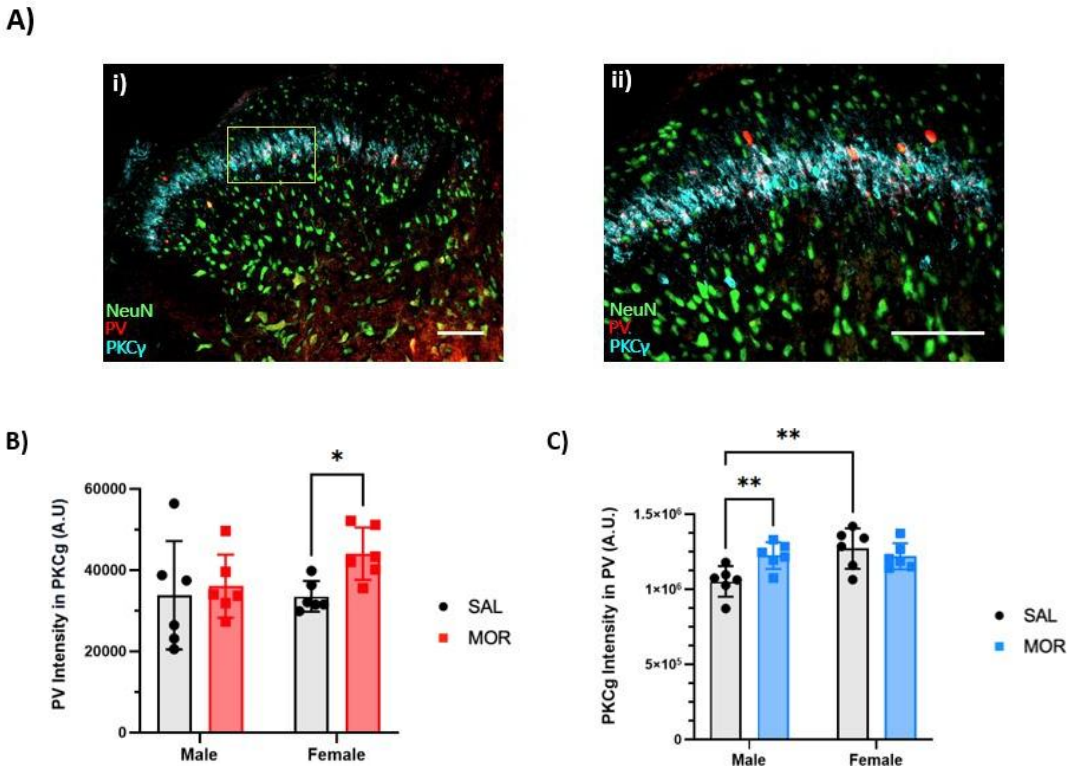


Figure 3.8: Colocalisation between parvalbumin and protein kinase c gamma between sexes and treatment groups of morphine or saline. A) (i) Representative image (X10) of triple labelling of NeuN, PV and PKCγ, (i) Representative image of triple labelling at X20 magnification, colocalisation seen in white. Scale bar= 100um. B) Histogram showing PV intensity in PKC gamma that illustrates a significant increase of PV intensity gamma found in female morphine treated rats relative to saline control female rats of PKCγ labelled regions (P=0.0460). C) Histogram displaying significant increase of PKCγ intensity within PV for male morphine exposed rats in comparison to male controls (saline) (P=0.0099). Additionally, significant sex differences seen by an increase of overall PKCγ intensity in PV labelling in female control rats relative to male control rats (P=0.0017). N=24, 12♂, 12♀ (12= morphine, 12= saline). Student's unpaired t-tests was used and for statistical comparisons between the treatment groups and sexes were made using 2-way ANOVA, as well as the Fisher's least significant difference (LSD) post hoc test. Data are presented as mean ± SD. *P < 0.05; **P < 0.01.

4.0 Discussion

In this study, during preadolescence, male and female rats were exposed to morphine for one week. Upon analysis at P42, we have shown that whilst there are no overall changes in DH area innervated by primary afferent sensory neurons, total number of cells or number of neurons, there were significant differences in IB4 and MOR immunoreactivity, which differed between sexes. We also discovered changes to the number of neurons within PV labelling in female rats as a result of morphine exposure. We further revealed that PKCγ labelling in the DH significantly increased upon morphine exposure in male rats. These data shed new light on the effect opioid exposure has during a critical period of postnatal pain processing development, and adds new knowledge regarding how early life interventions alter pain and nociception later in life.

4.1 Effect of morphine exposure on primary afferent termination patterns and MOR total intensity

The triple labelling of IB4, CGRP and MOR was to assess any changes that may have occurred to the primary afferent termination patterns and MOR as a result of early life opioid exposure. Although we expected little to no changes as the primary afferent fibre termination pattern, as in rats this tends to be mature by P21 (Fitzgerald et al. 1994), it was an important consideration. Whilst there were no significant differences between treatment groups, for either sex, in CGRP labelling area or total intensity, we found that male rats exposed to morphine exhibited significantly greater IB4 labelled area (um^2) and total intensity, than male control rats exposed to saline. These results indicate that non-peptidergic nociceptive afferents may be more sensitive to changes from opioid exposure during preadolescence. However, as this effect was not observed in female rats, it may be an oversimplification of the findings. Female controls did show a significantly greater

baseline level of IB4 total intensity when compared to male control rats. It may be that this higher baseline masks an opioid-dependent increase in IB4 in females or that there is no increase in non-peptidergic innervation zone in this group. It has previously been shown that females exhibit higher mechanical nociceptive thresholds in their primary afferent nociceptors (Hendrich et al. 2012). Nazarian et al. (2013) reported that female rats exhibited significantly greater activity of the neuropeptide Substance P (SP), demonstrated by increased NK1 receptor internalisation in the DH following formalin injection. This effect was hormone-dependent; removal of ovarian hormones reduced SP signalling, which was then subsequently restored by estradiol treatment. These findings suggest that sex hormones may amplify peptidergic signalling in females. It has also been shown that females have lower thresholds to mechanical pain in behavioural testing (Failla et al. 2024). Potentially, there is an association of this behaviour with an increased IB4 non-peptidergic C-fibre input. Our findings of male morphine exposed rats displaying significantly greater MOR intensity in IB4 labelling aligns with the increased IB4 labelling area seen in this treatment group. Overall, these comparisons highlight the complexity of interpreting sex differences in nociceptive signalling.

This study used three different measurements of MOR labelling in the DH to investigate any discrepancies between sex and treatment group. It is well established in the literature that sex plays a significant role on the interaction morphine has on pain processing. A clinical study revealed female patients required a greater dose (11% greater) of morphine to reach the same level of analgesia as male patients in the immediate postoperative period, and interestingly that this sex-specific effect was not observed in elderly patients (Aubrun et al. 2005). This is likely due to differences between sex at the anatomical level, but also indicates some degree of development as a factor to consider. Our findings revealed that morphine exposure had opposing effects on the sexes. The area of MOR

labelling decreased after opioid exposure in male rats, but had the reverse effect where it increased in female rats. This could suggest a difference in MOR expression; however, the total intensity of MOR staining showed no differences between sex or treatment group. Quantification of MOR protein levels in the DH would be valuable to undertake, or differences in transcript levels for MOR for further clarification. It is of course possible that MOR staining in our studies is not restricted to neurons, with MOR expression in glial cells. It would be valuable to label MOR with glial markers, as it could be neuroimmune interactions that are affected by the opioid exposure in males that explain the observed increase in total cell count.

Taken together, a change in MOR area, despite no change in intensity of labelling, could be justified by females simply exhibiting a broader receptor distribution across the DH, rather than any upregulation in MOR expression. Contrastingly, Loyd et al. (2008) found males had significantly higher expression of MOR in the ventral PAG (vlPAG) in male rats compares to females, and that morphine exposure elicited a significantly increased level of analgesia in males. From this, it suggests sex differences to opioid exposure is not necessarily occurring in the DH and that interactions of opioids and sex may be more susceptible to changes in the PAG-RVM.

4.2 Morphine exposure increased neuronal population in PV labelling in female rats only

The labelling of PV was primarily located in lamina IIi-III of the DH, which is in line with the current literature (Boyle et al. 2019). Our results revealed that there was a significant increase in NeuN+ve cells in PV labelling area or female rats exposed to morphine compared to female controls. This effect was not found in male rats and thus highlights the interaction sex may have on the observed response in neuronal population. Parvalbumin is a calcium-binding protein that is highly expressed in fast-spiking

inhibitory interneurons (Petitjean et al. 2015, Boyle et al. 2019). A critical study from Petitjean et al (2015) provided evidence into the important functional role PV neurons have in the modulation of sensory input and processing somatosensory signaling. This study found PV+ve interneurons essentially act as 'gate-keepers' that exert inhibitory control, and result in the prevention of innocuous touch mechanical sensations from activating nociceptive pathways. Ablation of PV+ve interneurons led to the development of mechanical allodynia (Petitjean et al. 2015). This highlights the functional role of PV and the impact an increased number of PV neurons could have in enhancing inhibitory control of morphine exposed female rats. The role of PV has further been examined beyond the DH in the DRG, where Fuller et al. (2023) highlighted PV neurons express the required enzymes and transporters to both synthesise and tonically release GABA. Together the literature shows the inhibitory control PV neurons can have on nociception, both within the DH and the periphery.

It is valuable to explore the effect morphine may have in changing the populations of NeuN+ve cells within the area of PV labelling. Repeated morphine exposure has been found to significantly reduce the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) in inhibitory neurons. This essentially suggests morphine exposure can indirectly inhibit inhibitory neurons by reducing the excitatory input these interneurons receive (Kearns et al. 2021). Morphine induced neuroplastic changes in the central nervous system is not unheard of, and has been associated with synaptogenesis and histone modifications in the dorsomedial prefrontal cortex (dmPFC), indicating an epigenetic effect (Liu et al. 2021), as well as neuroimmune responses (Terminel et al.2022). Although there is limited research on the direct effect morphine has on PV neurons in the DH, collating evidence from studies on the involvement of morphine in modulating GABAergic inhibitory transmission and neuroplastic offers a conceivable

interpretation of our change in neuronal population in the area of PV labelling.

We cannot ignore the important role sex plays in our findings. There was no significant change in male rats for PV neuron population and yet the majority of research into PV activity is completed in male rats only, offering little insight into the effect sex has on the interaction of PV and opioid exposure in the processing of nociception. As our findings did not show any significant difference between PV neuronal population between the sexes of our control groups, this leads us to speculate that potentially morphine exposure during the critical period of pain pathway maturation (Hathway et al. 2012), could be influencing hormonal-sensitive pathways that is contributing to the effect solely observed in female rats. Wu et al. (2014) found that in female mice, PV protein expression in the hippocampus gradually increased from postnatal weeks 3-12 and this was significantly correlated with serum 17β-estradiol levels, and that in male mice this effect was not observed. This provides understanding that sex steroid hormones could have a role in the regulation of PV expression during development. Although results are contrasting and often conducted in the brain not the DH, there are several studies that suggest ovarian steroids, in particular oestrogen, can modulate MOR expression and signalling (Sinchak et al. 2001, Torres-Reveron et al. 2009, Liu et al. 2007, Averitt et al. 2019). A study using a visceral pain model, discovered the potency of morphine was substantially greater in rats who had their ovaries removed, in comparison to rats with oestrogen replacement, with the measure of drug potency showing to be four times higher in ovariectomised rats (Ji et al. 2007). These various studies confirm the undeniable influence of oestrogen on efficacy of morphine and that it could be a hormonal role contributing to changes in the number of PV neurons in females exposed to morphine.

4.3 *The effect of opioid exposure to the PV & PKCg spinal circuit*

Our main motive of labelling PKCγ in the DH was based on the research from Petitjean et al. (2015) in which it describes a circuitry where inhibitory PV neurons synapse onto excitatory PKCγ neurons. This balance under normal conditions prevents innocuous touch from inciting nociceptive signalling. Blocking PKCγ interneurons attenuated mechanical allodynia that was previously a result of ablating PV interneurons, highlighting the dynamic between the two (Petitjean et al. 2015). We wanted to assess whether there were any interactions of sex or morphine exposure on this circuitry. Our results revealed that at baseline; female controls exhibited a greater PKCγ total intensity compared to male controls in the DH. This pattern was also found for the PKCγ intensity within the PV area of labelling, and though this doesn't explicitly indicate synaptic connections, it does indicate a level of overlap. In the context of nociception, this may suggest that females have increased excitatory output, and coincides with the notion that females tend to display greater sensitivity to pain (Fillingim et al. 2009).

Our results further showed that male rats who had been exposed to morphine in preadolescence, had significantly increased total intensity of PKCγ labelling compared to male controls. Research has implicated a role of PKCγ in the development of opioid tolerance (Mao et al. 1995, Mayer et al. 1995). In fact, there was a study that showed a significant increase in PKCγ labelling in the DH of morphine tolerant rats (Mao et al. 1995). However, this study was conducted in male rats only so we are unable to compare our findings of the lack of influence morphine had on female rats. However, it stresses that morphine exposure for seven consecutive days during preadolescence, resulted in similar findings to that seen with a greater dosage of morphine given to adult rats. This comparison emphasises the long-term influence opioids can have to the structure of nociceptive mechanisms in rats.

5.0 Future directions & limitations

This study demonstrates that morphine exposure during a critical period of postnatal maturation of the descending pain pathway, can lead to structural changes. Morphine is a commonly used drug for postoperative care and its analgesic properties, but as an opioid, it is also known for its adverse effects of addiction and drug dependence. Between 1999 to 2014, in utero infant exposure to opioids quadrupled, and in 2014 alone, over 25,000 infants in the US had been subject to prenatal opioid exposure (Haight et al. 2018). It would be interesting to assess the effect of in utero opioid exposure, and whether this causes implications on nociception later in life. For clinical relevance, methadone or buprenorphine as a replacement of morphine may be of clinical value for its use in treating opioid addiction in pregnancy (Tran et al. 2017). It has been shown in animal studies that have prenatal methadone exposure inutero can lead to altered pain behavioural responses (Enters et al. 1991), and it would therefore be interesting to explore if there are any structural changes that may occur in the spinal cord, as well as the PAG-RVM, for further insight and understanding. It is important to note that further research in pain studied should be conducted in both male and female subjects, as this study further confirms sex differences in nociception.

Our research used immunohistochemistry to identify pain markers in the DH that may have shown differences between sex and exposure to morphine, but it only displayed anatomical features that showed no significant interaction with sex and treatment group for total intensity of receptor expression. Future studies investigating phosphorylation of MOR, particularly at important regulatory sites like serine 375 that is important in the desensitisation and internalisation of MOR (Schulz et al. 2004). Adaptations in MOR expression within excitatory in contrast to inhibitory neurons would have differing functional implications. An increase in MOR expression on excitatory neurons would

typically lead to an enhanced analgesic effect by decreasing the transmission of nociceptive signalling, whilst an increase in MOR expression on inhibitory neurons typically results in disinhibition and enhanced pain sensitivity (Herman et al. 2024) Insight into these specific cellular markers would be valuable to interpret the potential long-term effect of morphine exposure on spinal circuitry.

Furthermore, although we know PV is primarily expressed in inhibitory interneurons, it can be expressed in excitatory neurons as well, therefore an additional immunofluorescence experiment would be valuable by colocalising PV expression with an inhibitory marker (such as VGAT) and excitatory marker (such as VGLUT2). This would allow us to determine the balance of excitatory compared to inhibitory in the DH and would be valuable in advancing our understanding of the impact from early life morphine exposure on later life pain processing.

6.0 Conclusion

This study sought to further understand the effect morphine exposure during the critical period of postnatal maturation of the descending pain pathway. Through the use of immunohistochemistry, we were able to identify various nociceptive markers in the DH and compare between sex and treatment groups for any significant changes. Our findings showed that there were alterations in IB4 labelling total intensity, marking the non-peptidergic C-fibre intensity, though this was specific to male rats only and was not found in peptidergic fibres. We found no change in MOR total intensity between groups, but did observe that morphine exposure caused opposing changes to the area of MOR labelling and total cell count within MOR labelling between sexes and treatment group, indicating a potential affiliation to glial cell activity and distribution of MORs. Furthermore, we found females exclusively showed an increase in NeuN+ve cells within PV labelling area after morphine exposure, indicating sex-specific interactions with morphine that led to

changes in PV. Furthermore, PKCγ intensity of labelling was increased in males exposed to morphine, an effect not seen in females as a result of opioid exposure, and could have implications in opioid tolerance. In summary, this data highlights importance in the inclusion of females in pain research as sex was a significant factor in the interaction of morphine on these various pain markers. It provides new insight into the impact early life opioid exposure in altering nociception later in life.

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Appendices

Appendix 1: Macro for IB4, CGRP, MOR

```
path = getDirectory("Choose a Directory");
files=getFileList(path);
for (fi=0; fi<files.length; fi++) {
        tag=files[fi];
        if (endsWith(tag, ".tif")==true) {
open(path+tag);
run("Set Measurements...", "area mean min display redirect=None decimal=2");

// Check brightfield image for artefacts
run("Channels Tool...");
Stack.setDisplayMode("color");
resetMinAndMax();
run("Enhance Contrast", "saturated=0.35");

// Split Channels
run("Split Channels");
    four = "C4-" + tag;
    three = "C3-" + tag;
    two = "C2-" + tag;
    one = "C1-"  + tag;


// Threshold MOR, measure area & MGI
selectWindow(four);
    run ("Duplicate...", " ");
    run("Auto Threshold", "method=Yen ignore_black white show");
    saveAs("tiff", path+tag+"C4threshold.tiff");
    run("Create Selection");
    roiManager("Add");
    roiManager("Select", 0);
    roiManager("Rename", path+tag+"MOR");
    roiManager("Save", path+tag+"MOR.roi.zip");
    selectWindow(four);
    roiManager("Select", 0);
    run("Measure");

//Count DAPI+ cells in MOR region
selectWindow(one);
resetMinAndMax();
run("Enhance Contrast", "saturated=0.35");
run("Enhance Contrast", "saturated=0.35");
roiManager("Select", 0);
run("Clear");
run("Select None");
setTool("multipoint");

waitForUser("Count number of DAPI+ nuclei, & record in spreadsheet. Click OK when finished.");

//record number of DAPI+ cells, save image & ROI
roiManager("Add");
roiManager("Select", 1);
waitForUser("DAPI+ count recorded?");
roiManager("Select", 1);
roiManager("Rename", path+tag+"DAPI");
roiManager("Save", path+tag+"DAPI.roi.zip");
roiManager("Select", 1);
run("Flatten");
saveAs("jpeg", path+tag+"DAPIcount.jpeg");
run("Close");
run("Select None");

// Threshold CGRP, measure area & MGI, & measure MOR within CGRP
selectWindow(three);
    run ("Duplicate...", " ");
    run("Auto Threshold", "method=Otsu ignore_black white show");
    saveAs("tiff", path+tag+"C3threshold.tiff");
```

```
    run("Create Selection");
    roiManager("Add");
    roiManager("Select", 2);

    roiManager("Rename", path+tag+"CGRP");
    roiManager("Save", path+tag+"CGRP.roi.zip");
    selectWindow(three);
    roiManager("Select", 2);
    run("Measure");
    selectWindow(four);
    roiManager("Select", 2);
    run("Measure");

// Threshold IB4, measure area & MGI, & measure MOR within IB4
    selectWindow(two);
    run ("Duplicate...", " ");
    run("Auto Threshold", "method=Triangle ignore_black white show");
    saveAs("tiff", path+tag+"C2threshold.tiff");
    run("Create Selection");
    roiManager("Add");
    roiManager("Select", 3);

    roiManager("Rename", path+tag+"IB4");
    roiManager("Save", path+tag+"IB4.roi.zip");

    selectWindow(two);
    roiManager("Select", 3);
    run("Measure");
    selectWindow(four);
    roiManager("Select", 3);
    run("Measure");

// Save results & thresholds, close all windows
    selectWindow("Results");
    saveAs("Results", path+tag+"Results.csv");
    run("Close");
    selectWindow("Log");
    saveAs("Results", path+tag+"Thresholds.csv");
    run("Close");
    selectWindow("ROI Manager");
    run("Close");

        }

    run("Close All");

    }


    run("Close All");
```

Appendix 2: Macro for NeuN, PV and PKCg

```
path = getDirectory("Choose a Directory");
files=getFileList(path);
for (fi=0; fi<files.length; fi++) {
        tag=files[fi];
        if (endsWith(tag, ".tif")==true) {
open(path+tag);
run("Set Measurements...", "area mean min display redirect=None decimal=2");

// Check brightfield image for artefacts
    run("Flip Vertically");
    run("Channels Tool...");
    Stack.setDisplayMode("color");
    resetMinAndMax();
    run("Enhance Contrast", "saturated=0.35");
    waitForUser("check BF image for damage or artefacts");
    setTool("freehand");

// Select grey matter in DH for analysis
    waitForUser("Draw ROI around DH");
    run("Clear Outside");
    roiManager("Add");
    roiManager("Select", 0);
    roiManager("Rename", path+tag);
    roiManager("Save", path+tag+"DH.roi.zip");
    run("Select None");

// Split Channels
```

```
run("Split Channels");
    five = "C5-" + tag;
    four = "C4-" + tag;
    three = "C3-" + tag;
    two = "C2-" + tag;
    one = "C1-"  + tag;

// Threshold PKC, measure area & MGI
selectWindow(five);
    run ("Duplicate...", " ");
    run("Auto Threshold", "method=RenyiEntropy ignore_black white show");
    saveAs("tiff", path+tag+"PKCthreshold.tiff");
    run("Create Selection");
    roiManager("Add");
    roiManager("Select", 1);
    roiManager("Rename", path+tag+"PKC");
    roiManager("Save", path+tag+"PKC.roi.zip");
    selectWindow(five);
    roiManager("Select", 1);
    run("Measure");

//Count DAPI+ cells in PKC region
    selectWindow(two);
    resetMinAndMax();
    run("Enhance Contrast", "saturated=0.35");
    run("Enhance Contrast", "saturated=0.35");
    roiManager("Select", 1);
    run("Clear");
    run("Select None");
    setTool("multipoint");

waitForUser("Count number of PKC DAPI+ nuclei, & record in spreadsheet. Click OK when finished.");

//Count NeuN+ cells in PKC region
    selectWindow(three);
    resetMinAndMax();
    run("Enhance Contrast", "saturated=0.35");
    run("Enhance Contrast", "saturated=0.35");
    roiManager("Select", 1);
    run("Clear");
    run("Select None");
    setTool("multipoint");

waitForUser("Count number of PKC NeuN+ cells, & record in spreadsheet. Click OK when finished.");

// Threshold PV, measure area & MGI, & measure PV within PKC
selectWindow(four);
    run ("Duplicate...", " ");
    run("Auto Threshold", "method=Triangle ignore_black white show");
    saveAs("tiff", path+tag+"PVthreshold.tiff");
    run("Create Selection");
    roiManager("Add");
    roiManager("Select", 2);
    roiManager("Rename", path+tag+"PV");
    roiManager("Save", path+tag+"PV.roi.zip");
    selectWindow(five);
    roiManager("Select", 2);
    run("Measure");
    selectWindow(four);
    roiManager("Select", 2);
    run("Measure");

//Count DAPI+ cells in PV region
    selectWindow(two);
    resetMinAndMax();
    run("Enhance Contrast", "saturated=0.35");
    run("Enhance Contrast", "saturated=0.35");
    roiManager("Select", 2);
    run("Clear");
    run("Select None");
    setTool("multipoint");

waitForUser("Count number of PV DAPI+ nuclei, & record in spreadsheet. Click OK when finished.");

//Count NeuN+ cells in PV region
    selectWindow(three);
    resetMinAndMax();
    run("Enhance Contrast", "saturated=0.35");
    run("Enhance Contrast", "saturated=0.35");
    roiManager("Select", 2);
    run("Clear");
    run("Select None");
    setTool("multipoint");

waitForUser("Count number of PV NeuN+ cells, & record in spreadsheet. Click OK when finished.");

// Save results & thresholds, close all windows
    selectWindow("Results");
    saveAs("Results", path+tag+"Results.csv");
    run("Close");
    selectWindow("Log");
    saveAs("Results", path+tag+"Thresholds.csv");
    run("Close");
    selectWindow("ROI Manager");
    run("Close");

        }

    run("Close All");

    }


    run("Close All");
```

Appendix 3: Macro for manual cell count in superficial, intermediate and deep DH

```
path = getDirectory("Choose a Directory");
files=getFileList(path);
for (fi=0; fi<files.length; fi++) {
        tag=files[fi];
        if (endsWith(tag, ".tif")==true) {
open(path+tag);
run("Set Measurements...", "area mean min display redirect=None decimal=2");

// Check brightfield image for artefacts
    run("Flip Vertically");
    run("Channels Tool...");
    Stack.setDisplayMode("color");
    resetMinAndMax();
    run("Enhance Contrast", "saturated=0.35");
    waitForUser("check BF image for damage or artefacts");
    setTool("freehand");

// Select grey matter in DH for analysis
    waitForUser("Draw ROI around DH");
    run("Clear Outside");
    roiManager("Add");
    roiManager("Select", 0);
    run("Measure");
    roiManager("Rename", path+tag);
    roiManager("Save", path+tag+"DH.roi.zip");
    run("Select None");

// Split Channels
run("Split Channels");
    five = "C5-" + tag;
    four = "C4-" + tag;
    three = "C3-" + tag;
    two = "C2-" + tag;
```

```
    one = "C1-"  + tag;

// DAPI Counts
    selectWindow(two);
    resetMinAndMax();
    run("Enhance Contrast", "saturated=0.35");
    run("Enhance Contrast", "saturated=0.35");
    makeRectangle(942, 72, 200, 100);
    run("Specify...", "width=200 height=100 x=413.54 y=31.61 scaled");
    roiManager("Add");
    roiManager("Select", 1);
    waitForUser("Position ROI in the superficial DH");
    run("Duplicate...", " ");
    setTool("multipoint");

waitForUser("Count number of DAPI+ nuclei in Superficial DH. Click OK when finished.");

    selectWindow(two);
    roiManager("Select", 1);
    waitForUser("Position ROI in the Intermediate DH");
    run("Duplicate...", " ");
    setTool("multipoint");

waitForUser("Count number of DAPI+ nuclei in Intermediate DH. Click OK when finished.");

    selectWindow(two);
    roiManager("Select", 1);
    waitForUser("Position ROI in the Deep DH");
    run("Duplicate...", " ");
    setTool("multipoint");

waitForUser("Count number of DAPI+ nuclei in Deep DH. Click OK when finished.");

// NeuN Counts
    selectWindow(three);
    resetMinAndMax();
    run("Enhance Contrast", "saturated=0.35");
    run("Enhance Contrast", "saturated=0.35");
    roiManager("Select", 1);
    waitForUser("Position ROI in the Superficial DH");
    run("Duplicate...", " ");
    setTool("multipoint");

waitForUser("Count number of NeuN+ cells in Superficial DH. Click OK when finished.");

    selectWindow(three);
    roiManager("Select", 1);
    waitForUser("Position ROI in the Intermediate DH");
    run("Duplicate...", " ");
    setTool("multipoint");

waitForUser("Count number of NeuN+ cells in Intermediate DH. Click OK when finished.");

    selectWindow(three);
    roiManager("Select", 1);
    waitForUser("Position ROI in the Deep DH");
    run("Duplicate...", " ");
    setTool("multipoint");

waitForUser("Count number of NeuN+ cells in Deep DH. Click OK when finished.");

// Save results, close all windows
    selectWindow("Results");
    saveAs("Results", path+tag+"Results.csv");
    run("Close");
    selectWindow("ROI Manager");
    run("Close");

        }

    run("Close All");

    }


    run("Close All");
```