Postnatal maturation of the opioid and endocannabinoid signalling systems within the descending pain pathway of the rat

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Abstract

Significant opioid- and endocannabinoid- dependent changes occur within the periaqueductal grey (PAG), rostroventral medulla (RVM) and spinal cord (DH) during postnatal development of the rat (Sprague Dawley). These changes are involved in the differential descending control of spinal excitability between young and mature rats. Microinjection of the μ-opioid receptor (MOR) agonist DAMGO (30ng) into the PAG of rats increased spinal excitability and lowered mechanical threshold to noxious stimuli in postnatal day (P)21 rats, but had inhibitory effects in adults and lacked efficacy in P10 pups. A tonic opioidergic tone within the PAG was revealed in adult rats by intra-PAG microinjection of CTOP (120ng, MOR antagonist) which lowered mechanical thresholds and increased spinal reflex excitability. Spinal adminstration of DAMGO inhibited spinal excitability in all ages yet the magnitude of this was greater in younger animals than in adults. The expression of MOR and related peptides were also investigated using TaqMan RT-PCR and immunohistochemistry. Proopiomelanocortin (POMC) peaked at P21 in the ventral-PAG, and MOR increased significantly in the DH as the animals aged. CB1/CB2 receptor activation by WIN55212 (4μg, CB1/CB2 agonist) and HU210 (4μg, CB1/CB2 receptor agonist) in the PAG, RVM and DH was anti-nociceptive in both young (P10, P21) and adult rats, but GPR55 receptor activation by LPI (12μg, endogenous GPR55 agonist) and AM251 (2.77μg, CB1 antagonist, GPR55 agonist) was exclusively inhibitory in young rats. Micro-injection of LPI into the adult RVM facilitated spinal reflex excitability, suggesting that GPR55 receptor activation in mature animals is pro-nociceptive. The expression of cannabinoid receptors and endocannabinoid-synthesising enzymes was investigated with immunohistochemical and TaqMan RT-PCR techniques. Overall the expression of CB1 receptors and the anandamide synthesising enzyme NAPE-phospholipase D (NAPE-PLD) increased within the descending pain pathway with age, whereas the expression of the 2-AG synthesising enzyme Diacylglycerol lipase α (DAGLα) decreased. These results illustrate that profound differences in the endogenous-opioidergic and endocannabinoid signalling systems occur within the descending pain pathway throughout postnatal development.

Chapter 1 General Introduction

1.1 What is pain?

Pain is a subjective experience. It represents a complex sensory modality encompassing physiological, affective, motivational and cognitive aspects. It is defined by the International Association for the study of Pain (IASP) as 'an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (H. Merskey, 1994). The term 'pain' takes into account both the emotional and cognitive responses to the physiological sensation of noxious or potentially noxious stimuli. The term 'nociception', which is typically described in animal studies, is defined by IASP as 'the neural process of encoding noxious stimuli', thus the emotional components of pain in these studies are inferred only.

1.2 Peripheral mechanisms of pain

1.2.1 Peripheral sensory transmission

Cutaneous receptors are sensory receptors in the skin that convey somatosensory information in order to initiate an appropriate response. They respond to a range of modalities, including mechanical stimuli such as pressure, touch and vibration, thermal, chemical as well as noxious stimuli (Andres and Düring, 1973). Upon tissue injury and damage, specialised sensory receptors for noxious stimuli known as nociceptors are activated. Nociceptors encode information for both the intensity and duration of the stimulus, as well as providing information relating to the location where injury has occurred (Peschanski et al., 1981, Handwerker et al., 1987, Cervero et al., 1988).

1.2.2 Primary afferent fibres

Somatosensory information detected by peripheral sensory receptors is passed onto the central nervous system via sensory neurones known as primary afferent fibres (PAFs). These fibres innervate the body and originate from cell bodies in the trigeminal and dorsal root ganglia (DRG)(Mesulam and Brushart, 1979, Lawson and Waddell, 1991). PAFs can be classed into three main subtypes (Aα/β, Aδ and C) according to their action potential conduction velocity, which is largely a consequence of the myelination and diameter of the axon (Boyd and Kalu, 1979). In this section, the functional and structural properties of PAFs in rats are described. Earlier research into the structural and functional properties of sensory neurones were originally conducted in cats (Hunt and Mcintyre, 1960), and they are conserved in other mammals. For a review of interspecies variations of these properties please see the following review (Djouhri and Lawson, 2004).

Myelinated Aα/β PAFs have neuronal diameters within the range of 5 to 14 μm. The conduction velocity for Aα fibres are normally >300m/s and for Aβ 14-30m/s (Harper and Lawson, 1985). Aδ fibres are also myelinated, but are smaller in diameter (2-5 μm)(Erdine et al., 2009) and have slower conduction velocities (12-30m/s)(Lawson and Waddell, 1991). Non-myelinated C-fibres are small in diameter (0.4-1.2 μm), and have conduction velocities of less than 2m/s (Woolf and Fitzgerald, 1983).

Some studies suggest that the reported conduction velocities for A-fibre types are controversial, as mid-range conduction velocities are either arbitrarily assigned as one type or the other, or differentiated on the basis of a range of properties such as stimulus modality responded to, or the waveform of resultant compound action potentials (Burgess and Perl, 1973, Villiere and McLachlan, 1996). Other confounding factors including the age of animals, temperature at which the recording was performed, and distance of recording site from the cell body of the neurone further complicate the results obtained in these experiments (Birren and Wall, 1956, Hopkins and Lambert, 1973, Harper and Lawson, 1985). Therefore, the ranges supplied above are generalised figures supplied for comparison only.

Under normal circumstances, Aδ- and C- fibres encode for nociceptive information; myelinated Aδ- fibres elicit a rapid, first phase of pain, which is sharp in nature, whereas unmyelinated C-fibres evoke a second wave of dull pain (Handwerker and Kobal, 1993, Belmonte and Cervero, 1996). Aα/β-fibres are responsive only to low threshold tactile stimulation, such as touch, vibration, pressure and other innocuous stimuli. However, there is evidence in several species, including cat, rat and guinea pig (Lawson, 2002) which shows that a subpopulation of rapidly conducting Aα/β-fibres exist and may contribute to nociceptive transmission. Brief activation of nociceptive C-fibres causes central sensitization by increasing the excitability of spinal neurones, which in turn lowers the nociceptive threshold of neurones within that receptive field, and allowing the recruitment of Aα/β PAFs (Cook et al., 1987, Hylden et al., 1989a). More information on the receptive field of spinal neurons is included in section 1.3.3. For further details on sensitisation and Aα/β- fibre mediated nociception please see review (Woolf and Doubell, 1994).

Aδ-fibres can be subdivided into type I and II. Type I Aδ-fibres are high threshold, rapidly conducting mechanoreceptors that are also weakly responsive to high intensity heat (>53ºC), cold and chemical stimuli (Handwerker and Kobal, 1993, Treede et al.,

1995, Simone and Kajander, 1997). However, repetitive thermal stimulation has been shown to cause sensitisation; following tissue damage, they become responsive to noxious heat and show sustained responses to thermal stimuli of long duration and slow latency (Treede et al., 1995). Type II Aδ-fibres are less common and are slower conducting. They display a lower threshold to heat (43 degrees) and therefore respond to heat preferentially compared to type I Aδ-fibres (Treede et al., 1995, Beydoun et al., 1996).

High threshold sensory receptors are localised on C-fibers and encode for nociceptive information relating to noxious cold, heat, chemical or mechanical stimuli. There is a subset of C-afferents that responds to all the modalities aforementioned, thus is termed polymodal (Torebjörk, 1974, Torebjörk and Hallin, 1974).

1.2.3 Neurochemistry of primary afferent fibres

Transduction of a particular sensory modality relies on the presence of receptors and ion channels on PAFs that can transform a stimulus into action potentials. Therefore, the categorization of PAFs can also be based on their neurochemistry. In general, small diameter C-fibres are classified as peptidergic or non-peptidergic (Hunt and Rossi, 1985). Non-peptidergic C-fibres express isolectin B4 (IB4) and the purinergic receptor $P2X_3$, while peptidergic C-fibres express the neuropeptide substance P (SP), calcitonin gene-related peptide (CGRP)(Averill et al., 1995, Molliver and Snider, 1997) and tyrosine kinase receptor Type I (TrkA), the high affinity receptor for the neurotrophin nerve growth factor (NGF)(Kaplan et al., 1991).

Approximately half of both peptidergic and non-peptidergic C-fibres express the transient receptor potential cation channel subfamily V member 1 (TRPV1), which responds to heat, capsaicin and protons (Chan et al., 2003). In addition, changes in tissue pH are detected by acid sensing ion channels expressed on C-fibres and cold sensation (change in temperature or chemically induced, e.g. menthol) in the skin is thought to be mediated by the ion channel TRPM8 (Kobayashi et al., 2005, Bautista et al., 2007). The exact mechanism of mechano-transduction in the periphery is less well understood. However, recent identification of the Piezo family as a mechano-transducer in drosophila may lead to a better understanding of these processes; behavioural responses to noxious mechanical stimulation evoked by von Frey hairs (vFh) are inhibited in Dmpiezo knock-out larvae, whilst responses to noxious heat and gentle touch are unaffected (Kim et al., 2012). The Piezo family (Piezo 1 and 2) are highly conserved transmembrane proteins and are known to be expressed in mouse neuroblastoma cells and dorsal root

ganglion (DRG) neurones (Coste et al., 2010), and on the membrane surface of Merkel cells, which is responsible for mediating slowly adapting responses of Aβ-fibres to encode fine touch sensations (Woo et al., 2014). Other potential candidate for mechano-transduction include Mdeg (also known as BNC1) (Driscoll and Tavernarakis, 2000) and Trek1 receptors (Tsunozaki and Bautista, 2009), both are cation channels that respond to stretching of the skin. For a recent review on mechano-sensory transduction please refer to (Delmas et al., 2011).

In contrast, the neurochemistry of A-fibres has not been extensively studied. It is known that CGRP is expressed in Aδ-fibres in the DRG (Lawson, 2002). Some studies showed that cutaneous Aα/β-fibres contain little or no SP (Lawson et al., 1997). However, under pathological conditions, Aβ-fibres may express and release SP (Noguchi et al., 1995, Malcangio et al., 2000). After peripheral spinal nerve ligation (SNL), SP content in spinal cord tissue was examined by radioimmunoassay and it was reported that significant SP is released in SNL animals even after selective activation of Aβ-fibres (Malcangio et al., 2000)

Other major neurotransmitters and neuromodulators present in PAFs include glutamate, the global energy source adenosine triphosphate (ATP), nitric oxide (NO), opioids (Ji et al., 1995, Martin-Schild et al., 1998), cannabinoids (Ahluwalia et al., 2000) and phospholipid metabolites such as prostaglandins and neurotrophins. For an extensive list of neurotransmitters involved in nociceptive processing on PAFs, please see review (Millan, 1999).

1.3 Central mechanisms of pain – spinal cord

1.3.1 Laminal organisation of the spinal cord

The dorsal horn (DH) of the spinal cord is the first site of integration of sensory information in the CNS; PAFs carrying sensory information from the periphery enter the DH via the dorsal root and synapse on intrinsic DH neurones (Mesulam and Brushart, 1979). The ventral horn (VH) is populated by motorneurones (MN), which are classed as efferent fibres and are important for the initiation and coordination of movement, particularly in withdrawal reflexes associated with noxious stimulation (Romanes, 1946). The spinal cord grey matter is divided into ten laminae, originally described in cryoarchitectural Nissl staining of the spinal cord in cats (Rexed, 1952, 1954), but

lamination of the spinal cord is also conserved in rats (Molander et al., 1984, Molander et al., 1989). A diagram showing the organisation of laminae in the spinal cord is included in Figure 1.1.

Lamina I (marginal layer), II (substantia gelatinosa), III and IV (nucleus propius), V and VI (deep layers) are collectively referred to as the DH. Lamina VII (intermediate grey area), VIII and IX form the medial and lateral VH. The region surrounding the central canal of the spinal cord is lamina X. The DH is also subdivided into the superficial laminae (I and the outer layer of II/II_o) and deep laminae (III-VI), these regions are particularly important in the detection, processing and transmission of nociceptive information from the periphery. Nociceptive information transmitted to the DH is then either relayed locally to reflex circuits and/or projected to supraspinal centres via the different types of intrinsic DH neurones (i.e. projection neurones), this will be discussed in the *section 1.3.3*.

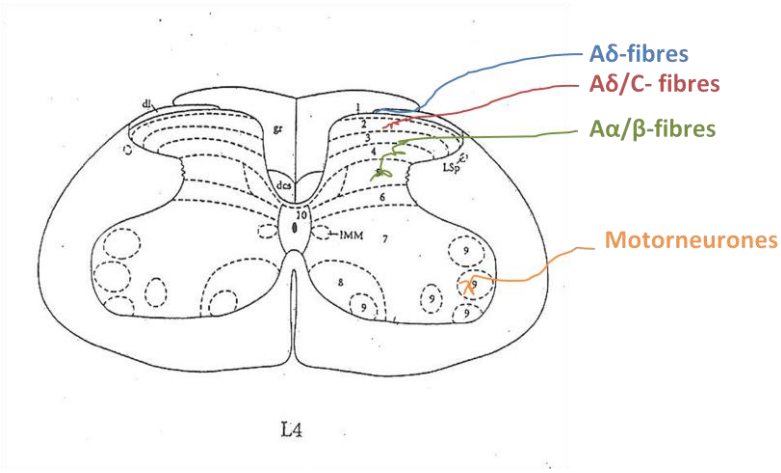


Figure 1-1 Organisation of the spinal cord dorsal horn (DH) laminar and primary afferent fibre terminals. *Nociceptive specific primary afferent fibres terminate in laminae I, II, II/III of the spinal cord dorsal horn.*

1.3.2 Spinal target of primary afferent fibres

PAFs are somatotopically distributed within the DH, such that the areas on the skin have specific representation within their central terminal fields in the spinal cord (Swett and Woolf, 1985, Molander and Grant, 1986). For example, PAFs innervating the rat hindlimb terminate in a strict topographic pattern in the superficial DH so that neighbouring skin areas are innervated by a slightly different, yet overlapping population of sensory neurones (Swett and Woolf, 1985). Specifically, transganglionic studies revealed that nerves supplying the rat hindlimb are organized in a mediolateral direction; the common

peroneal nerve, which supplies the dorsum of the foot terminates in the lateral third of the superficial DH (Woolf and Fitzgerald, 1986), whereas the tibial nerve, which supplies the plantar region of the foot terminates in the medial dorsal horn (Molander and Grant, 1986, Grant, 1993). In general, the more caudal the innervated skin area is, the more caudal the location of the spinal root of the nerve innervating that particular area of skin. In addition, this mediolateral pattern is conserved across the thoracic, lumbar and sacral segments of the spinal cord (Molander et al., 1984, Rivero-Melián and Grant, 1990). Horseradish peroxide (HRP)-based tracing studies revealed that spinal nerves in the thoracic segment branch and arborise in a longitudinal columnar fashion, such that the terminals of the nerves spanning across the thoracic segments display the same somatotopic arrangement (Ygge and Grant, 1983).

PAFs entering the DH can be distinguished based on fibre sizes and the sensory modalities they encode for (Todd, 2002). Finely myelinated (Aδ) or unmyelinated (C) small diameter PAFs primarily encode for high threshold nociceptive information, they terminate predominantly in laminae I and II, and to a lesser extent laminae V and VI (Light and Perl, 1979, Light et al., 1979, Sugiura et al., 1989). Specifically, C fibres project heavily to lamina II_o and limitedly to laminae I, V and X. Aδ fibres terminate mostly in lamina I but also weakly innervate laminae V and X. Some Aδ afferents innervate low threshold mechanoreceptors and arborise on either side of the laminae I to II border (Light and Perl, 1979, Light et al., 1979). Large diameter, low threshold Aα/β fibres display a distinctive pattern of arborisation and function predominantly as mechanoreceptors, they project to laminae III to IV and to a lesser extent laminae V and VI (Molander and Grant, 1986). In adult rats only a few Aβ terminals can be observed in laminae I and the inner layer of II (II_i), Aβ-fibres also do not innervate lamina II_o. However, in the early postnatal period, the immature superficial dorsal horn is dominated by Aβ-inputs (Fitzgerald and Jennings, 1999). The functional implications of this will be discussed in *section 1.7.2*.

MNs in the VH also display a somatotopic organization (Hardman and Brown, 1985); they are specified to innervate a particular muscle, and the location of the muscle is predictable from the position of the neurone within the MN pool in the VH. The types and organization of MNs within the VH will be discussed in further details in *section 1.3.4*.

1.3.3. Intrinsic dorsal horn neurones

Intrinsic DH neurones are responsible for the transmission of sensory information encoded by PAFs either onto reflex circuits or supraspinal sites. Although most PAFs

synapse with DH neuones in an ipsilateral fashion, a few of them may also cross over to the contralateral side (Culberson et al., 1979, Menetrey et al., 1989). All neurones within the DH possess a receptive field (RF), from which the activity of all the PAFs within the RF summate centrally to evoke action potentials (Price et al., 1977, Andersen et al., 1994). RFs in the DH are also organized somatotopically, for example, in the presence of tissue injury in the skin, an expansion in receptive field is observed, which represents either an increase in peripheral input or the occurrence of sensitisation (McMahon and Wall, 1984, Cook et al., 1987). Intrinsic DH neurones can be classified according to the nature of their response to sensory inputs (Menétrey et al., 1977), or their efferent destination.

Nociceptive specific (NS) neurones are activated by high threshold noxious stimulation only. They are found most concentrated in the superficial laminae of the dorsal horn and are innervated mostly by Aδ- and C- fibres (Cervero et al., 1976). Low threshold mechanoreceptive (LTM) neurones are found in the deeper laminae III and IV, they are also referred to as non-nociceptive because they do not respond to noxious stimulation, such as the application of mustard oil to the skin (Woolf and King, 1990), and are only responsive to tactile stimulation such as touch and pressure (Menétrey et al., 1977). LTM neurones are mostly excited by Aα/β mediated activity.

There is also a subset of DH neurones that respond to both noxious and innocuous stimulation (Handwerker et al., 1975, Menetrey et al., 1977, Menetrey et al., 1979). They are termed wide dynamic range (WDR) neurones, which refer to the wide variety of stimulus modalities they respond to, including touch, noxious chemical, heat and pressure. They are predominantly found in the deeper DH laminae IV to VI (Coghill et al., 1999), and sparsely in the superficial laminae (Woolf and Fitzgerald, 1983), lamina X and the ventral horn. WDRs respond to activity mediated by all three types of PAFs, and fire action potentials in a graded fashion depending on stimulation intensity (Mendell, 1966, Maixner et al., 1986). They also exhibit 'wind-up', a form of synaptic plasticity whereby continuous discharge and spontaneous bursting activity are observed in the neurone long after the onset of stimulation (Banna et al., 1986, Cata et al., 2006).

DH neurones can also be categorized by their efferent destination. Propriospinal (PS) neurones are responsible for communication between spinal segments and between the ipsilateral and contralateral DH, and are important for transmitting descending inhibitory signals following noxious stimulation (Alstermark et al., 1991). Moreover, propriospinal

neurones synapse with the MN pool in the VH, thus are involved in the coordination of movements by activating the local reflex circuits (Burke et al., 1992). The anatomical and functional organization of reflex circuits will be further discussed in *section 1.5*.

Projection neurones (PN) and interneurones (IN) also receive peripheral sensory information via PAFs. As their names suggest, PNs are involved in transmitting information to supraspinal centres (Trevino and Carstens, 1975), and are monosynaptically activated by A- and C- fibres and are located in laminae I, V and VI. INs are responsible for intra-laminal and inter-laminar modulation of PAF inputs (Jankowska and Lindström, 1972). Both PNs and INs can be of the WDR, NS and LTM subtypes. In addition, INs can also be subdivided into excitatory and inhibitory subtypes, depending on the expression of neurotransmitters of the respective IN. For a comprehensive review of the different functional IN subtypes please refer to (Millan, 1999).

1.3.4 Motorneurones and organisation of the ventral horn

MNs are responsible for the coordination of movement, they transmit signals from central sites to the periphery and formulate an appropriate response according to the nature of peripheral stimulation (Schieppati, 1987). Early work by Hunt and Kuffler in adult cats led to the identification of two MN subtypes, they are both myelinated and are differentiatied by their structure and functions (Hunt and Kuffler, 1951). α-MNs are large nerve fibres with diameters of 8-18μm and conduction velocities of 50-110 m/s, they are rapidly conducting and innervate skeletal muscle. γ-MNs are 3-8μm in diameter and they are slower conducting (15-50m/s), they provide information such as stretching and proprioception. The diameters and conduction velocities of motorneurones in cats are comparable to those of adult rats (Fraher and Kaar, 1985).

As aforementioned, VH is mostly comprised of MNs. Specifically, Romanes illustrated that the cell bodies of efferent nerves innervating the muscle of the cat are located longitudinally in the lateral VH and are known as spinal motor nuclei (Romanes, 1951). In rats, the MNs innervating a single muscle is also found in the longitudinal column of the lateral VH (Nicolopoulos-Stournaras and Iles, 1983). MNs in the lateral VH column are organized along the dorsal-ventral and mediolateral axes. Specifically, along the dorsal-ventral axis, the cell bodies of MNs innervating the proximal muscles of the limb are located in the ventral-most region of the VH, and those innervating the distal muscle of the limb are located in the dorsal regions (Romanes, 1951, Nicolopoulos-Stournaras and Iles, 1983). Along the medio-lateral axis, MNs innervating the medial muscles of

the thigh are located more rostrally compared to those innervating the lateral thigh muscles (Hardman and Brown, 1985). Therefore, similar to sensory afferent fibres, efferent MNs are also organized somatotopically and the central location of MN nuclei reflects the location of the muscle they innervate.

1.4 Central Mechanisms of pain – supraspinal centres

As mentioned briefly in the previous sections, nociceptive transmission requires interactions between neuronal populations in both spinal and supraspinal sites. This is due to the fact that the outcome of a peripheral stimulus is not determined solely at the spinal level. As demonstrated in both human and animal studies, a single discharge of an individual nociceptive fibre is not perceived as noxious and does not cause a nocifensive response, activity of multiple nociceptive fibres are required over a specific duration of time to elicit a noxious response (Vierck et al., 1997); and the strength of stimulus required to evoke a nocifensive response in rats is much higher than the threshold needed for eliciting firing of action potentials in a single nociceptive fibre: in tail-immersion noxious heat test, rats lifted their tails from the water at a threshold temperature of 43.7 ^{o}C, just above the threshold for lamina I neurones (42 ^{o}C) (Mitchell and Hellon, 1977). These observations indicate that the activity of nociceptive fibres within the spinal cord does not always correspond to the degree of pain measured behaviourally, and that activity within supraspinal sites must also contribute to the pain sensitivity in a whole animal.

1.4.1 The ascending pathway

PNs of the DH transmit sensory information received from the periphery onto supraspinal sites via ascending tracts, the major ones include spinothalamic (ST) tract, which projects directly to the thalamus, and the spinoparabrachial (SPB) tract which projects to the parabrachial area of the brainstem. The courses of these two tracts from the DH to the supraspinal centres are summarized in *Figure 1.2*.

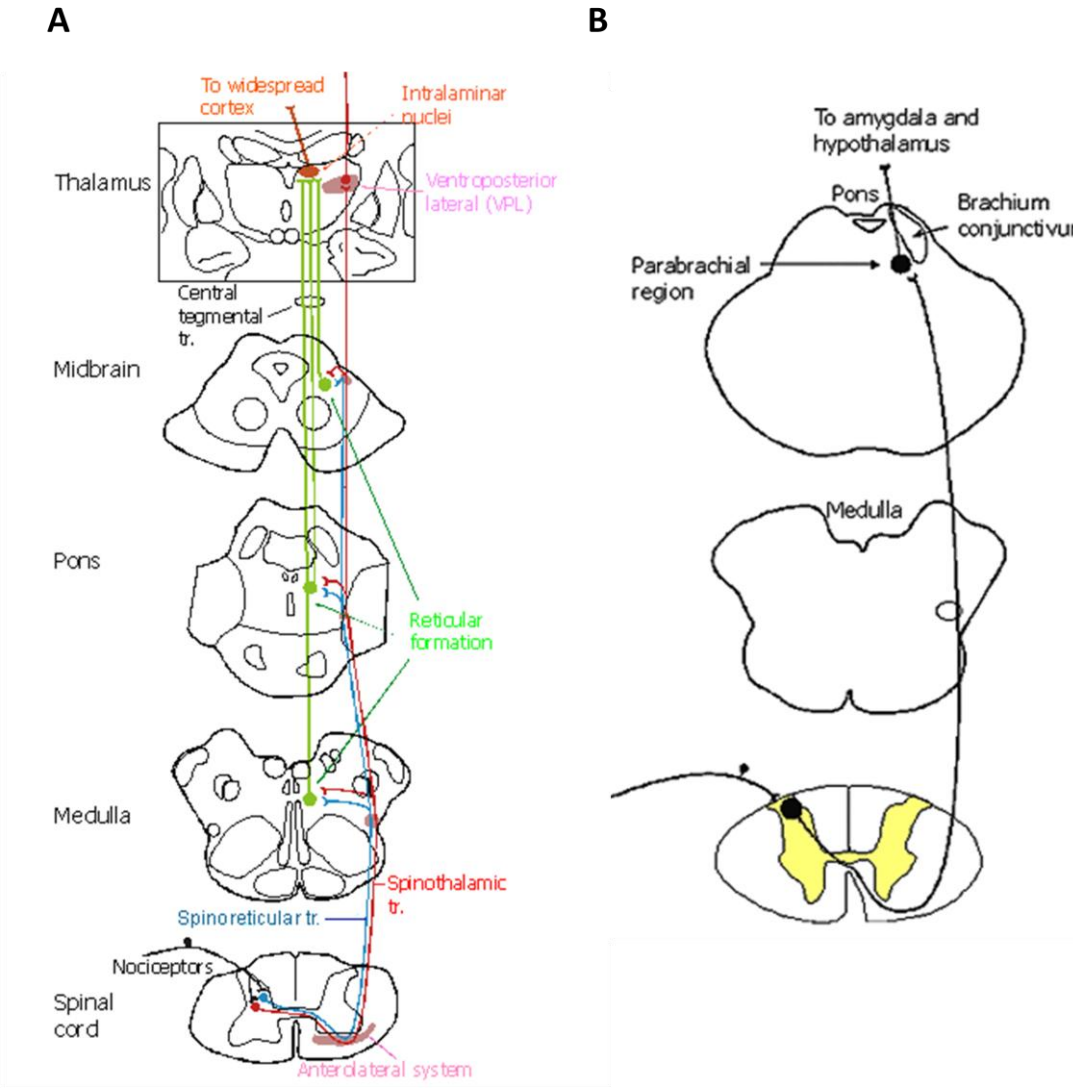


Figure 1.2 (A) The spinothalamic tract (B) The spinal parabrachial tract. Primary afferent fibres (PAFs) enter the spinal cord dorsal horn (DH) and transmit nociceptive information via ascending tracts. The thalamus and parabrachial region are important integrating sites, and have efferent projections onto other cortical areas. Diagram adapted from (Foreman et al., 1986).

The site of termination of the ST tract was examined using anterograde tracing techniques, the precise targets of the ST tract in the thalamus are the central lateral, posterior, supragenicuIate, limitans, submedius, medial dorsal, paracentral, central medial, reuniens and periventricular nucleus (Mantyh, 1983). Early studies using electrophysiological techniques identified the origin of ST tract neurones in the DH of both adult cats and rats, (Dilly et al., 1968), which are located in lamine I, V and VI. A later study using HRP as a reterograde tracer found that the ST tract ascend mostly in a contralateral fashion, and neurones that project to the lateral structures of the thalamus originate from lamina I of the DH and ascend via the ventrolateral funiculus, whereas

neurones that project to the medial structure of the thalamus originate from laminae V and VI of the DH, and ascend via the ventral funiculus (Giesler et al., 1981).

The thalamus is a key structure for the transmission of nociceptive information; the medial thalamus is involved in the affective and motivational aspects of pain whereas the lateral thalamus is responsible for the sensory and discriminatory components of noxious stimuli (Burstein et al., 1990). Moreover, the thalamus acts as a relay for the sensory discrimination of painful stimuli, it projects to somatosensory cortex, and the ST tract has collaterals to the parabrachial area in the brainstem, which is important for descending pain modulation (Hylden et al., 1989b).

Electrical stimulation of the parabrachial area (20–40 μA) inhibits firing activity of both A and C fibre in the trigeminal nucleus caudalis of the rat spinal cord evoked by electrical stimulation of cutaneous and deep tissue (Chiang et al., 1995).

Nociceptive afferents travel up to the parabrachial (PB) nucleus, which is located at the junction between the medulla and the pons in the lateral reticular formation. In reterograde tracing studies using wheat germ agglutinin-conjugated HRP, it was demonstrated that the origin of the SPB tract in the DH is located bilaterally in laminae I, V, and VII throughout the entire length of the spinal cord (Kitamura et al., 1993). The efferent connections of the PB nucleus are also well studied by anterograde autoradiographic methods, these regions include paraventricular nuclei of the thalamus, the dorsomedial, ventromedial, arcuate, lateral hypothalamic and the lateral preoptic areas of the hypothalamus and the anterior, central, medial, basomedial, posterior basolateral nuclei of the amygdala (Saper and Loewy, 1980). The amygdala is particularly important for the mediation of the attentional and emotional components of pain (Villemure and Bushnell, 2009), and the hypothalamus is essential for modulating autonomic functions affected by painful stimulus, such as heart rate and blood pressure (Bester et al., 1997).

In addition to the ST and SPB tracts, several spinobulbar pathways, including the spinoreticular (SR) and spinomesencephalic (SM) tracts are also involved in the transmission and modulation of nociceptive information. SM tract originates from neurones in lamina VI and VII (Menetrey et al., 1980), whereas the cell bodies of neurones forming the SR tracts are found in lamina I, IV to VI (Menétrey et al., 1982).

The efferent destinations of the spinobulbar pathways include the amygdala and the hypothalamus (Bernard et al., 1996), but they also project to the periaqueductal grey (PAG) via the PB nucleus (Hylden et al., 1986). The PAG is a major site of homeostatic and limbic motor output (Carrive et al., 1987, Bandler, 1988, Carrive and Bandler, 1991), as well as an integrating site for nociceptive information (Basbaum and Fields, 1979). The PAG is an important site within the descending pathways, which will be discussed in further details in section 1.4.3.

1.4.2 Cortical representation of pain

As mentioned in the previous section, the ascending tracts terminate in a variety of forebrain structures. Recent advances in neuroimaging techniques have enabled us to visualise how pain is represented in the forebrain (Brooks and Tracey, 2005). Cortical and subcortical structures that are activated by noxious stimulation include the thalamus, anterior cingulate cortex (ACC), insula, frontal cortices, primary somatosensory cortex (SI) and secondary somatosensory cortex (SII). Furthermore, using electrophysiology, spinal projections to the RVM, PB nucleus, PAG and reticular formation and subsequent efferent projections from these sites connecting the thalamus and SI are identified (Ab Aziz and Ahmad, 2006). These connections can be categorised along either a lateral or medial axis, depending on the location of their efferent terminations (Brooks and Tracey, 2005). The lateral system involves projections from the thalamic nuclei (ventral posterior lateral/VPL, ventral posterior medial/VPM and ventral posterior inferior/VPI) to the SI and SII, it is primarily responsible for the discrimination of the location and the intensity of noxious stimuli. The medial system involves projections from other thalamic nuclei (posterior part of the ventromedial nucleus (VMpo), ventrocaudal part of the medial dorsal nucleus (MDvc), parafasicular (Pf) and centrolateral (CL) nuclei) to the insula and the ACC. The ACC is mainly responsible for the affective component of pain, whereas the insula encodes the intensity and laterality of innocuous and noxious stimuli (Treede et al., 1999).

1.4.3 The descending pathway

Early research using electrophysiological techniques in cats and rats has shown that electrical stimulation of the PAG and the rostroventral medulla (RVM) produce analgesia (Mayer et al., 1971, Liebeskind et al., 1973, Mayer and Liebeskind, 1974, Rhodes and Liebeskind, 1978, Behbehani and Fields, 1979). This phenomenon is known as stimulation produced analgesia (SPA), and is mediated via the activation of the descending pathway. A lesion in the dorsolateral funiculus of the rat spinal cord abolishes the analgesic effects evoked by electrically stimulating the PAG (Basbaum et al., 1977), and electrical stimulation of the nucleus raphe magnus (NRM) of the RVM

inhibited activity of high threshold nociceptive neurones in laminae I, V and VI of the DH (Fields et al., 1977).

The perceived intensity of a painful stimulus is partially determined by the strength and duration of stimulation (Andrew and Greenspan, 1999, Gopalkrishnan and Sluka, 2000), but other factors, such as stress, expectation and arousal also modify the neural, behavioural and subjective responses to pain (Arena et al., 1990, Quintero et al., 2003). Stress-induced analgesia (SIA) is an in-built mammalian mechanism that contributes to the suppression of nociception, rats that were predisposed to stress by the forced swim paradigm display significantly less pain behaviours (i.e. jumping and flinching) induced by subsequent electric footshock (Bodnar et al., 1978). Stress induces neuronal activity in the medial prefrontal cortex, hippocampus, amygdala, and the hypothalamus (Stein-Behrens et al., 1994, Sousa et al., 2000, McGregor et al., 2004, Dedovic et al., 2009). Numerous studies have demonstrated that these regions modulate nociceptive responses via the descending pathway, for example, blockade of excitatory activity in the rat hippocampus abolished pain behaviours induced by subcutaneous injection of formalin to the hindpaw (McKenna and Melzack, 2001), and lesioning the central nucleus of the amygdala inhibited the antinociceptive effects of morphine in the formalin test (Manning and Mayer, 1995). For further information on SIA please refer to the review (Butler and Finn, 2009).

The PAG integrates descending sensory inputs from the frontal and insula cortices, amygdala, hypothalamus and various other brainstem regions such as the nucleus cuneiformis, pontine reticular formation and the locus coeruleus (Gebhart, 1982). Neurones in the PAG control nociceptive transmission via a spinobulbar loop; they descend into the spinal cord DH by entering the dorsolateral funiculus indirectly via the RVM and the dorsolateral pontine tegmentum (DLPT) (Basbaum and Fields, 1978). This pathway is summarized in Figure 1.3.

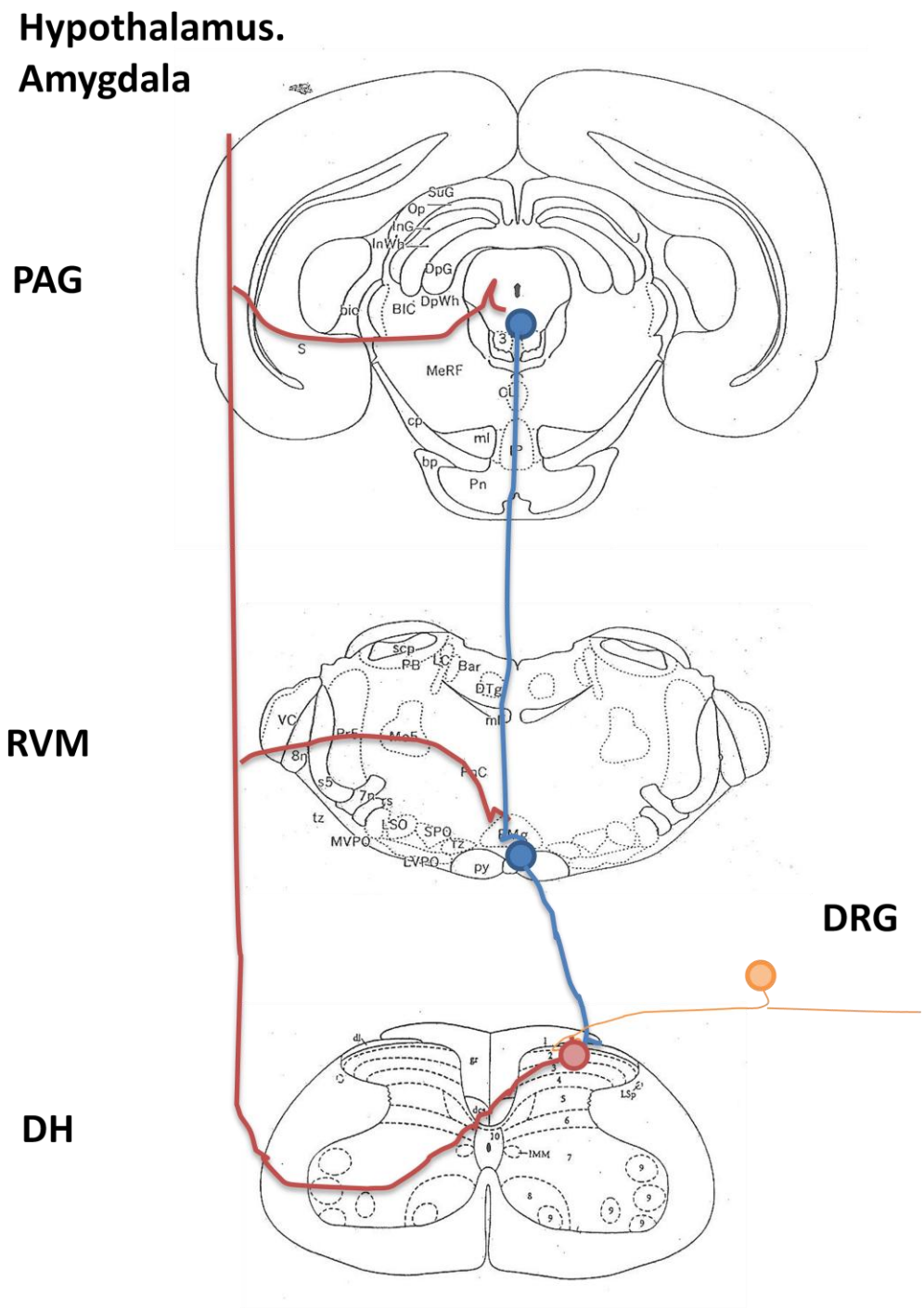


Figure 1.3 Information from supraspinal centres descend into the spinal cord via the spinobulbar loop, nociceptive information is integrated in the periaqueductal grey (PAG), and transmitted into the spinal cord dorsal horn (DH) indirectly via the rostroventral medulla (RVM). The descending tract terminates in the superficial laminae of the DH, and interacts with ascending pain transmission. The orange pathway represents the entrance of primary afferent fibres (PAFs) into the DH. The ascending pathway is depicted in red and the descending in blue. The red and blue pathways together constitute the spinobulbar loop.

Specifically, the PAG is divided into dorsal and ventral subregions. The ventral PAG (vPAG) projects to the NRM and adjacent reticular formation of the RVM, and also to the dorsolateral and ventrolateral pontine tegmentum (DLPT and VLPT respectively). The DLPT directly projects to the dorsolateral funiculus, and this pathway is crucial for mediating morphine or electrically induced analgesia (Basbaum and Fields, 1979, Tohyama et al., 1979b). A role of VLPT in antinociception was also demonstrated in a study where focal electrical stimulation to the VLPT inhibited the tail-flick flexion reflex upon noxious thermal stimulation in rats (Miller and Proudfit, 1990).

On the other hand, the dorsal PAG (dPAG) projects mainly to the pontine tegmentum and the ventrolateral medulla. In freely moving rats, electrical stimulation of the dPAG produces markedly less antinociceptive effects when compared to those seen in the vPAG (Fardin et al., 1984b), as measured by vocalisation to electrical stimulation of the tail. Moreover, in an earlier study Fardin and colleagues reported that in some cases, stimulation of the dPAG increase pain sensitivity; stereotypically pain related behavioural responses, such as gnawing and tremors were observed post-stimulation (Fardin et al., 1984a). Other studies have also found that stimulation of the dPAG produces mild antinociception, but these effects were accompanied by 'flight-like' autonomic responses, such as an increase in blood pressure and respiration rate, vasodilatation in hind limb muscle, pupillodilatation and widening of the palpebral fissure in anaesthetised rats (Lovick, 1985), 'wild running' is also observed in freely moving rats (Morgan et al., 1998). In general, the vPAG is responsible for the antinociceptive effects associated with SPA, and the dPAG mediates stress-induced autonomic responses.

There are very few direct axonal connections from the PAG to the DH, the majority of PAG efferent fibres descend into the DH via the brainstem nuclei including the NRM. Functional connection of the PAG-RVM-DH pathway is demonstrated by single unit recording, where electrical stimulation in the PAG directly affects the responses of NRM neurones, and antidromic firing spikes are observed in NRM neurones when the dorsolateral funiculus is stimulated (Pomeroy and Behbehani, 1979). Similarly in pharmacological experiments, blockade of endogenous opioid activity within the NRM reverses the inhibitory effect of morphine injected into the PAG on WDRs upon noxious pinch on their receptive field (Vasquez and Vanegas, 2000). Moreover, anatomical studies using anterograde tracing methods also reported that the PAG projects to the DH via other brainstem nuclei such as the locus coerulus (Cedarbaum and Aghajanian, 1978, Luppi et al., 1995).

Interestingly, the Pomeroy and Behbehani study also reported that electrical stimulation of the PAG can either facilitate, inhibit, simultaneously facilitate and inhibit or have no effect on firing activities of NRM neurones (Pomeroy and Behbehani, 1979). Subsequent studies using the same techniques identified two distinct classes of neurones within the RVM, neurones that exhibit an increase in firing activity just before the tail flick to noxious heat are termed ON cells, whereas those that decrease in firing are termed OFF cells (Fields et al., 1983). A third RVM cell type (Neutral) was identified in a later study, the firing activity of Neutral cells remains unchanged in the tail flick test, and does not respond to microinjection of morphine in the PAG (Cheng et al., 1986). Moreover, Fields and colleagues found that both ON and OFF cells project to the laminae I, II and V of the spinal cord (Fields et al., 1995), where most nociceptive PAFs terminate. The functional role of the PAG and the RVM will be further discussed in the following section.

1.4.4 Brainstem control of pain

Local application of morphine into the PAG produces analgesia (Sharpe et al., 1974, Lewis and Gebhart, 1977). In general, morphine and other opiates reduce neuronal excitability by hyperpolarisation of cells (Pepper and Henderson, 1980, Schneider et al., 1998, Svoboda and Lupica, 1998). Therefore, analgesia is unlikely to be achieved by direct excitation of PAG neurones. It has been proposed that stimulation of the PAG inhibits GABA-containing inhibitory INs (Stiller et al., 1996), which disinhibits efferent PAG neurones thus allowing descending inhibition (Basbaum and Fields, 1984, Depaulis et al., 1987).

In the previous section the three neuronal types of the RVM were described. The effect of pain modulation mediated by the RVM was intensely studied, and several research groups reported that electrical stimulation of the RVM evoke both facilitatory (McCreery et al., 1979, Haber et al., 1980) and inhibitory (Basbaum and Fields, 1984) responses to noxious stimulation. Later studies by Zhou and Gebhart found that low intensity electrical stimulation (5–25 μA) within the different sites of RVM, including the nucleus reticularis gigantocellularis (NGC), nucleus reticularis gigantocellularis pars alpha (NGCα) and the NRM enhanced firing acivity of DH neurones to noxious thermal and mechanical stimulation of the hindpaw and tail in rats, whereas high intensity electrical stimulation (50–200 μA) inhibited spinal transmission and produced profound analgesia (Zhuo and Gebhart, 1990, 1992, 1997). These studies demonstrate that the RVM is involved in the coordination of an appropriate reflex response to a noxious stimulus by exerting both facilitatory and inhibitory effects on DH neurones. Further, these findings provide a basis for the involvement of the RVM in chronic pain models: tail-flick reflex provoked by

application of mustard oil is inhibited in rats that received electrolytic lesions in the RVM (Urban et al., 1996); injection of local anaesthetic (ropivacaine) reverses hyperalgesia induced by repeated application of acidic saline to the muscle of the hindlimb (Tillu et al., 2008). For further details on the contribution of supraspinal sites to hyperalgesia, please refer to the review (Urban and Gebhart, 1999).

1.5 Functional organisation of reflex circuits

Previous sections have considered the functional anatomy of both the peripheral and central nociceptive systems. As illustrated in multiple studies, the withdrawal reflex, such as tail-flick, paw-flinch, and retraction of the limb are widely used as a measure of pain (Willer, 1977). This section will focus on the functional organization of both spinal and supraspinal circuits relating to withdrawal reflex behaviours.

Much of our understaning of reflex coordination and circuitry stemmed from Sherrington's initial characterization of the flexion withdrawal reflex (FWR) (Sherrington, 1910). In his study some key observations were made of the genesis, nature and coordination of FWR in mammals; 1) the whole hindlimb withdrawal reflex can be readily evoked from stimulating the hindpaw, particularly the toes; 2) there is a strong correlation between the intensity of stimulation and the strength of the resultant contraction and 3) the postural balance is maintained by counter-balancing the reflex flexion in the ipsilateral limb by extending the contralateral limb.

1.5.1 Spinal mechanisms of reflex activity

The organization of MNs in the VH is described in section 1.3.4. In this section, the focus is on the input/output and RFs of MNs in the VH.

The FWR is a polysynaptic, multi-segmental reflex that serves to withdraw a limb from a noxious or potentially tissue damaging stimulus (Woolf and Swett, 1984). The actual spatial distribution, specificity and modality characteristics of the FWR rely on the input/output relationship between cutaneous sensory and motor circuits. The mapping of cutaneous receptive field of the individual flexor and extensor muscles involved in hindlimb withdrawal reflexes was first performed by Hagbarth (Hagbarth, 1952). Responses of α-MNs to noxious pinch recorded in decerebrate cats revealed that even when a flexion response appeared 'pure', i.e. no discernible response was observed in the extensor muscle, the extensor was still activated, albeit to a lesser degree than the flexor muscles. These data suggest that the flexor muscles override the activity of the

extensor muscle. Using the same techniques, Hagbarth and colleagues recorded in γ-MNs and demonstrated that efferent activity can only be observed when the cutaneous area overlaying the muscle of interest is stimulated (Eldred and Hagbarth, 1954). These findings indicate that the hindlimb withdrawal reflex is highly organized and functionally selective: in simplified terms, the most important factor determining the actions of flexors and extensors is the location of stimulation on the skin (Megirian, 1962).

Further work on the functional organisation of the FWR led to the modular organisation theory (Schouenborg and Kalliomäki, 1990). This theory led to the construction of a comprehensive map of the RFs of individual motor units in the hindlimb of the rat. Electromyographic (EMG) responses to noxious pinch of the skin in the hindlimb were measured in halothane-anaesthetised rats. Twenty-five individual muscle fibres in the hindlimb were studied, of these, most of them responded to the stimulus, but there were also a subset that did not. This finding confirms the existence of both excitatory and inhibitory RFs for each muscle, which serves to either promote or inhibit movement accordingly. Moreover, for the muscles that were excited, it was shown that 1) the cutaneous RF of each muscle is highly organized so that if stimulated, a withdrawal response readily follows if the animal is on the ground 2) the most sensitive part of the skin (i.e. the toes) are most effectively withdrawn by that muscle and 3) muscles that have similar actions (for example, muscles on the ankle and knee that work against gravity) have similar respond thresholds and latencies (Schouenborg and Weng, 1994).

The specific relationship between sensory and motor neurones was examined extensively by Woolf and colleagues (Wall and Woolf, 1984, Woolf and Swett, 1984). They performed single unit recordings in the α-MNs innervating the bicep femoris and the principal head of the semitendinosus muscle in decerebrate rats and observed the following: 1) all α-MNs have cutaneous RFs that are ipsilateral to the foot stimulated (pinch, cold and hot temperatures, mustard oil); 2) they are not activated by innocuous stimuli (light touch, brush and vibration). Subsequent experiments were performed by simultaneously recording the activity of α-MNs whilst stimulating the sural nerve in the leg (Wall and Woolf, 1984, Woolf and Doubell, 1994), it was found that 1) Aβ-fibre strength stimulation produced a short latency (10ms) firing discharge; 2) Aδ-fibre strength stimulation produced a longer response lasting up to 1400ms and 3) increasing the strength of stimulus further recruits C-fibre activity, resulting in the longest response, with a after-discharge latency of 7s in α-MNs. These findings indicate that α-MNs receive input from all three types of PAFs, and activity of α-MNs correspond to the strength of stimulus applied, which can be evoked in a small, specific area of the skin.

FWR coordination in the spinal cord is mediated by reflex-encoding neurones located in lamiane V of the DH, the firing pattern of these neurones are highly correlated to the response patterns of the withdrawal reflex in a single muscle (Schouenborg et al., 1995b). Reflex-encoding neurones receive convergent input from PAFs and INs, and then synapse with MNs in the VH for sensory transmission (Schouenborg et al., 1995b). The summary of spinal reflex arc is provided in Figure 1.4.

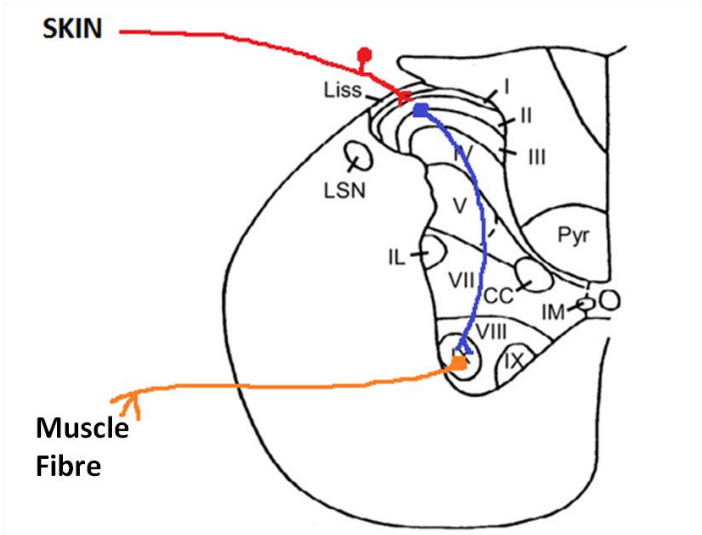


Figure 1.4 Anatomy of spinal reflex arc. Information from the skin is transmitted via primary afferent fibres (PAFs, in red), which terminates in the superficial laminae of the dorsal horn (DH). Information from the PAFs is transmitted intralaminally via reflex-encoding neurones (in blue), which terminates in lamina IX and synapses with motorneurones (MNs). MNs (in orange) innervate the muscle fibre, which contracts upon supra-threshold cutaneous stimulation.

1.5.2 Supraspinal mechanisms of reflex activity

The activity of the spinal reflex circuit is also driven by descending influences from supraspinal sites, as demonstrated by studies involving spinalised animals. Spinalisation is a technique often used to test the actions of analgesics on nociceptive responses, or local cellular mechanisms driving hyperalgesia in the absence of descending influences (Kehne et al., 1985, Sandkühler et al., 1995, Kauppila, 1997, Pitcher and Henry, 2000). It is typically performed by transecting the spinal cord at the thoracic level or the cervical level, leaving the lumbar segments intact but free from descending inputs from supraspinal sites. In spinalised rats, hindlimb withdrawal reflexes are enhanced when compared to control animals (Sherrington, 1910), accompanied by a decrease in mechanical threshold and an expansion of RFs in spinal neurones (Woolf and Swett, 1984).

A number of descending pathways controlling the reflex movements include the corticospinal (CST), rubrospinal (RST) and reticulospinal (RCST) tracts. The CST originate from pyramidal cells in the contralateral SI (Wise and Jones, 1977, Miller, 1987, Brosamle and Schwab, 1997) and terminate in laminae III-VI in the spinal cord DH (Brosamle and Schwab, 1997). Neurones of the CST synapse with MNs in the VH via INs (Alstermark et al., 2004). The majority of CST axons (75-90%) decussate in the medulla oblongata to form the lateral CST, which controls the distal musculature; the remaining axons form the anterior CST, which decussates at the spinal segments and controls the proximal musculature (Porter, 1985). Cell bodies of RST neurones projecting to cervical spinal segments originate in dorsal and medial portions of the red nucleus (a structure in the rostral midbrain, dorsal to the substantia nigra), while those projecting to lumbar segments are located ventrally and ventrolaterally within the red nucleus (Murray and Gurule, 1979). The RST axons terminate predominantly in laminae V–VI and in the dorsal part of lamina VII of the spinal cord DH at all levels and on both the ipsilateral and contralateral sides to the red nucleus (Antal et al., 1992). The RST descends into the ventral horn in parallel to the CST and interacts with MNs via both inhibitory and excitatory INs, the effect of electrical stimulation of the red nucleus typically results in excitation of the flexor and inhibition of the extensor muscles in the hindlimb (Hongo et al., 1969a, b). The RCST is mainly responsible for initiation of locomotor control (Cao et al., 2005). It originates from the mesencephalic reticular formation; descending projections arise from the cluster of cells located just lateral to the PAG and course through the anterior funiculus and ventral part of the lateral funiculus ipsilaterally (Tohyama et al., 1979a).

1.6 Neurotransmitter systems involved in brainstem pain modulation

The previous sections outlined the functional anatomy of pain pathways and withdrawal reflex circuits. In this section, an overview of the diverse neurotransmitter systems involved in the fine-tuning of nociceptive processing is provided.

1.6.1 Opioids

The observation that SPA can be partially blocked by naloxone indicates the existence of an endogenous opioid signalling system (Adams, 1976, Akil et al., 1976, Zorman et al., 1981). Endogenous opiates including enkephalin, β-endorphin and dynorphin act via four classes of opioid receptors: μ-opioid receptors (MOR), δ (DOR), K (KOR) and opioid receptor-like receptors (ORL-1) (Paterson et al., 1983).

Opioid receptors are highly expressed within the nociceptive pathways. Wihtin the spinal cord opioid receptors are most concentrated in the superficial laminae (Ninkovic et al., 1982). Specifically, MOR is expressed in laminae I, II and VIII; KOR is expressed in laminae I and II, and moderately throughout laminae III-VIII (Maekawa et al., 1994); and DOR is mostly expressed in laminae I and II (Zhang et al., 1997). Further studies indicate that opioid receptors are found on small diameter peptidergic PAFs (Atweh and Kuhar, 1977, Zhang et al., 1997), and both pre- and post-synaptic locations are identified (Besse et al., 1990, Arvidsson et al., 1995). Supraspinally opioid binding sites are concentrated in the brainstem, these regions include the RVM, superior colliculi, PAG, the raphe nucleus and the parabrachial nucleus (Cherubini et al., 1991). MOR mRNA expression is observed in the thalamus, striatum, locus coeruleus and nucleus of tractus solitaris (NTS), DOR mRNA was seen in the cortex, straitum and lateral reticular whereas KOR is seen in the hypothalamus, nucleus accumbens, substantia nigra, ventral tegmental area and the NTS (Pearson et al., 1982). More than 90% of spinally projecting RVM neurones respond to the selective MOR agonist DAMGO ([D-Ala2, N-MePhe4, Gly-ol]-enkephalin) and the KOR agonist U-69593 (Herlenius and Lagercrantz, 2004). PAG projection neurones to the RVM also express DOR and MOR mRNA (Jessell et al., 1978). It is noteworthy that significantly more PAG–RVM projection neurones were labelled for MOR mRNA in the ventrolateral subregion of the PAG than in the dorsomedial subregion (Jessell et al., 1978).

There are several mechanisms behind opioid mediated antinociception. It was proposed that opioids produced antinociception in the PAG by directly inhibiting tonically active GABAergic INs, thereby disinihibiting efferent RVM projecting neurones (Basbaum and Fields, 1984). It was shown that bath applied opioids in rat PAG slices inhibited both GABA-mediated IPSCs and glutamate-mediated EPSCs via presynaptic mechanisms; overall synaptic transmission was inhibited due to reduced probability of neurotransmitter release (Vaughan and Christie, 1997). Opioids also directly inhibit the firing of ON cells (Mantyh et al., 1997). Within the RVM opioid receptors are most likely expressed on ON cells: selective ablation of MOR expressing cells in the RVM by the dermorphin-saporin conjugate removed the descending facilitatory component (Porreca et al., 2001); and intravenous morphine (1.25–2.5 mg/kg) reduced spontaneous firing of all ON cells but not other cell types recorded (Bederson et al., 1990). Opioids may also indirectly modulate nociception by enhancing OFF cell activity. It was reported that the selective MOR agonist DAMGO (50-200 ng in 100 or 200 nl), when iontophoretically applied into the RVM inhibited the tail flick reflex in rats (Heinricher et al., 1994). This effect was thought to be mediated by disinhibition of OFF cells, as they never exhibited a

pause in firing after DAMGO was applied (Heinricher et al., 1994), and the firing activity of OFF cells are prolonged and continuous after local administration of bicuculline ($GABA_A$ receptor antagonist) in the RVM (Heinricher and Tortorici, 1994).

Opioid receptors mediate inhibitory effects at the cellular level by membrane hyperpolarisation and reduction of neuronal calcium currents (Moises et al., 1994). They are G protein-coupled and the MOR, DOR and KOR all preferentially couple to Gi/o (Giordano, 1997). The analgesic effects of MOR activation in particular are attributed to the Gβγ dimer released from Gi/o, which activates the G protein-activated inwardly rectifying potassium (GIRK) channels and in turn inhibits voltage-dependent calcium channels (VDCCs) (Rajaofetra et al., 1989).

Opiates can, however, produce 'paradoxical hyperalgesia'. Low dose morphine (0.1 to 1μg/kg) administered systemically in naive mice decreased the latency of tail flick in response to noxious heat stimuli, and this hyperalgesia was blocked by co-administration of an ultra-low dose (0.1ng/kg) naltrexone (MOR/KOR competitive antagonist) (Boucher et al., 1998). Opioid therapies are also associated with the development of tolerance and increased sensitivity to pain, the latter is a condition known as opioid-induced hyperalgesia (OIH)(Jakowec et al., 1995). In one study, chronic administration of morphine by subcutaneous implantation of two 75mg morphine pellets produced tactile allodynia and thermal hyperalgesia seven days after pellet implantation in adult rats (Monyer et al., 1994). This finding suggests that prolonged activation of opioid receptors induces plastic changes within the nociceptive pathways, which leads to altered pain sensitivity within the system.

Several mechanisms for opioid-induced paradoxical pain have been proposed. It was suggested to be related to NMDA receptor activity, as synergy between glutamate and morphine was observed: subcutaneous NMDA receptor antagonist MK801 (0.15 or 0.30 mg/kg) potentiated the antinociceptive effect of intravenous morphine (7.5mg/kg) (Senba et al., 1982). Interestingly, chronic morphine exposure was shown to alter G protein coupling from Gi/o to Gs. This may be the molecular mechanism underpinning opioid tolerance and dependence, and their attenuation by ultra-low-dose opioid antagonists (McGregor et al., 1982, Marti et al., 1987).

1.6.2 Cannabinoids

Pharmacological research on the plant *Cannabis sativa* and its terpenoid derivative Δ^9-tetrahydrocannabinol led to the identification of the cannabinoid CB1 receptor (Gaoni and Mechoulam, 1964). Since the successful cloning of the CB1 receptor (Matsuda et al., 1990), a family of lipid transmitters that serves as natural ligands at this receptor have been found, including 2-arachidonyl-glycerol (2-AG) and arachidonoylethanolamide (anandamide) (Crawley et al., 1993, Stella et al., 1997, Rodriguez de Fonseca et al., 2005). The immune-counterpart of CB1 receptor, CB2 receptor, was determined by sequence homology three years later (Munro et al., 1993). Endocannabinoids are released upon demand after cellular depolarisation or receptor stimulation in a calcium-dependent manner, are therefore synthesised de novo. They mainly act via CB1 and CB2 receptor, which are both coupled to Gi/o proteins. Endocannabinoids exert their inhibitory effects via both presynaptic and postsynaptic mechanisms: presynaptically they serve as retrograde signalling messengers in GABAergic and glutamatergic synapses and inhibit calcium channels (Rea et al., 2007), thereby indirectly control the release of neurotransmitter (Ryberg et al., 2007) and neuronal excitability. Postsynaptically they hinder neurotransmission by membrane hyperpolarisation via the opening of potassium channels (Mu et al., 1999, Piomelli, 2003, Pertwee, 2005).

Studies using CB1 and CB2 receptor knockout mice indicate the existence of novel cannabinoid binding sites (Hájos et al., 2001, Monory et al., 2002). The CB1/CB2 receptor agonist WIN55212 reduces the amplitudes of excitatory postsynaptic currents in hippocampal slices of both wild type and CB1 receptor knockout mice (Hájos et al., 2001). WIN55212 also induces $^{[35S]}$GTPγS binding in cerebellar homogenates of CB1 receptor knockout mice, and this effect is not reversible by the selective CB1 receptor antagonist SR141716A (Monory et al., 2002). The orphan G protein-coupledreceptor GPR55 is a likely cannabinoid binding site since it is capable of interacting with plant-derived and synthetic cannabinoid ligands (Ryberg et al., 2007). It is an atypical cannabinoid receptor because GPR55 receptors are phylogenetically distinct from CB1 and CB2 receptors, sharing a low amino acid identity (13.5% to CB1 and 14.4% to CB2) and they lack the classical 'cannabinoid binding pocket' present in both CB1 and CB2 receptors (Henstridge, 2012). Since the discovery and cloning of GPR55 receptors, L-α-lysophosphatidylinositol (LPI) was found to be an endogenous agonist, whereas one of the *Cannabis sativa* constituents cannabidiol (CBD) was found to be a GPR55 receptor antagonist (Sylantyev et al., 2013). The pharmacology of GPR55 receptors is enigmatic and several different subunits of G-protein coupling were proposed. Nevertheless it was agreed that upon agonist binding, intracellular concentration of calcium increased and

rapid phosphorylation of ERK was induced (Ross, 2009), which suggests that the effect of GPR55 receptor activation is excitatory at the cellular level.

Cannabinoid receptors are highly expressed in the rat central nervous system (Tsou et al., 1998). CB1 receptors in particular are the most abundant G protein-coupledreceptor in the brain, with binding densities about ten to fifty fold higher than those of opioid receptors (Rodriguez de Fonseca et al., 2005). CB1 receptors are mostly found in cortical structures such as the olfactory bulb, hippocampus, the caudate putamen, the substantia nigra and the amygdala (Herkenham et al., 1991). Within the brainstem, CB1 immunoreactivity is found in the PAG, very close to the lining of the aqueduct, and in the NTS and spinal trigeminal tract at the level of the medulla oblongata (Tsou et al., 1998). CB2 receptors are mostly found in the peripheral immune system, such as monocytes, polymorphonuclear neutrophils, T-lymphocytes and microglial cells (Rodriguez de Fonseca et al., 2005). However, reports suggest that CB2 receptor immunoreactivity is also found in neurones (Ashton et al., 2006). CB1 and CB2 immunoreactivity may be found in same brain structures, such as the amygdala and the PAG (Van Sickle et al., 2005), but the distribution patterns of neuronal elements where they are localised may differ (Gong et al., 2006). Within the spinal cord CB1 receptors are found in the dorsolateral funiculus, the superficial DH and lamina X, with the majority localised in spinal INs (Farquhar-Smith et al., 2000). Moreover, CB1 receptors are found on medium to large diameter cells in the DRG, and a subpopulation of these CB1 expressing neurones are capable of synthesising cannabinoid receptors and inserting onto the terminals of DH neurones (Hohmann and Herkenham, 1999).

Expression of GPR55 receptors is less prominent. Real-time PCR data showed that they are expressed in neuronal and glial cells, particularly in microglial cells (Pietr et al., 2009), which suggest a role in neuroimmunological regulation. Brain structures expressing GPR55 receptor mRNA include frontal cortex, striatum, hippocampus and brainstem (Ryberg et al., 2007), precise localisation, however, is not currently known.

The antinociceptive effects of cannabinoids are well described. Formalin-induced pain behaviours in mice are inhibited by intraplantar administration of the WIN55212 (500µg/kg), this effect is CB1-dependent as it can only be blocked by intravenous administration of SR141716A (CB1 antagonist, 0.1mg/kg) but not by naloxone or the CB2 antagonist SR144528 (Calignano et al., 1998). Intra-RVM microinjection of WIN55212 (0.25mg/kg) inhibits the tail-flick reflex (Meng et al., 1998). In a SIA

paradigm, inhibition of the tail flick reflex after brief electrical shock to the hindpaw is abolished by SR141716A injected directly in the dorsolateral PAG (Hohmann et al., 2005). In addition, intra-dorsolateral PAG microinjection of the CB1/CB2 agonist HU210 (0.1/1/5µg/rat) dose-dependently reduces formalin-induced pain behaviour (only the early phase) and c-Fos (marker of recent neuronal activity) immunoreactivity in the PAG (Finn et al., 2003). Antinociceptive activity of other cannabinoid agonists such as CP55940, into the dorsal raphe of the ventrolateral PAG is also documented (Lichtman et al., 1996).

The role of GPR55 receptors in nociception remains to be elucidated; both pro and anti-nociceptive effects have been reported. In GPR55 knockout mice, no mechanical hyperalgesia and inflammatory cytokine production are observed after spinal nerve ligation (Staton et al., 2008). It was also recently reported that response frequency to mechanical vFh stimulation in mice increases three to six days after intraplantar injection of Lysophosphatidylinositol (LPI, endogenous GPR55 receptor ligand) (3µM, 100nM and 5nM in 20µL), which is accompanied by an increase in phosphorylated ERK immunoreactivity in DRG neurones (Gangadharan et al., 2013). Conversely, peripheral administration of O-1602 (synthetic GPR55 agonist, 100µg in 100µL) reduces movement evoked C-fibre firing in a rat inflammatory joint pain model (Schuelert and McDougall, 2011). Together these findings suggest that GPR55 receptors may be a potential target for pain therapies, but more evidence is needed, and a clearer elucidation of its expression and function at the different sites is required.

1.6.3 Other major neurotransmitter systems expressed within the nociceptive pathways

Other major neurotransmitter systems involved in the modulation of pain include SP, glutamate, GABA and serotonin.

SP is a undecapeptide and mediates its effects on nociceptive transmission via the receptor neurokinin 1(NK1) (Regoli et al., 1987). SP is released by activation of small diameter PAFs following noxious stimulation (Wiesenfeld-Hallin et al., 1984, Villar et al., 1991). NK1 receptors are abundantly expressed in the superficial lamina of the DH, and moderate levels are detected in laminae II, IV and X (Shults et al., 1984). A high proportion of NK1 receptor positive neurons in the spinal cord are PNs (Todd, 2002), which suggest that SP released by PAFs bind to NK1 receptors on PNs, and transmit nociceptive information from the periphery to central sites. The expression of SP and NK1 receptors are relatively sparse in supraspinal sites when compared to the spinal cord. Nonetheless, autoradiographic localisation of SP binding sites include the medial

amygdala, dentate gyrus, superior colliculus, dorsal parabrachial nucleus, locus coeruleus, nucleus accumbens, striatum and PAG (Quirion et al., 1983). SP immunoreactivity is found in many parts of the brainstem (Ljungdahl et al., 1978), particularly in medullary neurones that project to the spinal cord (Johansson et al., 1981). NK1 receptors are typically coupled to Gq/11 proteins, which provoke an increase in intracellular Ca^{2+} ions via the phospholipase C (PLC) pathway (Womack et al., 1988, Heath et al., 1994). Activation of NK1 receptors is pronociceptive, and is involved in sensitisation mechanisms. Selective ablation of NK1 receptors in the spinal cord by the SP-saporin conjugate (SP-SAP) leads to a decrease in hyperalgesia induced by capsaicin, inflammatory and nerve injury (Mantyh et al., 1997). A recent study using the same techniques reported a decrease in spontaneous DH activity evoked by noxious mechanical and heat stimuli in SP-SAP treated rats (Khasabov et al., 2005). Moreover, the effect is maintained after the transection of the dorsolateral funiculus, which suggests that descending influences are at least in part mediated by NK1 positive spinal neurones.

Glutamate is the principle excitatory neurotransmitter in the CNS and it acts via a variety of receptors, which can be divided into ionotropic (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/AMPA, N-methyl-D-aspartic acid/NMDA and kainate) and metabotropic (group I mGluR1 and mGluR5) subtypes (Nakanishi and Masu, 1994, Conn and Pin, 1997). Ionotropic glutamate receptors are permeable to cations, whereas metabotropic receptors are coupled to Gq/11 proteins. In the spinal cord glutamate binding sites are most abundant in the substantia gelatinosa (Jansen et al., 1990, Shaw et al., 1991, Henley et al., 1993), and are found on both small and large diameter PAFs (De Biasi and Rustioni, 1988). Glutamate binding sites are also ubiquitously expressed in the brain, they include the hippocampus, dentate gyrus, superficial and deep layer of the cortex, caudate putamen, diencephalon, midbrain and the brainstem (Greenamyre et al., 1984, Ozawa et al., 1998). The role of glutamatergic transmission in nociception is well documented (Coderre and Yashpal, 1994, Karim et al., 2001), the expression of glutamate receptors are upregulated in chronic pain models (Harris et al., 1996). For further information on the relationship between glutamate and pain please refer to the review (Bleakman et al., 2006).

GABA is the major inhibitory neurotransmitter in the CNS and acts via two classes of receptors: the ionotropic $GABA_A$ receptor which is permeable to Cl^- ions and the metabotropic $GABA_B$ receptor which are coupled to Gi/o proteins (Barnard et al., 1998). GABA binding sites are ubiquitously found in the spinal cord, particularly in the

substantia gelatinosa, lamina III and IX (Frere et al., 1982, Persohn et al., 1991). They are localised on both small and large diameter PAFs and MNs (Frere et al., 1982, Price et al., 1987). In the supraspinal sites, autoradiographic localisation of GABA binding sites include the frontal cortex, the granule and molecular cell layer of the cerebellum, hippocampus, hypothalamus, midbrain, corpus striatum, olfactory bulb, interpeduncular nucleus, frontal cortex, and thalamic nuclei (Zukin et al., 1974, Bowery et al., 1987). GABAergic influences on nociceptive transmission are bidirectional; both excitatory and inhibitory effects were observed. The $GABA_A$ antagonists bicuculline and picrotoxin microinjected into the ventrolateral aspect of the caudal PAG inhibit the tail flick reflex by increasing the spontaneous activity of the OFF cells and decreasing firing of the ON cells in the RVM (Moreau and Fields, 1986). However, another study reported iontophoretically applied of bicuculline (5-30 nA) blocks the tail-flick-related pause in OFF cells (Heinricher and Tortorici, 1994). Immunohistochemical labelling of GABA receptors in the PAG-RVM-DH circuit reported that two-thirds of spinally projecting neurones from the RVM are GABAergic, and that 71% of PAG projections to these GABAergic reticulospinal neurones also contain GABA immunoreactivity (Morgan et al., 2008). More importantly, the same study also found that all ON cells are GABAergic, and receive GABAergic inputs from the PAG (Morgan et al., 2008). These findings confirm GABA mediate both anti- and pro-nociceptive influences via the descending pain pathways.

As mentioned in section 1.6.1, opioids in the PAG act indirectly via GABAergic INs (Vaughan et al., 1997). Most monoamine cell types within the CNS are under GABAergic control (Millan, 2002), which may also account for the GABA-mediated descending facilitation/inhibition. For instance, microinjection of the $GABA_B$ receptor agonist baclofen, into the NRM of the rat produces antinociception at doses of 0.1–1.0 ng and hyperalgesia at doses of 30–150 ng in the tail-flick test (Hammond et al., 1998). The antinociception is proposed to be caused by disinhibition of spinally-projecting neurons in this region that contain serotonin, and pronociception caused by direct inhibition of tonically-active pain inhibitory neurons in the NRM (Hammond et al., 1998). For a recent review on the relationship of GABA and other monoamine neurotransmitters please refer to (Benarroch, 2008).

Serotonin (5HT) is a monoamine biochemically derived from tryptophan (Fernstrom and Wurtman, 1971, Walther et al., 2003). 5HT-binding sites are densely located in the superficial lamina of the DH, and are expressed on PAFs, PNs and INs (Daval et al., 1987, Marlier et al., 1991, Morales et al., 1998). It was reported that brainstem nuclei

are the origin of spinally projecting 5HT-containing neurones (Bowker et al., 1981), these include the dorsal raphe nucleus and mesencephalic reticular formation of the PAG, the NRM and nucleus reticularis gigantocellularis of the RVM (Beitz, 1982). In the RVM only Neutral cells are immunoreactive for 5HT (Potrebic et al., 1994), and they receive monosynaptic inputs from the PAG (Mirnics and Koerher, 1995). Similar to GABA, 5HT transmission in the CNS exerts both excitatory and inhibitory influences over nociceptive processing (Aimone et al., 1987, Cui et al., 1999, Suzuki et al., 2004). The bidirectional effects of 5HT transmission signify the importance of underlying circuitry; depending on the location and subtypes of 5HT receptors the nociceptive response could be drastically different (Hoyer et al., 1994, Benn et al., 2001, Jeong et al., 2001). For reviews on the role of 5HT on pain and analgesia please refer to (Messing and Lytle, 1977, Sommer, 2004).

1.7 Developmental aspects of pain

The anatomical and functional significance of nociceptive pathways in mature animals have been provided so far in this introduction. It is known that sensory systems undergo significant developmentally regulated changes, in particular, nociceptive processing between young and adult animals are different (see reviews (Fitzgerald, 2005, Fitzgerald and Walker, 2009)). This section focuses on developmental events during the embryonic and postnatal period, with reference to neonatal pain behaviours. The literature on the development of the CNS is arguably more detailed in rats than humans, due to the availability of subjects and ethical considerations. When translating data from animal studies to human application, the relationship between rats' and humans' ages must be taken into consideration. Rats are born prematurely compared to humans and develop rapidly; gestation in rats on average takes 21.5 days, whereas in humans it takes approximately 38.5 weeks (Mittendorf, 1990). The general consensus of the relationship between rat and human age is that one day of the rat is approximately equivalent to 34.8 human days, it is also reported that rats become sexually mature at about six weeks old, and reach social maturity five to six months later (Sengupta, 2013).

1.7.1 Embryonic development of nociceptive pathways

The molecular pathways and mechanisms underlying the formation of nocifensive behaviours are related to those that control the genesis of nociceptors, and the formation of functional synapses in neural and reflex circuits. It is known that sensory neurones in the DRG commit to nociceptor fate early in the embryonic period (Marti et al., 1987). The generation of PAFs is dependent on the transient expression of basic helix-loop-helix (bHLH) transcription factors neurogenin 1 and 2 at around embryonic

day (E) 12 to 16 (Ma et al., 1999). This process takes place in two waves: TrkB (Trk = tyrosine kinase, receptor for neurotrophins) and TrkC-expressing large diameter sensory neurones, which relies on the expression of neurogenin 1 are generated prior to TrkA expressing small diameter neurones, which are neurogenin 2-dependent (Ma et al., 1999). Small diameter sensory neurones then proceed to become nociceptive C-fibres, and evidence suggests that the peptidergic population is generated before the non-peptidergic, IB4-expressing subtypes (Kitao et al., 1996). The ontogeny of peptidergic C-fibres depends on the release of nerve growth factor (NGF, which binds to p75 and TrkA receptors) from target tissue, whereas the non-peptidergic population loses its dependency on NGF at around the time of birth, and switch to glial-derived neurotrophic factor (GDNF, which binds to Ret receptors) signalling for survival and growth during the early postnatal period (Molliver et al., 1997, Stucky and Lewin, 1999, Franck et al., 2011).

In rats, DRG axonal connections to the periphery and spinal cord grey matter occur just before birth, and neurogenesis is dependent on the interaction between neurotrophins and Trk receptors (Johnson Jr et al., 1986, DiStefano et al., 1992). Sensory fibres innervating the skin project to the epidermis to form the cutaneous plexus, with A-fibres penetrating the plexus prior to C-fibres (Jackman and Fitzgerald, 2000). The plexus retracts as the epidermis thickens and specialized sensory organs (such as Merkel cells, responsible for light touch discrimination and Ruffini corpuscle, a slow-adapting mechanoreceptor) develop during maturation (Jackman and Fitzgerald, 2000). Sensory neurones directed to the lumbar segment of the spinal cord reach the DRG at E13, and after a significant delay penetrate the grey matter with A-fibres entering at E15-17 and C-fibres at E18-20 (Mirnics and Koerher, 1995).

Within the spinal grey matter, MNs are the first to reach maturity. Genesis of MNs are completed by the end of the neural plate stage (E9) (Yamada et al., 1993) and is dependent on the expression of brain-derived neurotrophic factor (BDNF) (Koliatsos et al., 1993). Specification of dorsal horn cells occurs along a ventrodorsal-gradient, such that neurones in the deeper laminae reach maturity prior to laminae I and II (Altman and Bayer, 1984). The generation of INs in the deep DH begins at E9.5 (Briscoe and Ericson, 2001). In the superficial lamina, neurogenesis of PSs and PNs began at E13 (Bice and Beal, 1997b), neurones with the longest axonal length, such as the ones that project to the forebrain, are generated before the ones that project to the brainstem (Bice and Beal, 1997a). Moreover, the generation of PNs in the superficial DH is completed by E14, 2 days before the generation of PS neurones (Bice and Beal, 1997a).

Interestingly, complete maturation of local INs circuit occurs after the generation of PNs and continues into the postnatal period (Bice and Beal, 1997a, b), which suggests that direct transmission between sensory neurones in the DH and supraspinal sites develops before the onset of local modulation by INs.

These findings, altogether, indicate that all the relevant axonal connections between the periphery and central sites are in place before birth, but functional projections continue to mature postnatally. The development events that occur during the postnatal period of the rat will be discussed in the following section.

1.7.2 Postnatal development of nociceptive pathways

Due to the late entry of C-fibres into the grey matter of the spinal cord, they are not detected in the DH until P5 onwards (Baccei et al., 2003). In addition, although C-fibres are functional from birth, evoked activity upon noxious stimulation cannot be detected until P10 onwards (Fitzgerald and Jennings, 1999). These indicate that in the early postnatal period dorsal horn is dominated by low threshold A-fibre inputs, which contribute to the lowered threshold and exaggerated dorsal horn activity seen in neonatal rats (Fitzgerald and Jennings, 1999, Woodbury and Koerber, 2003).

A-fibre terminals are found initially in the substantia gelatinosa of neonatal DH, they gradually withdraw from the surface of the dorsal horn and terminate in laminae III and IV by the third postnatal week (Fitzgerald et al., 1994). Postnatal increases in A-fibre mechanoreceptor firing lead to mismatched pre and postsynaptic firing (Fitzgerald, 1987a) and lamina II cells are increasingly being driven by C-fibre inputs as animals age, these events lead to a weakening in synaptic strength between A-fibres and cells within lamina II and retraction of A-fibres from the substantia gelatinosa. A later study confirmed that functional reorganization of A-fibres in the DH is driven by endogenous glutamatergic activity (Beggs et al., 2002). In this study, rats were chronically treated with the NMDA receptor antagonist MK801 from P0 with Elvax implant in the spinal cord. At eight weeks old, rats that were treated with MK801 exhibited an enhanced response to mechanical stimulation, enlarged cutaneous receptive field, greater A-fibre evoked responses and a failure of A-fibre retraction from the superficial DH (Beggs et al., 2002). These findings indicate that the rat CNS is highly plastic in the neonatal period; the development of nociceptive pathway during this time is activity-dependent.

At the cellular level PAFs are functional from birth, but mature stimulus-response evoked by the activation of nociceptors require a prolonged maturation phase extending into the

postnatal period (Fitzgerald, 1985). The tetrodotoxin (TTX)-resistant sodium channel Nav1.8, a voltage-gated sodium channel that regulates neuronal hyperexcitability is expressed in developing C-fibre neurons by E17, and adult levels can be observed by P7 (Benn et al., 2001). Moreover, modality-specific nociceptors such as the transient receptor potential cation channel subfamily member 1 (TRPV1) and the ATP receptor $P2X_3$ are found on C-fibres in the late embryonic period, and reach mature levels as early as postnatal day (P) 2 (Koltzenburg, 1999).

Centrally, within the supraspinal nociceptive circuits, the relevant connections are formed before birth but functional descending projection pattern and density cannot be observed before P21 (Bregman, 1987, Rajaofetra et al., 1989). Previous studies using a model of thoracic hemisectioning and spinal cord transection, examined degenerating axons and synaptic endings in the lumbosacral spinal cord at different stages of postnatal development in the rat. They found that 1) descending fibres are present from birth but do not penetrate the dorsal horn until P15 (Gilbert and Stelzner, 1979) and 2) spinal transection before P15 has markedly less impact on the development of descending projections than it does at older ages (Weber and Stelzner, 1977).

More importantly, functional brainstem-spinal cord connections develop relatively late at around the third postnatal week (Hathway et al., 2009). For instance, serotonergic descending fibres, although present in the dorsal horn from E19 onwards, do not reach adult patterns until P21 (Rajaofetra et al., 1989). In addition, SPA is not observed before P21 and the stimulation intensity required for the inhibition of tail-flick reflex evoked by focal radiant heat is much greater in P21 rats than adults (Van Praag and Frenk, 1991). Another study also demonstrated that diffuse noxious inhibitory control (DNIC: inhibition of nociceptive neuronal firing of spinal and trigeminal dorsal horn neurones by noxious stimulation of sites remote from the neurones excitatory receptive fields), which is dependent on descending inhibitory controls is absent before P21 (Boucher et al., 1998). These findings indicate that the neonatal DH lacks adequate descending control, which may be due to immature synaptogenesis between brainstem PNs and DH cells. During the embryonic and postnatal period, the pain modulation pathway is characterised by inadequate neurotransmitter signalling, immaturity of nociceptors and under-developed neuronal networks (Pan, 2012). A summary of both embryonic and postnatal development of pain pathways is included in Figure 1.5.

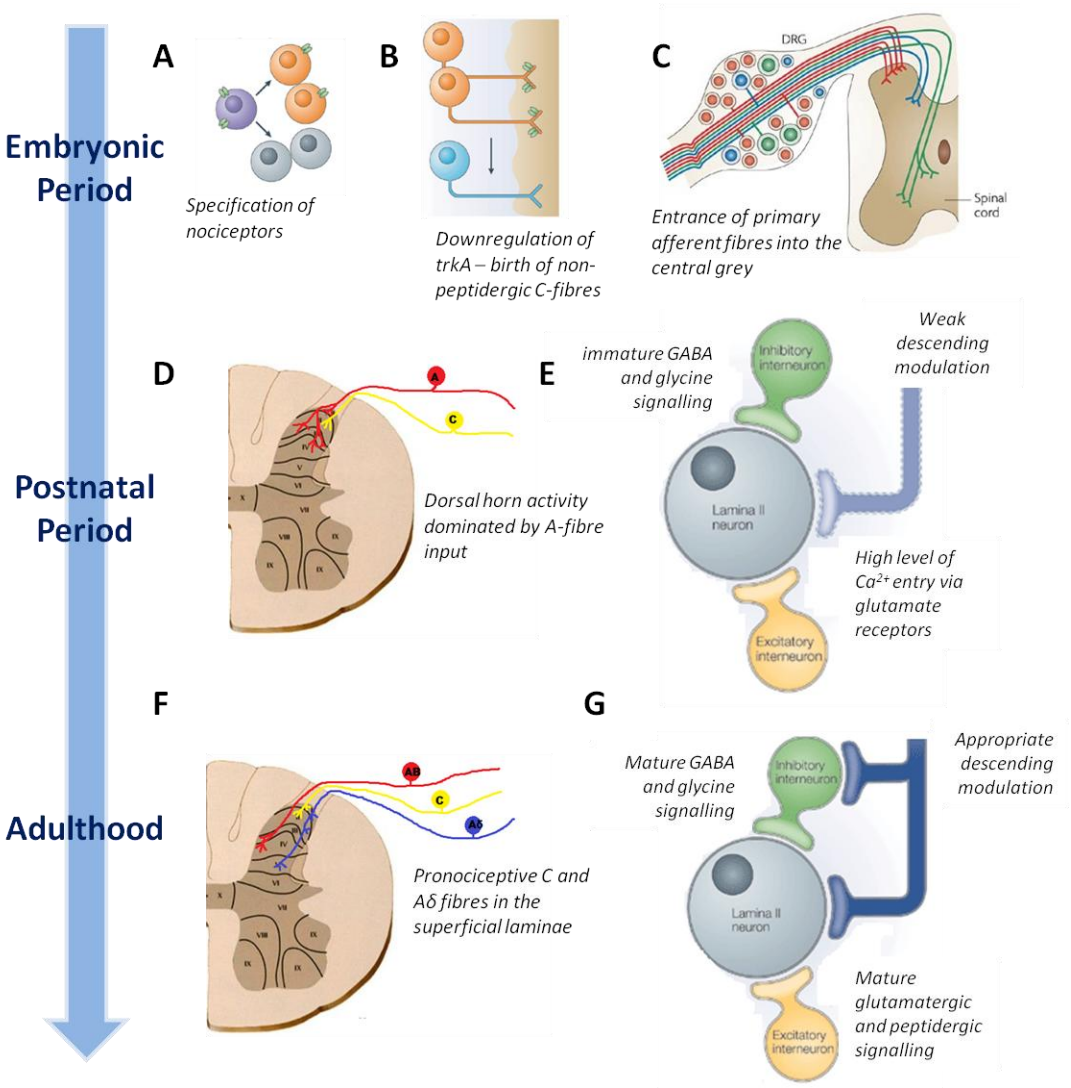


Figure 1.5 (A) Specification of nociceptors occurs very early in the embryonic period at around embryonic day (ED) 12 to 16. (B) Non-peptidergic C-fibres loses its dependency on NGF just before birth. (C) Primary afferent fibres from the dorsal root ganaglion (DRG) penetrate the grey matter of the spinal cord in two waves; large diameter fibres enter at around ED 15 to 17, small diameter fibres enter at around ED 18-20. (D) Neonatal superficial DH is dominated by A-fibre mediated input, as the animals mature they retract into the deeper laminae (III and IV). (E) In the early postnatal period (before postnatal day(P) 21) the spinal nociceptive circuit is characterised by immature descending modulation and neurotransmitter processing, and is hyperexcitable . Note that at this age, the influence of interneurones is also immature. (F) Large diameter A-fibres retract into the deeper laminae and small diameter Aδ and C-fibres are found in the superficial DH. (G) Spinal nociceptive circuits in the matured animals are governed by adequate descending modulation directly or via interneurones, which can be both facilitatory and inhibitory. Image adapted from Fitzgerald, 2005; Marmigere and Ernfors, 2007.

1.7.3 Neonatal pain behaviour

The development of nociceptive pathways during the embryonic and postnatal period of the rat is described in the previous two sections. In this section, the differences between young and adult pain behaviours are discussed, with specific reference to the functions of the PAG-RVM-DH circuitry during the developmental period.

It was once believed that due to the immaturity of the CNS, neonates do not experience pain to the same extent as adults (Hatch, 1987). Clinicians were also reluctant to prescribe analgesics to very young infants due to concerns over the potentially harmful effects of drugs (Hatch, 1987, Anand and Soriano, 2004). These factors compounded with poor judgement and pain assessment in children (Schechter and Allen, 1986), meant that neonatal pain was often under-treated. Fortunately, recent advances in developmental neurobiology have provided powerful evidence in disputing these misguided beliefs. Clinicians are now better at correlating facial expressions with indices of pain, especially in very pre-term infants (Craig et al., 1993). Long-term follow-up studies found that behavioural and cognitive outcomes improved in children who received adequate analgesics (morphine infusion) during Neonatal Intensive Care Unit (NICU) confinement (Anand et al., 1999a, van Lingen et al., 2002). Nonetheless, other research in humans and animal models has shown that pain and tissue damage in infancy have both immediate and long term consequences on the development of the CNS and future well-being (Anand et al., 1999b, Porter et al., 1999, Ren et al., 2004, Walker et al., 2009). These studies indicate that neonatal pain is a real clinical concern, and in order to provide better treatment and management our understanding of pain transmission in young children must improve.

Hospitalised infants are more likely to undergo repeated painful procedures compared to healthy, term-born infants (Taddio et al., 2002). Heel lance is a common procedure for blood sampling in infants (Shah and Ohlsson, 2001). In premature infants heel lance causes a prolonged crying time, increase in heart rate and other facial expressions such as brow bulge, eye squeeze, nasolabial furrow, and mouth opening (Owens and Todt, 1984, Grunau and Craig, 1987, Rushforth and Levene, 1994). One study sampled 50 human infants, ranging from 27.5 to 42.5 weeks postconceptional age, reported that the mean mechanical thresholds to vFh stimulation in 29 weeks old infants are lower when compared to 41 weeks old; it is possible to elicit the FWR from the whole limb as far up as the top of the thigh and buttock and muscle tensing in the contralateral limb is also observed (Andrews and Fitzgerald, 1994). The same study also reported that contralateral limb response begins to diminish by 30 weeks postconceptional age

(Andrews and Fitzgerald, 1994). These results indicate that neonatal nocifensive responses are exaggerated and often inappropriate and sensory processing continues to mature and develop in the postnatal period.

Results from studies performed in rat pups are consistent with those obtained from human infants. In adult rats, hindlimb withdrawal reflexes are well directed and functionally adapted; in both awake or decerebrate preparations they effectively move the limb away from the source of stimulation, evoked either by mechanical vFh or noxious thermal (CO_2 laser) stimulation (Holmberg and Schouenborg, 1996). The same study also reported that in young rats (below P25) FWR are poorly directed and tuned; both thermal and mechanical thresholds are lower compared to adults, and in the majority of rats tested they moved towards the source of stimulation. Mechanical threshold but not thermal threshold increased significantly after P25 (Holmberg and Schouenborg, 1996), which suggests that noxious thermal processing reaches maturity at around P25 but mechanical processing undergo a prolonged development in the postnatal period. In another study, tail-flick responses to noxious heat (hot water tail immersion) were tested in rats at P 3, 6, 9, 12, 15, 21 and 90 (Falcon et al., 1996). Thermal nociceptive thresholds are lower in neonates when compared to adults as tail-flick is elicited at 43.3°C in P12 rats and at 47.2°C in P90, by P21 thermal nociceptive responses are comparable to P90 (Falcon et al., 1996). Moreover, the same study also found that nociceptive thermal sensitivity in adult rats is enhanced in those that underwent spinal transection at P20. This indicates that 1) coordination of a matured reflex response is dependent on descending inputs from central sites, and 2) neonatal sensory processing reflects the immaturity of descending projections to the spinal cord.

EMG recordings provide a quantitative measurement for the size of FWRs in both human and animal studies and a clear correlation is demonstrated between the mechanical stimulus intensity and the latency and amplitude of the reflex (Andrews and Fitzgerald, 1999), which is also indicative of the size of cutaneous RF and spinal reflex excitability (Schouenborg and Kalliomäki, 1990, Schouenborg and Weng, 1994, Schouenborg et al., 1995a). EMG responses evoked by noxious stimuli are larger in neonatal subjects: upon threshold and supra-threshold mechanical vFh stimulation, an increase in response amplitude and post-stimulation spontaneous discharge are observed (Holmberg and Schouenborg, 1996, Andrews and Fitzgerald, 1999). In addition, studies using well-characterised animal pain models have demonstrated that immature nociceptive responses are exaggerated. Mature responses to inflammatory pain induced by intraplantar injection of formalin can be divided into two phases: an early phase

characterised by acute activation of C-fibres, which begins immediately after formalin administration and peaks within five minutes but rapidly declines and a second phase of sustained pain behaviours, which appears ten to fifteen minutes after formalin administration when sensory fibre activity is accompanied by inflammation and central sensitisation (Alreja et al., 1984). By using the intraplantar formalin model (Teng and Abbott, 1998), it was reported that low doses (0.3%, 0.6% and 0.9%) of formalin evoke a monophasic response in P3 rats, lasting for one hour post-injection; at P15, 0.5%, 1%, 2% formalin evoke only monophasic responses but the effects disappear within 30 minutes of injection; at P25, 2.5%, 5% and 10% formalin evoke the classic biphasic response, and sensitivity to formalin-induce pain, as measured by observations of flexing, shaking, licking, kicking, squirming and jerking are comparable to those seen in adults (Teng and Abbott, 1998). These studies demonstrate that the pain sensitivity and behaviour are higher and more pronounced in neonates when compared to adults, which in turn implies that immature nociceptive circuits are highly excitable and lack inhibition.

To further elucidate the activity of immature nociceptive circuits, one study investigated the RF properties and evoked responses of DH cells in both immature and adult rats to noxious and innocuous mechanical stimuli (Fitzgerald and Jennings, 1999). It was reported that before P21, the superficial DH is dominated by low threshold, A-fibre mediated activity, consequently RFs are larger and mechanical thresholds are lowered. After this age, A-fibres retract to the deeper laminae (Fitzgerald et al., 1994), nociceptive responses become more refined as RF size decreases and mechanical threshold increases (Fitzgerald and Jennings, 1999). As briefly mentioned in the previous paragraph, immature nociceptive circuits lack inhibitory inputs. Using whole cell patch clamp recordings in spinal cord slices from a range of neonatal rats (age: P0-2, P6-7 and P13-14), it has been revealed that the mean frequency of spontaneous inhibitory postsynaptic current (IPSC) increases as animals age (Baccei and Fitzgerald, 2004). The same study also reported that $GABA_A$ receptor-mediated miniature IPSC (mIPSC) are present from birth, but the exogenous application of the $GABA_A$ receptor agonist baclofen induces depolarisation in 40% of neurones recorded in the DH of P0-2 animals whereas only hyperpolarisation is evoked in the older rats (Baccei and Fitzgerald, 2004). Moreover, a later study showed that $GABA_A$ receptor antagonism by intrathecal application of bicuculline (10ng/g) dose-dependently decreases thermal nociceptive thresholds in P21 but increases them in P3 rats (Hathway et al., 2006). These findings show that during early postnatal development, $GABA_A$ receptor-mediated effects are depolarising, the DH is dominated by excitatory events and little inhibitory transmission can be observed.

As demonstrated in studies with spinalised animals, the output of spinal nociceptive transmission depends on descending inputs from supraspinal sites (Kehne et al., 1985, Sandkühler et al., 1995, Kauppila, 1997, Kauppila et al., 1998, Pitcher and Henry, 2000). Significant research has revealed the way in which spinal cord nociceptive circuits mature, and has increased our understanding of neonatal pain. Emerging evidence suggests that supraspinal sites also undergo significant postnatal refinement, which in turn influences the differential processing of noxious events in immature animals. In adults, low intensity electrical stimulation (5–10 μA) of the RVM facilitates EMG responses to mechanical vFh stimulation of the hindpaw whereas EMG responses and DH cell activity are inhibited at higher stimulation frequency (50-100 μA) (Zhuo and Gebhart, 1990, 1997, Hathway et al., 2009). However, in P3 and P21 rats, electrical stimulation of the RVM exclusively facilitates hindlimb withdrawal reflexes regardless of the intensity of stimulation, and activity of DH cells is never inhibited (Hathway et al., 2009). The same study also reported that RVM lesion in adults lead to a decrease in mechanical withdrawal threshold, whereas in P3 and P21 rats it leads to the opposite (Hathway et al., 2009). This finding suggests that descending influences, similar to spinal nociceptive transmission are also predominantly excitatory in the early postnatal period.

The immaturity in noxious processing of neonatal rats is further demonstrated by studies using hyperalgesic and neuropathic pain models (Jiang and Gebhart, 1998, Vega-Avelaira et al., 2012). Tail-flick latency to application of mustard oil (5.5μL) to the left hind leg is shorter in adult rats when compared to rats younger than P18 (Jiang and Gebhart, 1998). Carrageenan-induced hyperalgesia (as measured by the degree of reduction in mechanical threshold) is strongest in P21 rats when compared to P3 and P10 (Marsh et al., 1999). Moreover, rats do not develop symptoms associated with neuropathic pain in the first three weeks of life (Howard et al., 2005, Vega-Avelaira et al., 2012). Mechanical allodynia induced by spared nerve injury (SNI, the common peroneal and tibial branches of the sciatic nerve are ligated, leaving the sural nerve intact) are not observed in P3, 10 or 21 rats (Howard et al., 2005). In a later study, SNI procedures in P10 rat pups did not produce mechanical hypersensitivity until 21 days post-surgery (or age = P31) (Vega-Avelaira et al., 2012). Since the induction and maintenance of pain hypersensitivity depends upon descending mechanisms (Coderre et al., 1993, Lewin et al., 1994, Hsieh et al., 1995), the delay in onset of hyperalgesic mechanisms and neuropathic pain behaviours indicates that significant changes occur within the descending nociceptive pathways during postnatal development.

1.7.4 Role of opioids in postnatal development

Recent evidence suggests that the development of descending nociceptive pathways requires endogenous opioidergic activity, and that the opioidergic signalling system undergoes significant postnatal refinement. Opioids preferentially activate the descending facilitatory pathway in the immature CNS. Microinjection of DAMGO (30ng) into the RVM decreases mechanical withdrawal threshold in P21 rats, but the same dose of DAMGO microinjected into the RVM of adult rats has antinociceptive effects (Hathway et al., 2012). The same study also reported that the maturation of functional descending pain modulation relies on tonic opioidergic activity: blockade of endogenous opioids by naloxone hydrochloride (administered by subcutaneously implanted osmotic pump, 0.3μg/hour, 7 days) from P21-28 inhibited the formation of descending RVM inhibitory control of spinal nociceptive reflexes, whereas chronic administration of morphine (0.175μg/hour) accelerated the development of adult-like descending control (Hathway et al., 2012).

Findings from anatomical studies reveal that the expression of opioid binding sites undergoes alterations during postnatal maturation. Within the spinal cord, MOR and KOR are present from birth whereas DOR is not observed until P7 onwards (Rahman et al., 1998a). MOR is overexpressed on large diameter sensory neurones in the neonatal DRG (Nandi et al., 2004). The analgesic potency of systemic and intrathecal morphine is highest in neonates, and deceases as animals age (Bouwmeester et al., 2004). Since neonatal DH is dominated by large diameter A-fibre (see section 1.7.2), this may partly account for the higher potency of morphine observed. In addition, total MOR protein levels in the brainstem increases as the animals age, and the molecular weight of MOR switches from the 50kDa to the heavier isoform 70kDa from P15 onwards (Kivell et al., 2004). The change in expression and molecular weight of opioid binding sites may affect binding properties and efficacy of opioidergic ligands.

1.7.5 Role of endocannabinoids in postnatal development

Cannabinoid binding sites are ubiquitously distributed within the CNS (Tsou et al., 1998). Autoradiographic study of cannabinoid binding sites in humans revealed the existence of cannabinoid binding activity in foetal (33 weeks in gestation), neonatal (3 weeks old) and adult (21-28 years old) brains (Glass et al., 1997, Mato et al., 2003). Using the same technique, specific and saturable cannabinoid binding was found in P2 and P5 rat forebrains (de Fonseca et al., 1993). Another study also reported that cannabinoid binding increases from birth to P60, and within the hippocampus, adult levels of cannabinoid receptor binding are achieved by P21 (Belue et al., 1995). The findings of

these studies indicate that cannabinoid receptors are present from birth, but like opioid receptors, their expression and binding properties undergo significant development in the postnatal period.

Studies that examined the effects of cannabinoids in CNS development originally arose from observations that marijuana consumption in women who are pregnant, lactating, or both affect the neurobehavioural development of their children, either in utero or via breast feeding (Linn et al., 1983, Fried et al., 1984, Zuckerman et al., 1989). Marijuana use is highly correlated to shorter gestation length (Fried et al., 1984), and a decrease in birth weight and length of infants (Zuckerman et al., 1989). Longitudinal data showed that early postnatal exposure to marijuana via breast milk is associated with some cognitive and motor deficits, such as attention deficit hyperactivity disorder (ADHD), deficits and learning and motor tasks (Huizink and Mulder, 2006) and executive functioning, such as attentional behavior and visual analysis/hypothesis testing (Fried and Smith, 2001).

The effects of early cannabinoid exposure have been investigated in animal models. Consistent with findings from human studies, prenatal cannabinoid exposure is associated with shorter gestation (an average of 19 days instead of 21.5) and a decrease in size of pups (Abel, 1985). In addition, adult rats exposed to cannabinoids during the embryonic and early postnatal period exhibit persistent alterations in the behavioural responses to novelty, social interactions, locomotor and exploratory situations (Navarro and Rubio, 1995). In addition, exposure to cannabinoids in the gestational period alters the expression of other neurotransmitters, e.g. the number of tyrosine hydroxylase (enzyme responsible for converting L-tyrosine to L-DOPA) containing neurones are higher in the foetal brains that are chronically exposed to cannabinoids from gestation day (GD) 5 (Bonnin et al., 1996). An increase in expression of dopamine in the prosencephalic area of adult brains is associated with exposure to cannabinoids in the early postnatal period via breast-feeding (De Fonseca et al., 1992). These data suggest that alterations in cannabinoid levels during the embryonic and postnatal period lead to differential maturation of the CNS.

Recent studies have found that neurotrophic processes, such as neuronal fate determination, migration of progenitor cells, gliogenesis and neurogenesis occurring in normal maturation of the CNS requires endocannabinoid signalling (Keimpema et al., 2011). 2-AG is critical for the migration of mouse neuroblasts in the subventricular zone

(Oudin et al., 2011a). Prenatal exposure to WIN55212 (pregnant female rats received the drug daily via subcutaneous injection) alters the development of cerebral cortex, specifically, the migration of GABAergic and glutamatergic neurones from the cotical plate is impaired (Saez et al., 2014). Figure 1.6 summaries the proposed roles of endocannabinoids during CNS development.

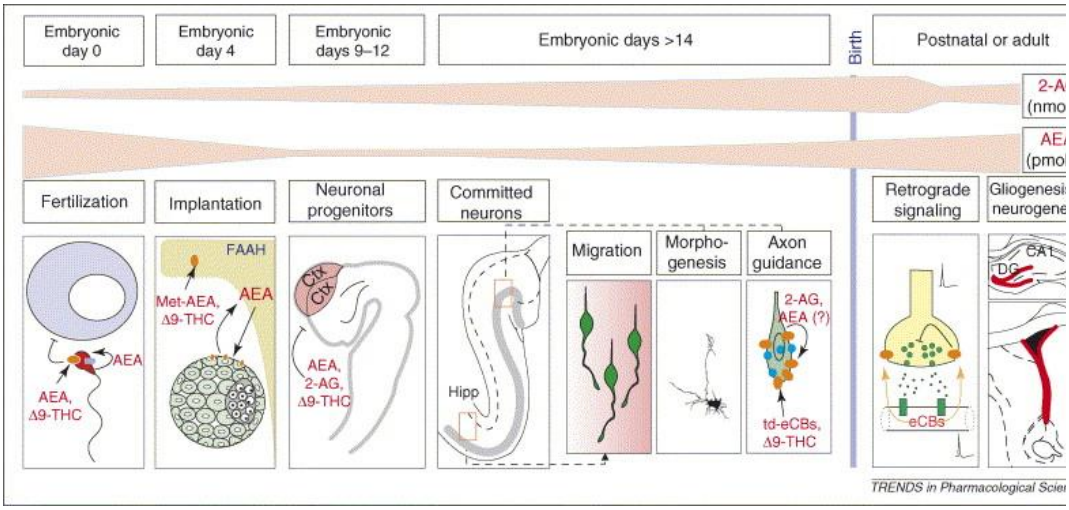


Figure 1.6 Proposed roles of endocannabinoids during CNS development. Top panel shows the corresponding age in the embryonic and postnatal period. The 2 following panels represent the levels of endocannabionoids (2-AG and anadamide/AEA) expressed during the different periods. Bottom panel: processes mediated by the endocannabinoids. Prenatally, anandamide emerges early, present in embryos and important for successful fertilization and implantation in utero. 2-AG is present in the late embryonic period and expression increases until adulthood. 2-AG is important for successful gliogenesis, neurogenesis and subsequent synaptogenesis. Image adapted from Harkany et al., 2007.

It is evident that the endocannabinoid signalling system is plastic during the developmental period. Since endocannabinoid signalling is also important for nociception, it may also be implicated in the maturation of nociceptive circuits. One study, investigated tail flick latencies in P24 and P50 rats that were exposed to cannabinoids in the perinatal period (born of mothers who received a daily dose of Δ^9-THC, 5mg/kg orally) and found that these animals exhibit a longer latency, indicating a higher baseline nociceptive threshold (Vela et al., 1995). A later study also reported that THC-treated animals have elevated β-endorphin and methionine-enkephalin levels in whole brain homogenates (Kumar et al., 1990). The findings of these studies suggest a role for cannabinoids in development of nociceptive circuits. However, research on the

effects of cannabinoids on neonatal pain is limited and further studies in this area will be beneficial.

1.8 Hypothesis

Previous studies have shown that pain behaviours are exaggerated and inappropriate during the early postnatal period (Owens and Todt, 1984, Andrews and Fitzgerald, 1994, Holmberg and Schouenborg, 1996), which reflects the immaturity of the pain circuitry, in particular the descending pain pathway during the early developmental period (Fitzgerald and Jennings, 1999, Hathway et al., 2006, Koch et al., 2008b, Hathway et al., 2009). Other studies have also shown that both the endogenous opioid (Rahman et al., 1998a, Kivell et al., 2004, Hathway et al., 2012) and cannabinoid (Belue et al., 1995) signalling pathways undergo significant postnatal refinement. I hypothesise that significant postnatal changes occur in both the endogenous opioid and cannabinoid systems within the descending pain pathway during postnatal development, and that these changes are crucial to the maturation of pain modulation during this period.

1.9 Aims of thesis

1) To identify the functional role of MOR within the descending pain pathway during postnatal development
2) To characterize the underlying changes in expression of MOR and related peptides during postnatal development
3) To identify the functional role of cannabinoid receptors in the descending pain pathway during postnatal development
4) To illustrate the expression of the components of the endocannabinoid signalling system within the descending pain pathway during postnatal maturation

Chapter 2 General Methods

2.1 In vivo surgery

2.1.1 Animals

Postnatal day (P) 3, 14 and adult (male, 240-260g) Sprague Dawley rats were purchased from Charles River, UK. Pups (female and male, depending on the litter) were housed with their dams in individually ventilated cages in an in-house animal facility. Free access to food and water was available throughout. All experiments were performed in P10, P21 and adult (minimum age = P40) rats during the animals' light cycle. Experimental procedures were carried out under the Home Office License 40/3647 and in accordance with the Animals (Scientific Procedures) Act 1986 and IASP guidelines.

2.1.2 Anaesthesia

When choosing a general anaesthetic for electrophysiological studies, care must be taken to ensure the anaesthetic selected will not significantly affect the responses (Jinks et al., 2003). The anaesthetic agent chosen must be constant and not vary throughout the duration of the experiment. Isoflurane (Baxter, UK), vaporised and carried by 100% oxygen was the choice of anaesthetic in this study because both the induction and calibration time is fast, allowing ease of control of the depth of anaesthesia during surgical procedures and recordings. Furthermore, it does not suppress spinal activity to the extent of other gaseous anaesthetics such as halothane (Jinks et al., 2003).

Anaesthesia was induced in a plastic box, at 5% (isoflurane) with a 2L/min oxygen flow rate, controlled by the oxygen regulator and flowmeter. Once the animal became areflexic (corneal reflexes and muscle tone of the hindpaw were checked), it was transferred onto a fitted nose cone for surgical procedures. Anaesthesia was maintained during surgery at 2%. After surgery, anaesthesia was further reduced to 1.3%. At this anaesthetic depth, the animal remained unconscious but hindpaw withdrawal reflex remained intact upon mechanical stimulation. This set-up allows the optimum condition for electromyographic recordings (EMG), as previously described by other studies (Hathway et al., 2006, Koch et al., 2008b, Hathway et al., 2012).

2.1.3 Maintenance of anaesthesia via tracheal cannulation

After induction of anaesthesia, tracheal cannulation was performed in P21 and adult rats to allow stable maintenance of anaesthesia throughout the long period of recording. The skin overlaying the front of the neck/throat was cut open with a scalpel. The fat patch and muscle covering the trachea was removed by blunt dissection using small blunt-tip scissors. The smooth muscle layer surround it was removed using a pair of iris scissors. Care was taken not to cut through any veins or vessels, as blood loss at this stage has

significant impact on the overall health of the animal, thus affecting the withdrawal reflex responses. Once the trachea was revealed, an incision was made using a pair of iris scissors, parallel to the orientation of cartilage rings, to allow the insertion of the cannula. The cannulae were made with polyethylene tubing (2.1mm diameter, Portex). The cannulae were then secured by sutures; one tied around the trachea and the cannula, one tied the cannula to the skin. These two sutures were tied together to ensure the positioning of the cannula was secure. The wound was then closed by suturing up the skin, which helped to both minimise heat loss and maintain the positioning of the cannula.

After tracheotomy was performed, the cannula was connected to a ventilator pump (model number 50-9703, Harvard Apparatus), which could be adjusted to match the lung volume and breathing rate of the animals (lung volume = approximately 1mL/100g; breathing rate = 80 breaths/minute). As lung sizes do not differ between P21 and adult rats (Burri et al., 1974), the same volume and breathing rate were used for experiments performed in these two age groups. The vaporiser is directly connected to the ventilator, and the ventilator has two outputs, one to the animal via the tracheal cannula and one for excess to scavenge (Cardiff Aldasorber, Vincent Works Sheffield). The tube connecting the ventilator and the tracheal cannula bypass a water cylinder (approximately 15cm of water in the cylinder), which helps to protect the lungs from overinflating by offering a resistance equal to normal lung pressure (Tremblay et al., 1997). This technique ensures the experimenter has complete control over the amount of anaesthetic delivered to the animal, which was crucial as any fluctuation in anaesthetic depth impact on the quality of EMG recordings (King and Rampil, 1994, Rampil and King, 1996).

2.1.4 Maintenance of anaesthesia in P10 rats

Blood loss during surgical procedures has detrimental consequences on the well-being of the animal and subsequently the quality of electromyographic (EMG) recording is compromised. Since P10 rats have a much smaller blood volume, and less fur to help insulate the body and retain heat, it was decided that the best way to maintain anaesthesia in these animals was via a fitted facemask, rather than surgical insertion of tracheal cannula. The facemask is designed to fit onto the bite bars of the stereotaxic frame. The facemask has a small conical area, which was made to fit the nose tip of neonatal rats. It also has one air inlet and one air outlet, which allows free flowing of gas and excess to be scavenged.

2.1.5 Stereotaxic placement of animals

P10, P21 and adult rats were all secured onto the stereotaxic frame with conventional ear bars and bite bars. The body temperature of animals (37ºC) was maintained by a heating blanket unit. For P21 and adult rats, the ear bars were inserted into the ear canals so that the head of the animal was well secured and levelled to the frame, with its nose pointing directly forward. The bite bar was positioned between the animal's incisors to minimise movements of the head throughout the experiment.

For P10 rats, it was not possible to use ear or bite bars because their ear canals were not fully developed. To secure the animals, their heads were supported with cotton wool and swabs, and the bodies were secured onto the stereotaxic frame with electricians tape.

For both P10 and P21 rats bodies were raised with a special platform attachment (Model 901, adjustable Stage Platform, Kopf instruments) for the stereotaxic frame. This was because most stereotaxic frames for rats were designed for adult sized rats, rather than neonates or adolescents. If their bodies were not raised, their thoracic cavity would be stretched, which affects their breathing during the experiment.

2.1.6 Laminectomy

The skin on the back was cleaned with alcohol swabs. An incision to the skin was made parallel to the base of the rib cage using a scalpel. The positioning of this incision was to allow access to the lumbar segment (L)4-5 of the spinal column. The fat and connective tissue holding the skin and underlying muscle was removed using a scalpel. The connective tissue covering the vertebrae was also removed using a scalpel. Once the vertebrae were visible, two incisions were made: one along each side of the vertebrae to allow the spinal column to be lifted up with a pair of rat-toothed forceps. After the column was lifted up, two to three vertebrae were removed using a small pair of rongeurs. In order to prevent obstruction during surgery bleeding was immediately stopped by applying gentle pressure to the bone. The dura was lifted up with a pair of fine forceps, and cut off using a pair of iris scissors. Once the spinal cord was exposed, physiological saline was dripped onto the cord to keep it moist. This procedure allowed drugs to be administered directly onto the spinal cord, and can be carried out in rats of all ages.

2.1.7 Craniotomy

The skin overlying the skull was cleaned with alcohol swabs and an incision along the midline was made. The connective tissue covering the skull was removed. Bregma and

Lambda were located and the coordinates were recorded using the stereotaxic arm attachment for holding the Hamilton syringe. The coordinates for the ventral-periaqueductal grey (vPAG; both adult and P21: medial-lateral [M-L] 0.5mm; anterior-posterior [A-P] -7.8 mm; dorsal-ventral [D-V] -6.0mm; P10: [M-L] 0.5mm; [A-P] -7.8mm; DV -4.5mm) and rostroventral medulla (RVM; adult: [M-L] 0mm; [A-P] -9.7mm; [D-V] -10mm; P21: [M-L] 0mm; [A-P] -9.2mm; [D-V] -10mm; P10: [M-L] 0mm; [A-P] -8.7mm; [D-V] -8mm) were calculated and marked on the skull using a marker pen. A hole was then drilled on the skull using a micro bone drill; this allowed the needle to be inserted for the injections.

The injection sites were examined post-mortem. The brains were quickly dissected out and kept on ice, then cut coronally and lesion tracts identified. The location of the end of lesion tracts were recorded and results are presented in *chapters 3 and 5*.

2.2 Electromyographic (EMG) recordings

The fur overlying the biceps femoris muscle on the hind limb was trimmed and a bipolar concentric needle EMG recording electrode (a modified 27 gauge hypodermic needle with one wire (A) down the middle of the needle and another wire (B) wrapped around the inside of the needle; Ainswork, Coventry, UK) was inserted into the belly of the muscle. Such recording electrodes ensure that recorded activity (A minus B) is restricted to local muscle activity in small animals. The EMG electrode was connected to a NeuroLog head-stage (module NL100AK; Digitimer, Welwyn Garden City, UK), signals were amplified x2000 (module NL104A), band-pass filtered between 10-1000 Hz (module NL125) before being sampled at 2kHz using LabChart software via a PowerLab data acquisition unit (ADInstruments Ltd., Oxford, UK). Rats were left to equilibrate to anaesthesia for thirty minutes before recording.

2.2.1 Mechanical stimulation

EMG activity of the bicep femoris muscle was evoked by mechanical stimulation of the plantar hindpaw using von Frey hairs (vFh). The vFh is a device that measures mechanical sensitivity/threshold (Chaplan et al., 1994); it is a series of fine plastic monofilaments of varying diameters that have progressively stronger bend strengths. The force required to bend each monofilament is calibrated so that measurements can be standardised from one individual animal to another.

Responses to two sub-threshold vFh (T-1, T-2), and the threshold hair (T) and a supra-threshold hair (T+1) were recorded and the same four hairs used in all subsequent stimulation conditions for data analysis. Thresholds were determined in each animal as the vFh that produced an EMG response that was more than 10% greater than the resting EMG activity. Each hair was applied three times, and the mean and standard error of mean (SEM) reading for each of the three presentations were calculated and recorded.

2.2.2 Calculation of spinal reflex excitability and change in mechanical threshold

A stimulus-response curve of the integral EMG magnitude versus mechanical stimulus intensity (grams, as indicated on vFh) was plotted and the area under curve (AUC) was calculated to provide an integrated measure of spinal reflex excitability. An example of this calculation is provided in Figure 2.1. The value obtained was considered as the pre-drug response of the animal. Subsequent responses following pharmacological manipulations were normalised to this because of variations in background (nonevoked) EMG activity. vFh stimulation were carried out at regular ten minute intervals. Pre-drug responses were normalised as 100%, therefore a value greater than 100% indicates facilitation, whereas a value of less than 100% means inhibition of spinal reflex excitability (analgesia). The mechanical thresholds was also normalised as 100%; subsequent post-drug values was presented as a percentage change of the pre-drug value plus 100%. Therefore a value greater than 100% would indicate an inhibition of nociceptive responses (increase in threshold; analgesia), whereas a less than 100% value would indicate facilitation (decrease in threshold; hyperalgesia).

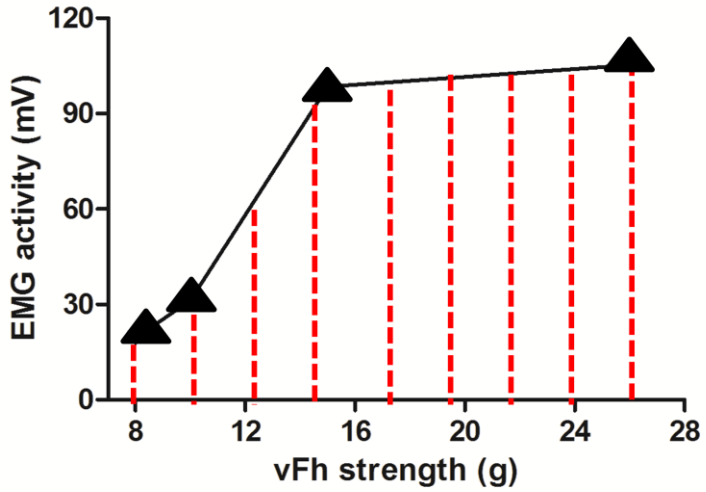


Figure 2.1. An example of EMG activity in response to von Frey hairs (vFh) in a P10 rat. EMG activity increased according to the strengths of vFh, a total of four hairs were used (indicated as triangles on the graph). The shaded area (dotted red lines) is the area

under curve (AUC). AUCs for both before and after drug administration in each animal were calculated to provide an integrated measure of spinal reflex excitability.

2.3 Immunohistochemistry

2.3.1 Animals

Different cohort of rats was used for immunohistochemical experiments. For details of age and husbandry of animals please refer to section 2.1.1.

2.3.2 Perfusion and tissue collection

P10, P21 and adult rats were overdosed with intra-peritoneal (i.p.) injection of sodium pentobarbital (P21 and adults, 2mL; P10, 1mL). Once animals were areflexic and breathing had ceased, the chest cavity was quickly opened up to reveal the heart. The heart should still be beating in order to obtain the best results with perfusion. A needle connected to a perfusion device (adults and P21, perfusion pump; P10, a 50mL syringe) was inserted into the left ventricle, and a small incision was made in the right atrium. Rats were then transcardially perfused with 150mL (50mL for P10 rats) of physiological saline (0.9% sodium chloride in double distilled water), followed by 200mL (100mL for P10 rats) of 4% Paraformaldehyde (PFA, Sigma Aldrich) solution. The rats should be pale and stiff after the perfusion. The brains and spinal cords were quickly dissected out and post-fixed in 4% PFA solution overnight. Afterwards the brains and spinal cords were stored in 30% sucrose solution in 0.1M phosphate buffered saline (PBS) containing 0.01% azide (Sigma Aldrich) for at least one day for cryoprotection before sectioning.

The PAG and the RVM were dissected out by removing the forebrain, the cortex and the cerebellum. The remaining brainstem was then subdivided accordingly into the PAG and the RVM. The lumbar section of the spinal cord (where the enlargement is located) was cut off from the remaining spinal cord.

2.3.3 Tissue sectioning

The PAG, RVM and lumbar sections of the spinal cord were sectioned on the freezing microtome (Leica, SM2010R) set at a nominal thickness of 40 µm. Dry ice was used to freeze and mount the tissue onto the stage of where the tissue sectioning takes place. It was ensured that the blade was well frozen before the cutting. Tissue slices were collected onto 96 well plates filled with sucrose azide solution and stored at 4 degrees Celsius.

2.3.4 Immunofluorescent staining

Tissue was blocked with 3% serum solution with 0.3% triton X100 (Sigma Aldrich) for 1 hour before incubation with primary antibody solution. Lists of primary antibodies and their incubation conditions are included in the methods sections of the respective results chapters. Before incubation with secondary antibodies the tissue was washed three times in 0.1M PBS, for ten minutes each wash to ensure that the tissue was well rinsed. Secondary antibodies (AlexaFluor conjugated) were typically left bathing the tissue at room temperature, concentration 1:500, in the dark (due to the attached fluorophore) for two hours. The choice of secondary antibodies depended on the species of blocking serum used and the species that the primary antibodies were raised in. For example, if goat serum was used in the blocking solution, and the primary antibody was raised in rabbit, the secondary antibody would be goat-anti-rabbit.

In a subset of experiments instead of the protocol above, tyramide signal amplification (TSA, Life Technologies, T20922) methods were used to provide a better signal. For TSA, tissue was rinsed three times for ten minutes after primary antibody incubation. After rinsing, tissue was initially incubated with a horseradish-peroxidase solution (1:100 in blocking solution) for one hour at room temperature, which was then rinsed and then labelled with AlexaFluor conjugated tyramide solution (1:100 in hydrogen peroxide amplification buffer).

Finally the tissue were rinsed and mounted onto microscopy slides, they were air dried, before coverslipping with fluoromount (Sigma Aldrich) and edges sealed with clear nail polish.

2.3.5 Microscopy and quantification

Immunofluorescent sections were observed with a Leica IRE2 fluorescence microscope fitted with Hammamatsu OrcaER monochrome camera and captured using Volocity 6.1 software (Perkin Elmer, UK). For presentation purposes, some images were captured with a confocal head, which has a better resolution than the conventional timelapse microscope. Image J 1.29 (NIH) was used to adjust brightness and contrast of the images post-acquisition. For each anatomical region (vPAG, RVM and DH), the area of interest was outlined and selected by using the ROI (region of interest) function of the Volocity software and the motorised stage (Prior Scientific, UK). 10X, 20X and 40X images were taken for each section. The same exposure time of image acquisition was used for each sections staining for the different antibodies from the different animals to ensure consistent brightness in images. The 40X images (raw tiles) were then stitched

together at 40% overlapping to form a composite image of the area of interest; the raw tiles were saved into a separate folder for quantification.

Systematic random sampling and unbiased stereological methods were used for quantification as adapted from previously published studies (M Gu, 2007, ML Leong, 2011). Six raw tiles were chosen from each area of interest for quantification, the first raw tile was always included, and five other raw tiles were selected at regular intervals according to the number of tiles collected for the section. Number of cells and staining intensity were accounted for as follows. A counting frame was superimposed onto the raw tile, the right and upper boundary of the tile were 'forbidden lines', the left and bottom boundary were 'acceptance line'. An example of a counting frame superimposed onto the images is shown in *Figure 2.2*. Number of cells was only counted if they either lie entirely within the counting frame or cross an acceptance line without also crossing a forbidden line (CV Howard, 1998, ML Leong, 2011). The staining intensity was estimated with Image J. The data obtained were added together to form an estimate of immuno-positive cells or fibres. This process was repeated for each animal. A summary of microscopy and quantification methods is included in *Figure 2.2*.

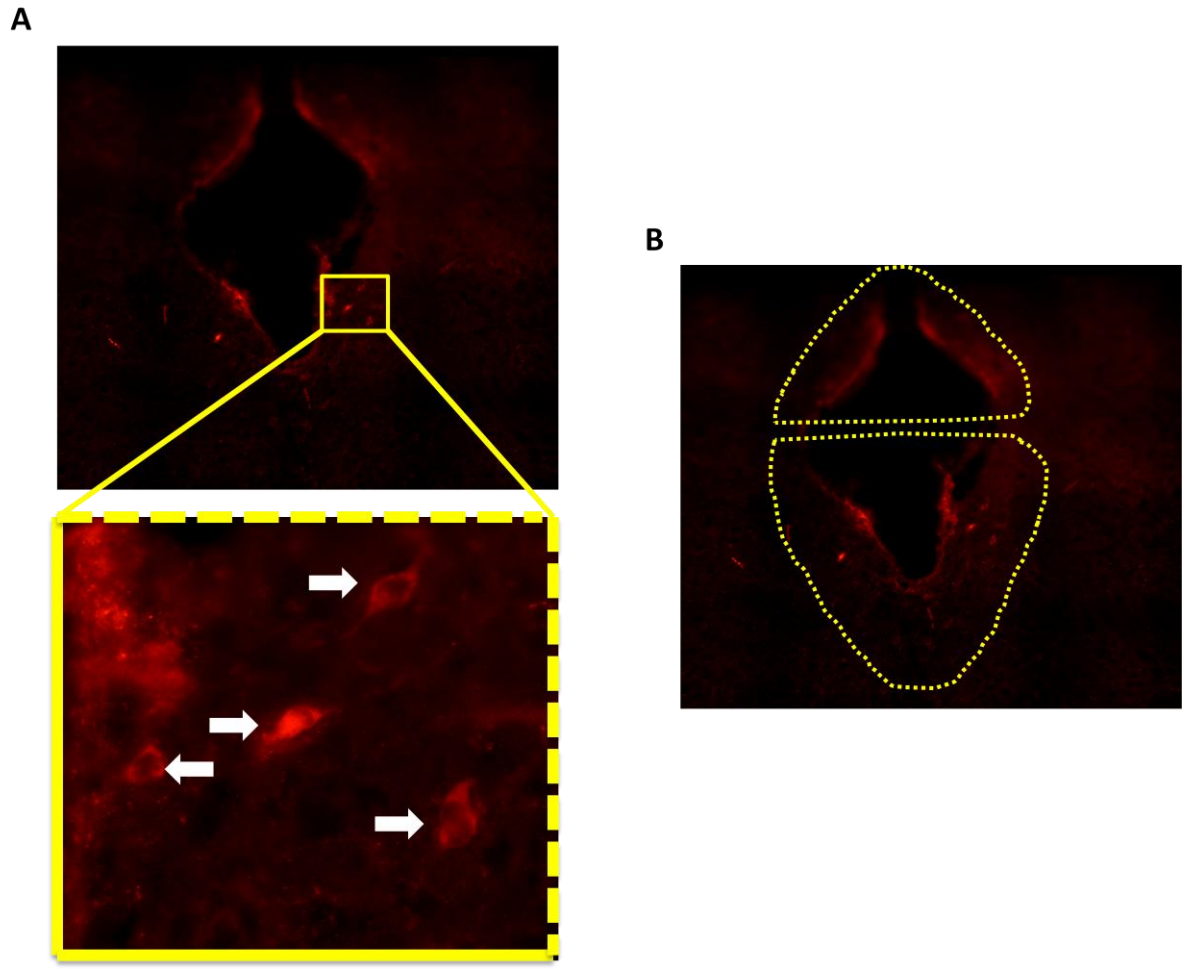


Figure 2.2 Stereology methods. (A) The top panel shows the composite of stitched 40X images of enkephalin immunofluorescent in P21 PAG. The bottom panel shows a single 40X image (raw tile), and it corresponds to the area outlined on the top panel by the yellow square. In the bottom panel, enkephalin immuno-positive cells are labelled with white arrows. The dotted lines represent the 'acceptance lines' whereas the solid lines represent the 'forbidden lines'. If a cell crossed any of the forbidden lines, it was not counted. (B) The regions of interest (dorsal (top) and ventral (bottom) PAG) are outlined. Mean staining intensity was measured using the 'measure' function of Image J. Staining intensity was calculated by the following equation: mean intensity/area.

2.4 Taqman real-time polymerase chain reaction (RT-PCR)

2.4.1 Animals

Different cohort of animals was used in TaqMan RT-PCR experiments. For details of age and husbandry of animals please refer to section 2.1.1.

2.4.2 Fresh tissue collection

P10, P21 and adult rats were overdosed with intra-peritoneal (i.p.) injection of sodium pentobarbital (P21 and adults, 2mL; P10, 1mL). Their brains and spinal cords were quickly dissected out on ice. Tissue from the PAG, RVM and lumbar (L3-L5) spinal cord

were isolated (see section 2.3.2 for details of dissection) with the PAG and spinal cord further subdivided into dorsal and ventral parts with a scalpel blade. Samples were put into separate cryovials for flash freezing in liquid nitrogen and stored at -80ºC.

2.4.3 RNA extraction

50mg of the frozen tissue was homogenized in 2 ml of ice cold Tri reagent (Sigma Aldrich). The homogenate was allowed to stand for 5 minutes at room temperature to allow for the complete dissociation of nucleo-protein complexes. 400µl of bromochloropropane (BCP) was added to the homogenate and mixed thoroughly, which was then centrifuged at 10,000g for 10 minutes. RNA was precipitated from the aqueous medium by the addition of 250µl of 2M sodium acetate (Sigma Aldrich) and 700µl 2-isopropanol (Sigma Aldrich) and chilled at -20^{o}C for up to an hour, followed by centrifugation at 10000g for 10 minutes at 4^{o}C. The supernate was discarded and the pellet washed x3 in 500µl, 70% ethanol solution and re-suspended in 50µl of RNAse free water.

Total RNA clean up and on-column DNAse digestions were performed using RNeasy purification kit (Qiagen) following the manufacturer's instructions. The RNA integrity and purity were analysed on a nanodrop spectrophotometer (nanodrop technologies, USA) by determining the ratio of absorbances at 260/280 and 260/230 respectively. Ratios of between 1.8-2.0 for both integrity and purity were deemed acceptable. RNA concentration in nanograms per microlitre was also determined by the nanodrop spectrophotometer.

2.4.4 cDNA synthesis

A cDNA synthesis kit (Invitrogen) was used following the manufacturer's instructions. Initially, 10 µl of total RNA equivalent to 500ng, 1µl of random primers (100ng) and 1µl of dNTP mix (10mM each dATP, dGTP, dCTP and dTTP at neutral pH) was added to 13µl of DEPC treated water and heated for five minutes at 65^{o}C. The mixture was chilled on ice for at least one minute after heating. The contents of the tube were collected by brief centrifugation followed by the addition of 4µl of 5x first strand buffer, 2µl 0.1M dithiothreitol (DTT), 1µl RNAse inhibitor (Invitrogen) and 1µl of SuperScript^{TM} III reverse transcriptase. The contents of the tube were gently mixed and incubated at 25^{o}C for five minutes, and then at 50^{o}C for 1 hour. The reaction was inactivated by heating at 70^{o}C for 15 minutes.

2.4.5 Primers and probes

All gene primers and probes were designed using primer 3 software (Life Technologies). All FAM-TAMRA (5'end with 6-carboxyfluorescein/FAM and at the 3'end with tetramethylrhodamine/TAMRA) conjugated probes, forward and reverse primers were synthesised by Eurofins Scientific. The MGB (minor groove binder) probes were synthesised by ABI Biosystems. The DNA sequence file of target gene was loaded onto the software dialogue box, the intron and exon boundaries were marked onto the sequence, which allow the design of primers and probes. The sequences chosen must adhere to the following rules

- Primer length: within 50 to 150 bases long
- Probe length: 13-30 bases for FAM-TAMRA, 13-25 for MGB
- Melting temperature: 68 to 70 ^{o}C
- %G/C (guanine residue content to cytosine residue content): 30%-80%

2.4.6 Real-time polymerase chain reaction (RT-PCR)

Taqman RT-PCR was performed using a StepOneTM plus real-time PCR system (Applied Biosystems). A master mix of forward primer, reverse primer, probe, TaqMan Rox-UDG mix and HPLC (high-performance lipid chromatography grade) water was added to 3µL of cDNA samples to make up a total reaction volume of 13µL. All PCR assays were performed in triplicates. A reaction mixture without cDNA was included in all runs as a no template control (NTC). The cycle was performed in two stages as follows: Stage 1: 50^{o}C for 2 minutes, 95^{o}C for 10 minutes (melting temperature) Stage 2: 95^{o}C for 15 seconds (40 times), and 68^{o}C for 1 minute (annealing temperature).

2.4.7 Quantification of target gene expression

The relative standard curve method based on Taqman qRT-PCR was used for quantifying each gene expression. In this method, a pool of the samples to be analysed was generated and diluted 4 fold to yield a NEAT sample. The NEAT sample was subsequently diluted serially in four-fold dilutions to generate a 5 point standard curve. The slope of the standard curve represents the number of cycles for amplification of the gene and is also an indication of the efficiency of the amplification process. For these experiments slopes within the range (-3.2 to -3.6) were deemed acceptable with a slope of -3.32 indicating a hundred percent efficiency of the amplification reaction. A standard curve was included for each plate run and each gene expression studied. Target gene expression was quantified relative to the expression of the corresponding reference gene.

2.4.8 Selecting an appropriate target gene

B-actin, cyclophilin A and GAPDH were assessed for their suitability as reference genes. The expression of B-actin (Figure 2.3A) and cyclophilin A (Figure 2.3B) decreased throughout postnatal development, preventing their use as a reference gene. One-way ANOVA revealed a significant effect of age $F(2,57)=5.83$, $P<0.01$, and Bonferroni post-tests revealed a significantly higher B-actin mRNA transcript level in P10 when compared to adults ($P<0.01$). For cyclophilin A, one-way ANOVA revealed a significant effect of age $F(2,57)=3.46$, $P<0.05$, and Bonferroni post-tests revealed a significantly higher cyclophilin A mRNA transcript level in P10 when compared to adults ($P<0.05$). By contrast, there were no developmentally related changes in the expression of GAPDH ($F(2,57)=0.21$, $P=0.81$; one-way ANOVA with Bonferroni post-tests; Figure 2.3C). Therefore, GAPDH was chosen for the subsequent studies.

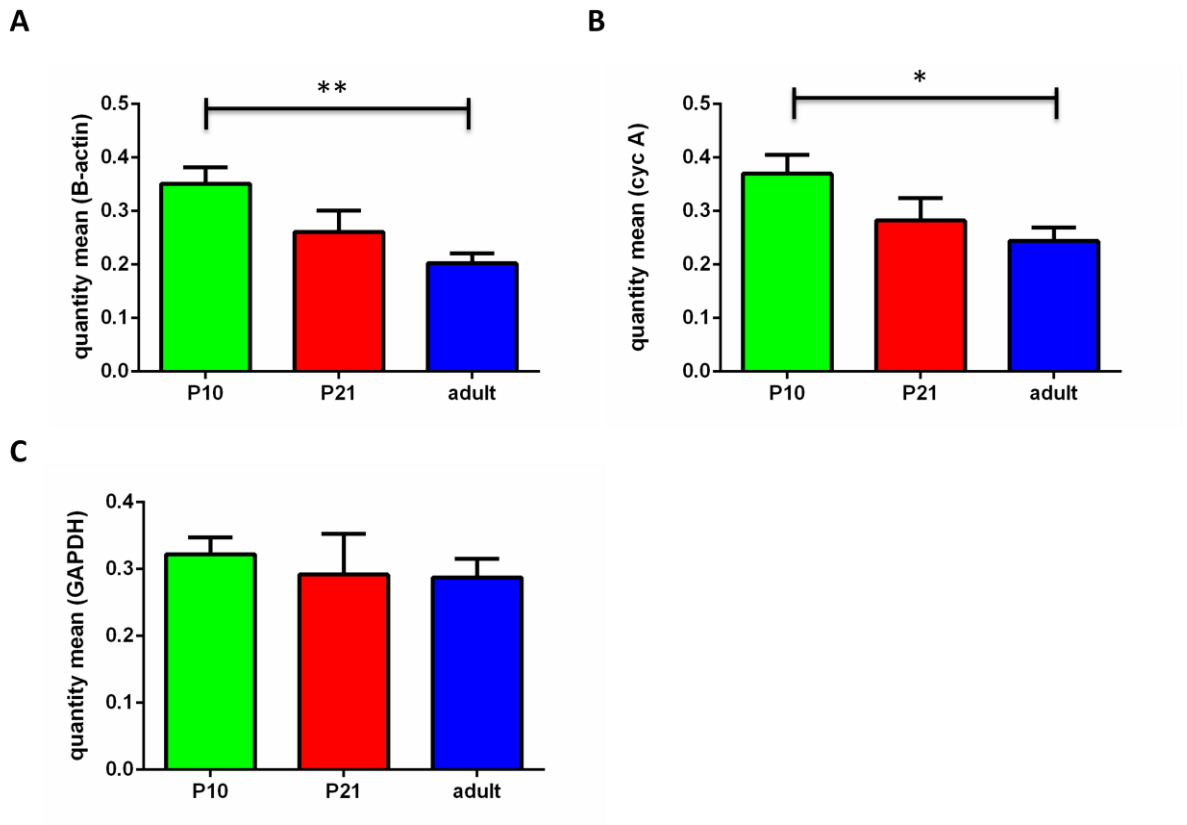


*Figure 2.3 The quantity means of mRNA transcripts for B-actin (A), cyclophilin A (cyc A, B) and GAPDH (C) of P10, P21 and adult rats. The expression of B-actin and cyc A decreased as the animals mature. * = P<0.05, ** = P<0.01; one-way ANOVA with Bonferroni post-tests.*

Chapter 3 The functional role of μ-opioid receptors in the immature descending pain pathway

3.1 Introduction

Nociceptive processing in rodents and humans is subjected to developmental regulation (Koch et al., 2008b, Man et al., 2012). As mentioned in the *Chapter 1 section 1.7.3*, nociceptive transmission in the early postnatal period is predominantly excitatory; as a result of this, nociceptive responses in young animals are exaggerated and un-coordinated. As the CNS matures, activity within the nociceptive pathways becomes increasingly inhibitory; thus, a fine-tuned balance of facilitatory and inhibitory control of spinal excitability is formed (Hathway et al., 2009, Hathway et al., 2012).

Significant data exist detailing the maturation of nociceptive circuits within the spinal cord. However, the development and influences of immature supraspinal nociceptive pathways are less well known, even though spinal excitability can be governed by supraspinal inputs. Previous studies provided evidence that supraspinal pain pathways undergo significant postnatal refinement; for instance, GABA-mediated excitation in P3 rats is reversed by spinalisation at the upper thoracic level (Hathway et al., 2006, Hathway et al., 2009), and chronic low-dose morphine (0.175mg/kg) administered systemically during the second postnatal week accelerates the maturation of the RVM (Hathway et al., 2012). Significant descending inhibition evoked by RVM stimulation was observed in P21 rats after chronic morphine treatment in the second postnatal week, but this effect was not normally observed in rats until P40 onwards. These findings indicate that maturation of supraspinal pain circuits affects nociceptive processing in the postnatal period, and that the opioidergic signalling system has a trophic role. The factors that drive the development of supraspinal nociceptive pathways throughout normal maturation are currently unclear (Hathway et al., 2012, Li et al., 2013a). Given the complexity of pain mechanisms, factors underlying the switch from facilitation to inhibition during development are most likely to be multifaceted (Pan, 2012).

This study therefore, aimed to explore the role of opioids within the descending pain pathway, with an emphasis on the PAG and the spinal cord, to fully elucidate the events that occur during normal postnatal development.

3.1.1 Role of the rostroventral medulla (RVM) in the differential pain processing between adults and neonates

It has been found that micro-stimulation of RVM networks produces differential effects upon spinal excitability between adults and juvenile rats (P21) (Hathway et al., 2009). By measuring spinal reflex excitability, it has been shown that low intensity RVM

electrical stimulation (5-20A) facilitation, and high intensity stimulation (50-200A) leads to inhibition in adult rats. The same study also showed that RVM ablation in adults resulted in decreased mechanical thresholds in the hindpaw, which confirmed that tonic descending influences from the RVM in adults are predominantly inhibitory. On the other hand, in P21 rats spinal reflex excitability can never be inhibited by RVM electrical stimulation, regardless of stimulation intensity. Moreover, RVM ablation by kainic acid (0.5μg/rat; non-NMDA ionotropic glutamate/kainate receptor agonist) in P3 and P21 rats results in an increase in mechanical withdrawal threshold, suggesting that excitatory input was removed (Hathway et al., 2009). Hence, the functioning of the RVM in immature animals is pronociceptive, and switches to a biphasic pain facilitation and inhibition as the animals reach maturity.

The RVM is crucial for nociceptive processing in both young and mature animals. It relays descending information from the PAG to the spinal cord: blockade of endogenous opioid activity within the NRM reverses the inhibitory effect of morphine injected into the PAG on WDRs upon noxious pinch on their receptive field (Vasquez and Vanegas, 2000). Therefore, mature functioning of the RVM requires PAG input and the development of PAG during the postnatal period warrants further investigation.

3.1.2 Possible role of MOR in early development

A later study reported that activation of MOR in the RVM preferentially activates descending facilitation of spinal excitability in young animals (Hathway et al., 2012); microinjection of the MOR agonist [D-Ala2, N-MePhe4, Gly-ol]enkephalin (DAMGO, 3, 10 and 30ng) into the RVM of lightly anaesthetised adult rats produces a dose-dependent decrease in mechanical threshold and nociceptive hindlimb reflex electromyographic (EMG) activity, whereas the same dose of DAMGO in juvenile rats only produces reflex facilitation. Additionally, they reported that blockade of endogenous opioidergic activity by systemic administration of naloxone hydrochloride (3mg/kg per day) from P21 to P28 in rats prevented the normal development of descending RVM inhibitory control of spinal nociceptive reflexes in adults. Descending nociceptive processing developed normally if the block was made before or after the third postnatal week (P21-28). These results indicate that the developmental transition from RVM monophasic descending facilitation to biphasic inhibition and facilitation of spinal excitability is controlled by opioidergic activity within the pain modulatory circuit at a critical period of around P21.

Although focal supraspinal microinjection of opioids is pronociceptive in juvenile rats, systemic administration of opioids is analgesic in very young animals (Nandi and

Fitzgerald, 2005). Reflex withdrawal to mechanical stimulation in P3, P10 and P21 rats is inhibited by systemic administration of morphine, but the analgesic potency of morphine is significantly greater in neonates when compared to adults. This parallels observations in neonatal clinics, where morphine requirements increased from 5μg/kg per hour at birth to 16μg/kg per hour at 1-3 years old for cardiac surgery (Bouwmeester et al., 2003a, Bouwmeester et al., 2003b, Bouwmeester et al., 2004). These findings further suggest that the effects of opioidergic signalling are significantly different between young and adult animals, and the sensitivity of opioid receptors during the postnatal period warrants further investigation. It is noteworthy that other studies have independently found that molecular weight of MORs is developmentally regulated. Using immunoblotting and immunohistochemical techniques Kivell and colleagues showed between P6 and P15, MOR switched from a 50kDa to a 70kDa isoform (Kivell et al., 2004), which may have an impact on the binding properties of the receptor or its intracellular signalling properties.

Opioid exposure in early life may also lead to long-term alterations in pain processing as the animals mature into adulthood. Early injury induced by intraplantar administration of carrageenan in rats at P0 caused hypoalgesia at P60, which was reversed by direct administration of naloxone (MOR, to a lesser extent DOR and KOR antagonist) into the PAG ten minutes prior to behavioural testing at P60 (LaPrairie and Murphy, 2009). This finding suggests that early injury alters the endogenous opioid tone in the PAG. Another study found that systemic morphine administration in early life (once a day for seven days form P8 onwards) leads to an increase in nociceptive responses in adult life, which implies that opioids can induce neuroplastic changes in the nociceptive circuits (Rozisky et al., 2008).

3.1.3 Effects of opioid administration in the PAG during the neonatal period

There are numerous reports of opiate-mediated inhibition of cellular activity and integrated pain responses within the adult PAG (Jensen and Yaksh, 1986, Smith et al., 1988, Vaughan et al., 2003), but few investigated the activity of opioid peptides in younger rats. Recently a study has demonstrated potent opioid-mediated antinociception elicited from the PAG, RVM or dorsolateral pons (DLP) in P3, 10 and 14 rats (Barr and Wang, 2013). DAMGO injected into the PAG, RVM and DLP reduced withdrawal reflexes in both fore- and hindimbs in all ages tested. DPDPE (δ opioid receptor agonist) produced modest analgesia in P10 and P14 rats whereas U50, 488H (κ opioid receptor agonist) had no effects in all ages tested. However, experiments in this

study were performed in unanaesthetised, freely moving rats. It is impossible to rule out confounding factors such as handling stress, novel testing environment, and perhaps most importantly, the effects of a protracted period of maternal separation which are known to significantly affect pain responding in neonates via an opioid-dependent pathway (Kehoe and Blass, 1986a, b).

Although existing evidence suggests that the maturation of the pain modulation pathway is under the influence of endogenous and exogenous opioidergic activity, much of this work has been done at the level of the RVM. Given that the PAG was one of the first sites to be identified as a critical site for μ-opioid receptor-mediated analgesic effects, and neonatal injury can significantly alter opioidergic tone in the adult PAG (LaPrairie and Murphy, 2009), it is surprising that the role of opioidergic activity in the PAG throughout postnatal development has been neglected. Only a small number of studies have been conducted to investigate the relationship between opioids and nociceptive processing at the level of PAG in neonatal animals (Barr and Wang, 2013). Moreover, the effect of intrathecal opioid administration on spinal reflex excitability during postnatal development has not been demonstrated to date. This study, therefore, aimed to elucidate the acute effects of opioids administered focally to both the vPAG and the spinal cord at the different ages, and demonstrates that the functions of opioidergic activity within the descending pain modulation pathway undergo significant alterations as the animals reach maturity.

3.2 Aims

This study aimed to show that MOR-mediated activity undergoes significant postnatal refinements. This was achieved by spinally or focally administering the MOR agonist DAMGO and antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2 (CTOP) into the PAG, and measuring the EMG responses upon vFh stimulation post-drug administration in each age (P10, P21 and adults). Spinal MOR activation by DAMGO is antinociceptive in all ages but most efficacious in the younger animals (P10). As with the RVM, spinal excitability is differentially modulated by intra-PAG microinjection of DAMGO in P10, P21 and adult rats. In particular, supraspinal application of DAMGO will be pronociceptive in P21 but anti-nociceptive in older rats. Since the endogenous opioidergic signalling system may have a trophic role, endogenous opioid activity is expected within the PAG, but the age at which it occurs remains to be elucidated.

3.3 Methods

3.3.1 Drugs

DAMGO (MOR-agonist, 30ng, Tocris) and CTOP (MOR-antagonist, 100ng, Tocris) were administered at doses determined from previously published studies in adult brainstem (Hathway et al., 2009). Saline was administered in separate sets of animals as vehicle control, and it was confirmed that saline either injected into the PAG or spinally applied had no significant effect on spinal reflex excitability. The experimenter was blinded to the drug administered. Sites of injection in the PAG were confirmed by examining the lesion tracts post-mortem after EMG recordings. Brains were quickly dissected and kept on dry ice, they were then coronally sectioned on a freezing microtome (Leica). The lesion sites were visually observed and recorded. All DAMGO injection sites lay within the ventral PAG (vPAG). Data from two adult rats following CTOP microinjection were excluded because the injection sites fell outside of the vPAG. For injection sites in the PAG please see Figure 3.4A of this chapter.

3.3.2 Spinal and intra-PAG drug application

Laminectomy Animals were anaesthetised and mounted on a stereotaxic frame. A laminectomy was performed to expose the L4-5 segments of the spinal cord. The dura mater was carefully removed, leaving the pia mater intact. This method allows the drugs to be applied directly onto the spinal cord. Only one dose of drug was administered to each animal, the volume applied was 25µL for P10 and P21 rats and 50µL for adults.

PAG microinjection Animals were anaesthetised with isoflurane (Baxter, UK) and mounted on a stereotaxic frame (Kopf Instruments, Tujunga, California). The skull was exposed and bregma located. Stereotaxic coordinates for the ventral-PAG (vPAG) were calculated (both adult and P21: left-right [L-R] 0.5mm; anterior-posterior [A-P] -7.8 mm; dorsal-ventral [D-V] -6.0mm; P10: L-R 0.5mm; A-P -7.8mm; DV -4.5mm) and a 26 gauge 2.5µL syringe (Hamilton, Reno, Nevada) was inserted through a drilled hole in the skull. Drugs were injected over a five-minute period, after which the syringe was removed and the wound was closed. Total volume of drug administered into the PAG was 1µL, only one drug was administered per animal.

For detailed description of laminectomy and craniotomy, please refer to *Chapter 2 section 2.1.6 and 2.1.7.*

3.3.3 Statistics

All individual data points were represented as mean ± standard error of mean (SEM). EMG data were normally distributed. Comparisons between the pre-drug values and those obtained following intra-PAG microinjections or spinal administrations within each individual animal (age-matched) were made using repeated measures one-way analysis of variance (RM one-way ANOVA) with Bonferroni post-tests. Statistical comparisons between the age groups and drugs were made using two-way ANOVA with Bonferroni post-tests.

3.4 Results

3.4.1 Baseline EMG activity does not change significantly between ages

Baseline EMG activity in P10, P21 and adult rats were not significantly different (Figure 3.1). After adjusting electrical noise levels with both low-pass and high-pass filter modules baseline EMG activity in all ages remains within the +/- 10mV range.

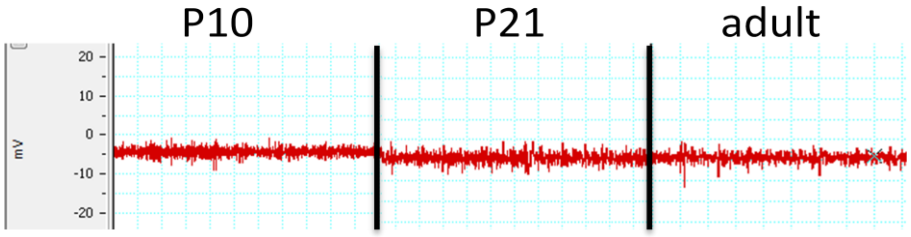


Figure 3.1. Raw baseline EMG signal recorded by LabChart. Scale bar was inserted on the left and background grid included to demonstrate that EMG activity did not surpass the +/- 10mV range in all ages.

3.4.2 Mechanical threshold significantly increased as the animals aged

Mechanical threshold is defined by the amount of force (g, applied by vFh) needed to elicit at least one withdrawal response out of three presentations of the same force. As shown in other studies, neonatal animals have significantly lower mechanical thresholds than older animals in both PAG and spinal application studies ($F(2,14)=9.61$, $P<0.01$ and $F(2,10)=24.34$, $P<0.001$ respectively, one-way ANOVA, Figure 3.2). Bonferroni post-tests revealed that mechanical threshold of P10 was significantly lower when compared to P21 and adults in the PAG ($P<0.01$), which was also true in the spinal study ($P<0.01$ vs P21; $P<0.001$ vs. adult). Therefore different ranges of stimuli were used in the different age groups: for P10, 8g, 10g, 15g and 26g (strength of vFh) were used; for P21 and adults, 26g, 60g, 100g and 180g were used.

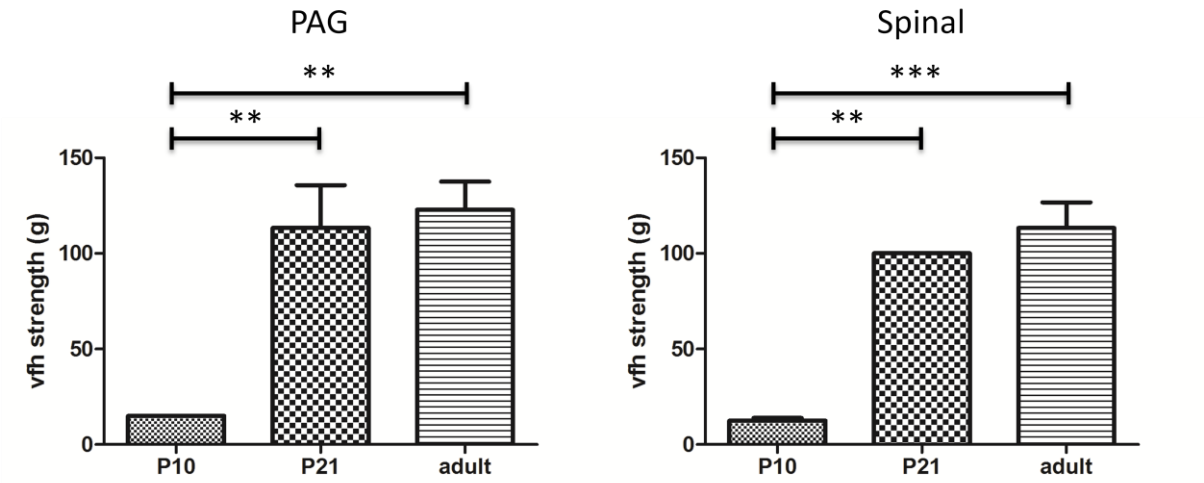


*Figure 3.2. Mechanical thresholds (g) of P10, P21 and adult rats in the PAG and spinal studies, both studies are shown here because different cohorts of rats were used. Data shown here represents the mean ± SEM for each age group in each study, and were analysed with one-way ANOVA with Bonferroni post-tests. P10 rats had significantly lower mechanical thresholds when compared to P21 and adults. Mechanical thresholds of P21 and adult rats were not significantly different (** P<0.01, ***P<0.001).*

3.4.3 Spinal MOR activation causes a decrease in nociceptive responses in all ages

Figure 3.3A shows the EMG activity of P10, P21 and adult rats after spinal application of the MOR agonist DAMGO (30ng) and antagonist CTOP (120ng). EMG responses to the threshold vFh before drug application in each age group are also included for comparisons.

Direct application of DAMGO onto the L4-5 spinal cord of lightly anaesthetised rats (n for P10 = 4, P21 = 8, adult = 5) produced a reduction in spinal reflex excitability (two-way ANOVA, drug x age interaction: $F(4,135)=4.43$, $P<0.01$; Figure 3.3B). Significant reductions in spinal excitability were observed at all age groups between saline and DAMGO treated animals ($P<0.0001$ for P10 and adults and $P<0.001$ for P21 rats). The degree of inhibition observed was significantly greater in the P10 rats compared to adults (mean ± SEM; P10 = 96.03±0.97% vs. adult = 49.24±8.79%, $P<0.01$), illustrating the greater potency of opioidergic ligands in younger animals. The MOR antagonist CTOP applied spinally had no effect on reflex excitability in all ages (n for P10 = 4, P21 = 7, adult = 7) when compared to saline or to pre-drug responses (P10: $F(3,9)=3.82$, $P=0.51$; P21: $F(3,18)=0.76$, $P=0.53$; adult: $F(3,18)=1.39$, $P=0.28$; Figure 3.3B).

Changes in reflex excitability were also accompanied by a parallel changes in mechanical threshold (Figure 3.3C). Mechanical thresholds significantly increased after DAMGO was

applied spinally (two-way ANOVA, drug x age interaction: $F(4,135)=5.70$, $P<0.01$; Figure 3.3C). Comparisons between the different drug treatment groups showed that DAMGO significantly increased thresholds compared to saline in P10 and P21 rats ($P<0.0001$) but not in adults. As with changes in EMG excitability, comparisons between the age groups showed that DAMGO mediated increases in threshold were larger in P10 and P21 rats when compared to DAMGO treated adults (both $P<0.01$; Figure 3.3C). Spinal application of CTOP had no effect on mechanical threshold at any age when compared to saline treated groups or pre-drug responses, but in P10 and P21 animals post-CTOP responses were significantly different from post-DAMGO responses ($P<0.0001$; Figure 3.3C).

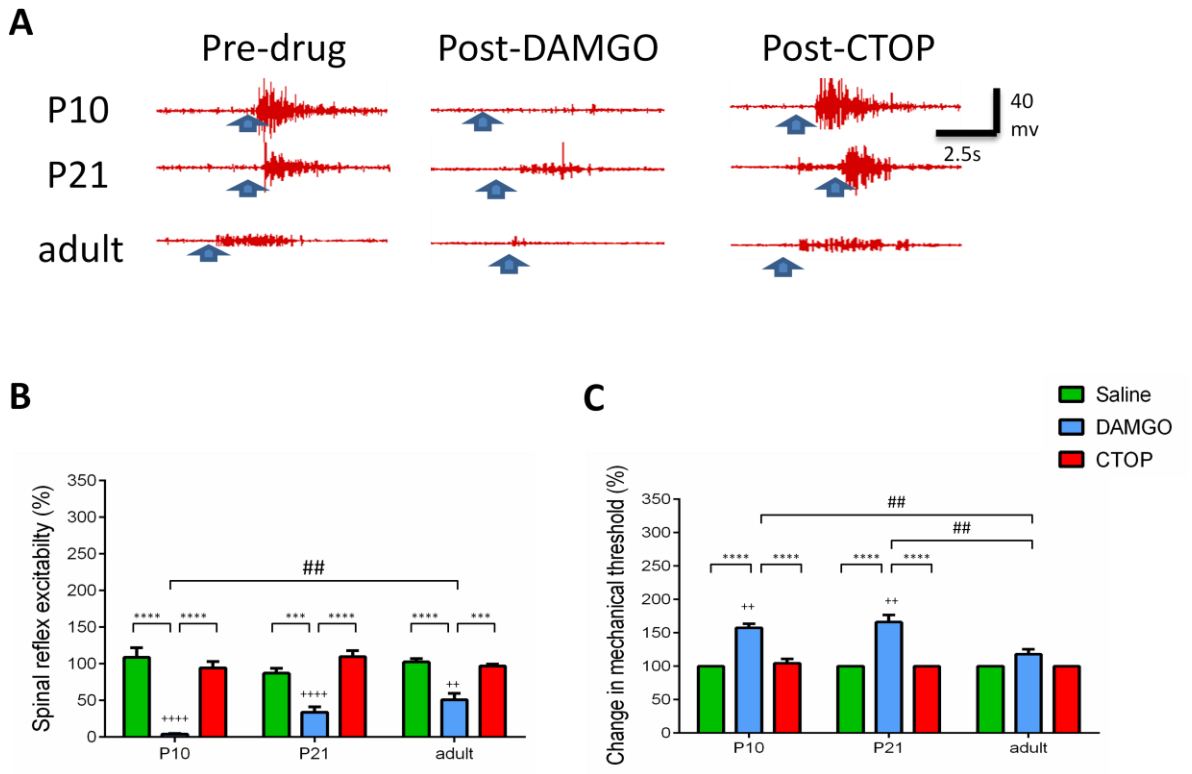


Figure 3.3. The effect of spinally applied opioids on EMG responses to mechanical vFh stimulation. (A) Raw EMG traces, blue arrow indicates the start of stimulus (vFh). Left hand panel shows threshold responses before drug application (P10: 16g; P21 and adults: 100g). Middle and right hand panel shows responses to the same strength of hair in each age group after spinally applied DAMGO (30ng) and CTOP (120ng), respectively. Spinal reflex excitability (B) and change in mechanical threshold (C) in P10, P21 and adult rats after spinally applied saline (n = 4 for P10, 3 for P21 and 6 for adults), DAMGO (n = 4 for P10, 8 for P21 and 5 for adults) *and CTOP* (n = 4 for P10, 7 for P21 and7 for adults). (B) Spinally applied DAMGO decreased spinal reflex excitabilities in all ages tested, but this effect was stronger in younger rats. CTOP did not alter spinal reflex excitabilities in all ages tested. (C) Spinally applied DAMGO significantly increased mechanical thresholds in P10 and P21 rats. CTOP did not alter mechanical thresholds in all ages tested. ++ = $P<0.01$, ++++ = $P<0.0001$, one-way ANOVA, pre-drug vs. post-drug responses; *** = $P<0.001$, **** = $P<0.0001$, two-way ANOVA with Bonferroni post-tests, between drug comparison; ## = $P<0.01$, two-way ANOVA with Bonferroni post-tests, between age comparison.

3.4.4 Intra-PAG MOR activation facilitates nociceptive responses in immature rats but inhibits them in adults

MOR activation in the RVM is pro-nociceptive in P21 rats but analgesic in adults (Hathway et al., 2012). It was suggested that the differential effects of MOR activation in the supraspinal sites were responsible for the hyperexcitability seen in younger animals. The PAG is the major source of afferent input to the RVM; in this experiment the effects of MOR activation in the PAG across the ages were examined.

Figure 3.4A shows the sites of injection of saline, DAMGO (30ng) and CTOP (120ng) in each age group. Figure 4B shows the EMG activity of P10, P21 and adult rats after intra-PAG microinjection of the DAMGO and CTOP. EMG responses to the threshold vFh before drug application in each age group are also included for comparisons.

Administration of DAMGO (n for P10 = 4, P21 = 6, adult = 7) and CTOP (n for P10 = 4, P21 = 7, adult = 5) produced differential responses in the different age groups with respect to reflex excitability (two-way ANOVA, drug x age interaction: $F(4,126)=17.14$, $P<0.0001$; Figure 3.4C) and mechanical thresholds (two-way ANOVA, drug x age interaction: $F(4,126)=84.89$, $P<0.0001$; Figure 3.4D). In P10 rats neither DAMGO nor CTOP had any effect on spinal excitability when compared to saline-treated animals or pre-drug responses (DAMGO: $F(3,9)=3.32$, $P=0.07$; CTOP: $F(3,9)=1.52$, $P=0.28$). DAMGO however significantly facilitated spinal excitability in P21 rats (saline vs. DAMGO: 100.64 ± 1.98% vs. 266.06 ± 40.22%, $P<0.001$; pre-DAMGO vs. post-DAMGO: 100% vs. 266.06 ± 40.22%, $P<0.05$; Figure 3.4C) and this was accompanied by a significant reduction in mechanical thresholds when compared to saline-treated rats (saline vs. DAMGO: 100% vs. 49.26%, $P<0.0001$; Figure 3.4D). Comparisons of the effects of intra-PAG DAMGO between P21 and both P10 and adult rats showed that DAMGO is pro-nociceptive in P21 rats only. Spinal reflex excitability after DAMGO injection into the PAG in P21 was significantly higher when compared to P10 and adult rats (P10: 102.94 ± 11.88%, P21: 266.06 ± 40.22%, adults: 73.37 ± 15.30%; $P<0.001$ vs. P10; $P<0.0001$ vs. adults; Figure 3.4C). Mechanical threshold was also significantly lower in P21 when compared to adults (mean ± SEM; P21 vs. adults: 50.44 ± 2.81% vs. 204.76 ± 11.97%; $P<0.0001$; Figure 3.4D).

CTOP (120ng) significantly increased spinal excitability in adults when compared to saline- and DAMGO- treated rats(post-saline: 96.51 ± 2.49%; post-DAMGO: 72.78 ± 11.42%; post-CTOP: 281.83 ± 52.07%; $P<0.0001$ vs. saline and DAMGO; Figure 3.4C).

In P21 rats reflex excitability was increased compared to pre-drug responses only (pre-CTOP vs. post-CTOP: 100% vs. 113.2 ± 6.85%; $P<0.05$; Figure 3.4C). No effect was observed in P10 when compared to saline-treated age-matched controls or pre-drug responses. The increase in adult spinal excitability was accompanied by a significant reduction in mechanical threshold when compared to saline (post-saline vs. post-CTOP: 100% vs. 64.44 ± 4.75%; $P<0.05$) or changes in threshold in P10 or P21 rats (P10: 100.33 ± 6.93%; P21: 100%; adults: 64.44 ± 4.75%; P10 vs. adults, P21 vs. adults, both $P<0.01$; Figure 3.4D). These data show that MOR mediated signalling in the PAG produces differential responses over postnatal development. At P21 DAMGO in the PAG is pro-nociceptive, whereas it is inhibitory in adult rats. Surprisingly, DAMGO microinjected into the PAG had no effects in P10 rats. Furthermore, antagonising MOR has significant effects in adult rats indicating the presence of a tonic opioidergic tone within the mature PAG.

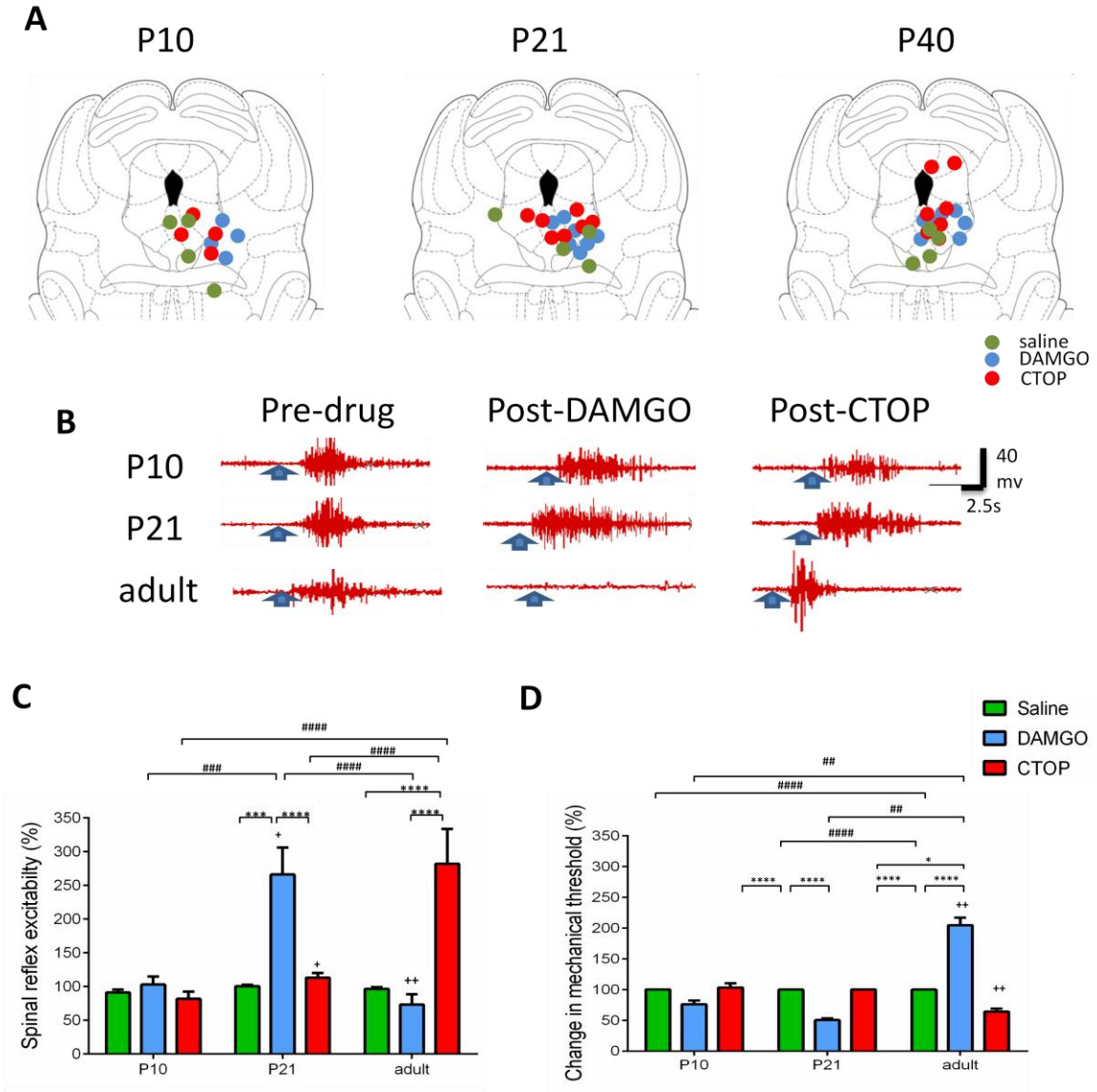


Figure 3.4. The effect of intra-PAG opioids on EMG responses to mechanical vFh stimulation. (A) Injection sites of saline, DAMGO and CTOP in the PAG of P10, P21 and adult rats (B) Raw EMG traces, blue arrow indicates the start of stimulus (vFh). Left hand panel shows threshold responses before drug application (P10: 16g; P21 and adults: 100g). Middle and right hand panel shows responses to the same strength of hair in each age group after intra-PAG microinjection of DAMGO (30ng) and CTOP (120ng), respectively. Spinal reflex excitability (C) and change in mechanical threshold (D) in P10, P21 and adult rats after intra-PAG microinjected saline (n = 4 for P10, P21 and adults), DAMGO (n = 4 for P10, 6 for P21 and 5 for adults) *and CTOP* (n = 4 for P10, 7 for P21 and 5 for adults). (C) Intra-PAG DAMGO was pronociceptive in P21 but antinociceptive in adult rats. CTOP did not alter spinal reflex excitabilities in the younger rats, but facilitated responses in adults. (D) Intra-PAG DAMGO significantly also produced differential responses on mechanical thresholds between the ages tested. CTOP did not alter mechanical thresholds in P10 or P21 rats. In adult rats, intra-PAG CTOP led to a reduction in mechanical threshold. + = P<0.05, ++ = P<0.01, one-way ANOVA, pre-drug vs. post-drug responses; * = P<0.05, *** = P<0.001, **** = P<0.0001, two-way ANOVA with Bonferroni post-tests, between drug comparison; ## = P<0.01, ### = P<0.001, #### = P<0.0001, two-way ANOVA with Bonferroni post-tests, between age comparison.

3.5 Summary

Collectively these data reveal that the endogenous opioid system in the descending pain modulatory pathway undergoes crucial refinements during postnatal development. Efficacy of spinally applied opioids decreases as the animals aged. Supraspinally in the PAG, effect of MOR activation is pro-nociceptive in P21, and there is tonic opioidergic activity in matured animals. This study also showed that the PAG and the RVM (*see Supplementary Figure 3.1, pg.* 79) undergo similar developmental refinements during the postnatal period, and P21 is a critical timepoint for the postnatal maturation of the opioidergic signalling system within the descending pain pathway.

3.6 Discussion

The descending pain modulation pathway, comprising of the PAG, RVM and the DH has important opioidergic components. It was previously observed that focal administration of DAMGO, a mu opioid receptor (MOR) agonist, into the RVM of juvenile (P21) rats is pro-nociceptive. The cellular mechanisms responsible for the DAMGO-mediated facilitation of spinal excitability are currently unknown and are the focus of on-going experimental research. There are several studies which report facilitatory effects of opioid agonists (Devillers et al., 1995, Hathway et al., 2012), with the MOR particularly being shown to be able to signal via Gs rather than the typical Gi protein second messenger systems (Talbot et al., 2005). The underlying mechanisms by which this biased signalling occurs are currently unknown but have significant implications for the study of opioids in neurodevelopment and for the utility of these drugs as analgesic agents. In this study, I illustrated that functional changes also occur within the opioidergic signalling of the PAG and the spinal cord during the different timepoints of postnatal maturation (P10, P21 and adult).

3.5.1 MOR-mediated inhibition in the spinal cord is stronger in younger rats

In this study, application of DAMGO to the spinal cord produced profound analgesia in all ages tested. These anti-nociceptive effects of DAMGO, both in terms of spinal reflex excitability and mechanical threshold were significantly greater in P10 and P21 pups when compared to adults (Figure 3.2). These findings suggest that opioid-mediated signalling in the spinal cord is stronger in the younger rats when compared to adults, which is in agreement with findings from previously published studies (Bouwmeester et al., 2003a, Bouwmeester et al., 2003b, Bouwmeester et al., 2004, Nandi et al., 2004, Nandi and Fitzgerald, 2005).

The enhanced opioid sensitivity in the spinal cord of immature animals could be the result of various anatomical developments during the postnatal period. Opioid receptors and related peptides are present on both primary sensory afferents and intrinsic neurons within the adult DH (Dickenson, 1994). These are often co-localised with calcitonin-gene related peptide (CGRP) (Abbadie et al., 2001) and substance P (Beaudry et al., 2011). In neonatal animals, mu-opioid receptor (MOR) binding sites in the spinal cord are equally concentrated in the superficial and deeper laminae, and as animals age their expression becomes refined to the superficial DH (Alvares and Fitzgerald, 1999). Calcium imaging in cultured dorsal root ganglia has shown that neonatal sensory neurones of all fibre types (C, Aδ and Aβ) are sensitive to morphine whereas only small calibre fibres are sensitive in adult DRG (Nandi et al., 2004b). It is perhaps this functional reorganisation of MORs that leads to the differences in sensitivity and selectivity of opioidergic actions in the spinal cord between neonatal and adult rats.

3.5.2 MOR activation in the PAG is pro-nociceptive in adolescent but not neonatal or adult rats

The PAG and RVM represent the major supraspinal centres controlling spinal excitability (Pomeroy and Behbehani, 1979, Luppi et al., 1995, Vasquez and Vanegas, 2000). It was previously observed DAMGO facilitates nociceptive transmission in the RVM (Hathway et al., 2012), it was also implied that the third postnatal week is the critical period for the maturation of opioidergic signalling within the descending pain modulation pathway (Hathway et al., 2012). However, this study did not demonstrate the effects of intra-RVM DAMGO microinjection in P10 rats. In light of this, a separate experiment was performed in P10 rats to investigate the effects on spinal nociceptive processing when DAMGO was injected into the RVM. It was found that CTOP injected into the RVM of P10 rats had no effect, but DAMGO was anti-nociceptive (please see appendix 1 for detailed analysis).

Here we have demonstrated that the functions of the endogenous opioidergic system within the PAG undergo significant alterations during the postnatal period of the rat. Pharmacological activation of the MOR via DAMGO (30ng/rat) in the PAG is pro-nociceptive in P21 rats, anti-nociceptive in adults and lacks an effect in P10s. More importantly, the facilitatory effects of DAMGO were only observed in P21 but not in P10 rats, indicating that opioidergic transmission is different at around P21 specifically, highlighting this period as the critical timepoint for postnatal refinement of opioidergic signalling. The PAG exerts descending control to the spinal cord dorsal horn indirectly via the RVM (Basbaum et al., 1977, Basbaum and Fields, 1978, Basbaum and Fields, 1979)

and the concurrent reversal of the predicted effects of DAMGO within both the PAG and RVM indicates that these structures undergo comparable postnatal modifications.

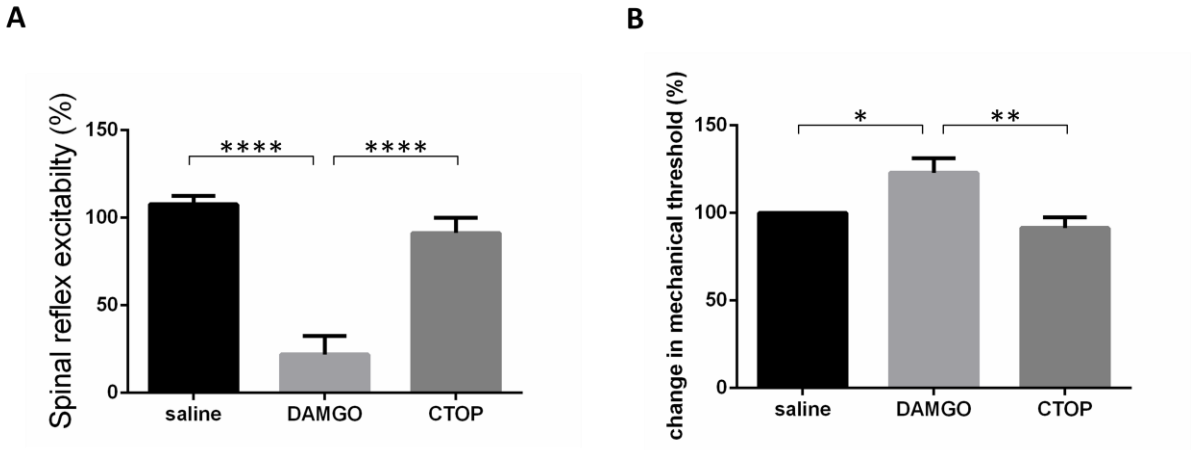
3.5.3 Tonic MOR activity is absent in younger rats

Results obtained by intra-PAG microinjection of CTOP showed that tonic opioidergic control of neurotransmission exists in adult rats. Although a small increase in spinal reflex excitability was seen in P21 rats when CTOP was injected into the PAG, there were no significant differences between post-CTOP and post-saline responses in both reflex excitability and mechanical threshold. On the other hand in adult rats, CTOP injected into the PAG increased spinal nociceptive reflex excitability and decreased mechanical thresholds (P40 on Figure 3.4D). These results suggest that supraspinal MORs are tonically active in the healthy, mature pain modulation circuit.

It is noteworthy that tonic opioidergic tone was found in the RVM of P21 rats (Hathway et al., 2012), whereas my results indicate that tonic opioidergic tone exists at a much later age (greater than P40) in the PAG. Research into the role of tonic opioidergic transmission within the central nervous system is currently sparse, but recently Corder and colleagues showed that tissue injury produced a MOR constitutive activity, and that this activity was crucial for the initiation of analgesic signalling and a compensatory opponent process that generates endogenous opioid dependence (Corder et al., 2013). It is also important to take into consideration that ON cells are the only physiologically defined subpopulation of RVM cells to directly respond to MOR agonists (Heinricher et al., 1992) and are activated just prior to a nocifensive reflex responses (Heinricher and Roychowdhury, 1997). Given that ON cells in the RVM receive efferent input from the PAG (Morgan et al., 2008), perhaps tonic opioidergic transmission from the PAG to the RVM is necessary for the coordination of nociceptive cellular activity within the RVM.

Supplementary Figure 3.1

Effects of opioids injected into P10 RVM on nociceptive processing



*Histograms showing spinal reflex excitability (A) and change in mechanical threshold (B) in P10 rats when DAMGO (30ng) and CTOP (120ng) were injected into the RVM (injection volume = 1µL, n=4 per drug group). Data here are presented as mean ± SEM and were analysed by one-way ANOVA with Bonferroni post-tests. Overall the effects of drugs on spinal reflex excitability and change in mechanical threshold were significant ($F(2, 33)=29.39$, $P<0.0001$; $F(2, 33)=7.523$, $P<0.01$ respectively). A) Post-tests revealed that DAMGO significantly inhibited spinal reflex excitability when compared to saline and CTOP (both ****=$P<0.0001$). B) Post-tests revealed that DAMGO significantly increased mechanical threshold when compared to saline and CTOP (*=$P<0.05$, **=$P<0.01$ respectively).*

Chapter 4 Age-dependent changes in the expression of MOR and related peptides within the descending pathway

4.1 Introduction

4.1.1 Age-related differential pain processing upon MOR receptor activation

In Chapter 3 I have demonstrated that the function of opioid signalling system within the descending pain modulation pathway undergoes significant refinement over the postnatal period. It is known that the rate of maturation within the spinal cord is slightly faster than the brain, by the second postnatal week the spinal components of nociception are comparable to adults: glutamatergic transmission (mEPSP) induced by noxious chemical (menthol and capsaicin) (Baccei et al., 2003) and GABAergic transmission (mIPSP) are present from birth and reach maturity by P14 (Baccei and Fitzgerald, 2004). In addition, PAF termination in the DH exhibits a similar shape and pattern to adults by P14 (Granmo et al., 2008). However, it is also known that the analgesic potency of morphine administered intrathecally decreases as animals age (*Chapter 1, section 1.7.2*). In agreement with this, results illustrated in *Chapter 3* showed that sensitivity to opioids within the spinal nociceptive circuit decreased as the animals aged, and that the MOR agonist DAMGO was most efficacious in P10 rats compared to P21 and adults (C*hapter 3, section 3.4.3*). Similar to the RVM, at around P21 MOR activation in the PAG was pro-nociceptive (*Chapter 3, section 3.4.4*). Interestingly, in P10 rats intra-PAG microinjection of DAMGO had no effect on both the amplitude and threshold of mechanical withdrawal reflex response, whereas intra-RVM microinjection of DAMGO significantly inhibited hind limb reflexes (*Chapter 3, appendix 1*). These results support the view that 1) P21 represents the critical age during the development of supraspinal opioidergic processing, 2) maturation of opioidergic pain modulation is developmentally regulated, and 3) underlying changes within the PAG-RVM-DH circuitry, such as cellular expression and distribution of opioid receptors and related endogenous peptides may contribute to the functional refinement of nociceptive behaviours.

4.1.2 Expression of MOR during postnatal development

There are numerous reports on the distribution and expressional levels of opioid receptors within pain modulation pathways (Basbaum and Fields, 1984, Rahman et al., 1998b, Chen et al., 2008, Little et al., 2012, Martins et al., 2012). Studies using microinjection techniques have shown that the potent anti-nociceptive effects of opioids are mediated by receptor specific action at a number of sites within the brain, including the PAG (MOR), the RVM (MOR and delta/DOR receptors), the substantia nigra (MOR) and within the DH (MOR, DOR and Kappa/KOR receptors) (Yaksh, 1997). On the other hand, there are significantly fewer reports on the expression of opioid receptors during

postnatal development. Studies using radioligand binding indicate that opioid receptors are present from the late embryonic period (Kent et al., 1981), with MOR and KOR existing in the early neonatal periods, and DOR appearing from P7 onwards (Bayon et al., 1979a, Leslie et al., 1982). However, most of these studies examined the localisation of opioid receptors in brain homogenates; therefore it is not possible to precisely distinguish between the regions.

In addition to early expression, opioid receptors are functional from birth and mediate the developmental actions of both exogenous and endogenous opiates (Kornblum et al., 1987). MOR was found to switch from a lighter 50kDa to a heavier 70kDa isoform as the animals mature (Kivell et al., 2004). It is unclear whether there are any differences between the binding properties of the two isoforms, but it was reported that binding affinities of radiolabelled MOR, DOR and KOR agonists in brain homogenates between neonates (P6) and adults (P60) did not change (Kornblum et al., 1987). It has also been found that the number of MOR immunopositive cells in the RVM did not change between the third postnatal week and adult in rats (Hathway et al., 2012).

There are relatively fewer studies investigating the expression of opiate peptides. It is known that endogenous opioid peptides, such as β-endorphin (MOR ligand), enkephalin (DOR ligand) and dynorphin (KOR ligand) are expressed widely in the pain pathways of adult rats, specifically, the hypothalamus, thalamus, midbrain and spinal cord (Yoshikawa et al., 1984). It is also known that β-endorphin immunoreactivity is detectable early in the embryonic period (E13), and reach adult levels of anatomical distribution and staining intensity by P2 (Loughlin et al., 1985). However, enkephalin immunoreactivity cannot be detected in the embryonic brain until E16, and does not reach adult levels of distribution and intensity until P25 onwards (Bayon et al., 1979b). Similarly, dynorphin immunoreactivity is not detectable until P2, it is more sparsely distributed compared to enkephalin and β-endorphin immunoreactivity and staining intensity increased throughout postnatal development (Loughlin et al., 1985). In all these studies, the regions of positive immunoreactivity were not specified, and the ages provided were given as a range. Therefore, the precise timing of expression and distribution of opioid peptides within the PAG, RVM and DH throughout postnatal development is yet to be elucidated.

Currently it is not known whether both spatial and temporal differences in the expression of MOR occur elsewhere within the descending pain modulation circuit. Since maturation of the CNS is activity driven (Ren et al., 2004, Grunau et al., 2006b, Lowery et al.,

2007), expression levels of endogenous ligands, as well as receptor density during the postnatal period may play a crucial role in refinement of receptor function. By using a combination of molecular biology techniques, this study sought to investigate the expression of MOR and related peptides during the postnatal period. A previously published study (Hathway et al., 2012) only used rats as young as P21, therefore P10 is included in this study to provide a more comprehensive overview of the maturation of MORs within the descending pain modulation circuit.

4.2 Aims

The aim of this study is to characterise changes in the opioid signalling system within the descending pain pathway (PAG, RVM and DH) during postnatal development. In *Chapter* 3 I have shown functional changes within the opioid signalling system, in this chapter I will determine whether anatomical changes mirror changes seen in physiological studies. Previous studies found no changes in number of MOR in the RVM (Hathway et al., 2012), however, expression of related peptide levels may undergo significant alterations. Immunohistochemistry and Taqman RT-PCR will be performed to characterise the changes in expression of MOR, pro-opiomelanocortin (POMC, precursor polypeptide of the endogenous MOR agonist β-endorphin) and enkephalin (an endogenous opioid pentapeptide) during postnatal development at P10, P21 and adults.

4.3 Methods

4.3.1 Antibodies

The primary antibodies used were mouse anti-NeuN (Millipore, 1:100), rabbit anti- POMC (Phoenix Pharmaceuticals Inc. USA, 1:100), rabbit anti-MOR (Neuromics, 1:1000 with tyramide signal amplification (TSA) protocol), mouse anti-GAD67 (Millipore, 1:500) and mouse anti-enkephalin (Fitzgerald, USA, 1:100). Sections were incubated with these primary antibodies overnight at room temperature, and separate cohort of rats were used for each antibody. Both POMC and enkephalin immunohistochemistry were performed following the direct staining protocol.

4.3.2 TSA indirect amplification

The detection of MOR immunoreactivity was performed using the TSA indirect amplification protocol. A specific HRP-tyramide conjugate was added to the secondary antibody matrix to enhance the fluorescent signal. For further details, please refer to *General methods section* 2.3.4.

4.3.3 Sequences of primers and probes

Primers and probes for GAPDH (NCBI reference sequence NM_017008.3), POMC (NCBI reference sequence NM_139326.2), enkephalin (ENK, NCBI reference NM_017139.1) and MOR-1A (MOR, NCBI reference sequence NM_001038597.2) were designed on Primer Express 3 (Applied Biosystems). The POMC and GAPDH probes were labelled at the 5'end with 6-carboxyfluorescein (FAM) and at the 3'end with tetramethylrhodamine (TAMRA). The MOR-1A and ENK probes were labelled at the 5'end with FAM and the 3'end with dihydrocyclopyrroloindole tripeptide minor groove binder (MGB). The probes were specifically designed to span across an intron-exon boundary in order to avoid potential amplification of genomic DNA in the analysed samples.

The Taqman RT-PCR primers and probes sequences are summarised in table 1 below.

	Forward primer	Reverse primer	probe
GAPDH	GAA GAT GTC CCT TTG GGT AGG A	TGG ACT GTG GTC TAG AAA GCA TAG A	TGC CCT GCA AGA CCT CAC CCA TTG
POMC	CAC TGA ACA TCT TCG TCC TCA GA	CTG TAG CAG AAT CTC GGC ATC TT	CCT TTC CGC GAC AGA GCC TCA GC
MOR	CTC CAA AGA AAA GGA C	GAG CGT TCG CAT GCT ATC G	GGG TGA TCC TGC GAC GAT
Enkephalin	GCT TTC TCT GCA GCC TGT GTA C	CAG CTG CCC TTC ACA TTC G	TTT GCA GGC ATG CAC A

4.4.4 Statistics

Statistical comparison between the age groups for the expression of various endogenous opioid targets in TaqMan RT-PCR and immunohistochemical experiments were made by one-way ANOVA with Bonferroni post-tests.

4.4 Results

4.4.1 Age-related differences in NeuN immunoreactivity in the PAG, RVM and DH during postnatal development

Apoptosis (programmed cell death, may also be known as synaptic pruning) is a naturally-occurring process during the development of the central nervous system (CNS) and has important implications for formation of functional synapses (Yuan and Yankner, 2000). Pruning in humans begins at around the time of birth and is completed around the time of sexual maturation (Iglesias et al., 2005). In mammals it was estimated that as many as 25% of cells are lost per day due to apoptosis (Bursch et al., 1990). One study using TUNEL assay, a method designed to detect of apoptotic cells, found that the rate of apoptosis in the brainstem of the rat gradually decreased from P1 onwards, and reached a basal plateaux by P90 (White and Barone, 2001). This indicates that the number of cells in the immature central nervous system may be higher when compared to adults, and this in turn may confound the interpretation of anatomical differences that occur during the postnatal period.

To ensure that studies were not cofounded by the changes in the number of cells, the established marker NeuN (Arvidsson et al., 2002, Jeon et al., 2012) was used to qualitatively and quantitatively characterise the age-related changes in the overall neuronal population in the PAG, RVM and DH of rats during postnatal development. Four rats were included in each age group, and two to four slices within the areas of interests were stereologically processed to give an estimated neuronal cell count for each of the regions at each ages. Epifluorescent images of neuronal staining (NeuN, in green) in the PAG, RVM and DH of P10, P21 and adult rats are presented in Figure 4.1 below, and quantified neuronal cell count are shown in Figure 4.2.

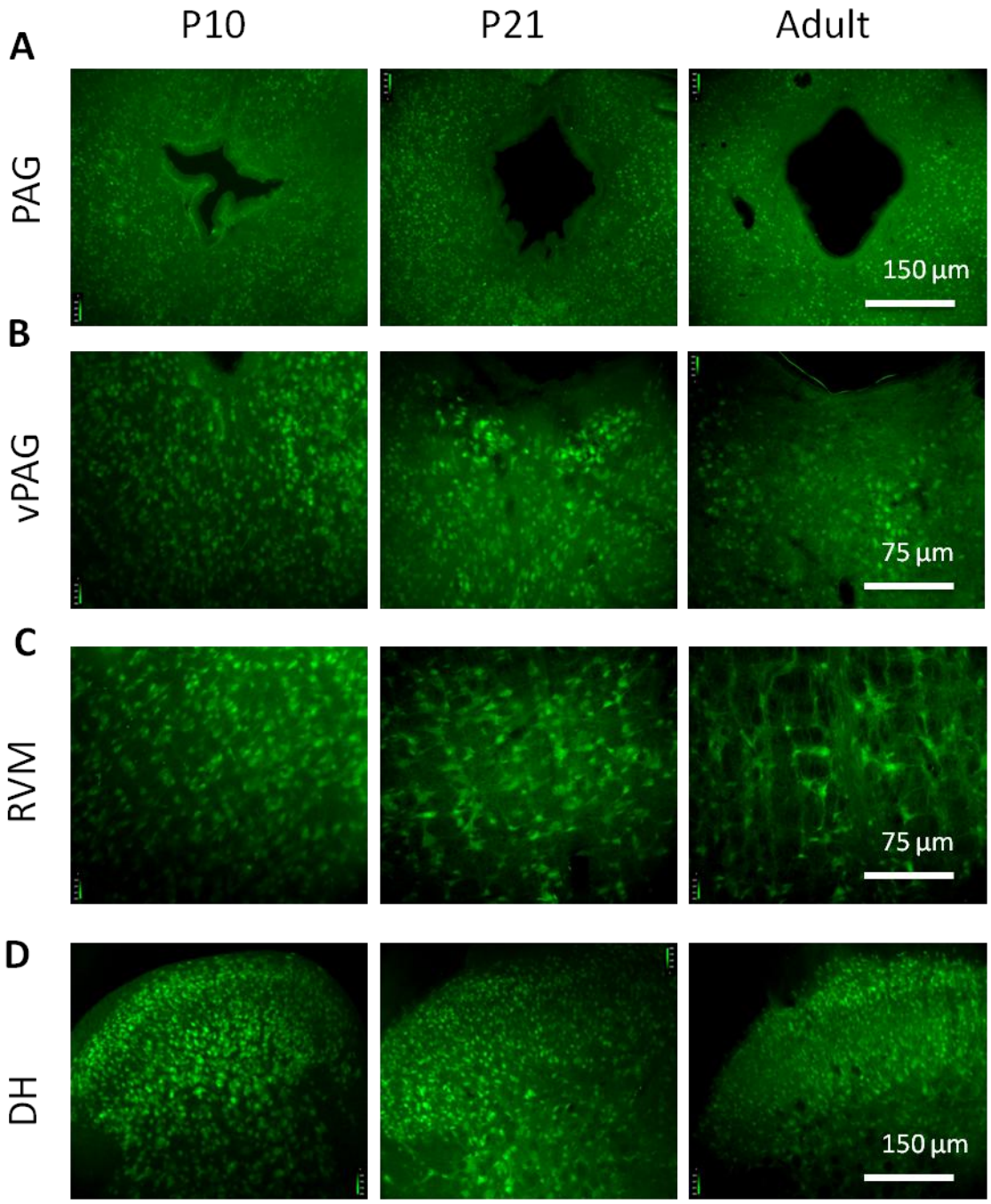


Figure 4.1. Epifluorescent images of NeuN staining in the PAG (A), vPAG (B), RVM (C) and DH (D) in P10, P21 and adult rats. PAG and DH images were taken on a timelapse microscope with a 10X objective, the RVM and vPAG with a 20X objective. Scale bar: 1cm = 75µm or 150µm

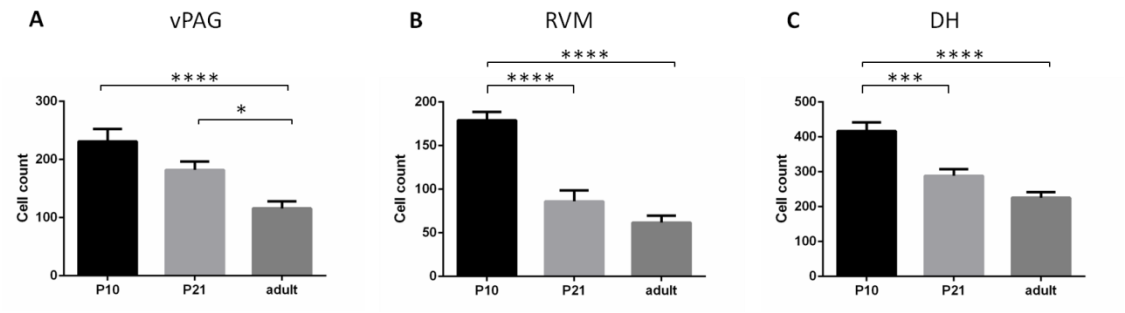


*Figure 4.2. Estimated neuronal cell count in the vPAG (A), RVM (B) and DH (C) in P10, P21 and adult rats. The bars represent the mean ± SEM in each group. Data were analysed with one-way ANOVA with Bonferroni post-tests. In all the regions tested neuronal count was highest at P10, which gradually decreased as the animals aged. *=P<0.05, ***=P<0.001, ****=P<0.0001*

NeuN immunoreactivity was observed ubiquitously throughout the PAG, RVM and DH in all ages (Figure 4.1). Cell counting was performed in the vPAG, RVM and DH. Only cells in the vPAG were counted as this is the region where most opioidergic RVM projecting neurones originate (Osborne et al., 1996). Statistical analysis showed that neuronal cell count decreased in all regions as the animals aged (PAG: $F(2,35)=12$, $P<0.001$; RVM: $F(2,31)=23.89$, $P<0.0001$; DH: $F(2,29)=19.55$, $P<0.0001$; Figure 4.2). Bonferroni post-tests revealed that in the vPAG, neuronal cell count was higher in P10 and gradually decreased to adult levels (mean±SEM; P10: 231.1±21.3; P21: 182.1±14.4; adult: 115.8±12.3; P10 vs. P21, $P<0.0001$; P21 vs. adult, $P<0.05$). In the RVM and DH, neuronal cell count was highest at P10 and rapidly decreased to adult levels by P21 (RVM: P10: 179±9.7; P21: 86.1±12.5; adult: 61.8±7.8; P10 vs. P21 and adult, $P<0.0001$. DH: P10: 416.8±24.7; P21: 288.3±19.46; adult: 225.8±16.1; P10 vs. P21, $P<0.001$; P10 vs. adult, $P<0.0001$).

4.4.2 Age-related differences in the expression of MOR and related peptides in the PAG

In this set of experiments I sought to investigate the expression of MOR and related peptides specifically within the PAG during CNS development. Immunoreactivity for MOR, POMC and enkephalin was found in both the dorsal side (dPAG) and the ventral side of the PAG (vPAG) in all ages tested. There was less staining in the dorsal region, compared to the ventral region, and there were no significant differences in POMC, enkephalin and MOR immunoreactivity in the dPAG between the ages (Figure 4.3).

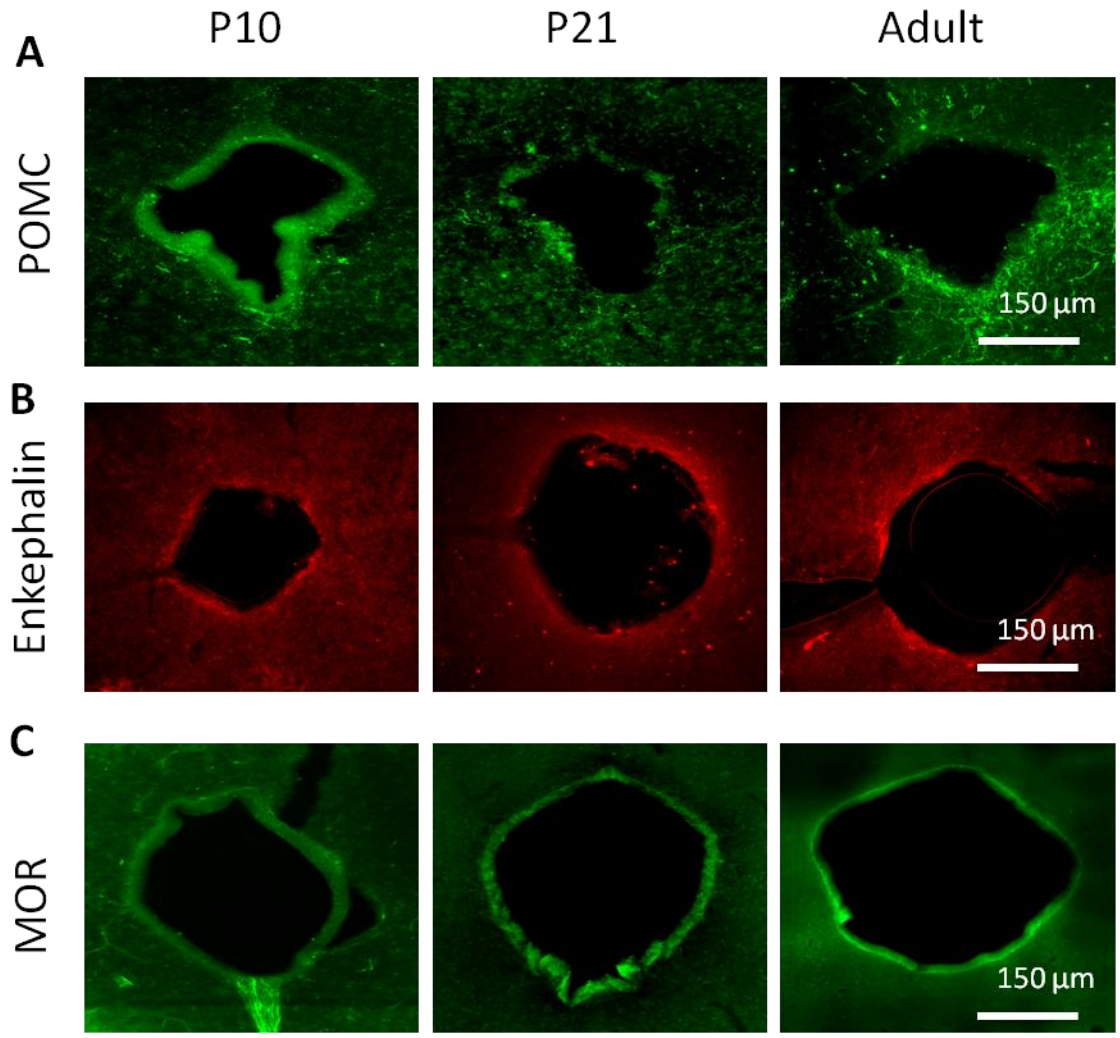


Figure 4.3. Epifluorescent images of POMC (A), enkephalin (B) and MOR (C) in the PAG of P10, P21 and adult rats. There were no specific expressional changes in the dorsal side of the PAG (dPAG), but staining for POMC in particular became more intense in the ventral side of the PAG (vPAG) as the animals matured. Images were taken with a 10x objective with a confocal head. Scale bar: 1cm =150µm

At P10 fibre staining for POMC was present in the vPAG, which intensified and became more restricted to the ventral side of the aqueduct at later ages (Figure 4.3A). In particular, staining of POMC in P21 was diffusely scattered around the medial geniculate body (MG), dorsal (DTg) and ventral tegmental nucleus (VTg), extending sideways into the superior cerebellar peduncle (SCP) within the regions of the PAG. As the animals aged, staining of POMC became restricted to only the DTg and VTg (see Figure 4.4A). Staining intensity analysis showed that POMC immunoreactivity significantly increased as the animals became older (one-way ANOVA, $F(2,9)=23.91$, $P<0.001$; Figure 4.5A). Post-tests revealed that POMC immunoreactivity was the lowest in P10 (P10 vs. adult and P21 both $P<0.001$).

Both enkephalin immunoreactive fibres and cells (indicated by white arrows; Figure 4.4B) were observed, and there were less enkephalin immunoreactivity in the PAG of all ages compared to POMC. Enkephalin immunoreactivity was mostly found in the ventral side of the aqueduct along the VTg. Since only fibre staining could be found in the majority of sections investigated staining intensity was measured, but no statistically significant changes in staining intensity were detected (Figure 4.5B). MOR staining was also observed at all ages, but particularly more so in the ventral parts of the vPAG, spread across both laterally and ventrally from the DTg and VTg (Figure 4.4C). MOR immunoreactivity was found in cells and this was quantified by cell counting, which found no significant differences between the ages (Figure 4.5C).

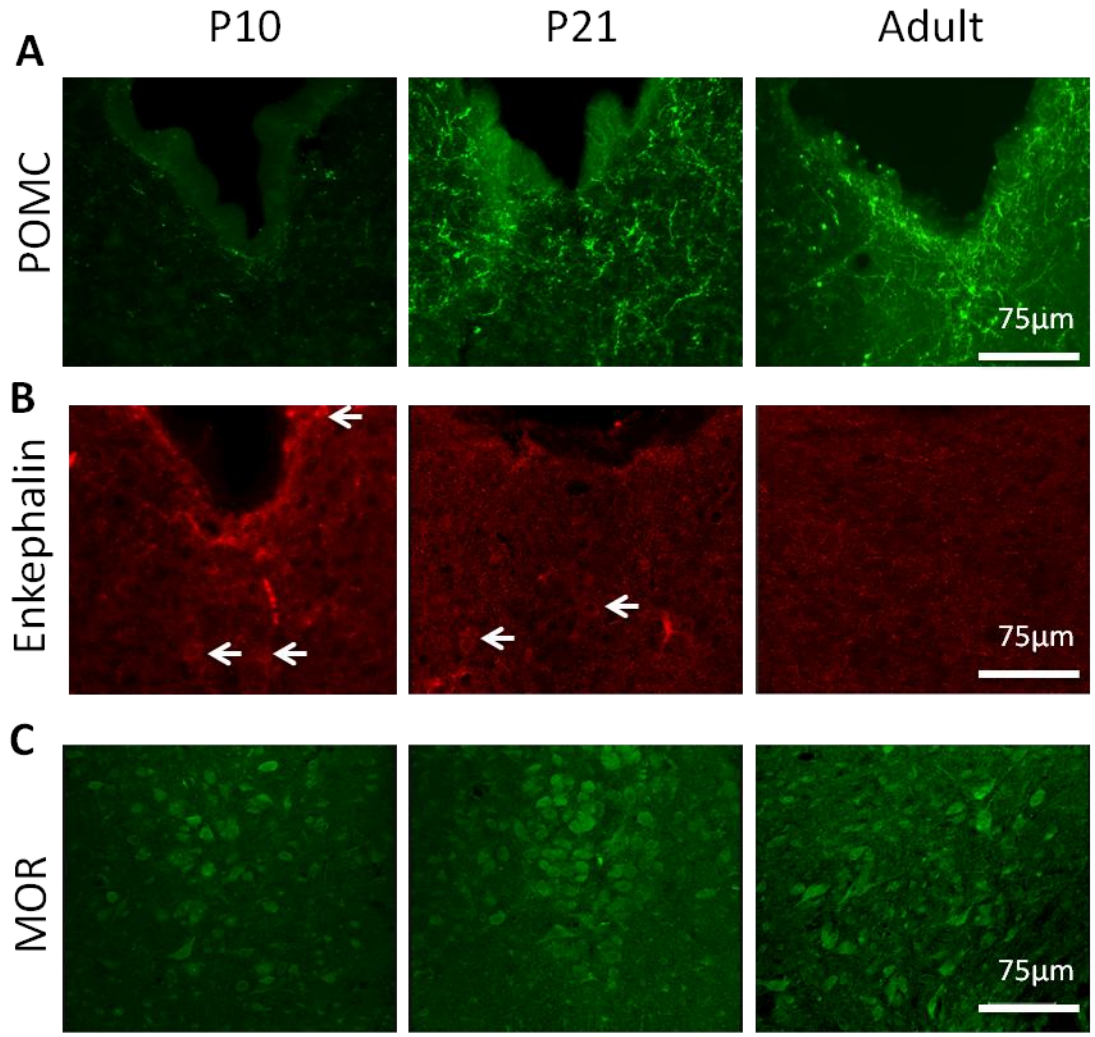


Figure 4.4. A) Epifluorescent images showing POMC immunoreactive fibre staining of the vPAG in P10, P21 and adult animals. B) Enkephalin immunoreactivity in the vPAG of P10, P21 and adult animals. C) MOR staining in the vPAG, the location was more ventral when compared to POMC and enkephalin images because that was where we found most MOR immunoreactive cells. All images shown were captured with a 40x objective with a confocal head. Scale bar: 1cm = 75µm.

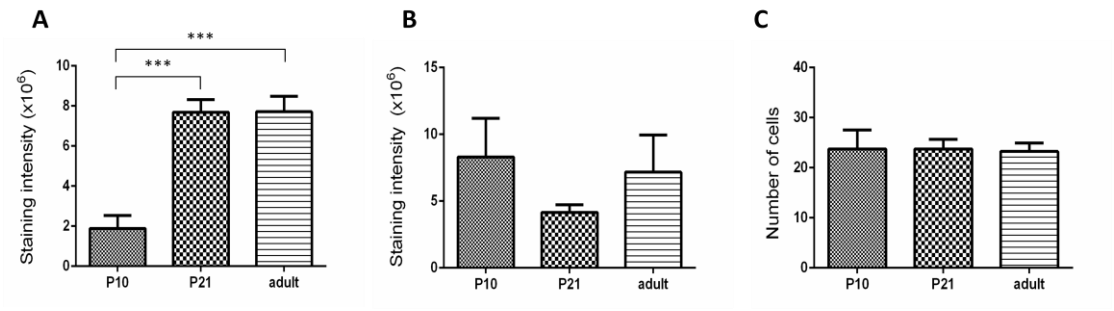


*Figure 4.5. Staining intensity for POMC (A), enkephalin (B) and cell count for MOR (C) immunoreactivity in the vPAG. There were no significant changes detected with either enkephalin or MOR immunoreactivity. POMC staining intensity was the lowest at P10 when compared to both P21 and adults (***=P<0.001).*

Taqman RT-PCR data shared similar findings to immunohistochemistry studies. Copy numbers of mRNA for MOR, POMC or enkephalin within the dPAG were not significantly different between the ages (Figure 4.6). Significant changes in POMC mRNA copies were found in the vPAG between the three ages (one-way ANOVA, F(2,16)=3.65, P =<0.05; Figure 4.7B). In particular, post-tests revealed there were significantly more POMC mRNA copies in P21 vPAG when compared to adult rats (P<0.05). There were no changes in MOR and enkephalin mRNA copy numbers in the vPAG throughout postnatal development (Figure 4.7).

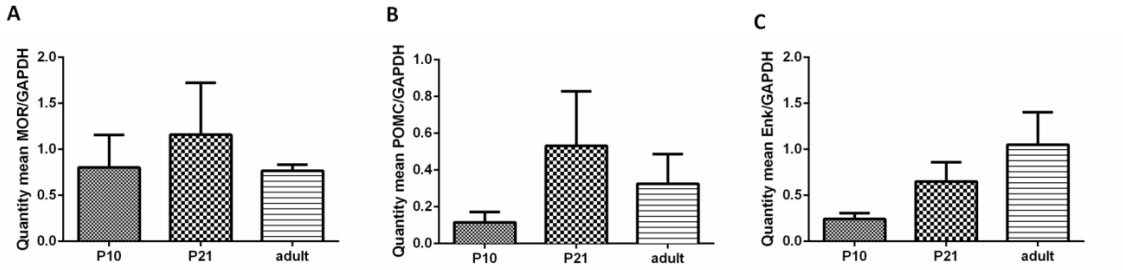


Figure 4.6. mRNA transcript levels of MOR (A), POMC (B) and enkephalin (C) in the dPAG of P10, P21 and adult (P40) rats. All target genes were normalised to GAPDH. There were no significant changes detected.

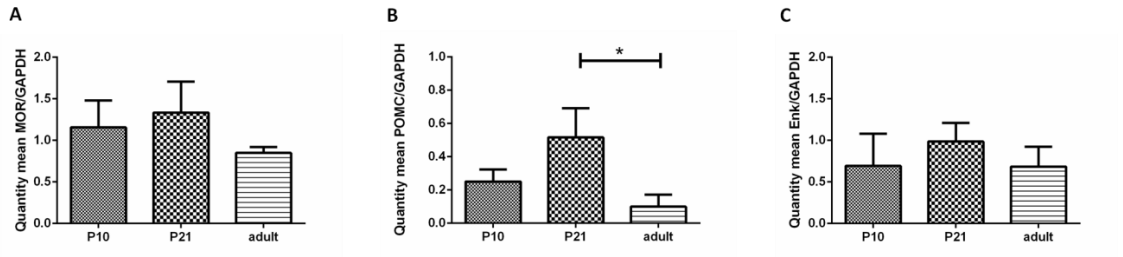


Figure 4.7. mRNA copy numbers of MOR (A), POMC (B) and enkephalin (C) in the vPAG of P10, P21 and adult (P40) rats. Significantly more POMC mRNA copies were found in P21 vPAG when compared to adult rats (=P<0.05). All target genes were normalised to GAPDH. There were no significant changes detected in the expression of enkephalin and MOR.*

4.4.3 Age-related differences in the expression of MOR and related peptides in the RVM

In this set of experiments the expression of MOR and related peptides in the RVM was investigated. Abundant POMC, MOR and to a lesser extent enkephalin immunoreactivity was observed in all ages (Figure 4.8). Most staining was found to be concentrated at the raphe magnus nucleus (RMg), a structure within the RVM where most spinal projection neurones are found (Hjornevik et al., 2008).

Since MOR, POMC and enkephalin staining were found predominantly in cell bodies, quantification of immunoreactivity were carried out by cell count (Figure 4.9). There was significantly less enkephalin immunoreactivity in all age groups when compared to POMC and MOR. The number of POMC immunopositive cells increased as the animals aged (one-way ANOVA, $F(2,9)=6.101$, $P<0.05$; Figure 4.9A), post-hoc Bonferroni analysis revealed that there were significantly more POMC immunoreactivity in P21 when compared to P10 ($P<0.05$). Enkephalin immunoreactivity followed a similar pattern to POMC, where enkephalin immunopositive cells increased as the animals aged (one-way ANOVA, $F(2,9)=6.912$, $P<0.05$; Figure 4.9B) and was most abundant at P21 (P10 vs. P21, $P<0.05$). It was previously reported that there were no alterations in the expression of MOR in the RVM between P21 and adult rats (Hathway et al., 2012), in this study I also found no changes overall in MOR immunoreactivity in the RVM throughout postnatal development (Figure 4.9C).

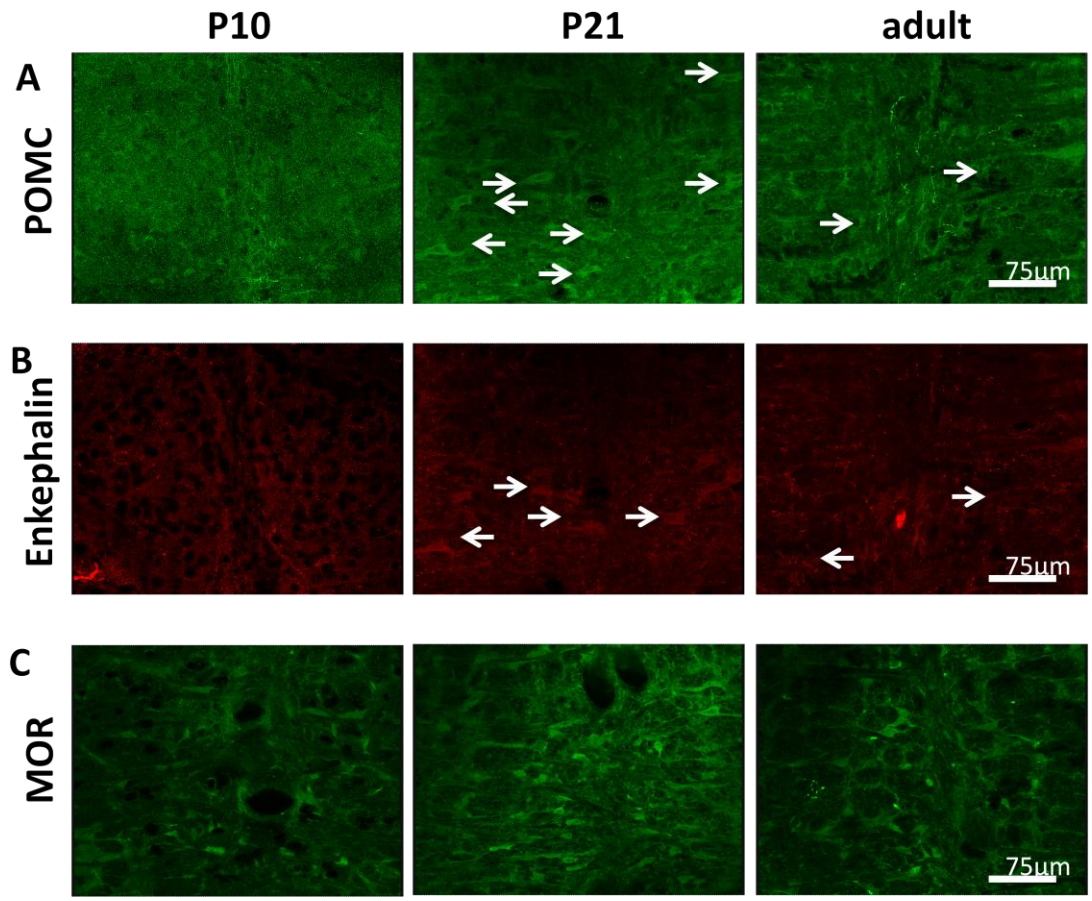


Figure 4.8. Epifluorescent images showing POMC (A), enkephalin (B) and MOR (C) in the RVM of P10, P21 and adult rats. Positive MOR immunoreactivity was found in cells only; both fibre and cell staining were observed for POMC and enkephalin. There were no changes found in MOR staining, but there were significant alterations in POMC and enkephalin immunoreactivity. In particular, most staining was observed at P21. All images shown were captured with a 40x objective with a confocal head. Scale bar: 1cm = 75µm.

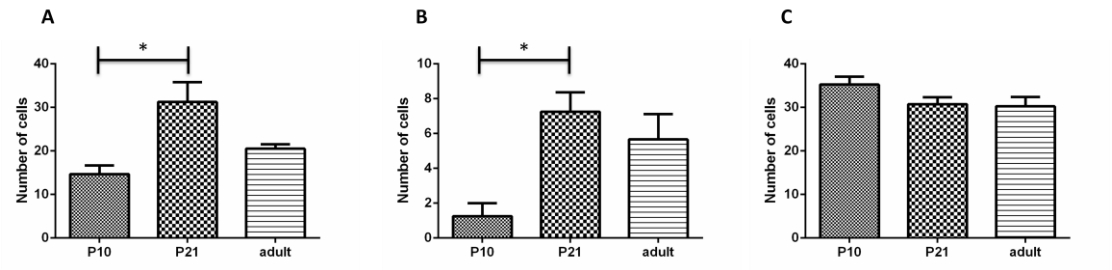


Figure 4.9. POMC(A), enkephalin (B) and MOR (C) immunoreactivity (cell counts) in the RVM. There were no changes in MOR-positive cells during postnatal development. For both POMC and enkephalin, number of immunopositive cells were higher in P21 rats when compared to P10 (=P<0.05).*

Taqman RT-PCR showed some contradictory data about the expression of MOR and related peptides in the RVM (Figure 4.10). Unlike what was seen in immunohistochemical studies, there were no changes detected in the mRNA expression of POMC and enkephalin. Moreover, the mRNA level of MOR were found to be increasing as the animals aged (one-way ANOVA, $F(2,9)=6.269$, $P<0.05$; Figure 4.10A), post-hoc Bonferroni tests revealed that there is significantly more MOR mRNA in adults when compared to P10 rats ($P<0.05$).

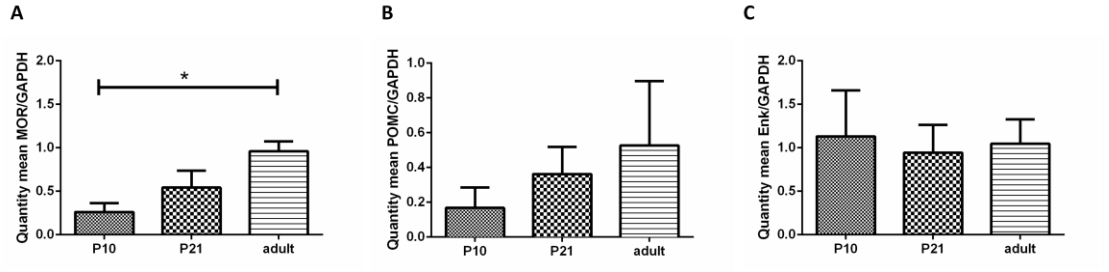


Figure 4.10. mRNA copy numbers of MOR (A), POMC (B) and enkephalin (C) in the RVM of P10, P21 and adult rats. No changes were found in the expression of POMC and enkephalin throughout postnatal development. However, the expression of MOR increased as the animals matured, and post-hoc analysis revealed that there were significantly more MOR mRNA in adults when compared to P10 rats (=P<0.05).*

4.4.4 Age-related differences in the expression of MOR and related peptides in the spinal cord

The expression of MOR and related peptides in the spinal cord was studied. GAD67 (glutamate decarboxylase, 67kDa isoform, responsible for the synthesis of GABA via decarboxylation of glutamate) immunoreactivity was also investigated to quantify the changes in inhibitory component within the spinal circuits during postnatal development. All targets were observed in the DH and exhibited significant postnatal refinement.

POMC-positive cell staining was seen in both superficial and deeper laminae of the DH. As the animals aged, staining became more localised to the superficial lamina, and fibre staining could be seen throughout lamina I of adult rats (indicated by white arrows, Figure 4.11A). Overall, the intensity of staining decreased as the animals aged ($F(2,9)=9.16$, $P<0.01$; Figure 4.12A). Post-tests revealed that POMC immunoreactivity was the highest in P10 (P10 vs. P21, $P<0.05$; P10 vs. adult, $P<0.01$, Figure 4.12B).

ENK immunoreactivity in the DH (shown in Figure 4.11B) significantly increased as the animals aged ($F(2,13)=8.61$, $P<0.01$). Post-tests revealed that ENK immunoreactivity

was the highest in adult (P10 vs. adult and P21 vs. adult, both $P<0.05$, Figure 4.12B). Most ENK fibre staining in the DH was observed in the superficial laminae (lamina I and II) in all of the ages, but immunoreactivity intensified as the rats reached maturity.

MOR immunopositive cells were found throughout the dorsal horn. In the younger animals MOR staining was mostly found in laminae I, II and as the animals aged, staining was also observed in the deeper laminae. Significantly more MOR immunoreactive cells were found in the DH of older rats when compared to P10 animals ($F(2,15)=9.64$, $P<0.01$, Figure 4.12C). Post-tests revealed that there was more MOR immunoreactivity in the adult and P21 when compared to P10 in the DH ($P<0.05$ and $P<0.01$ respectively).

No spatial differences were found since GAD67 immunoreactivity could only be observed as fibre staining in the superficial laminae of the DH in all three ages (Figure 4.11D). However, similar to enkephalin immunoreactivity, GAD67 staining significantly intensified as the animals matured ($F(2,15)=41.8$, $P<0.0001$, one-way ANOVA) and post-tests revealed that GAD67 staining intensity was highest in adults (P10 vs. adults, $P<0.0001$; P21 vs. adults, $P<0.01$; P10 vs. P21, $P<0.001$; Figure 4.12D).

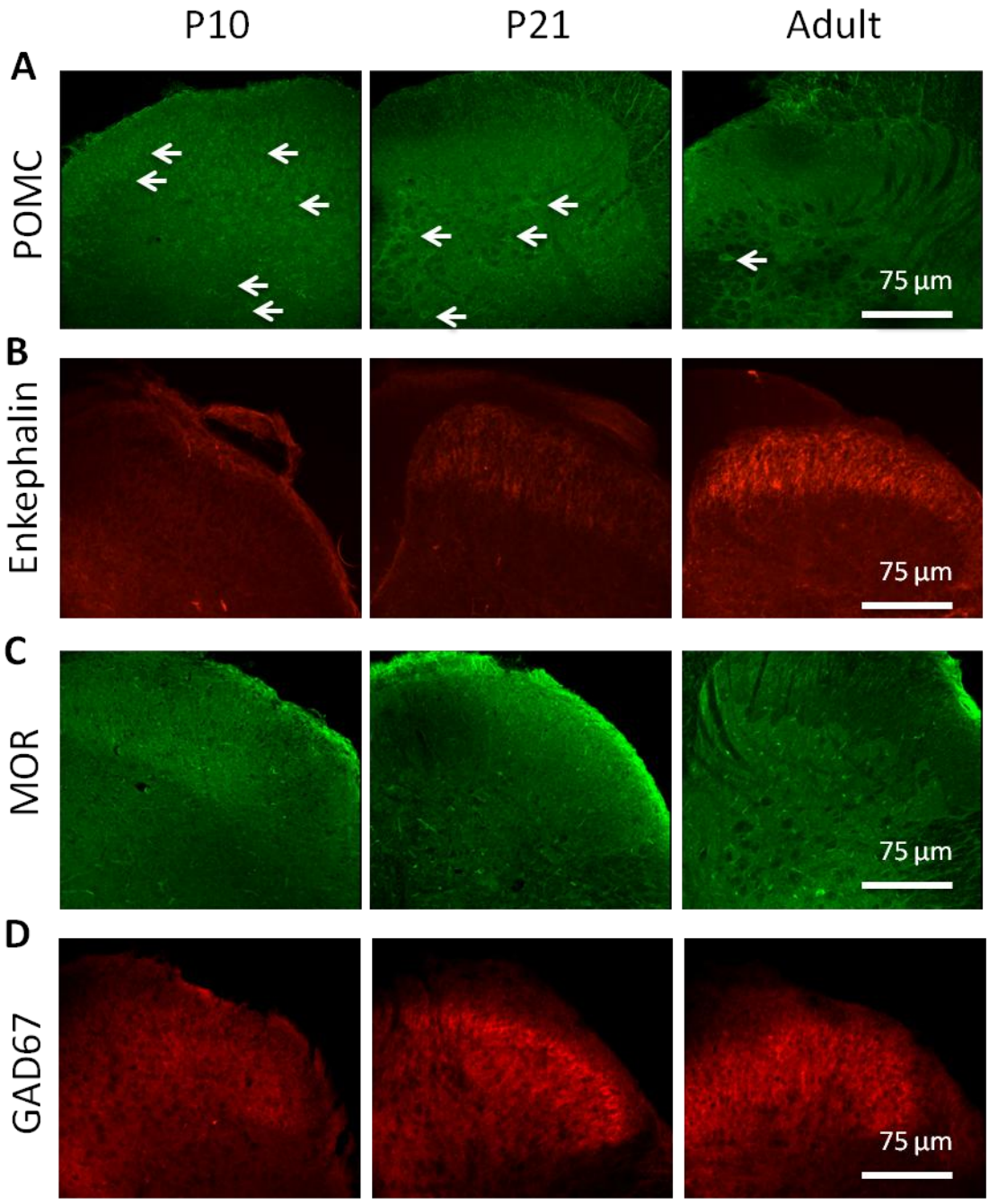


Figure 4.11. Epifluorescent images showing immunoreactivity of POMC (A), enkephalin (B), MOR (C) and GAD67 (D) in the spinal cord DH. Significant postnatal refinement of these targets was observed. Most immunoreactivity was found within the superficial laminae (I and II), although MOR and POMC labelled cells and fibres were also seen in the deeper laminae. All images were captured with a 10x objective on a timelapse microscope. Scale bar 1cm=75µm.

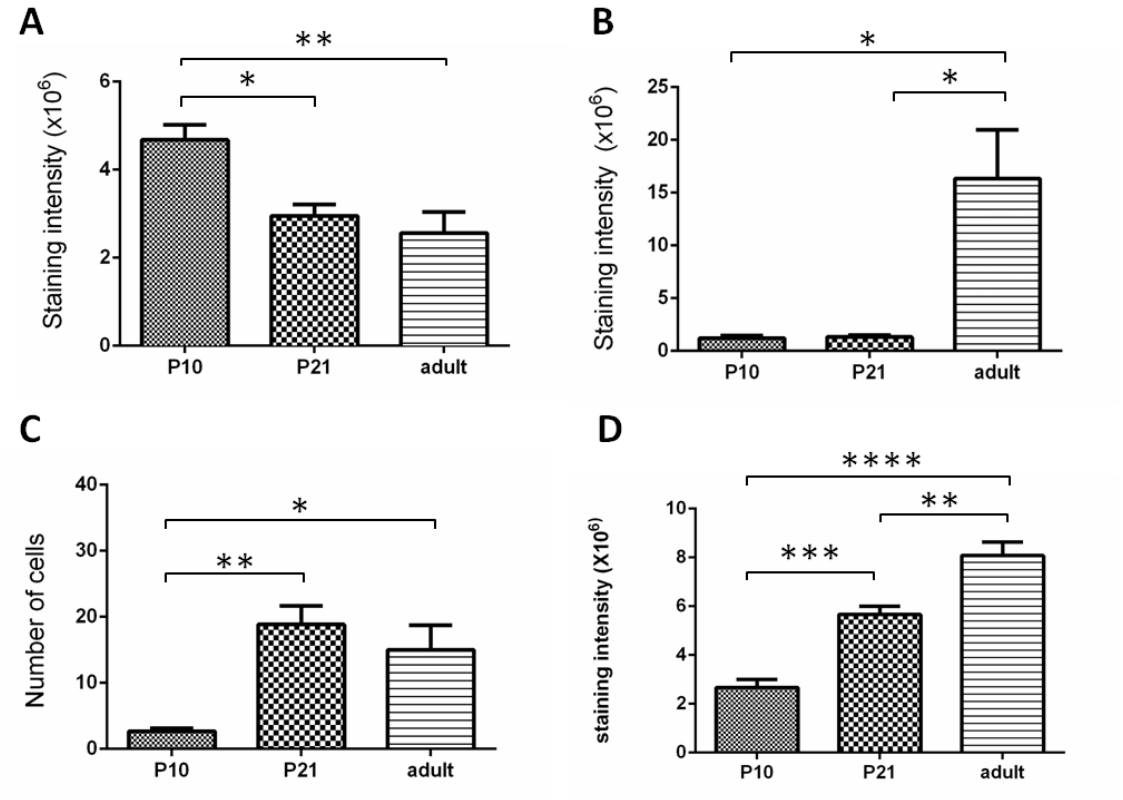


*Figure 4.12. Quantification of immunoreactivity of POMC (A), enkephalin (B), MOR (C) and GAD67 (D) in the spinal cord DH. Data shown here are mean±SEM, and analysed by one-way ANOVA with Bonferroni post-test. Immunoreactivity of POMC, enkephalin and GAD67 were measured by staining intensity and MOR by counting labelled cells. Apart from POMC, the expression of all the targets increased as the animals aged. *=P<0.05, **=P<0.01, ***=P<0.001, ****=P<0.0001.*

Using Taqman RT-PCR techniques, no changes were found in mRNA expression of POMC and MOR in the DH during the postnatal period. However, enkephalin mRNA expression were found to be decreasing as the animals aged (one-way ANOVA, $F(2,9)=5.982$, $P<0.05$; Figure 4.13C).

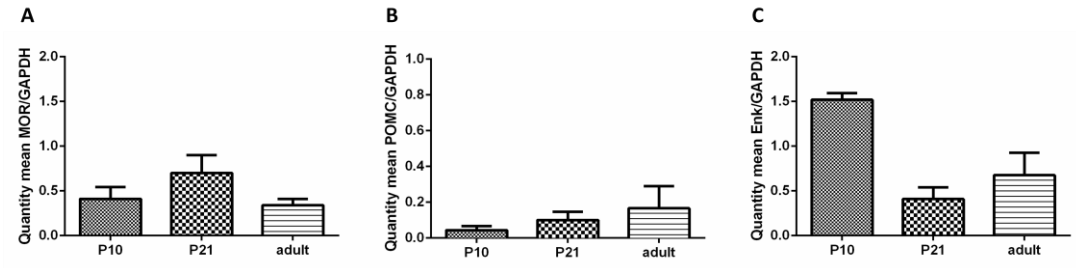


Figure 4.13. mRNA expression of MOR (A), POMC (B) and enkephalin (C) in the spinal cord dorsal horn (DH) of P10, P21 and adult rats. There were no alterations detected with MOR and POMC. The expression of enkephalin decreased as the animals aged.

4.5 Summary

This study examined the expressional profiles of MOR and related peptides at both protein (immunohistochemistry) and mRNA (TaqMan RT-PCR) levels. Contrary to previous findings (Hathway et al., 2012), MOR mRNA transcript levels in the RVM and MOR protein levels increased as animals aged. Moreover, protein levels of endogenous opioidergic peptides (enkephalin and POMC) are upregulated in the later stages (from P21 onwards) of postnatal development.

4.6 Discussion

Maturation requires precise orchestration of cell growth, differentiation, apoptosis and formation of synapses. The endogenous opioid system was previously implicated to play a role in the regulation of cell growth and differentiation in the developing brain (IS Zagon, 2002). It was shown that the targeting of MOR on the plasma membrane of dendrites and spines parallels the peak period of synaptogenesis during the third postnatal week in the rat caudate-putamen (Wang et al., 2003). Moreover, it was recently shown that sufficient levels of endogenous opioids are required for the maturation of pain pathways during a critical period between P21 and P28. When tonic endogenous opioidergic activity was blocked by naloxone hydrochloride (a MOR antagonist that crosses the blood brain barrier) administered via osmotic mini-pumps between P21-P28, descending inhibition failed and this effect last well into adulthood. Naloxone administered either before or after this period had no effect on long term nociceptive processing.

Taking into account the results from Chapter 3, in which opioidergic processing within the descending pain modulation pathway was shown to undergo a significant developmental switch as the animals mature (i.e. from facilitation to inhibition in the PAG and a decrease in opioid sensitivity in the DH), it strongly argues for a role of opioids in mediating the processes that occur during normal maturation of the CNS. It has been postulated that the underlying cellular expression and distribution of opioid receptors within the circuit, and/or epigenetic control of genes that code for downstream signalling proteins, may play in a role in the functional differences seen in opioidergic activity during postnatal development (Pan, 2012). The majority of previous studies investigating the ontogeny of the opioid peptides have relied upon SYBR Green (Hathway et al., 2012) or radioimmuno-assays (Rahman et al., 1998b) but both have lower spatial resolution compared to immunohistochemistry, and lack the sensitivity of TaqMan RT-PCR used in this study (Freeman et al., 1999). One major difference between conventional RT-PCR and TaqMan is the inclusion of a fluorophore-conjugated probe,

which was designed to span the intron-exon boundary of the target gene and thus further increase the accuracy of binding and allow real-time detection.

4.5.1 Neuronal cell count decreased as the animals aged

As mentioned in the results section, apoptosis is a naturally occurring process during CNS development, in which only cells that formed accurate synapses typically survive (Landmesser and Pilar, 1978). In this study I found neuronal cell count decreased with age within the descending pain modulation pathway. NeuN labelled cells were most abundant at P10 in the PAG, RVM and spinal cord DH, and gradually decreased as the animals aged. The decrease in neuronal cell count seemed more rapid within the RVM and the DH when compared to the PAG; significant decreases in neuronal cell count between P10 and P21 were observed but not between P21 and adults. In light of this, perhaps a more effective analytical approach would be the normalisation of immunohistochemical expressions of target proteins to that of NeuN. Interpretation of immunoreactivity of MOR and related peptides must take into consideration that any loss of expression may be a naturally occurring phenomenon during development, rather than an intrinsic functional role. On the other hand, it is true that in this study the overall expression of MOR and related peptides increased as the animals aged. The data will be discussed in details in the sections below.

4.5.2 Expression of MOR during postnatal development

Overall, there were no significant differences in the expression of MOR in the PAG at both mRNA and protein levels. In the RVM, MOR mRNA level increased as the animals aged. This was however, not mirrored by immunohistochemistry, as immunoreactivity of MOR in the RVM remained unchanged between the three different timepoints. Similarly, in the DH, MOR immuno-labelled cells increased as the animals aged, and this trend was not replicated with TaqMan techniques. It is noteworthy that different cohorts of rats were used in these studies and the product measurements of these techniques are different. Expression of mRNA should in theory, precede the translation of proteins, but the relationship between mRNA and protein expression is not linear: expression levels of proteins cannot be deduced simply by measuring the number mRNA transcript within the system of interest (Gygi et al., 1999) which may explain this discrepancy.

Nonetheless, contrary to what was shown previously (Hathway et al., 2012), these data suggest that the expression of MOR, particularly in the RVM and the spinal cord DH increases as animals age. In addition, taken into account that total neuronal count decreased as the animals aged, this indicates a greater density of MOR positive neurones

in both the RVM and DH with increasing age. The differences between findings in this thesis and results from *Hathway et al., 2012* may reflect the effects of differential rearing environments; animals used in this thesis were purchased from *Charles River, UK*, which meant that they were transported to the animal facility in *University of Nottingham*, whereas animals used in the *Hathway et al., 2012* study were bred in-house at *University College London*. Recent studies have shown that environmental manipulations, such as enriched housing regulates the development of sensory cortices (Zheng et al., 2014), another study reported that rats that were exposed to noise from birth had significantly elevated sound frequency discrimination thresholds compared with age-matched naive rats (Zhu et al., 2014). Therefore, during the postnatal maturation period, the development of the CNS is a plastic process that is at least partly influenced by environmental factors.

4.5.3 Increase in POMC expression in adolescent rats

POMC expression increased in both PAG and RVM as the animals aged. In particular, as demonstrated by both immunoflourescence and TaqMan RT-PCR, expression level of POMC in the vPAG was highest at P21. The significant increase in POMC expression around P21 coincides with the critical period of the development of supraspinal pain pathways (Hathway et al., 2012), at which the net result of activating these pathways changes from being primarily facilitatory to increasingly inhibitory.

Since there were no changes in the expression of both enkephalin and MOR in the PAG, it is reasonable to speculate that POMC, or the POMC metabolite β-endorphin is the crucial mediator for the maturation of supraspinal opioid signalling. In opioid withdrawal states it was reported that hyperalgesia might be caused by overstimulation of MOR by opiate abuse (Crain and Shen, 2000). The underlying mechanism was related to a change in secondary messenger coupling, from the normal inhibitory Gi/o to the stimulatory Gs. The surge in POMC expression at P21 could have functional resemblance to what was seen in opioid withdrawal states: overstimulation of MOR in the PAG by endogenous POMC/ β-endorphin could cause a switch in Gi/o to Gs coupling, which then lead to an opioid induced facilitatory effect. In light of this, further immunohistochemical study investigating the expression of β-endorphin in the PAG, RVM and DH throughout postnatal development will be beneficial.

POMC containing neurones in the PAG most likely originate from the hypothalamus (Meister et al., 2006). The hypothalamus is an important neural substrate for a variety of functions (Hsieh et al., 1996), including stress-induced analgesia (SIA). It was

reported that ablation of specific subregions within the hypothalamus disabled SIA (Millan et al., 1980). Coincidentally, in rats SIA begins to form at around P21 (*General introduction, section 1.7.3*) and this increase in POMC around this period may be associated with postnatal development of SIA. It would therefore be interesting for further studies to explore the effects of opioids in the hypothalamus during postnatal development.

The link between the increase in endogenous POMC and opioid-induced facilitation of nociceptive responses was not fully demonstrated here and further studies will be needed. The link could be established by characterising the behavioural effect of either blocking the MOR in the PAG using chronic focal administration of naloxone, or by depleting POMC at around P21.

4.5.4 Increase in enkephalin expression as rats approach adulthood

Immunofluorescence data show that enkephalin and GAD67 significantly increased in the DH during postnatal development. These results suggest that enkephalin and GABA become more available in the spinal cord as animals age, which is logical because 1) enkephalin and GABA are crucial for anti-nociception and 2) as the central nervous system matures, the output of the descending pain pathway becomes increasingly inhibitory. However, the TaqMan RT-PCR data shows that mRNA copy numbers were the highest in P10 DH. It should be remembered that tissue taken for TaqMan will not include the cell bodies of the neurones whose enkephalin positive fibres are prominent in the adult DH. The origin of these fibres is currently unknown yet it is likely that these fibres are primary afferent nerve terminals rather than terminals of descending fibres; a study using the complete Freund's adjuvant (CFA) model showed that localised chronic pain leads to an increase in enkephalin immunoreactivity in the ipsilateral DH, specifically in laminae that receive direct projecting from the inflamed paw (Faccini et al., 1984).

4.5.5 Possible implications of the anatomical differences in the opioid signalling system

This study is the first of its kind aimed at unravelling the underlying differences in the expression of MOR and related peptides that may explain the functional differences. As described in the *Chapter 3*, activation of MOR in the PAG and the RVM at around P21 is pronociceptive, and MOR agonism at the DH becomes increasing less inhibitory as the animals mature. It has been shown in this study that all MOR, POMC and enkephalin undergo significant postnatal refinement in expression, which I believe provides strong

evidence supporting the view that opioidergic activity in the descending pain modulation circuit is crucial for normal maturation. Although not an exhaustive list of opioid receptors and related peptides, MOR was chosen because they are known to be central to the function of the PAG; being abundantly expressed within the region (Osborne et al., 1996, Gutstein et al., 1998) and to play a significant role in pain modulation (Gogas et al., 1991, Bellgowan and Helmstetter, 1998, Wang and Wessendorf, 2002). To further strengthen this study, it is important to elucidate the functional role of POMC and its associated metabolites such as β-endorphin in the PAG at P21. It would also be useful to investigate whether the delta or kappa opioid signalling undergo parallel postnatal refinement.

Chapter 5 The functional role of cannabinoid receptors in the immature descending pain pathway

5.1 Introduction

5.1.1 Pharmacology of cannabinoid receptors

As described previously in the general introduction, CB1 and CB2 receptors are primarily coupled to the inhibitory Gi/o protein which, when activated, cause an inhibition of adenylyl cyclase and protein kinase A activity, thus reducing intracellular calcium concentration and cellular excitability (Pertwee, 2005). Although Gs coupling of CB1 receptors has been observed in both Chinese hamster ovary (CHO) (Calandra et al., 1999) and primary striatal neurone culture lines (Glass and Felder, 1997), actions of CB1 receptors in vivo are predominantly inhibitory. Accumulating evidence suggests that GPR55 receptors are coupled to Gq, and increase cellular activity by enhancing intracellular calcium levels and rapid phosphorylation of related proteins, such as extracellular signal-regulated kinase (ERK) (Lauckner et al., 2008).

Due to the extensive functional profile of cannabinoids, numerous ligands have been discovered and developed as potential therapeutics. An overview of some of these compounds is provided below (Pertwee et al., 2010) (Yao and Mackie, 2009). Numerous cannabinoid compounds have overlapping affinities at both CB1 and CB2 receptors (non-selective CB1/CB2 agonists). The four classes of cannabinoid ligands, based on chemical structures, are classical, non-classical, aminoalkylindoles and eicosanoids. Classical cannabinoids are ABC-tricyclic benzopyrans, and non-classical compounds include CP55940. It is noteworthy that both classical and non-classical compounds are structurally similar to Δ^9-tetrahydrocannabinol (THC). The widely studied WIN55212 is an example of an aminoalkylindole and was originally developed as an anti-inflammatory and analgesic drug. Eicosanoids are derived from the either omega-3 (ω-3) or omega-6 (ω-6) fatty acids, derivatives of eicosanoids form endocannabinoids such as anandamide and 2-arachidonoyl glycerol (2-AG). Selective CB1 agonists include arachidonyl-2'-chloroethylamide (ACEA), which is a synthetic analog of anandamide; whereas AM1241, GW405833 and JWH133 are CB2 selective agonists.

Some cannabinoid antagonists bind to both CB1 and CB2 receptors, but most of them exhibit some selectivity. Compounds with highest affinity for CB1 receptors include AM251, AM281, rimonabant (SR141718A) and CP55940. AM630 and SR144528 are more selective for CB2 receptors.

Since GPR55 receptors have only been discovered and cloned in the last decade, their pharmacology and physiological functions are currently unclear. However, it is known that the bioactive lipid L-α-lysophosphatidylinositol (LPI) and the synthetic O-1602 are potent GPR55 agonists and cannabidiol is an antagonist. Moreover, a recent review suggests that the CB1/CB2 agonist WIN55212 is inactive at GPR55 sites (Ross, 2009).

5.1.2 Role of cannabinoids in descending pain modulation

Cannabinoid receptors, particularly CB1 receptors, are found ubiquitously within the adult mammalian CNS, including in pain modulatory sites such as the superficial DH and the PAG and RVM in the brainstem (Tsou et al., 1998, Hohmann and Herkenham, 1999, Farquhar-Smith et al., 2000). Activation of cannabinoid receptors is antinociceptive, as demonstrated by systemic administration of WIN55212 (CB1/CB2 receptor agonist): it inhibits the noxious pressure evoked firing of WDR neurones (Hohmann et al., 1995), reduces formalin-evoked Fos expression (Tsou et al., 1996) and C-fibre mediated after-discharge of DH neurones (Strangman and Walker, 1999). Supraspinal cannabinoid receptors contribute to the anti-nociceptive effects in models of acute/chronic pain (Sagar et al., 2009). Specifically, micro-injection of WIN55212 and HU210 (CB1/CB2 receptor agonist) into the RVM significantly increased tail-flick reflex latency (Martin et al., 1998, Meng et al., 1998). Microinjection of CP55940 (non-selective cannabinoid agonist) into the dorsal raphe of the ventrolateral PAG also increases tail-flick reflex latencies, although catalepsy and hypothermia are observed post CP55940 administration (Lichtman et al., 1996). It was also reported that administration of HU210 into the dorsal PAG significantly reduces the second phase of formalin-evoked nociceptive behaviour (Finn et al., 2003), which is intriguing because traditionally the dorsal region of the PAG is thought to be more related to fear and anxiety processing rather than nociception (Graeff et al., 1993, Bellgowan and Helmstetter, 1996). Impaired mobilisation and synthesis of endocannabinoids in the RVM was implicated in the genotype-dependent hypersensitivity of Wistar-Kyoto (WKY) rats, in particular, WKY rats display enhanced nociceptive behavioural responses (biting, flinching and licking) to the formalin-injected paw compared to Sprague Dawley rats, and mass spectrometry analysis revealed that WKY has decreased levels of anandamide in the RVM (Rea et al., 2014). These data support a role of the cannabinoid receptor system at the level of the spinal and supraspinal sites in mediating antinociceptive effects in models of pain.

5.1.3 Impact of cannabinoid signalling in the late embryonic/early postnatal period of the rat

It is known that endocannabinoids are present in the rat brain from the foetal period: 2-AG levels remain constant, whereas anandamide levels increase throughout postnatal

development until adulthood (Berrendero et al., 1999). Cannabinoid receptors are transiently expressed in areas where they are not found in adults, including the corpus callosum, anterior commissure, stria terminalis, fornix and white matter areas of the brainstem (Romero et al., 1997), which indicate that the endocannabinoid signalling system is developmentally regulated (Borcel et al., 2004, Fernandez-Ruiz et al., 2004). A trophic role of the endocannabinoids has been described, including migration of neuronal and glial cells, or axonal elongation and synaptogenesis (Fernandez-Ruiz et al., 2004). 2-AG is present in maternal milk, and the demonstration that a single injection of SR141716A (CB1 receptor antagonist) in newborn mice inhibited milk ingestion and subsequent growth in most pups (Fride, 2004), suggests that the endocannabinoid system is crucial to neonatal development and survival.

Although a role in antinociception is widely documented in adult animals, studies investigating the potential analgesic effects of the cannabinoids in the neonatal/early postnatal period are limited. Subcutaneous injection of WIN55212 (3mg/kg) significantly increased response latencies in tail immersion test in both pre-weaned P20 and weaned P25 rats (Borcel et al., 2004). Subcutaneous injection of CP55940 inhibited tail immersion-related nociceptive behaviour in P40 rats, but effects were not dose-dependent (Romero et al., 2002). Experiments using P10-14 spinal cord slices demonstrated cannabinoid mediated attenuation of glutamatergic transmission in the superficial trigeminal caudal nucleus, which led to a significant decrease in cellular excitability (Liang et al., 2004).

Given that the maturation of pain signalling system is developmentally regulated, and that the endocannabinoid system plays a significant role in neurodevelopment, it is surprising that the regulation of nociceptive processing by the endocannabinoid system in the neonatal/early postnatal period has not been widely studied. There is some evidence to suggest that CB1 receptor activation in the immature CNS is antinociceptive, but this has not been tested in a) models other than the tail immersion test and b) ages that are at critical developmental timepoints postnatally. Romero et al., (2002), studied tail-flick responses to hot water immersion at P40, but other studies suggest that by this age rats exhibit structural and functional properties of mature adults (Hathway et al., 2012). Moreover, the effect of focal cannabinoid application into the discrete sites within the descending pain modulation pathway in younger animals has yet to be performed.

5.2 Aims

The aim of the work in this chapter was to determine the function of the endocannabinoid signalling system within the descending pain pathway during postnatal development. The effects of a range of cannabinoid compounds injected into the ventrolateral region of the PAG, the RVM and lumbar spinal cord on spinal reflex excitability, quantified as changes in electromyographic (EMG) responses and mechanical thresholds, in lightly anaesthetised P10, P21 and adult rats were determined.

5.3 Methods

P10, P21 and adult Sprague Dawley rats were used in this study. For detailed information on the husbandry of rats, please refer to the *General Methods* section 2.1.1. Craniotomy, laminectomy and EMG recordings were performed as described in the *General methods* section 2.1.6, 2.1.7 and 2.2.

5.3.1 Drugs

WIN55212 (CB1/CB2 agonist, 4µg, Tocris), HU210 (CB1/CB2 agonist, 4µg, Tocris), AM251 (CB1 antagonist, GPR55 agonist, 2.77µg, Tocris) and LPI (GPR55 agonist, 12µg, Sigma-Aldrich) were administered at doses determined from previously published study (Finn et al., 2003). 60% DMSO (vehicle for WIN55212, HU210, AM251) and saline (vehicle for LPI) was administered in separate sets of animals as vehicle control, and it was confirmed that vehicles had no effect when injected into the PAG, RVM and spinal cord. Only one dose and one drug was administered per animal, and the injection volume was 1µL. The changes in spinal reflex excitability (as indicated by EMG responses) and mechanical withdrawal threshold were recorded for 2 hour post drug administration. Injection sites in the PAG and RVM were examined as previously described in Chapter 3, and are shown in part A of the following figures.

5.3.2 Statistics

Data presented in this study are represented by mean±SEM. For detailed description of calculation for spinal reflex excitability and change in mechanical threshold, please refer to the Chapter 2 section 2.2.2. One-way ANOVA with Bonferroni post-test was used to compare pre-drug to post-drug responses. Comparisons between age and drug groups were made using two-way ANOVA with Bonferroni post-tests.

5.4 Results

5.4.1 Activation of CB1 and CB2 receptors in the vlPAG is antinociceptive in both adult and immature rats

As a preliminary experiment, WIN55212 (4μg) was injected into the vPAG of P21 and adult rats (n=7 for P21; 4 for adults; Figure 5.1A). This dose of WIN55212 significantly inhibited spinal reflex excitability in P21 rats (pre-drug = 100%, post-drug = 49±5%; one-way ANOVA; $F(3,20)=6.26$; $P<0.01$; Figure 5.1B). Although WIN55212 reduced spinal reflex excitability in adult rats (post-drug = 57±6%; Figure 5.1B), statistical significance was not reached. Two-way ANOVA revealed that there were no significant differences in the effects of WIN55212 in P21 and adult rats (drug x age interaction: $F(1,212)=0.01$; $P=0.91$; Figure 5.1C). However, post hoc analysis revealed that in both P21 and adult rats, the effects of WIN55212 on spinal reflex excitability were significantly different from vehicle (n=4 for P21, 3 for adult; post-vehicle vs. post-WIN55212; P21: 95±3% vs. 49±5%; adult: 102±2% vs. 57±6%; two-way ANOVA Bonferroni post-test, both $P<0.0001$).

The reduction in spinal reflex excitability suggests that intra-PAG WIN55212 is antinociceptive in P21 and adult rats. To confirm this view, changes in mechanical threshold were also assessed. Two-way ANOVA revealed that intra-PAG WIN55212 significantly increased mechanical threshold in both P21 and adult animals, compared to post-vehicle responses. Bonferroni post-tests showed that in P21 rats, intra-PAG vehicle had no effect on mechanical threshold but WIN55212 significantly increased mechanical threshold (vehicle: 100%, WIN55212: 134±13%; $P<0.001$). Similar effects were observed in adults (vehicle: 100%, WIN55212: 193±41%; $P<0.0001$). The magnitude of increase in mechanical threshold after intra-PAG microinjection of WIN55212 was higher in adult rats, when compared to P21 rats (P21 vs. adults; 134±13% vs. 193±41%; drug x age interaction: $F(1,212)=21.57$; $P<0.0001$; Figure 5.1D).

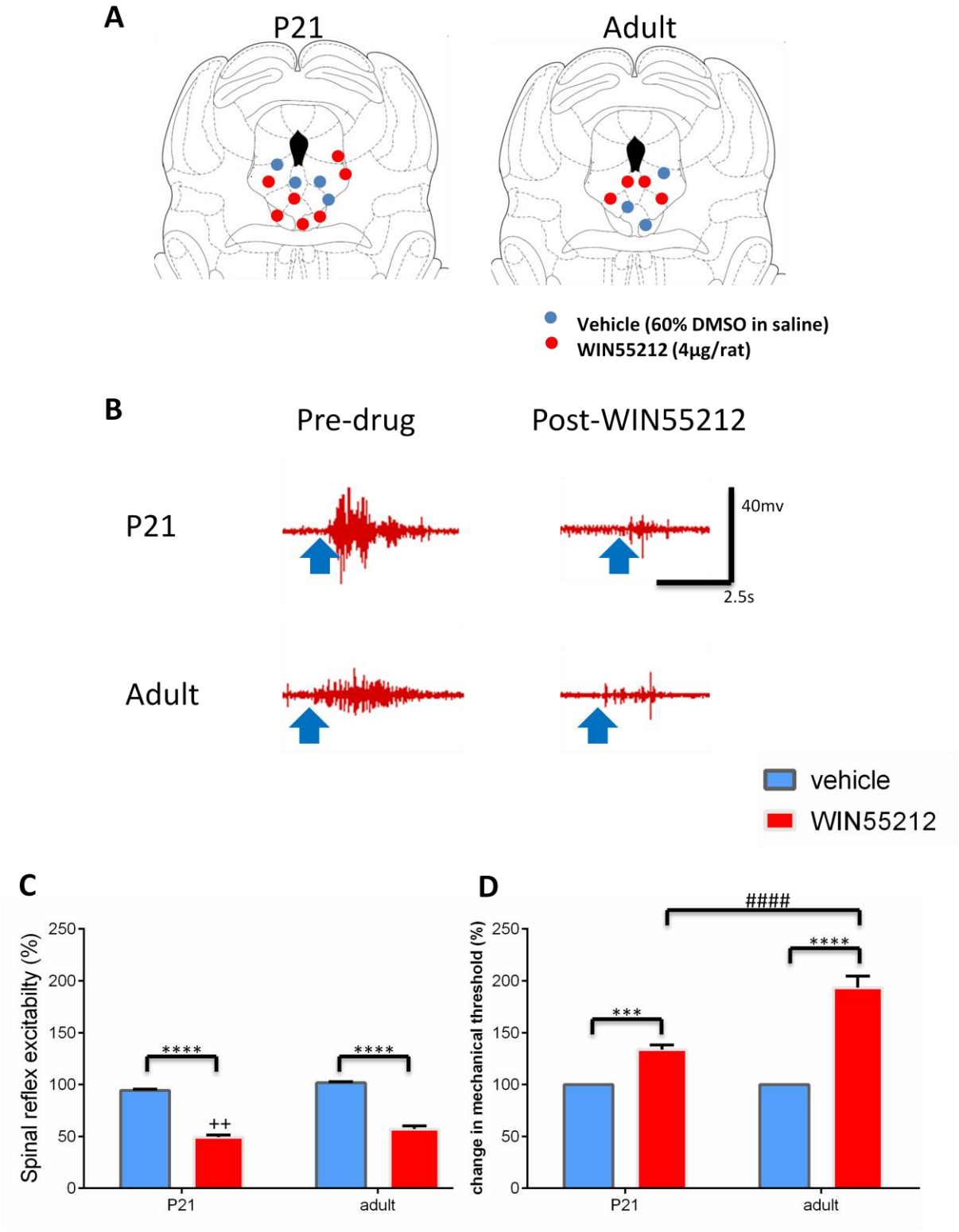


Figure 5.1. Effect of intra-PAG microinjection of WIN55212 on EMG responses in P21 and adult rats. (A) Injection sites for vehicle and the CB1/CB2 receptor agonist WIN55212 in P21 and adult PAG. (B) Raw EMG traces, blue arrow indicates the start of stimulus (von Frey hair). Left hand panel shows threshold responses before drug application (P21: 60g; adult: 100g). Right hand panel show responses to the same strength of hair in each age group after intra-PAG WIN55212 microinjection. Spinal reflex excitability (C) and change in mechanical threshold (D) in P21 and adults rats after intra-PAG microinjection of vehicle (n= 4 for P21 and 3 for adult) or WIN55212 (n= 7 for P21 and 4 for adult) per

*animal. (C) In both P21 and adults spinal reflex excitability was decreased post-WIN55212 injection. (D) In both P21 and adults mechanical threshold increased post-WIN55212 injection, and the increment was greater in adults compared to P21. ++ = P<0.01, one-way ANOVA, pre-drug vs. post-drug responses; ***, **** = P<0.001 and P<0.0001 respectively, two-way ANOVA Bonferroni post-test, between drug comparison; #### = P<0.0001, two-way ANOVA Bonferroni post-test, between age comparison.*

These data suggest that CB1/CB2 receptor activation is antinociceptive in both P21 and adult rats. To explore this further, the effects of another CB1/2 receptor agonist (HU210, 4μg) and a CB1 antagonist (AM251, 2.77μg) injected into the PAG of P10, P21 and adults was studied (Figure 5.2A, representative EMG responses before and after drug administration in each age groups; Figure 5.2B). Administration of HU210 (n = 3 for P10, 4 for P21 and adults; Figure 5.2A) and AM251 (n = 4 for P10, P21 and adults; Figure 5.2A) produced differential effects in the different age groups with respect to reflex excitability (two-way ANOVA, drug x age interaction: $F(4,399)=13.94$, $P<0.0001$; Figure 5.2C) and change in mechanical threshold (drug x age interaction: $F(4,399)=11.73$, $P<0.0001$; Figure 5.2D).

Similar to WIN55212, HU210 inhibited spinal reflex excitability in all ages tested (Figure 5.2B). Post-HU210 spinal reflex excitabilities were 42±8%, 36±7%, 30±6% for P10, P21 and adults respectively, which were significantly reduced when compared to pre-drug (pre-drug = 100%; one-way ANOVA; P10: $F(12,39)=4.72$, $P<0.001$; P21: $F(12,39)=6.34$, $P<0.0001$; adult: $F(12,39)=5.95$, $P<0.0001$; Figure 5.2C). Post-HU210 responses were also smaller when compared to post-vehicle and post-AM251 responses in all three ages, as revealed by two-way ANOVA Bonferroni post-tests (all $P<0.0001$).

The change in spinal reflex excitability was reflected in changes in mechanical threshold; post-HU210 mechanical thresholds were 119±21%, 160±4% and 155±19% for P10, P21 and adults respectively, which were higher when compared to pre-drug, but this effect was only statistically significant in P21 rats (pre-drug = 100%; one-way ANOVA; P21: $F(12,39)=5.55$, $P<0.0001$; Figure 5.2D). In addition, two-way ANOVA Bonferroni post-tests revealed that post-HU210 mechanical threshold was significantly higher when compared to post-vehicle responses in P21 rats ($P<0.0001$), and in adults post-HU210 mechanical threshold was significantly higher when compared to both post-vehicle and post-AM251 values (both $P<0.0001$).

On the other hand, AM251 did not have an effect in adults (post-AM251 spinal reflex excitability = 101±9%; Figure 5.2C), but significantly inhibited spinal reflex excitability in P10 and P21 rats. Responses were significantly reduced post-AM251 in P10; (pre-drug = 100%, post-AM251 value = 66±3%; one-way ANOVA, $F(12,39)=5.57$, $P<0.0001$), and although responses were reduced post-AM251 in P21 rats (71±8.%), this effect was not statistically significant. Two-way ANOVA Bonferroni post-tests revealed that post-AM251 responses were significantly different to post-vehicle responses in both P10 and P21 rats (both $P<0.0001$). Interestingly, adult post-AM251 responses were also significantly different to P10 and P21 post-AM251 responses (both $P<0.0001$), in which spinal reflex excitability following intra-PAG AM251 microinjection in adults were higher compared to both P10 and P21.

Similarly, mechanical threshold was increased post-AM251 when compared to post-vehicle values in the PAG of P21 rats (post-vehicle = 117±17%, post-AM251 = 138±15%; two-way ANOVA Bonferroni post-tests; $P<0.01$; Figure 5.2D). In addition, post-AM251 mechanical threshold in adults was significantly lower when compared to P10 and P21 post-AM251 values (adult post-AM251 mechanical threshold = 99±1%, P21 = 138±15%, P10 = 109±7%; both $P<0.0001$).

These data confirmed an antinociceptive role of CB1/CB2 receptor activation throughout postnatal development. However, it was unexpected that blockade of the CB1 receptor in the PAG in P10 and P21 also produced antinociceptive effects. Since AM251 is also a GPR55 receptor agonist, these data may indicate a role of GPR55 at this level in neonatal and juvenile analgesia. Hence, an analogue of the endogenous GPR55 receptor agonist LPI (12µg) was injected into the PAG of P10, P21 and adult rats to unravel the potential role of this receptor in nociception.

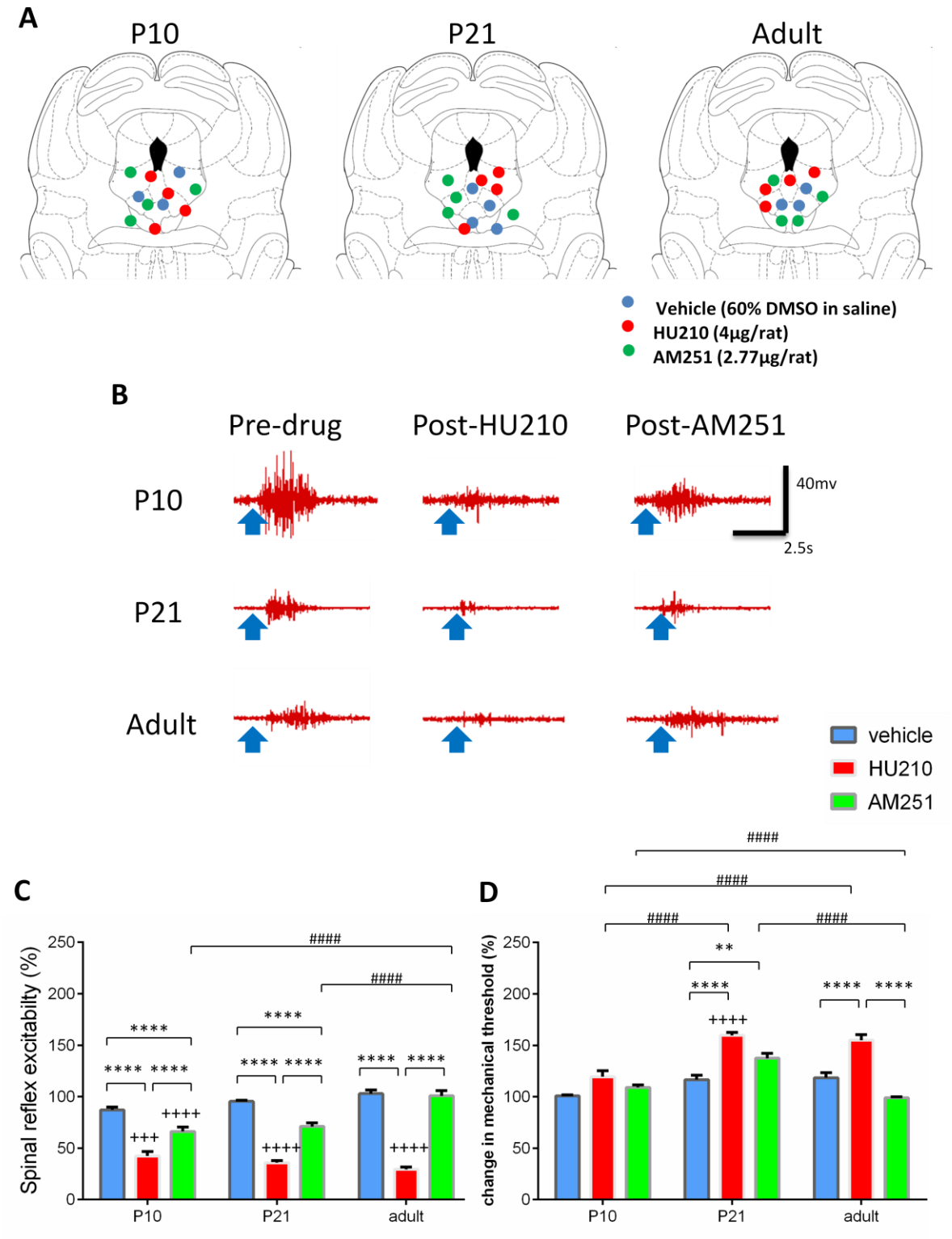


Figure 5.2. Effects of intra-PAG microinjection of HU210 and AM251 on EMG responses in P10, P21 and adult rats. (A) Injection sites for vehicle, CB1/CB2 receptor agonist HU210 and CB1 receptor antagonist/GPR55 receptor agonist in P10, P21 and adult PAG. (B) Raw EMG traces, blue arrow indicates the start of stimulus (von Frey hair). Left hand panel shows threshold responses before drug application (P10: 15g; P21: 60g; adult: 100g). Middle and right hand panel show responses to the same strength of hair in each age group after intra-PAG HU210 and AM251 microinjection respectively. Spinal reflex excitability (C) and change in mechanical threshold (D) in P10, P21 and adult rats after

*intra-PAG microinjection of vehicle (n = 3 for P10, 4 for P21 and 3 for adult), HU210 (n = 4 for P10, P21 and adult) and AM251 (n = 4 for P10, P21 and adult). (C) HU210 significantly reduced spinal reflex excitability in all ages; post-AM251 reduction was only seen in P10 and P21 rats. (D) HU210 increased mechanical threshold in both P21 and adults, AM251 also increased mechanical threshold in P21 rats. +++, ++++ = P<0.001 and P<0.0001 respectively, one-way ANOVA, pre-drug vs. post-drug responses; **, **** = P<0.01 and P<0.0001 respectively, two-way ANOVA with Bonferroni post-test, between drug comparison; #### = P<0.0001, two-way ANOVA with Bonferroni post-test, between age comparison.*

5.4.2 Activation of GPR55 receptors in the PAG is antinociceptive in immature rats only

In this set of experiments LPI (12µg) or the vehicle (saline) was microinjected into the PAG of P10, P21 and adult rats. The injection sites and the representative EMG responses before and after drug administration in each age are shown in Figure 5.3A and 5.3B, respectively. LPI (n = 4 for P10, 7 for P21 and 6 for adults) produced differential effects in the different age groups with respect to reflex excitability (two-way ANOVA, drug x age interaction: $F(2,330)=40.62$, $P<0.0001$; Figure 5.3C) and change in mechanical threshold (drug x age interaction: $F(2,330)=14.37$, $P<0.0001$; Figure 5.3D).

As seen with AM251, intra-PAG microinjection of LPI significantly inhibited spinal reflex excitability in P10 and P21 rats, compared to pre-drug (pre-drug = 100%, P10 post-LPI = 55±6%, P21 = 58±5%; one-way ANOVA, P10: $F(12,39)=16.19$, $P<0.01$; P21: $F(12,78)=2.83$, $P<0.01$; Figure 5.3C) and compared to post-saline values (P10 post-saline = 110±2%, P21 post-saline = 88±6%; two-way ANOVA Bonferroni post-tests, both $P<0.0001$). Moreover, adult post-LPI spinal reflex excitability was higher when compared to P10 and P21 post-LPI responses (adult post-LPI = 102±3%; both $P<0.0001$).

Post-LPI mechanical thresholds were also higher in P21, compared to adult rats. In P21, post-LPI mechanical threshold was significantly higher when compared to post-saline (post-saline = 117±13%, post-LPI = 166±20%; two-way ANOVA Bonferroni post-test, $P<0.0001$; Figure 5.3D). P21 post-LPI mechanical threshold were also significantly different to P10 post-LPI and adult post-LPI values (P10 post-LPI = 108±5%, adult post-LPI = 100%; both $P<0.0001$). Thus, intra-PAG injection of LPI had no effect on nociception in adult rats, but significantly inhibited nociceptive responses in P10 and P21 rats. Together the findings suggest that cannabinoidergic actions in the PAG, particularly GPR55 receptor mediated actions are developmentally regulated.

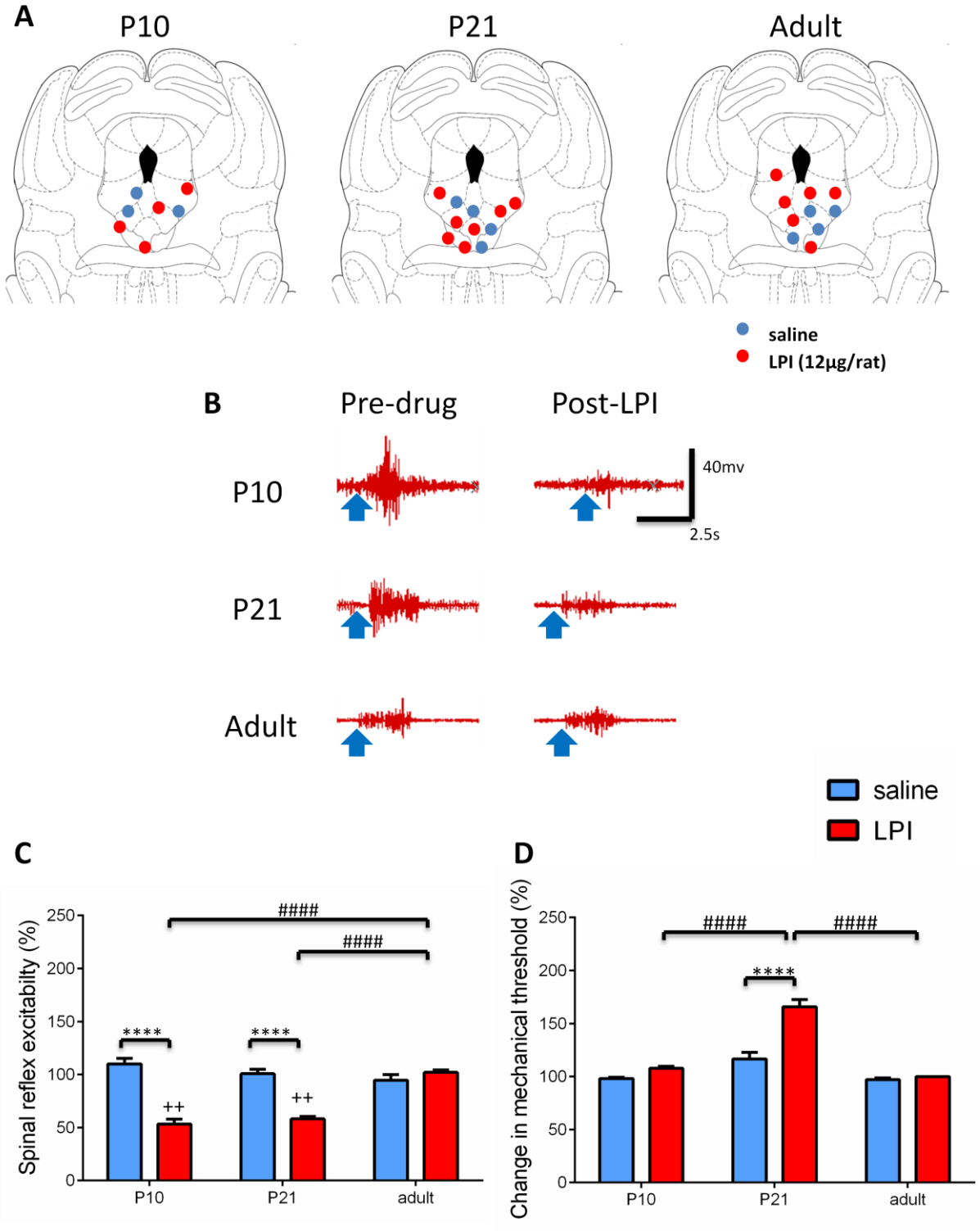


Figure 5.3. Effects of intra-PAG LPI microinjection on EMG responses in P10, P21 and adult rats. (A) Injection sites for vehicle and the GPR55 receptor agonist LPI in P10, P21 and adult PAG. (B) Raw EMG traces, blue arrow indicates the start of stimulus (von Frey hair). Left hand panel shows threshold responses before drug application (P10: 15g; P21: 60g; adult: 100g). Right hand panel show responses to the same strength of hair in each age group after intra-PAG LPI microinjection. Spinal reflex excitability (C) and change in mechanical threshold (D) in P10, P21 and adult rats after intra-PAG microinjection of saline (n = 3 for P10, 4 for P21 and adult) and LPI (n = 4 for P10, 7 for P21 and 6 for adult). (C) Intra-PAG LPI produced differential responses between young

*and mature nociceptive processing; spinal reflex excitability was decreased post-LPI in P10 and P21 but remained unchanged in adults. (D) Mechanical threshold post-LPI was significantly increased in P21 rats only. ++ = P<0.01, one-way ANOVA, pre-drug vs. post-drug responses; **** = P<0.0001, two-way ANOVA with Bonferroni post-test, between drug comparison; #### = P<0.0001, two-way ANOVA with Bonferroni post-test, between age comparison.*

5.4.3 Activation of CB1/CB2 receptors in the RVM of both mature and immature rats is antinociceptive

From previous experiments I have shown that CB1/CB2 receptor agonism with WIN55212 and HU210 in the PAG inhibited spinal reflex excitability and increased noxious mechanical threshold in P10, P21 and adult rats. However, the influence of CB1/CB2 receptors in the RVM on nociception throughout postnatal development is currently unknown. In this set of experiments, HU210 (4µg; n = 3 for P10, 4 for P21 and adults; Figure 5.4A) and AM251 (2.77µg; n = 4 for P10, 3 for P21 and 4 for adults; Figure 5.4A) were microinjected into the RVM. The EMG responses before and after drug administrations in each age groups were shown in Figure 5.4B. It was found that these drugs produced differential responses in spinal reflex excitability (two-way ANOVA, drug x age interaction, $F(4,375)=27.56$, $P<0.0001$; Figure 5.4C) and mechanical threshold (drug x age interaction, $F(4,375)=8.6$, $P<0.0001$; Figure 5.4D).

Administration of HU210 into the RVM significantly reduced spinal reflex excitability in all ages tested when compared to pre-drug (pre-drug = 100%; P10 = 31±4%; P21 = 15±6%; adult = 58±12%; one-way ANOVA; $F(12,24)=23.64$, $P<0.0001$ $F(12,36)=14.17$, $P<0.0001$, $F(12,36)=3.02$, $P<0.01$ respectively; Figure 5.4B). Post-HU210 spinal reflex excitabilities in adults were also significantly lower compared to post-vehicle values (P10 post-vehicle = 95±5%, P21 post-vehicle = 84±8%, adult post-vehicle = 100±0.4%; two-way ANOVA Bonferroni post-test, all $P<0.0001$; Figure 5.4C). Interestingly, post-HU210 spinal reflex excitability was significantly higher when compared to P10 and P21, indicating that HU210 was more efficacious in the younger age groups (two-way ANOVA Bonferroni post-test, P10 vs. adult, P21 vs. adult, both $P<0.0001$).

There was also a similar change in mechanical threshold post-HU210, which was significantly higher when compared to post-vehicle values in all ages (post-vehicle vs. post-HU210; two-way ANOVA Bonferroni post-test; P10: 104±3% vs. 151±10%, $P<0.01$; P21: 100% vs. 208±66%, $P<0.0001$; adult: 98±2% vs. 143±18%, $P<0.01$; Figure 5.4D). The effects of intra-RVM microinjection of HU210 were also significantly

stronger in P21 when compared to P10 and adult rats (two-way ANOVA with Bonferroni post-tests; $P<0.0001$ and $P<0.05$ respectively).

Similar to what was seen in the PAG, AM251 had no effect on either spinal reflex excitability or mechanical threshold in adult rats, as it did not differ from either pre-drug or post-vehicle responses. However, spinal reflex excitability was significantly reduced in P10 and P21 rats after intra-RVM AM251 microinjection when compared to pre-drug (pre-drug = 100%; one-way ANOVA; P10 post-AM251: 28±4%; P21 post-AM251: 29±14%; both $P<0.0001$; Figure 5.4C) and post-vehicle values (two-way ANOVA Bonferroni post-test; both $P<0.0001$). Moreover, P10 and P21 post-AM251 responses were significantly lower when compared to adult post-AM251 (adult post-AM251 spinal reflex excitability = 101±4%; two-way ANOVA Bonferroni post-test; P10 vs. adult, P21 vs. adult; both $P<0.0001$).

In both P10 and P21, post-AM251 mechanical threshold was higher than post-vehicle responses (post-vehicle vs. post-AM251; two-way ANOVA Bonferroni post-test; P10: 104±3% vs. 155±8%, $P<0.0001$; P21: 100% vs. 141±5%, $P<0.05$). In adult rats, post-AM251 mechanical threshold was lower than post-HU210 (post-AM251 vs. post-HU210: 101±3% vs. 143±18; two-way ANOVA Bonferroni post-test; $P<0.01$). In addition, effects of AM251 on mechanical threshold in adults were significantly different to P10 and P21 (two-way ANOVA Bonferroni post-test; P10 vs. adult, $P<0.0001$; P21 vs. adult, $P<0.05$).

These results indicate that CB1/CB2 agonism in the RVM is antinociceptive in all ages tested, but similar to the PAG, GPR55 receptors in the RVM may also have a role in antinociception in the younger ages.

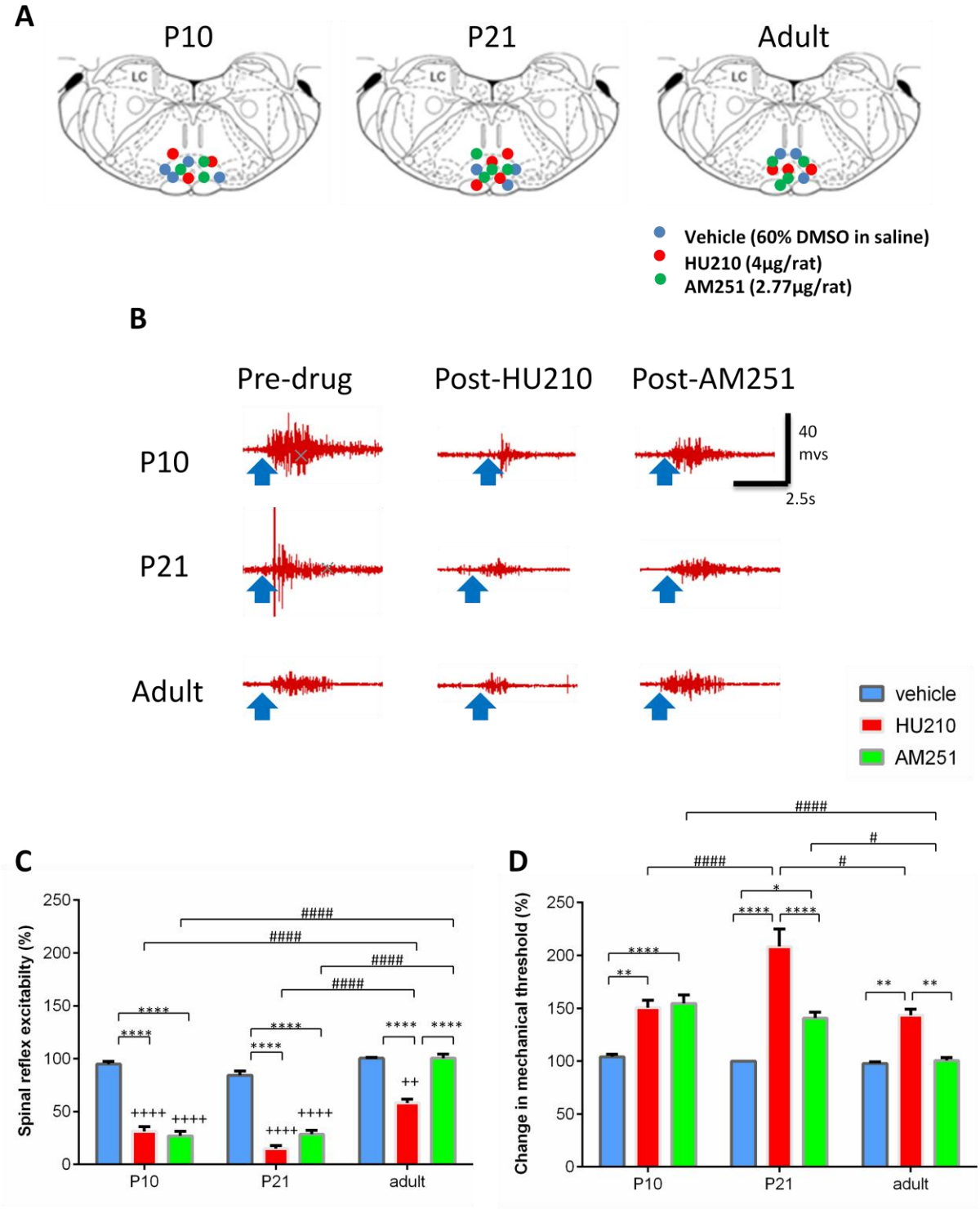


Figure 5.4. Effects of intra-RVM HU210 and AM251 microinjection on EMG responses in P10, P21 and adult rats. (A) Injection sites for vehicle, CB1/CB2 receptor agonist HU210 and CB1 receptor antagonist/GPR55 receptor agonist in P10, P21 and adult RVM. (B) Raw EMG traces, blue arrow indicates the start of stimulus (von Frey hair). Left hand panel shows threshold responses before drug application (P10: 15g; P21: 60g; adult: 100g). Middle and right hand panel show responses to the same strength of hair in each age group after intra-RVM HU210 and AM251 microinjection respectively. Spinal reflex excitability (C) and change in mechanical threshold (D) in P10, P21 and adult rats after intra-RVM microinjection of vehicle (n = 4 for P10, 3 for P21 and adult), HU210 (n = 3 for P10, 4 for P21 and adult) and AM251 (n = 4 for P10, 3 for P21 and 4 for adult). (C)

*Similar to observations in the PAG, HU210 in the RVM strongly reduced spinal reflex excitability in all ages, AM251 inhibited responses in P10 and P21 rats only. (D) Mechanical threshold was significant increased by intra-RVM HU210. AM251 enhanced mechanical threshold in P10 and P21 rats only. ++ = P<0.01, ++++ = P<0.0001, one-way ANOVA, pre-drug vs. post-drug responses; *, **, **** = P<0.05, P<0.01 and P<0.0001 respectively, two-way ANOVA Bonferroni post-test, between drug comparison; #, #### = P<0.05 and P<0.0001 respectively, two-way ANOVA Bonferroni post-test, between age comparison.*

5.4.4 Activation of GPR55 receptors in the RVM is antinociceptive in immature rats, but pronociceptive in adults

In this set of experiments, LPI (12μg, n = 4 for P10, P21 and adults; Figure 5.5A) was administered in the RVM. The representative EMG responses before and after drug administration in each age groups were shown in Figure 5B. It was found that intra-RVM LPI produced differential responses in the different age groups with respect to reflex excitability (two-way ANOVA, drug x age interaction: $F(2,246)=55.68$, $P<0.0001$; Figure 5.5C) and change in mechanical threshold (drug x age interaction: $F(2,246)=47.3$, $P<0.0001$; Figure 5.5D).

In both P10 and P21, post-LPI spinal reflex excitabilities were smaller compared to post-saline values (post-saline vs. post-LPI; two-way ANOVA Bonferroni post-test; P10: 82±5% vs. 26±4%; P21: 94±3% vs. 28±4%, both $P<0.0001$; Figure 5.5C). In particular, spinal reflex excitability after intra-RVM LPI microinjection was significantly reduced when compared to pre-drug responses in P10 (pre-drug = 100%; one-way ANOVA; $F(12,36)=5.42$, $P<0.0001$; Figure 5.5B). In contrast, LPI significantly increased spinal reflex excitability in adults when compared to post-saline responses (post-saline vs. post-LPI; 104±2% vs. 125±7; two-way ANOVA Bonferroni post-test; $P<0.05$). In addition, post-LPI responses in adults were significantly higher when compared to P10 and P21 (two-way ANOVA Bonferroni post-test; P10 vs. adult, P21 vs. adult, both $P<0.0001$).

Intra-RVM LPI microinjection also increased mechanical threshold in P10 and P21 rats when compared to post-saline responses (post-saline vs. post-LPI; two-way ANOVA Bonferroni post-test; P10: 101±2% vs. 123±4%, $P<0.0001$; P21: 98±2% vs. 150±4, $P<0.0001$; Figure 5.5D). It was found that the increase in mechanical threshold in P21 rats after LPI administration was significantly greater than P10 ($P<0.0001$). Intra-RVM LPI microinjection had no effect on mechanical threshold in adults (post-LPI response = 93±2), but it was significantly different to P10 and P21 (two-way ANOVA Bonferroni post-test; P10 vs. adult, P21 vs. adult, both $P<0.0001$).

Together the results from these experiments indicate that much like the PAG, activation of GPR55 receptors in the RVM is antinociceptive in the younger age groups. It was recently shown that GPR55 receptor agonism is pronociceptive (Gangadharan et al., 2013, Sylantyev et al., 2013). In this study, I have also demonstrated that LPI, when injected into adult RVM is pronociceptive, as it caused an increase in spinal reflex excitability. The functional differences of GPR55 receptor across the different timepoints of postnatal development will be discussed further in the *Discussion* section of this chapter.

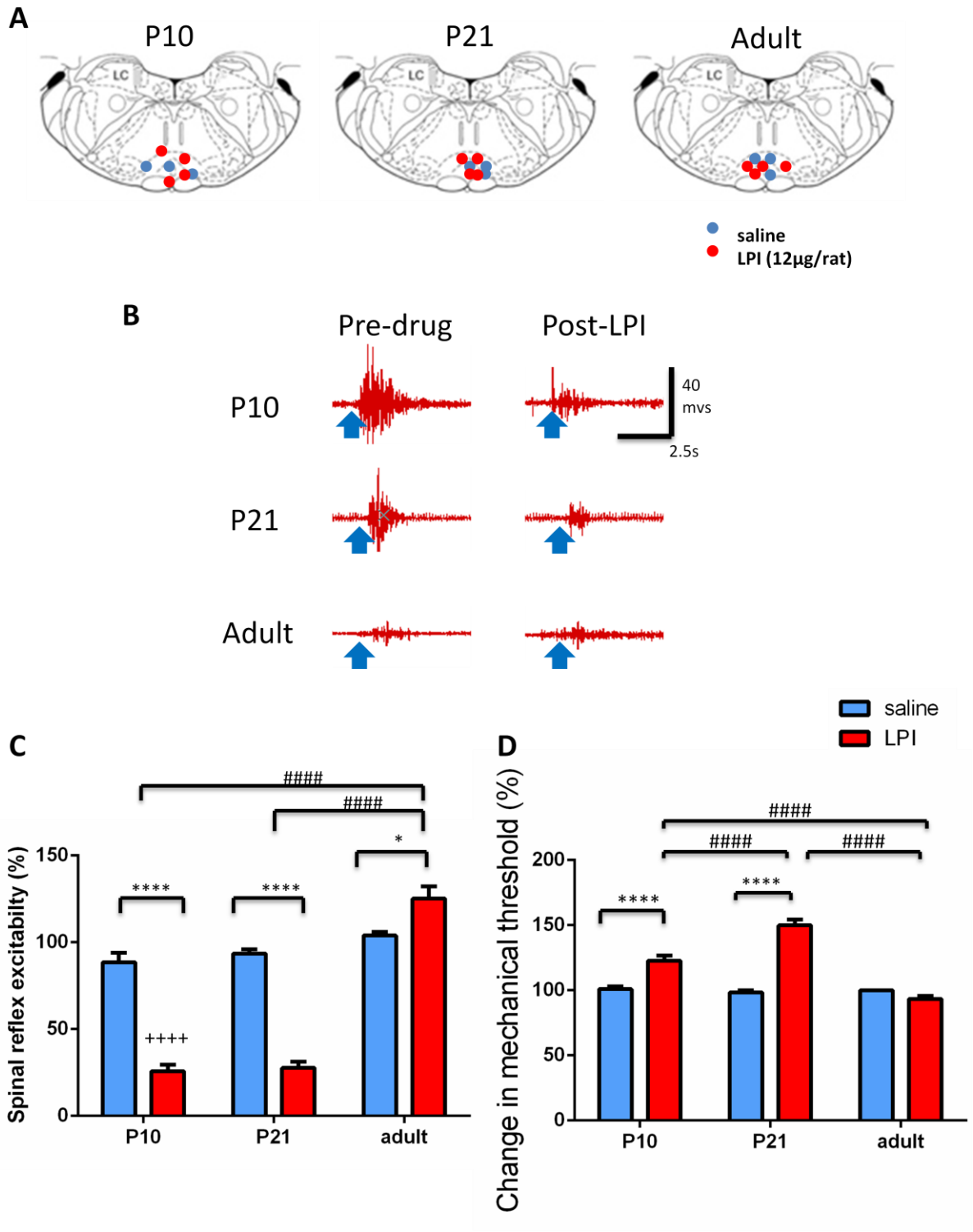


Figure 5.5. Effects of intra-RVM LPI microinjection on EMG responses in P10, P21 and adult rats. (A) Injection sites for vehicle and the GPR55 receptor agonist LPI in P10, P21 and adult RVM. (B) Raw EMG traces, blue arrow indicates the start of stimulus (von Frey hair). Left hand panel shows threshold responses before drug application (P10: 15g; P21: 60g; adult: 100g). Right hand panel show responses to the same strength of hair in each age group after intra-RVM LPI microinjection. Spinal reflex excitability (C) and change in mechanical threshold (D) in P10, P21 and adult rats after intra-RVM microinjection of saline (n = 3 for P10, P21 and adult) and LPI (n = 4 for P10, P21 and adult). (C) Intra-RVM LPI produced differential responses between young and mature

*rats; spinal reflex excitability was reduced in P10 and P21 whereas it was increased in adult rats. (D) Mechanical threshold was increased post-LPI in P10 and P21 rats only. ++++ = P<0.0001, one-way ANOVA, pre-drug vs. post-drug responses; *, **** = P<0.05, P<0.0001 respectively, two-way ANOVA with Bonferroni post-test, between drug comparison; #### = P<0.0001, two-way ANOVA with Bonferroni post-test, between age comparison.*

5.4.5 Activation of CB1/CB2 receptors in the spinal cord is antinociceptive across the different timepoints of postnatal development

In this last set of experiments, I sought to determine the effects of CB1/CB2 receptor activation in the spinal cord throughout postnatal development, as inputs from supraspinal sites descend and converge at the level of DH, and spinal nociceptive circuits undergo significant postnatal refinement.

The representative EMG responses before and after drug administration in each age groups are shown in Figure 5.6A. Administration of HU210 (4µg; n = 4 for P10, P21 and adult) and AM251 (2.77µg; n = 4 for P10, P21 and adult) onto the spinal cord produced differential effects both in terms of spinal reflex excitability (two-way ANOVA; drug x age interaction; $F(4,411)=15.79$, $P<0.0001$; Figure 5.6B) and mechanical threshold two-way ANOVA; drug x age interaction; $F(4,411)=8.97$, $P<0.0001$, Figure 5.6C).

Spinal application of HU210 reduced spinal reflex excitability when compared to post-vehicle responses in all ages tested (post-vehicle vs. post-HU210; two-way ANOVA Bonferroni post-test; P10: 90±7% vs. 7±3%; P21: 101±4% vs. 26±3%; adult: 95±5 vs. 59±6%; all $P<0.0001$). In particular, spinal reflex excitabilities in P10 and P21 rats were significantly lower when compared to pre-drug responses (pre-drug = 100%; one-way ANOVA; P10: $F(12,39)=11.41$; P21: $F(12,39)=11.72$, both $P<0.0001$; Figure 5.6B) and adults (two-way ANOVA Bonferroni post-test; P10 vs. adult; P21 vs. adult; both $P<0.0001$).

Comparable post-HU210 changes were also seen in mechanical threshold, where it was significantly increased when compared to post-vehicle responses in all ages (post-vehicle vs. post-HU210; P10: 103±1% vs. 169±5%; P21: 117±4% vs. 157±3%; adult: 107±3% vs. 135±6%; all $P<0.0001$). In P10 and P21 rats mechanical thresholds after spinal application of HU210 were significantly higher when compared to pre-drug responses (pre-drug = 100%; one-way ANOVA; P10: $F(12,39)=9.84$, $P<0.05$; P21: $F(12,39)=34.33$, $P<0.0001$) and adults (two-way ANOVA Bonferroni post-test; P10 vs. adults, $P<0.0001$; P21 vs. adults, $P<0.01$).

Similar to what was observed in the PAG and the RVM, spinal application of AM251 did not alter spinal reflex excitability or mechanical threshold in adults, but significantly inhibited these nociceptive behaviours in the younger rats. Spinal reflex excitability post-AM251 in P10 and P21 rats were smaller compared to post-vehicle responses (post-vehicle vs. post-AM251; two-way ANOVA Bonferroni post-test; P10: 90±7% vs. 22±3%, P21: 101±4% vs. 61±5%; both $P<0.0001$) and adults (adult post-AM251 = 105±7%; P10 vs. adult, P21 vs. adult, both $P<0.0001$). In P10, post-AM251 spinal reflex excitability was also significantly lower compared to pre-drug responses (pre-drug = 100%, one-way ANOVA, $F(12,39)=4.36$, $P<0.001$), which suggest that spinal AM251 was most inhibitory in P10 rats compared to the other age groups tested.

On the contrary, mechanical threshold was significantly elevated compared to post-vehicle responses in P10 rats only (post-vehicle vs. post-AM251; two-way ANOVA Bonferroni post-test; 103±1% vs. 132±3%, $P<0.0001$). It was also found that P10 post-AM251 mechanical threshold were significantly higher compared to P21 post-AM251 responses (P21 post-AM251: 109±4%; two-way ANOVA Bonferroni post-test; $P<0.01$), which further suggest that spinal AM251 were likely to be most effective in antinociception in P10 rats but not in the older ages.

Since spinal application of HU210 lowered spinal reflex excitability and increased mechanical threshold in all age groups, CB1/CB2 receptor activation within the spinal cord is antinociceptive throughout postnatal development. Interestingly, spinal HU210 was more efficacious in the younger age groups, as post-HU210 responses were reduced compared to both post-vehicle and pre-drug responses, whereas in adults a significant reduction was seen when compared to post-vehicle responses only.

In parallel to the PAG and RVM, AM251 in the younger age groups was also antinociceptive. This suggests that there might also be GPR55 mediated activity within the immature spinal nociceptive circuits. Intriguingly, the efficacy of AM251 seemed to decrease as the animals aged, in P10 rats post-AM251 responses were significantly inhibited compared to both pre-drug and post-vehicle responses, whereas in P21, where AM251 was also antinociceptive, the effect was only statistically significant when compared to post-vehicle responses.

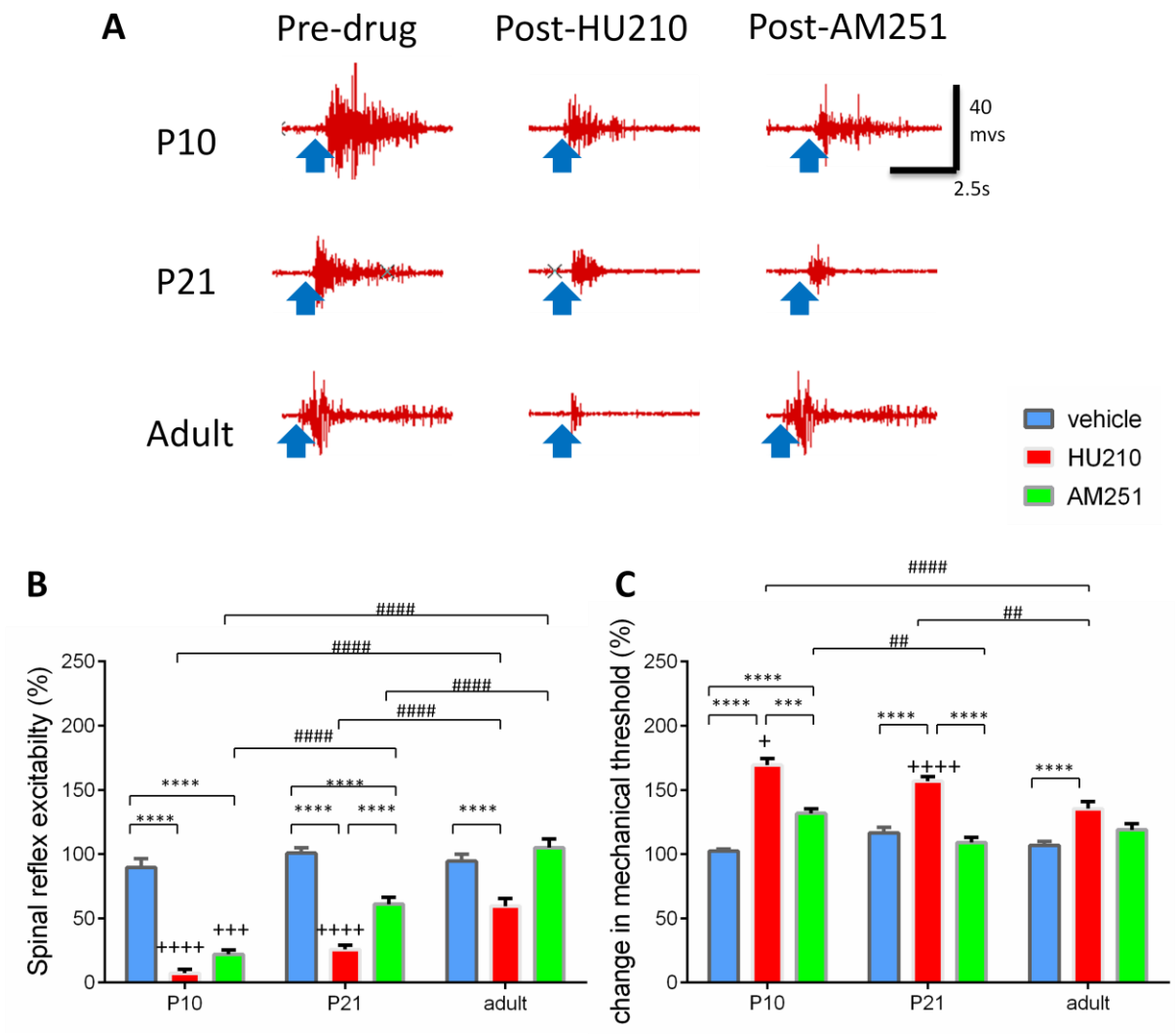


*Figure 5.6. (A) Raw EMG traces, blue arrow indicates the start of stimulus (von Frey hair). Left hand panel shows threshold responses before drug application (P10: 15g; P21: 60g; adult: 100g). Middle and right hand panel show responses to the same strength of hair in each age group after spinal application of HU210 and AM251 respectively. Spinal reflex excitability (B) and change in mechanical threshold (C) in P10, P21 and adult rats after spinal application of vehicle (n = 3 for P10, 4 for P21 and adult), HU210 (n = 4 for P10, P21 and adult) and AM251 (n = 4 for P10, P21 and adult). (B) Spinally applied HU210 significantly reduced spinal reflex excitability in all ages tested. AM251 reduced spinal reflex excitability in P10 and P21 rats only. (C) HU210 significantly increased mechanical threshold in all ages tested. AM251 increased mechanical threshold in P10 rats only. +, +++, ++++ = P<0.05, P<0.001, P<0.0001 respectively, one-way ANOVA, pre-drug vs. post-drug responses; ***, **** = P<0.001, P<0.0001 respectively, two-way ANOVA with Bonferroni post-test, between drug comparison; ##, #### = P<0.01, P<0.0001 respectively, two-way ANOVA with Bonferroni post-test, between age comparison.*

5.5 Summary

Collectively these data suggest that either CB1 or CB2 receptors are effective analgesic targets in both immature and mature animals. Initial experiments with intra-PAG

microinjections of the CB1/CB2 receptor agonist WIN55212 revealed that CB1/CB2 receptor activation inhibited spinal reflex excitability in both P21 and adult rats. Further experiments with another more potent CB1/CB2 receptor agonist HU210 confirmed the analgesic effects of CB1/CB2 receptor agonism in P10, P21 and adult rats.

Interestingly, in the younger animals (P10, P21 rats), it was observed the CB1 receptor antagonism by AM251 was also antinociceptive. On one hand, this highlighted the complexity in the pharmacology of the cannabinoid signalling system. On the other hand, since AM251 is also a GPR55 agonist, this prompted questions about the functions of GPR55 receptors in the transmission of pain. Experiments illustrated in this chapter showed that intra-PAG and RVM microinjection of the endogenous GPR55 receptor agonist LPI reduced spinal reflex excitability in P10 and P21 rats, but facilitated nociceptive responses in adult rats when LPI was injected into the RVM. These data provided important evidence to support the role of GPR55 receptors in nociception.

5.6 Discussion

It is known that nociceptive processing is subjected to developmental regulation, as demonstrated in other studies and *Chapter 3 and 4* of this thesis. The role of cannabinoids in antinociception and normal brain maturation are described elsewhere, the work described in this chapter investigates for the first time the function of cannabinoid receptors, within the descending pain modulation pathway during postnatal development.

5.6.1 The role of CB1 and CB2 receptors in nociception during postnatal development

Initially WIN55212 was injected into the PAG of P21 and adult rats, and was antinociceptive in both ages. HU210 was used in subsequent experiment as a CB1/CB2 agonist because HU210 has about a hundred fold more affinity at the CB1 receptors than WIN55212 (Ki for CB1 receptors, HU210 = 0.061nM, WIN55212 = 62.3nM) (Pertwee et al., 2010). Application of HU210 in the PAG, RVM and spinal cord reduced nociceptive behaviours in all ages tested; a decrease in spinal reflex excitability and an increase in mechanical threshold were observed post-HU210 in all ages, indicating that CB1/CB2 agonism is analgesic throughout postnatal development. This is in line with observations from previous studies, where systemic administration of CB1/CB2 agonists, including WIN55212, CP55940 and HU210 inhibited nociceptive responses in young and adult rats

(Iversen and Chapman, 2002, Romero et al., 2002, Finn et al., 2003, Borcel et al., 2004).

Moreover, in the RVM and the spinal cord the inhibitory effects of HU210 were greater in the younger ages, compared to adults. In the spinal cord, post-HU210 mechanical thresholds were also significantly higher in both P10 and P21 than adults. This observation is similar to the observation that morphine/DAMGO are more efficacious in younger rats (*Chapter 3, section 3.4.3*). that the increased effect of HU210 in the younger age groups could be attributed to either an alteration in receptor expression or receptor coupling.

The most surprising finding is that AM251, which primarily is a CB1 receptor antagonist, was antinociceptive in P10 and P21 rats. While intra-PAG, intra-RVM and spinal application of AM251 had no effect on spinal reflex excitability and mechanical threshold in adults, nociceptive responses were significantly inhibited in the younger age groups. Indeed, as the rats aged the inhibitory effects of spinally applied AM251 gradually disappeared; greater reduction in spinal reflex excitability and enhancement in mechanical threshold was observed in P10 rats when compared to P21, but these effects were never detected in adults (Figure 5.6B). It was then postulated that the orphan GPR55 receptor, which was recently described as a novel, atypical cannabinoid receptor may play a part in nociception, especially in the younger age groups.

5.6.2 The role of GPR55 receptors in nociception during postnatal development

As mentioned previously in the *general introduction section 1.6.2*, GPR55 receptor pharmacology and physiology is not currently well characterised, but the general consensus is that GPR55 receptor-mediated activity is most likely to be pronociceptive in mature animals; it is coupled to the Gq proteins, and when activated causes an increase in intracellular calcium levels and synaptic excitability (Sylantyev et al., 2013). It was also shown that intraplantar injection of LPI (2 pmol) led to allodynia in mice (Gangadharan et al., 2013). Therefore, in line with observations from previous studies, intra-RVM microinjection of LPI caused an increase in spinal reflex excitability in adult rats (Figure 5.5C). This was accompanied by a slight decrease in mechanical withdrawal threshold (Figure 5.5D), however this failed to reach statistical significance. Intra-PAG LPI microinjection had no effects on nociceptive behaviours in the adults, this may be due to low expression of GPR55 receptors in the PAG, or that they are not found on neurones that form the descending pathways originating from the PAG.

On the other hand, LPI injected either into the PAG or the RVM was antinociceptive in both P10 and P21 rats. Post-LPI spinal reflex excitabilities were significantly lower compared to both pre-drug and post-saline responses in the younger rats, an enhancement in mechanical withdrawal was also observed. Interestingly, there was more inhibition when LPI was injected into the RVM compared to the PAG; for instance, post-LPI spinal reflex excitability in the RVM was 25.79±3.79% and 27.63±3.61% for P10 and P21 respectively, which were lower compared to 53.34±5.55% and 58.22±5.34% observed in the P10 and P21 PAG. It is unlikely that receptor pharmacology of GPR55 is different between these two regions, but GPR55 receptors may be expressed in higher levels in the RVM than the PAG (*Chapter 6*). Since both LPI and AM251 inhibited nociceptive responses in young rats, GPR55 receptors must be expressed within the descending pain modulatory pathway, and play a role in antinociception in the immature CNS.

To fully elucidate the physiological functions of GPR55 receptors in the descending pain modulation pathways during postnatal development, spinal application of LPI must be carried out as it was not included in this study. Nonetheless, results from this study indicated that LPI in adult RVM was pronociceptive whereas in the immature PAG and RVM were antinociceptive, which suggests a switch in GPR55-mediated actions throughout postnatal development. It is possible that GPR55 receptors switch from an inhibitory G protein coupling to an excitatory one as animals age, but it is also possible that expression of GPR55 receptors undergo significant functional redistribution throughout postnatal refinement. At the present stage, the lack of identified GPR55 selective agonists might be hindering, but further studies on G protein coupling using techniques like GTP-gammaS assays in both neonatal and adult tissue could clarify some of the questions posed in this study.

5.5.3 Comparison between CB1/CB2 and GPR55 receptor-mediated responses

Another noteworthy observation is that in young rats in which both HU210 and AM251 exerted inhibitory effects on nociceptive responses, the level of inhibition mediated by HU210 was always greater than ones mediated by AM251. Especially in the PAG and spinal cord, post-HU210 responses were always significantly larger than post-AM251 responses in P10 and P21 rats (Figure 5.2 and 5.6). This suggests that CB1/CB2 receptor agonists might be more effective analgesics than GPR55 receptor agonists. However, it is also known that cannabinoids exert undesirable psychoactive side effects in most patients (Campbell et al., 2001). Since there are virtually no reports of GPR55 receptor

mediated psychoactive effects, and GPR55 receptor activation is only antinociceptive in young rats, it represents a unique opportunity to therapeutically exploit GPR55 ligands as potential treatment for neonatal and juvenile pain pathologies.

5.5.4 Selectivity of cannabinoid ligands

In this study, a wide variety of cannabinoid drugs were administered to the regions within the descending pain modulation pathway: the PAG, the RVM and the spinal cord. The doses were determined from previously published studies. Body temperature was closely monitored and no hypothermic effects were observed in all experiments. It would be ideal to focus on CB1 receptors only, given its dense expression in all the aforementioned areas, whereas only moderate levels of CB2 receptors were detected in the PAG and spinal trigeminal tract (Svíženská et al., 2008). It is important to mention that most of the effects described in this study cannot be attributed to a single subtype of cannabinoid receptor. AM251 and HU210, apart from their binding affinities at the CB1 or CB2 receptors, are both efficacious at the GPR55 receptor (EC50 HU210 = 26nM, EC50 AM251 = 39nM) (Ryberg et al., 2007). In addition, ACEA, which is currently known as a CB1 selective agonist would be unsuitable for in vivo electrophysiological experiments because it is a homolog of anandamide, and is thus subjective to rapid metabolic degradation by fatty acid amide hydroxylase (FAAH). Perhaps another way to elucidate cannabinoid functions within the descending pain pathway is to focally inject drugs that specifically target the endocannabinoid-degrading enzymes, such as URB597 (potent and selective FAAH inhibitor), JZL184 (monoacylglycerol lipase/MAGL inhibitor) or JZL195 (dual FAAH and MAGL inhibitor), which in turn raise the concentration of endocannabinoids within the region. Studies investigating the effect of FAAH and MAGL inhibitors on pain transmission are inconclusive: oral administration of URB597 (1-50mg/kg, once daily for 4 days) produced a dose-dependent reduction in nociceptive behaviours (paw withdrawal latency) to noxious mechanical and thermal stimuli in the mouse model of chronic constriction injury (CCI) induced neuropathic pain (Russo et al., 2007), whereas another study reported repeated URB597 treatment (0.3mg/kg, once daily for four days) did not attenuate the development of mechanical hyperalgesia in a rat model of carrageenan-induced inflammatory pain (Okine et al., 2012). The difference in the effect of URB597 between these two studies could be attributed to the difference in dosage used. Nonetheless, elevation in endocannabinoid content may induce plastic changes within the CNS, which in turn may alter nociceptive processing (Okine et al., 2012). Therefore, the use of endocannabinoid-degrading enzyme inhibitors, or the discovery of more selective ligands in the future will be hugely beneficial for this type of study.

5.5.5 Future directions

Apart from the lack of selective ligands and data on efficacy of LPI in the spinal cord, there are some technical issues that future studies should address. Firstly, only a single dose of drug was administered to each animal, therefore dose-response data and effect of antagonist on actions of agonists at the receptor were lacking. Secondly, due to the difficulty in obtaining stable recordings in neonatal animals, the n-numbers of some experiments are quite small. These issues will be addressed in more details in the *General Discussion* section. The next chapter will describe the expression of receptors and related proteins involved in the endocannabinoid system, and investigate the underlying anatomical changes which may reflect some of the cannabinoid mediated differential noxious processing described in this study.

Chapter 6 Expression of the endocannabinoid system within the descending pain pathway during postnatal development

6.1 Introduction

6.1.1 The synthesis and degradation of endocannabinoids

As mentioned in the introduction, the two known endocannabinoids in both rats and humans are anandamide and 2-arachidonyl-glycerol (2-AG). Anandamide and 2-AG are lipids in nature and differ from classical peptide neurotransmitters in terms of chemical structures, mechanisms of synthesis and release (Piomelli et al., 2000): anandamide and 2-AG, unlike the other neurotransmitters are not synthesised in neuronal cytosol and stored within vesicles; they are produced upon demand by receptor-stimulated cleavage of membrane-bound lipid precursors and are released immediately after production (Di Marzo et al., 2004).

The synthesis of anandamide involves enzymatic cleavage of the membrane-bound lipid precursor N-arachidonoyl-PE (NAPE) by a phospholipase D (PLD). The brain has very low level of NAPE (20-40 pmol/g) (Piomelli, 2003) but it can be replenished by catalysis of phosphatidylethanolamine (PE, another class of phospholipids found in biological membranes) by the enzyme N-acyltranferase (NAT). The production of anandamide can be triggered by depolarisation-induced intracellular increase in calcium, as activation of the calcium ionophore ionomycin, the ionotropic kainate receptor and the potassium channel blocker 4-aminopyridine increases accumulation of [^{3}H]anandamide in cultures of rat striatal neurones (Dimarzo et al., 1994, Cadas et al., 1996). Alternatively, G protein-coupled receptor signalling can trigger the synthesis of anandamide; it can interact with the Rho family of small G proteins to stimulate the activity of PLD, or it can recruit the NAT/NAD pathway by mobilising calcium ions from intracellular stores (Di Marzo et al., 1994).

2-AG is classed as a monoacylglycerol and it is about 170-200 fold higher in concentration than anandamide in rat brain tissue (Stella et al., 1997). The synthesis of 2-AG involves generation of 1,2-diacylglycerol (DAG) and phosphatidylinositol (4,5)-biphosphate (PIP_2) by phopholipase C (PLC) mediated mechanisms. DAG is then converted to 2-AG by diacylglycerol lipase (DAGL) (Piomelli, 2003). There are two isoforms of DAGL, α and β, which are closely related: they are both localised at the dendritic terminals of neurones, with DAGLα showing higher expression than β in the murine subventricular zone, a site important for neurogenesis in mature animals (Goncalves et al., 2008). An alternative way to synthesise 2-AG is through catalysis of PIP_2 into 2-arachidonoyl-lysophospholipid by phospholipase A1 (PLA1), which in turn can

be hydrolysed to 2-AG by lyso-PLC (Piomelli, 2003). Similar to anandamide, 2-AG production can be elicited by increases in intracellular calcium.

Endocannabinoids released into the extracellular space are taken up intracellularly via facilitated diffusion, a process driven by transmembrane concentration gradient rather than ATP or sodium ion-dependent mechanisms (Pacher et al., 2006). Once anandamide and 2-AG enters the cell, they are subjected to rapid degradation by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) respectively. Recently, a cytoplasmic protein, which is structurally similar to FAAH, but lacks catalytic functions, was discovered. Due to its capability to bind anandamide and subsequently increase its intracellular concentration, this protein is identified as an anandamide transporter and is termed FAAH-1-like anandamide transporter (FLAT) (Fu et al., 2012, Marsicano and Chaouloff, 2012). The non-synaptic release mechanisms and short life spans of endocannabinoids suggest that the main biological function of these compounds is to regulate the effects of other neurotransmitters and hormones by acting near the site of their synthesis (Piomelli et al., 2000).

Other putative endocannabinoids include virodhamine, noladin-ether and N-arachidonoyldopamine (NADA). These all act on cannabinoid receptors and their role in analgesia has been investigated (Walker et al., 2002). They are all fatty acid derivatives related to both anandamide and 2-AG. Although all of them have been detected in rat and bovine brains, their precise mechanisms of action, synthesis and degradation remain to be elucidated.

A summary of the synthesis, release and degradation of endocannabinoids (anandamide and 2-AG) is provided in Figure 6.1.

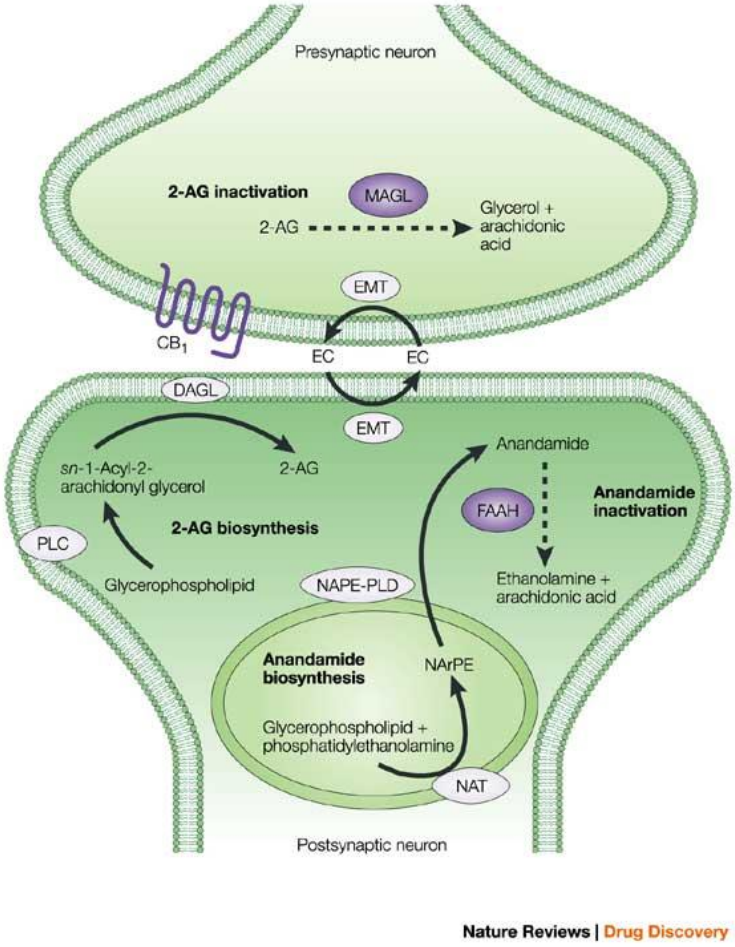


Figure 6.1 The synthesis, release and degradation of anandamide and 2-acylglyerol (2-AG). Anandamide is synthesised from the precursor N-arachidonoyl-PE (NAPE) by a phospholipase D (NAPE-PLD). NAPE is released from membrane phospholipids by N-acyltransferase (NAT). 2-AG is synthesised by diacylglycerol lipase (DAGL) via a phospholipase C (PLC)-dependent pathway. Endocannabinoids synthesised intracellularly are transported across the synapse by an endocannabinoid membrane transporter (EMT), which is likely to be FAAH-1-like anandamide transporter (FLAT) (Marsicano and Chaouloff, 2012). Endocannabinoids are subjected to rapid degradation, anandamide is degraded by Fatty Acid Amide Hydrolase (FAAH) and 2-AG is degraded by Monoacylglyerol Lipase (MAGL). Figure adapted from (Di Marzo et al., 2004).

6.1.2 The expression of receptors, ligands and related enzymes of the endocannabinoid system within the CNS

As well as pain, endocannabinoids participate in a wide range of other pathophysiological conditions, such as obesity, movement disorders, epilepsy and substance abuse (Pacher et al., 2006), which is concurrent with the finding that cannabinoid receptors are ubiquitously expressed within the CNS. CB1, CB2 and GPR55 receptors are all found within the descending pain modulation circuitry; immunohistochemical studies show that CB1 receptors are found in the PAG, spinal trigeminal nuclei and the RVM (Tsou et al.,

1998), whereas PCR studies show that CB2 receptors are predominantly found in peripheral tissue (Svíženská et al., 2008) and GPR55 receptors are predominantly expressed in microglial cells (Pietr et al., 2009). It was also reported recently that CB2 receptors are found in microglial and neuronal cells in the rat spinal cord, and are upregulated in a model of osteoarthritis (Burston et al., 2013).

All the cannabinoid receptors are G protein-coupled and govern synaptic excitability and neurotransmitter release via secondary messenger signalling systems; CB1 receptors can be located both pre- and postsynaptically (Ohno-Shosaku et al., 2001, Yoshida et al., 2006), CB2 are predominantly postsynaptic (Gong et al., 2006, Svíženská et al., 2008) and the precise localisation of GPR55 receptors are not known. For further details of the expression of cannabinoid receptors please see *general introduction section 1.6.2*.

There have been a few studies investigating the concentration of endocannabinoids in different tissues, utilising techniques involving lipid extraction and high pressure lipid chromatography (HPLC) (Fontana et al., 1995, Berrendero et al., 1999). In these studies both anandamide and 2-AG were found in the brainstem, diencephalon and striatum (Bisogno et al., 1999). However, the precise localisation of endocannabinoids is difficult because both anandamide and 2-AG, once accumulated intracellularly are subjected to rapid degradation.

In contrast, expression and localisation of related endocannabinoid synthesising or degrading enzymes are better characterised. NAPE-PLD (the enzyme responsible for the conversion of NAPE to anandamide) mRNA has been found in the whole rat brain; specific regions include the olfactory bulb, brainstem, cerebellum, hippocampus and highest in the thalamus (Morishita et al., 2005). Using immunohistochemistry and in situ hybridisation, DAGLα (the enzyme responsible for synthesis of 2-AG) has been found in the rat forebrain, and it is most abundantly expressed in the amygdala, hippocampus and the hypothalamus (Suarez et al., 2011). These studies indicate that within the regions of pain pathways, the neurones have the appropriate enzymes for the synthesis of endocannabinoids.

The catalytic enzyme FAAH is mainly present on large principal neurones, such as the pyramidal cells of the cerebral cortex and the hippocampus (Rodriguez de Fonseca et al., 2005). Interestingly, Egertova and colleagues using immunohistochemical techniques

revealed that FAAH-positive cell bodies are complementarily localised within axonal terminals that are also CB1 receptor positive (Egertova et al., 1998). Moreover, it was reported that FAAH is mostly located postsynaptically (Di Marzo, 2009). This observation implies that FAAH controls the duration of anandamide-mediated actions closely. On the other hand, MAGL is mainly located presynatically in the hippocampus, cortex, cerebellum and the anterior thalamus (Dinh et al., 2002). The spatial segregation of these two enzymes within the synapse indicated that anandamide mediates its actions via a postsynaptic mechanism whereas 2-AG is more actively involved in endocannabinoid retrograde signalling (Rodriguez de Fonseca et al., 2005).

6.1.3 Postnatal development of the endocannabinoid system

The endocannabinoid system is critical for the normal maturation of the CNS. Its roles in pruning (Fernandez-Ruiz et al., 2004), neurogenesis in the subventricular zone (Goncalves et al., 2008, Oudin et al., 2011b) and synaptogenesis between glutamatergic (Saez et al., 2014) and GABAergic (Berghuis et al., 2007) terminals are well described. Deficits in endocannabinoid signalling in early life lead to detrimental behavioural and motor consequences (Keimpema et al., 2011).

Chromatography studies have shown that in rat whole brain tissue, concentrations of anandamide and its precursor NAPE steadily increase as animals (P1, P5 and adults) age, whereas 2-AG levels peak at P1, then decrease to adult levels from P5 onwards (Berrendero et al., 1999). Simultaneously, the boost in concentration of anandamide throughout postnatal development corresponds to Western blot data that shows that expression of NAPE-PLD in brain homogenates increases as animals age. Within the brainstem, NAPE-PLD protein level was higher in P56 rats when compared to P14 (Morishita et al., 2005). In particular, a surge in NAPE-PLD activity (as measured by specific binding of NAPE in rat (Wistar, P1, P4, P7, P10, P14, P21, P28, P42, P56) brain homogenates) occurs between the ages P14 to P21 and NAPE-PLD activity increases steadily from P21 onwards (Morishita et al., 2005).

Autoradiographic, immunohistochemical and GTP-binding assays revealed that the mRNA levels for CB1 receptors and binding activity undergo significant postnatal alterations. In the caudate putamen, specific binding of CB1 receptors is low in the neonatal (P1 and P5) period but high in adult rats (Berrendero et al., 1999, Fernandez-Ruiz et al., 2004). Immunohistochemical distribution of CB1 receptors in the rat dentate gyrus increases from P20 onwards (Morozov and Freund, 2003). It was also reported that specific CB1 binding in the hippocampus was highest in adult rats compared to neonates (Berrendero

et al., 1999). On the contrary, CB1 mRNA levels in the brainstem are highest in gestational rats (E16-21), and rapidly decrease as animals approach adulthood (Berrendero et al., 1999, Fernandez-Ruiz et al., 2004).

The aforementioned findings provided strong evidence that the expression and anatomical distribution of the endocannabinoid system undergo significant postnatal alterations. Much of this work was concerned with the ontogeny of endocannabinoids in corticolimbic development (Lee and Gorzalka, 2012). Changes in expression of CB1 receptors and NAPE-PLD protein levels were reported in the brainstem, but the location of the precise brainstem regions were not specified. Therefore, in this chapter, the focus is on expression of the various components of the endocannabinoid system within the descending pain pathway (PAG, RVM, spinal cord DH) at the three developmental timepoints.

6.2 Aims

This study aims to investigate the developmentally related changes in the endocannabinoid system within the regions of the descending pain pathway (PAG, RVM and DH), which will be achieved using immunohistochemical and TaqMan RT-PCR techniques. The expression of DAGLα, NAPE-PLD, CB1 receptor and GPR55 receptors are expected within the regions of interest, but any age-related differences will remain to be characterised.

6.3 Methods

6.3.1 Antibodies

The primary antibodies used were goat anti-CB1 (Frontier Institute, Japan, 1:200), rabbit anti-GPR55 (gift from Prof. Ken Mackie. A total of 2 vials (each containing 50μL of antibody) was received in September 2010 and June 2012 respectively, 1:1000 with tyramide signalling amplification (TSA) protocol), rabbit anti-NAPE-PLD (Cayman Chemical, 1:1000), goat anti-DAGLα (abcam, 1:2000), mouse anti-NeuN (1:100, Millipore) and rabbit-anti CGRP (1:100, Millipore). Sections were incubated with these primary antibodies overnight at room temperature (except for GPR55, which was incubated for 72 hours at 4ºC). Separate cohorts of rats were used for each antibody. CB1, NAPE-PLD and DAGLα immunohistochemistry were performed following the direct staining protocol.

6.3.2 TSA indirect amplification

The detection of GPR55 immunoreactivity was performed using the TSA indirect amplification protocol. A specific HRP-tyramide conjugate was added to the secondary antibody matrix to enhance the fluorescent signal. Spleen tissue from adult Sprague Dawley rats was included as positive tissue control. For further details, please refer to *general methods section 2.3.4*.

6.3.3 Sequences of primers and probes

Primers and probes for GAPDH (NCBI reference sequence NM_017008.3), CB1 (NM_012874.4), GPR55 (XM_006245494.1), DAGLα (NM_006133.2) and NAPE-PLD (NM_199381.1) were designed on Primer Express 3 (Applied Biosystems, Forster City, CA). All probes were labelled at the 5'end with 6-carboxyfluorescein (FAM) and at the 3'end with tetramethylrhodamine (TAMRA). The probes were specifically designed to span across an intron-exon boundary in order to avoid potential amplification of genomic DNA in the analysed samples. The Taqman RT-PCR primers and probes sequences are summarised in table 6.1 below.

	Forward primer	Reverse primer	probe
GAPDH	GAA GAT GTC CCT TTG GGT AGG A	TGG ACT GTG GTC TAG AAA GCA TAG A	TGC CCT GCA AGA CCT CAC CCA TTG
CB1	CCA AAA GTG GAG AGC GAC AAC	CGT CTC GAA GGT CCC AAT GT	CAT CCA GAT CAC CAT GCC GTT CAC A
GPR55	TCC ATA TTC CAG CAG ACC ACC TA	GCA CAA ACC TTG GGT GAA ACA	TCA ATC ACT TCC GGT CCC CCA GG
DAGLα	ACC TGC GGC ATC GGT TAG	CTT TGT CCG GGT GCA ACA G	CAG CTG GTC CCG CCG TCT AAA AGT G
NAPE-PLD	CTG GAG GAG GAC GTA ACC AA	TCA AGC TCC TCT TTG GAA CC	TAT CCC AAA CGT GCT CAG ATG GCT

Table 6.1. Sequences of forward primer, reverse primer and probe for the genes of interest, GAPDH, CB1, GOR55, DAGLα and NAPE-PLD.

6.3.4 Statistics

Statistical comparison between the age groups for the expression of various endogenous opioid targets in TaqMan RT-PCR and immunohistochemical experiments were made by one-way ANOVA with Bonferroni post-tests.

6.4 Results

6.4.1 Changes in CB1 receptor expression during postnatal development of the descending pain pathway

The expression of CB1 receptors within the PAG, RVM and DH was investigated in P10, P21 and adult rats using immunoflourescence (n = 4 for P10, P21 and adults) and Taqman RT-PCR (n = 4 for P10, P21 and adults). Similar to previously reported findings (Tsou et al., 1998), immunohistochemical expression of CB1 receptors was found in the PAG, and it was concentrated around the aqueduct (Figure 6.2A). No changes in CB1 immunoreactivity was found in the dPAG (Figure 6.2A, C). Within the vPAG, only fibre staining was observed and staining intensity significantly decreased as the animals aged ($F(2,9)=79.44$, $P<0.0001$; one-way ANOVA; Figure 6.2B), Bonferroni post-tests revealed that it was highest at P10 (P10 vs. P21 and P10 vs. adults, both $P<0.0001$).

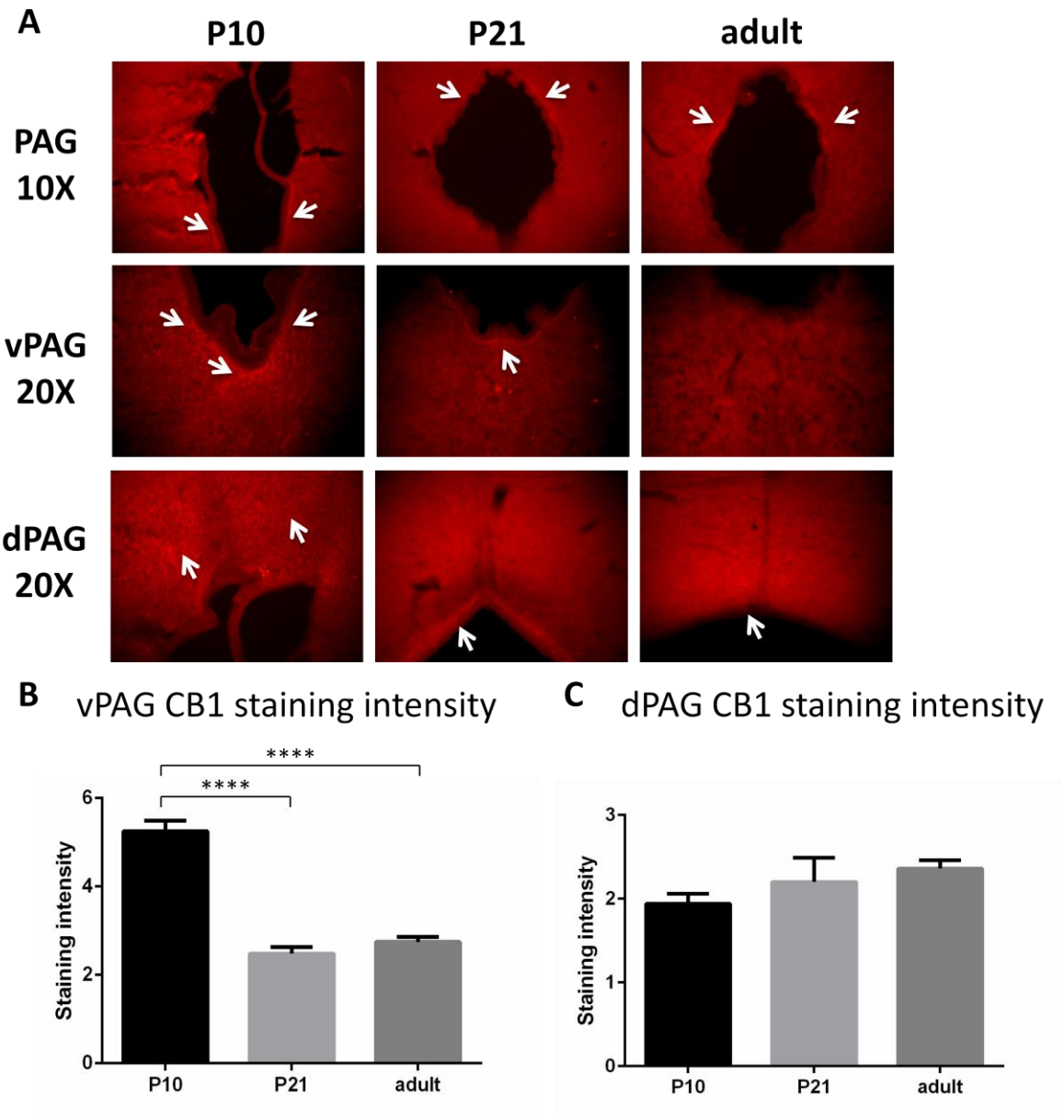


*Figure 6.2. (A) Epifluorescent images of CB1 receptor immunoreactivity in the PAG of P10, P21 and adult rats. The white arrows depict where fibre staining was observed. All images shown were captured with either a 10x or 20x objectives fitted with a Confocal head. (B) Quantified CB1 staining intensity in the vPAG. (C) Quantified CB1 staining intensity in the dPAG. Data are presented as mean ± SEM. CB1 expression was highest in P10. ****=P<0.0001.*

In contrast to the expression of CB1 receptor in the vPAG, CB1 receptor immunoreactivity in the RVM increased as the animals aged. Both fibre and cell body staining was observed along the midline within the NRM (Figure 6.3A), therefore both cell count (Figure 6.3B) and staining intensity (Figure 6.3C) were performed. At P10, there were sparse fibre staining along the midline, by P21, cell body staining was distributed within the NRM. There were no differences in staining intensity, but there

were significantly more CB1 positive cells detected within the RVM as the animals aged ($F(2,9)=24.49$, $P<0.001$; one-way ANOVA; Figure 6.3B). Bonferroni post-test revealed the lowest cell count at P10 (P10 vs. P21, $P<0.01$; P10 vs. adult, $P<0.0001$).

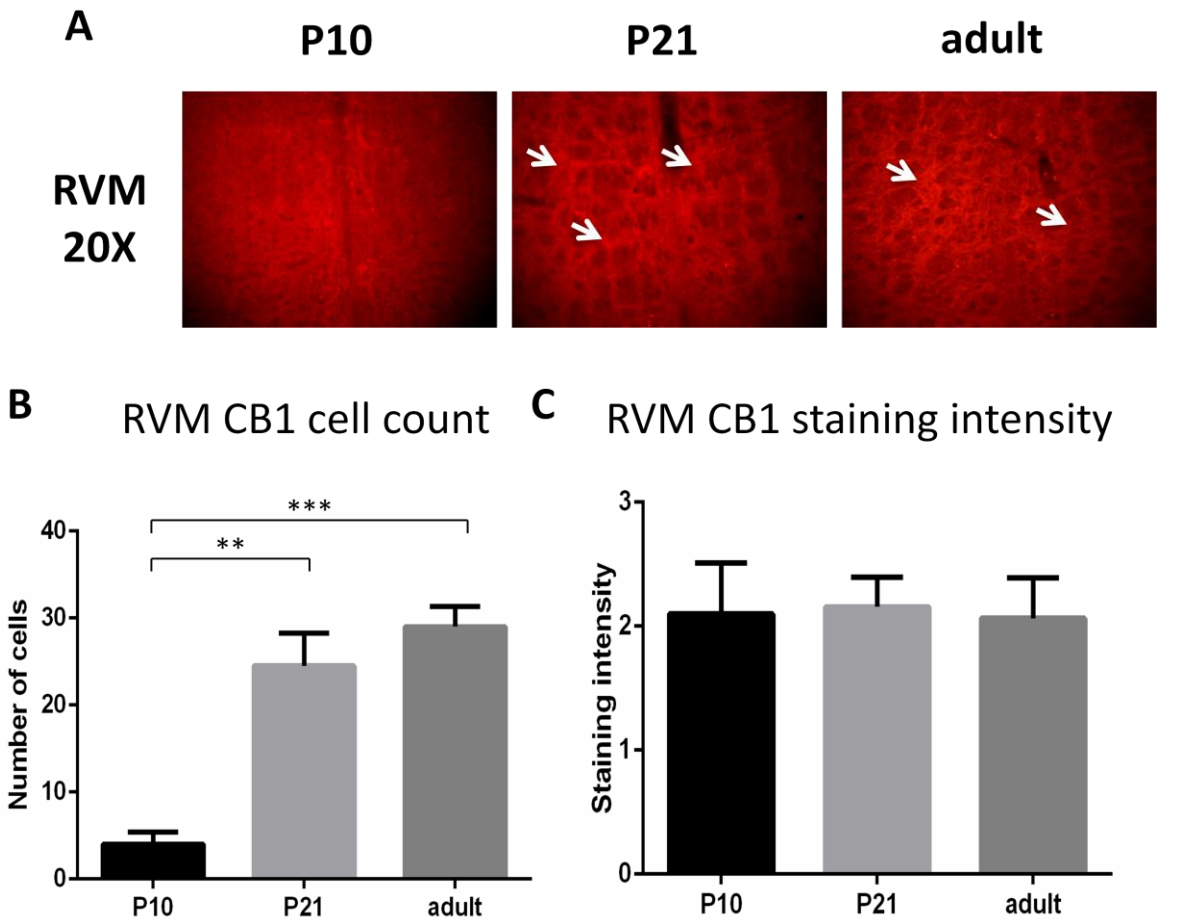


*Figure 6.3 (A) Epifluorescent images of CB1 receptor immunoreactivity in the RVM of P10, P21 and adult rats. The white arrows depict where fibre or cell body staining was observed. All images shown were captured with a 20x objectives fitted with a Confocal head. (B) Quantified CB1-positive cell count in the RVM. Number of CB1 containing cells in the RVM increases as animals aged. (C) Quantified CB1 staining intensity in the RVM. No changes were detected. Data are presented as mean ± SEM. **=P<0.01, ***=P<0.001.*

CB1 receptor immunoreactivity was seen in the superficial laminae of the DH (Figure 6.4A), only fibre staining was observed in this region. Similar to the RVM, staining intensity of CB1 receptors in the DH increased as the animals aged ($F(2,9)=25.84$, $P<0.001$; one-way ANOVA; Figure 6.4B), Bonferroni post-test revealed CB1 receptor immunoreactivity was the lowest in P10, and reached adult levels by P21 (P10 vs. P21; P10 vs. adults; $P<0.001$). In addition, there were no spatial differences in CB1 receptor staining as it was only found in the superficial laminae in all age tested, and colocalised

with CGRP immunoreactivity (Figure 6.4C). This indicated that CB1 receptors were found on peptidergic C-fibres throughout postnatal development.

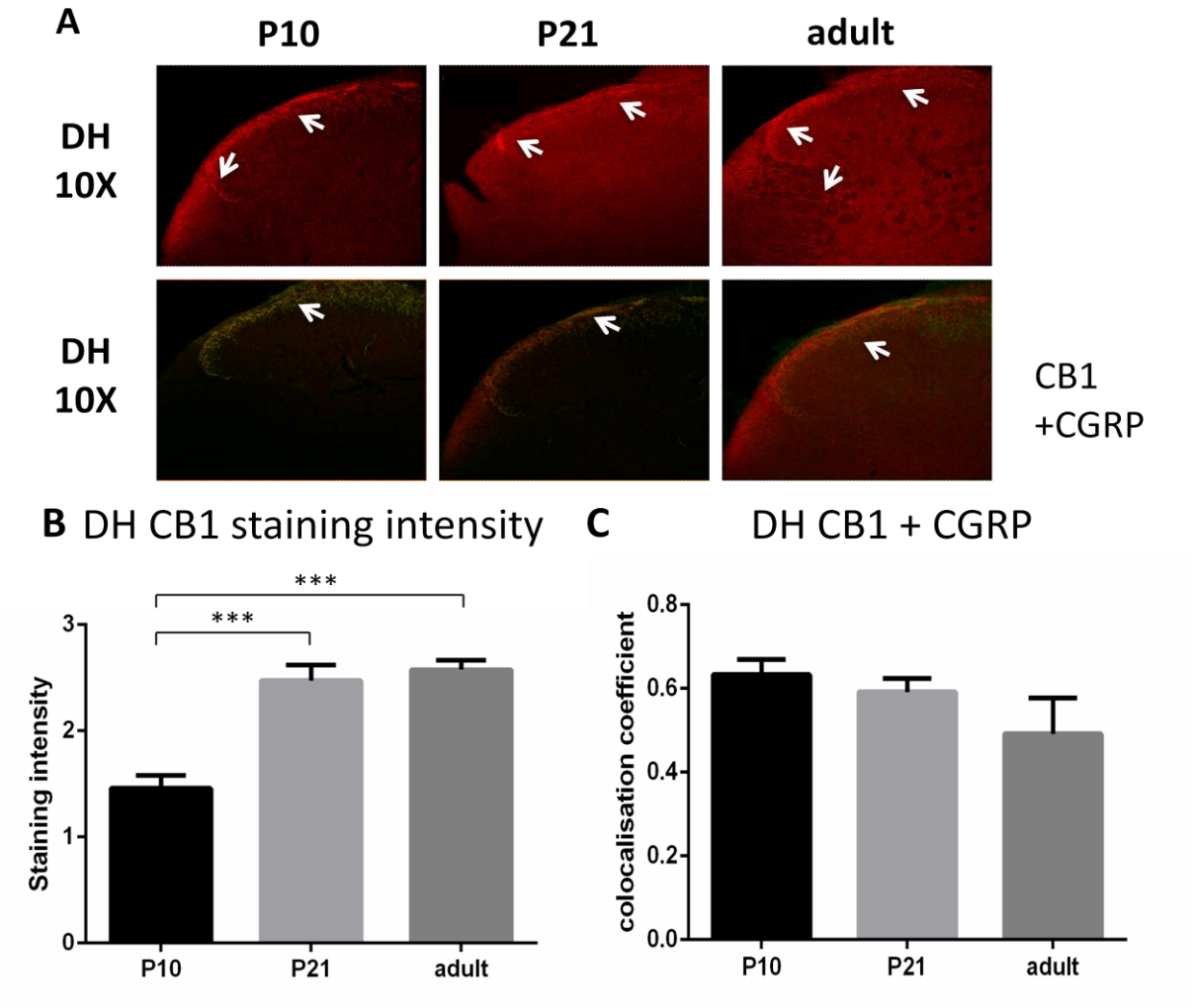


*Figure 6.4. (A) Epifluorescent images of CB1 receptor immunoreactivity in the DH of P10, P21 and adult rats. The white arrows depict where fibre or cell body staining was observed. CB1 immunoreactivity was shown in red and CGRP in green, yellow depicts colocalisation. All images shown were captured with a 10x objectives fitted with a Confocal head. (B) Quantified CB1 staining intensity in the DH. CB1 immunoreactivity in the DH increased as animals aged. (C) Colocalisation coefficient for CB1 and CGRP staining intensity in the DH, no specific changes were detected as animals aged. Data are presented as mean ± SEM. ***=P<0.001.*

There were no significant differences in the expression of CB1 mRNA (Figure 6.5) in all regions tested. Although a trend of decreasing CB1 receptor mRNA transcript was observed in the vPAG, this difference was not statistically significant ($F(2,9)=1.98$, $P=0.1936$; one-way ANOVA). CB1 receptor mRNA expression in the DH was highest at P21, but age-related differences did not reach statistical significance (Figure 6.5).

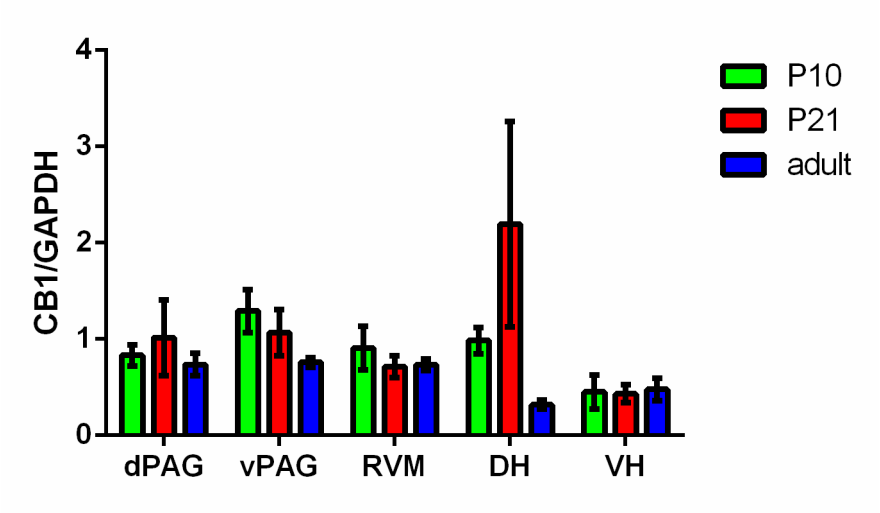


Figure 6.5. CB1 mRNA expression within the descending pain modulation circuit in P10, P21 and adult rats. CB1 expression was normalised to GAPDH. Data was presented as mean ± SEM and analysed by one-way ANOVA with Bonferroni post-tests. No statistically significant differences were observed between the ages in all regions tested.

6.4.2 Changes in NAPE-PLD expression within the descending pain pathway during postnatal development

NAPE-PLD is an essential enzyme involved in the production of anandamide. As previous reports suggest that anandamide levels increase as the animals aged, a parallel increase in the expression of NAPE-PLD was expected.

Figure 6.6A shows the spatial and temporal distribution of NAPE-PLD immunoreactivity in the PAG of P10, P21 and adult rats. Only cell body staining was observed, which suggested that NAPE-PLD was localised in neuronal cell bodies. In the PAG, NAPE-PLD immunoreactivity was higher in the vPAG compared to the dPAG, and NAPE-PLD expression exhibited postnatal refinement. Significant increase in NAPE-PLD cell body staining was found in the dPAG as the animals aged (n = 4 for P10, P21 and adults; $F(2,9)=5.208$, $P<0.05$; one-way ANOVA; Figure 6.6C). In particularly, Bonferroni post-test revealed that NAPE-PLD immunoreactivity was significantly higher in adult dPAG than P10 (P10 vs. adults, $P<0.05$). Similarly, within the vPAG, the number of NAPE-PLD positive cells increased as the animals aged (n = 4 for P10, P21 and adults; $F(2,9)=34.99$, $P<0.0001$; one-way ANOVA; Figure 6.6B), Bonferroni post-test revealed that NAPE-PLD immunoreactivity in the vPAG reached adult levels by P21 (P10 vs. P21, P21 vs. adults, both $P<0.001$).

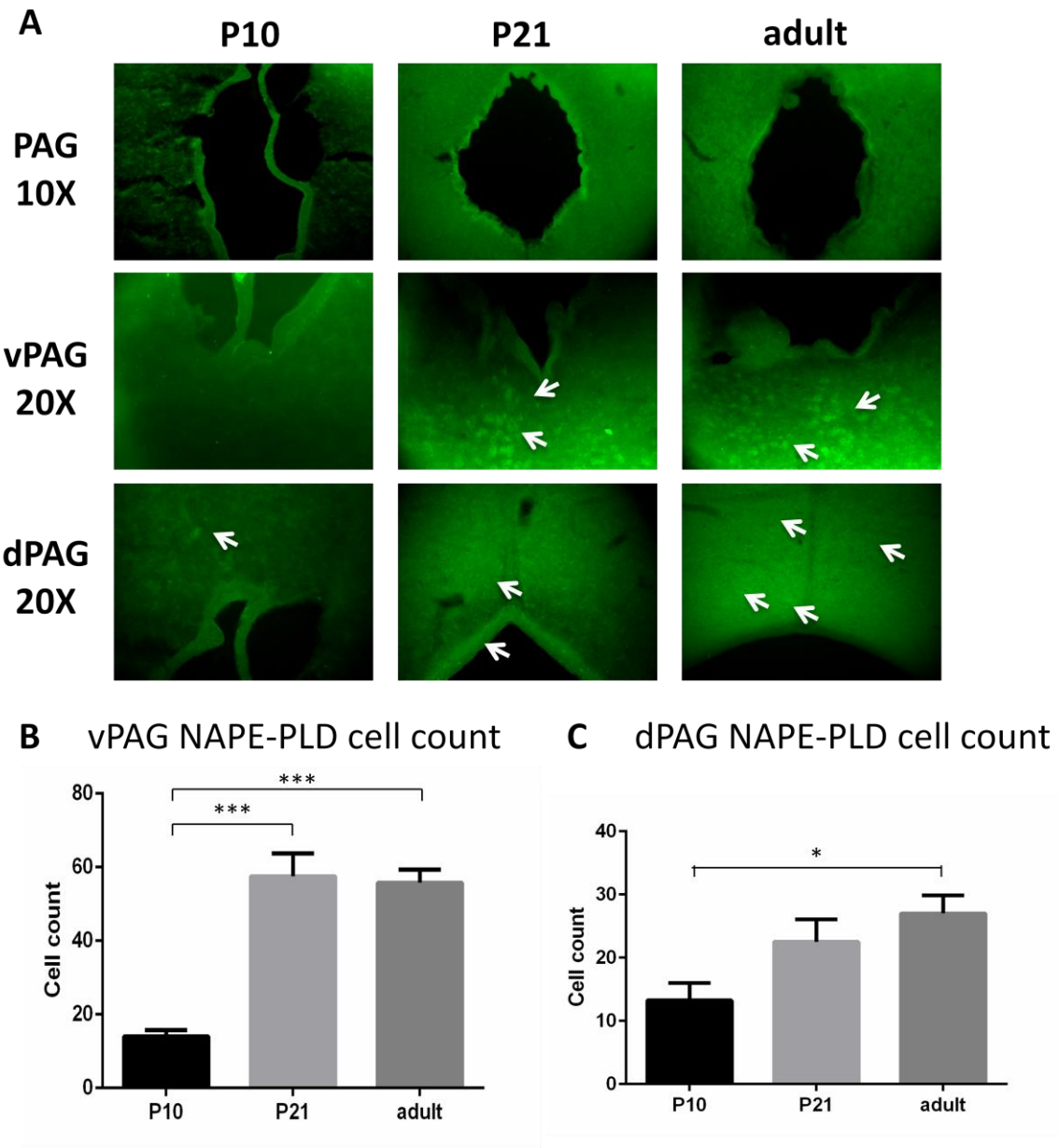


*Figure 6.6. (A) Epifluorescent images of NAPE-PLD immunoreactivity in the PAG of P10, P21 and adult rats. The white arrows depict where cell body staining was observed. All images shown were captured with either a 10x or 20x objectives fitted with a Confocal head. (B) Quantified NAPE-PLD cell count in the vPAG. (C)Quantified NAPE-PLD cell count in the dPAG. Data are presented as mean ± SEM. NAPE-PLD immunoreactivity increased as animals aged. ***=P<0.001.*

Within the RVM, NAPE-PLD expression also underwent significant postnatal refinement (Figure 6.7A). Immunohistochemical study showed that NAPE-PLD cell count was increasing as the animals aged (n = 4 for P10, P21 and adults; $F(2,9)=21.16$, $P<0.001$; one-way ANOVA; Figure 6.7B), Bonferroni post-tests revealed that NAPE-PLD immunoreactivity reached adult levels by P21 (P10 vs. P21, $P<0.001$; P10 vs. adults, $P<0.01$). Moreover, as the animals matured NAPE-PLD immunoreactivity colocalised with

CB1 (n=2 for P10, 3 for P21 and 4 for adult; F(2,6)=22.66, P<0.01; one-way ANOVA; Figure 6.7C), Bonferroni post-tests revealed that the least overlapping immunoreactivity between CB1 and NAPE-PLD in P10 rats (P10 vs. P21, P10 vs. adult; both P<0.01).

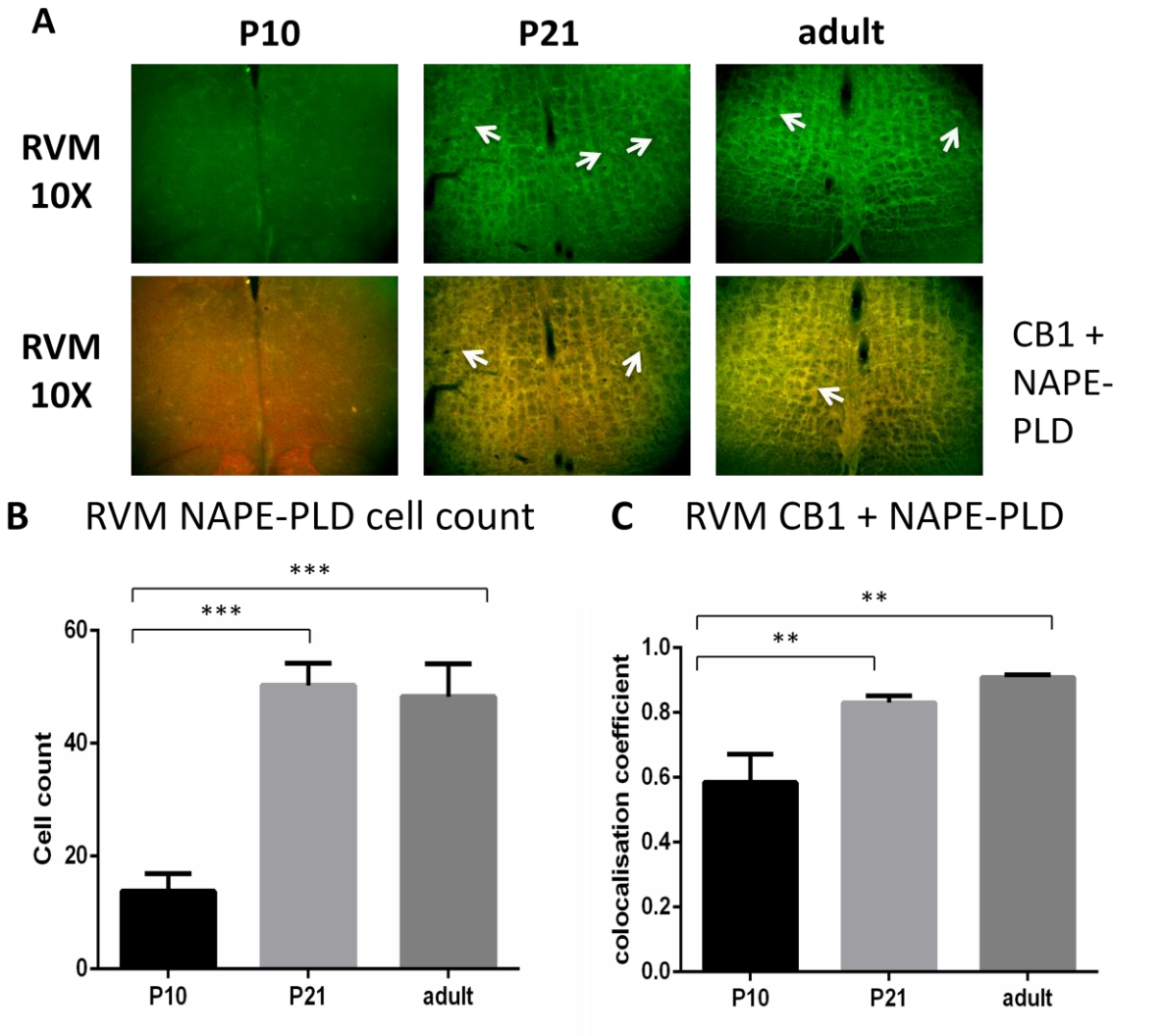


*Figure 6.7 (A) Epifluorescent images of NAPE-PLD immunoreactivity in the RVM of P10, P21 and adult rats. The white arrows depict where fibre or cell body staining was observed. CB1 immunoreactivity was shown in red and NAPE-PLD in green, yellow depicts colocalisation. All images shown were captured with a 10x objectives fitted with a Confocal head. (B) Quantified NAPE-PLD-positive cell count in the RVM. NAPE-PLD immunoreactivity in the RVM increased as animals aged. (C) Colocalisation coefficient for CB1 and NAPE-PLD staining intensity in the RVM, significant more colocalisation between CB1 and NAPE-PLD was observed as animals aged. Data are presented as mean ± SEM. **=P<0.01, ***=P<0.001.*

In the DH, immunohistochemical studies showed the NAPE-PLD staining was found in cells throughout the DH in superficial and deeper laminae (Figure 6.8A). In P10 and adult rats very few NAPE-PLD positive cells were observed, and immunoreactivity was

highest at P21 (n = 4 for P10, P21 and adults; F(2,9)=14.01, P<0.01; one-way ANOVA; Bonferroni post-tests: P10 vs. P21, P21 vs. adult, P<0.01; Figure 6.8B).

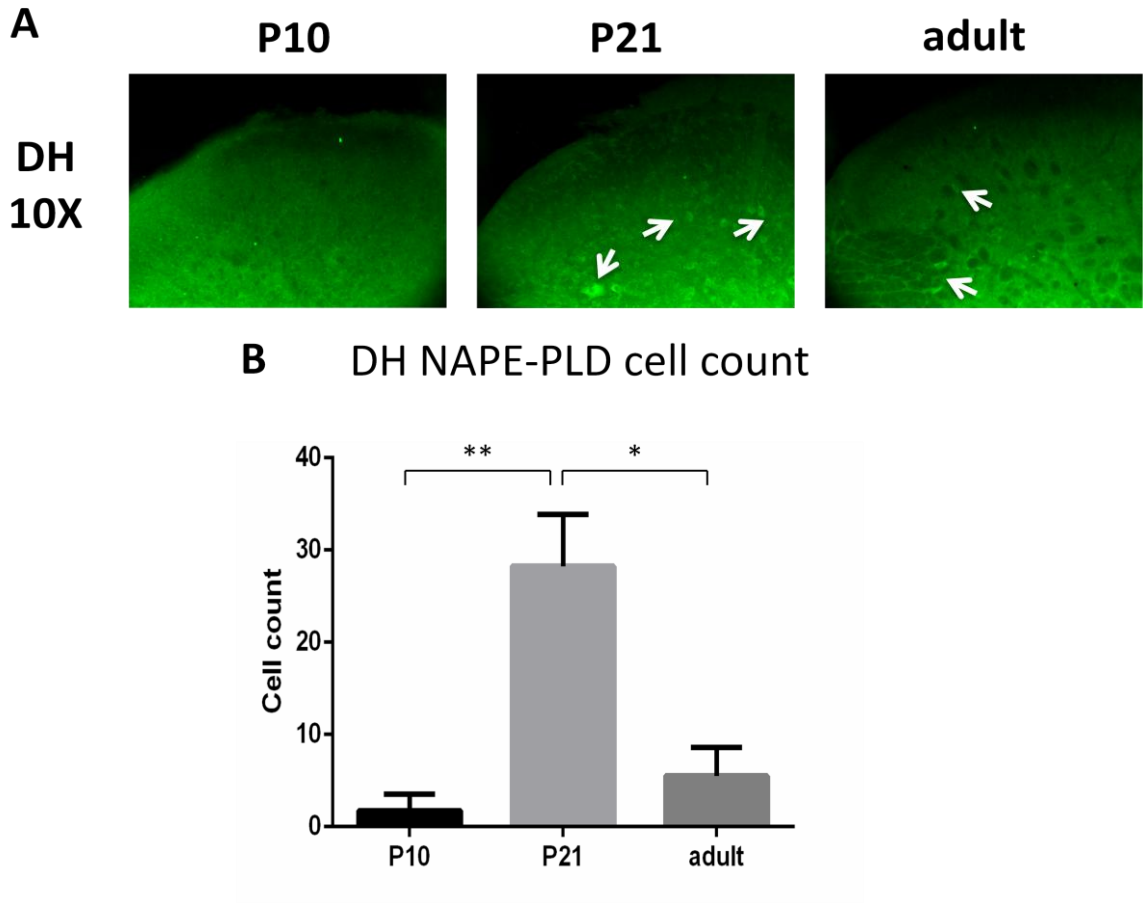


*Figure 6.8. (A) Epifluorescent images of NAPE-PLD immunoreactivity in the DH of P10, P21 and adult rats. The white arrows depict where cell body staining was observed. All images shown were captured with a 10x objectives fitted with a Confocal head. (B) Quantified NAPE-PLD staining intensity in the DH. NAPE-PLD immunoreactivity was highest at P21. Data are presented as mean ± SEM ***=P<0.001.*

Taqman RT-PCR experiment also showed an increase in NAPE-PLD mRNA transcript level in the vPAG as the animals aged (n = 4 for P10, P21 and adults; F(2,9)=4.64, P<0.05; one-way ANOVA; Figure 6.9), and Bonferroni post-tests revealed higher levels in adult when compared to P10 (P10 vs. adult, P<0.05). In the RVM, NAPE-PLD mRNA levels increased as the animals aged, however this trend was not statistically significant (n = 3 for P10 and P21, 4 for adult; F(2,7)=3.95, P=0.07; one-way ANOVA; Figure 6.9). NAPE-PLD transcript level in the DH was also highest at P21 (n = 3 for P10, P21 and adult; F(2,6)=15.12, P<0.01; one-way ANOVA; Bonferroni post-tests: P10 vs. P21, P<0.01; P21 vs. adult, P<0.05; Figure 6.9).

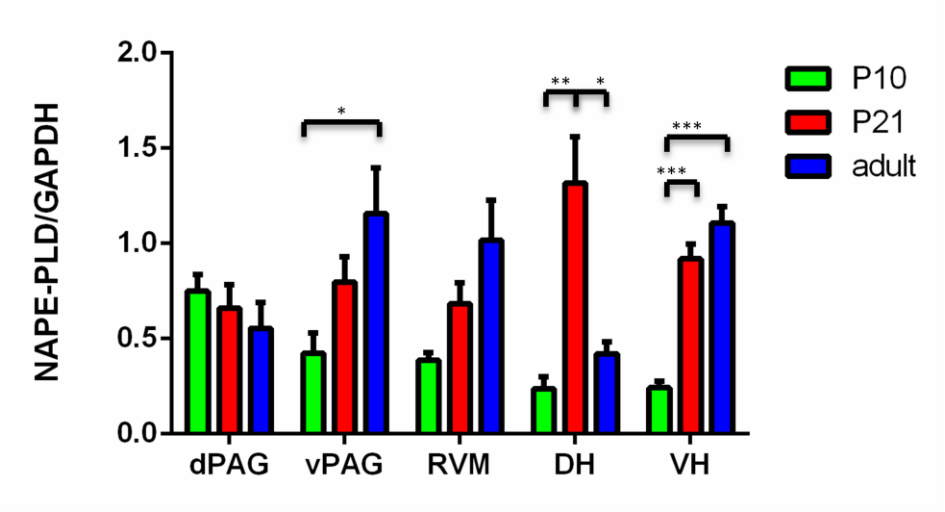


*Figure 6.9. NAPE-PLD mRNA expression within the descending pain modulation circuit in P10, P21 and adult rats. NAPE-PLD expression was normalised to GAPDH. Data was presented as mean±SEM and analysed by one-way ANOVA with Bonferroni post-tests. Within the vPAG and the VH, NAPE-PLD mRNA transcript level increased as the animals aged. In the DH, NAPE-PLD mRNA transcript number was highest at P21. *=P<0.05; **=P<0.01; ***=P<0.001.*

6.4.3 Changes in DAGLα expression within the descending pain pathway during postnatal development

DAGLα is an enzyme crucial to the synthesis of 2-AG. Since 2-AG level is high in the brain, at about 170 to 200 fold that of anandamide, it was expected to find DAGLα ubiquitously expressed in all the regions investigated. Moreover, other reports have shown that 2-AG levels sustain a similar level regardless of age.

In line with previously published studies, DAGLα was highly expressed in all regions tested using both immunohistochemical and PCR techniques. No change in DAGLα expression was found in the PAG. Immunohistochemical study showed that DAGLα staining was localised within neuronal cell bodies, and could be observed in both dPAG and vPAG (Figure 6.10A). There were no age-related differences in DAGLα immunoreactivity within the vPAG (n = 3 for P10, 4 for P21 and adult, $F(2,8)=1.86$, $P=0.23$, one-way ANOVA, Figure 6.10B).

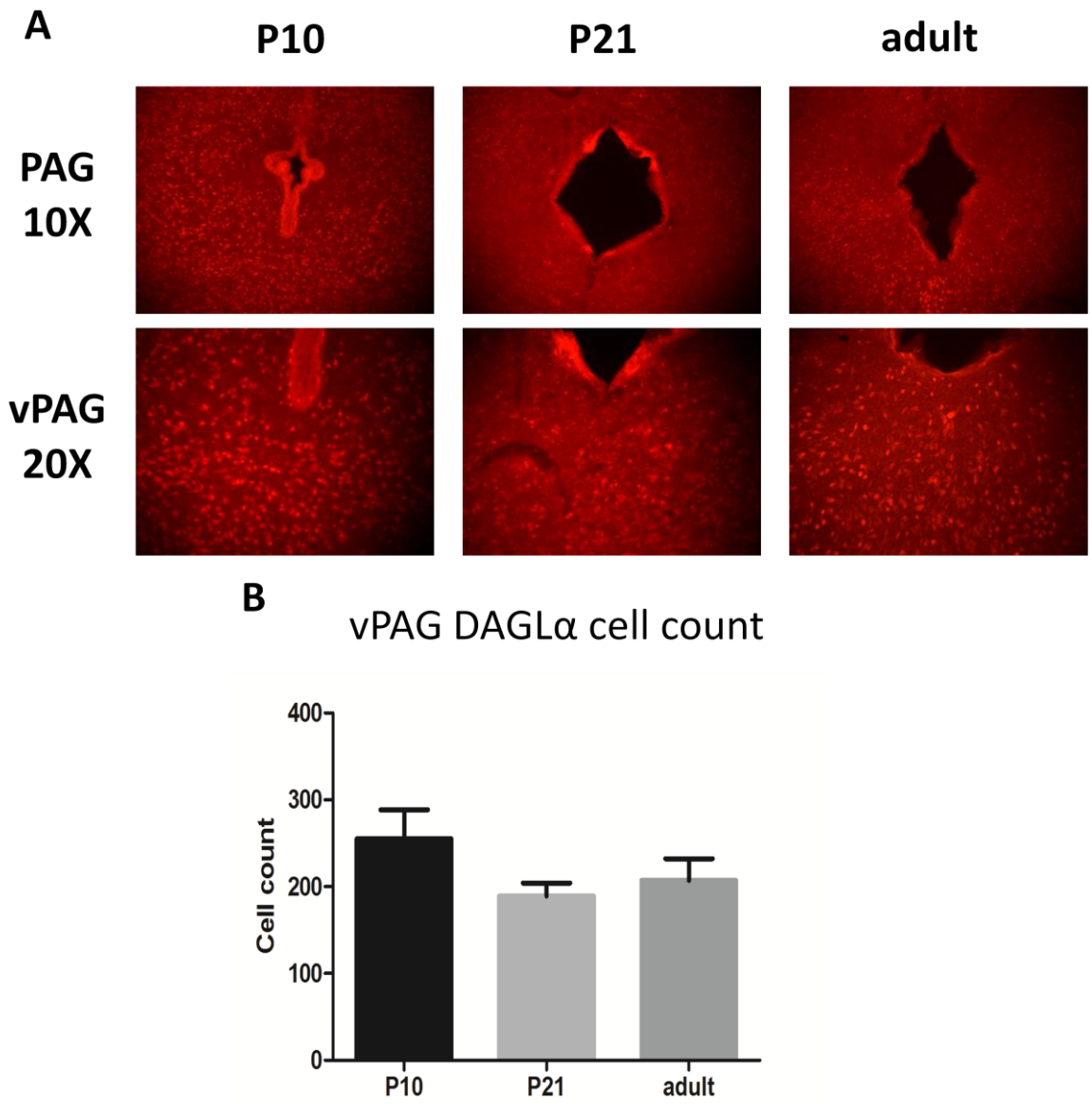


Figure 6.10. (A) Epifluorescent images of DAGLα immunoreactivity in the PAG of P10, P21 and adult rats. Cell body staining was observed in ages, ubiquituously distributed in the PAG. All images shown were captured with either a 10x or 20x objectives fitted with a Confocal head. (B) Quantified DAGLα *staining intensity in the vPAG. Data are presented as mean ± SEM. No age-related differences was observed.*

DAGLα immunoreactivity in the RVM was also found in neuronal cell bodies, it colocalised with the neuronal marker NeuN (Figure 6.11A). Intensive DAGLα staining could be observed, throughout the whole section of the RVM but particularly concentrated within the NRM region. As the animals aged, the number of DAGLα immunoreactive cells decreased (n = 3 for P10, 4 for P21 and adult; $F(2,8)=8.81$, $P<0.05$; one-way ANOVA; Figure 6.11B). Bonferroni post-tests revealed lowest cell count in P10, and by P21 DAGLα positive cells decreased to adult levels (P10 vs. P21; P10 vs. adult; both $P<0.05$).

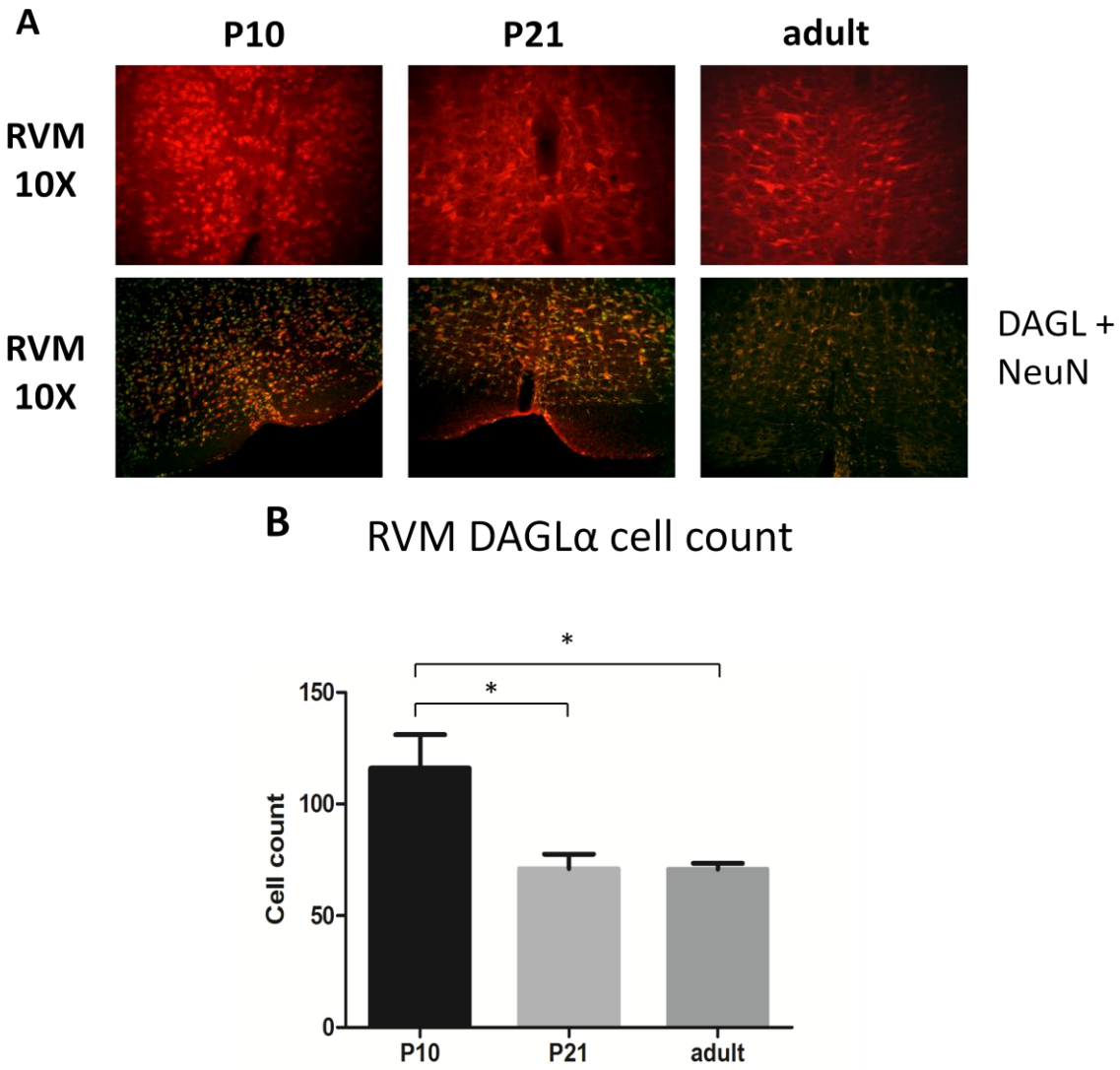


*Figure 6.11 (A) Epifluorescent images of DAGLα immunoreactivity in the RVM of P10, P21 and adult rats. DAGLα immunoreactivity was shown in red and NeuN (neuronal marker) in green, yellow depicts colocalisation. All images shown were captured with a 10x objectives fitted with a Confocal head. (B) Quantified DAGLα-positive cell count in the RVM. NAPE-PLD immunoreactivity in the RVM decreased as animals aged. Data are presented as mean ± SEM. *=P<0.05.*

In the spinal cord, DAGLα immunoreactivity was detected in the DH. At P10, there were numerous cell bodies staining in laminae I and II and the deeper laminae (IV, V). As the animals aged staining became more restricted to the deeper laminae, as significantly less DAGLα positive cells were observed in the superficial laminae of P21 and adult rats (Figure 6.12A). Quantitative analysis by cell count suggests that the number of DAGLα positive cells decreased as the animals mature (n = 4 for P10, 3 for P21 and 4 for adult; $F(2,8)=5.23$, $P<0.05$; one-way ANOVA; Figure 6.12B), and Bonferroni post-tests revealed a significant decrease of immunoreactivity in adult DH when compared to P10 ($P<0.05$).

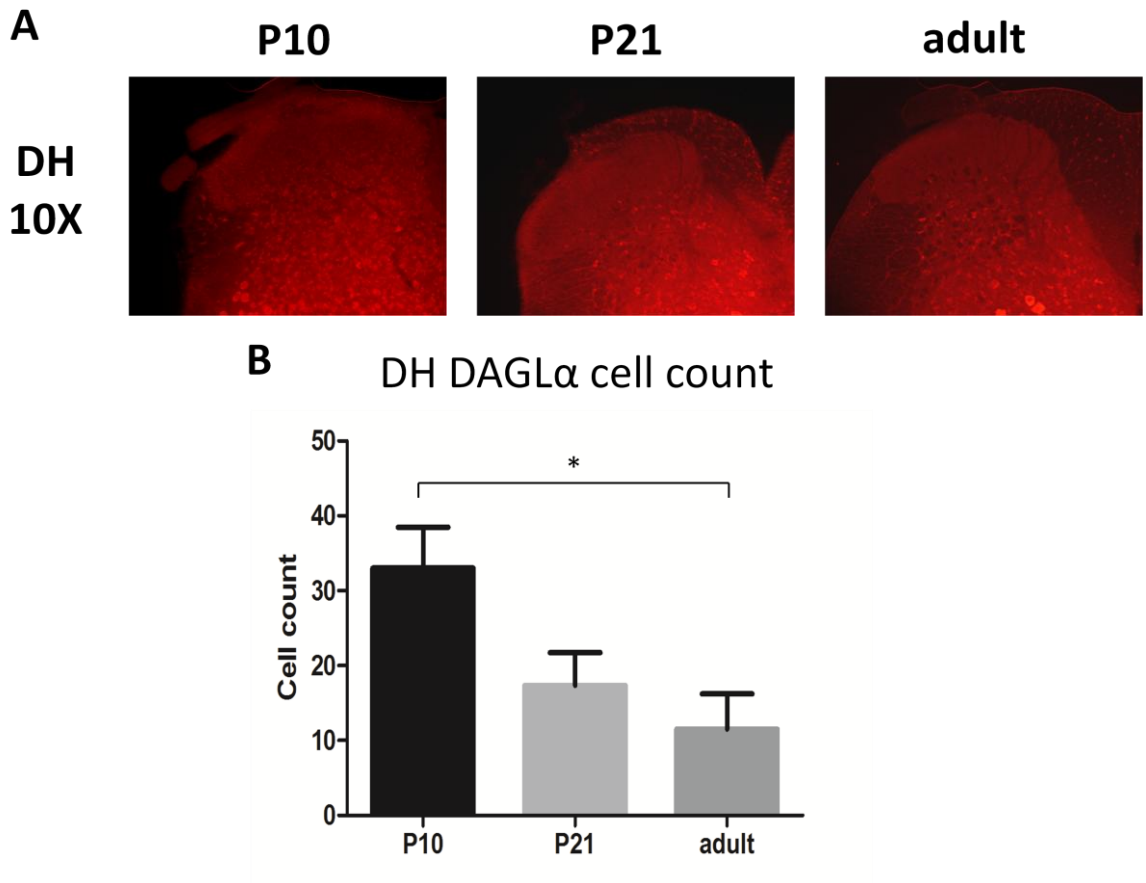


*Figure 6.12. (A) Epifluorescent images of DAGLα immunoreactivity in the DH of P10, P21 and adult rats. All images shown were captured with a 10x objectives fitted with a Confocal head. (B) Quantified DAGLα staining intensity in the DH. DAGLα immunoreactivity decreased as animals aged. Data are presented as mean ± SEM *=P<0.05.*

There were no changes in DAGLα mRNA level in the vPAG (n = 4 for P10, 3 for P21 and adult; $F(2,7)=2.25$, $P=0.18$, one-way ANOVA, Figure 6.13). However, DAGLα mRNA transcript levels decreased in the dPAG as animals aged (n=3 for P10, P21 and adult; $F(2,6)=7.56$, $P<0.05$), Bonferroni post-test revealed higher DAGLα mRNA level when compared to adults (P10 vs. adult, $P<0.05$). In the RVM, DH and VH there were no changes in DAGLα mRNA levels between the ages (Figure 6.13).

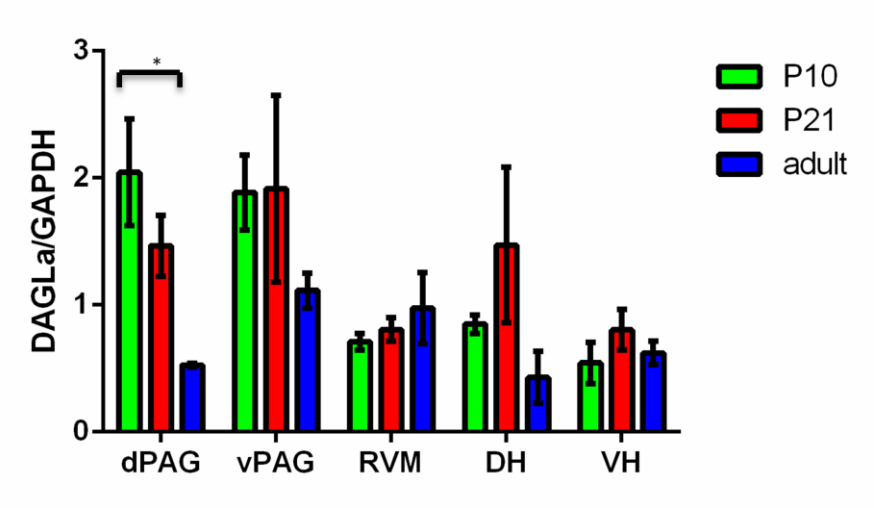


*Figure 6.13. DAGLα mRNA expression within the descending pain modulation circuit in P10, P21 and adult rats. DAGLα expression was normalised to GAPDH. Data are presented as mean ± SEM and analysed by one-way ANOVA with Bonferroni post-tests. Within the dPAG, DAGLα mRNA transcript level decreased as the animals aged. *=P<0.05.*

6.4.4 Expression of GPR55 receptors throughtout postnatal development

The expression of GPR55 receptors was investigated using Taqman RT-PCR techniques. It is known from previously published studies that GPR55 receptors are expressed in the brain. Although there were some inconsistencies about precise localisation and the amount of GPR55 protein expressed (Ryberg et al., 2007, Pietr et al., 2009), nonetheless, results obtained from Chapter 5 suggest that GPR55 receptors are functional and modulate responses to mechanical vFh stimulation, thus it was hypothesised that GPR55 receptors are expressed within the regions of descending pain modulation. It was also found in my previous study that the functions of GPR55 could differ between the younger and older rats; intra-RVM microinjection of the endogenous agonist LPI facilitated pain-related reflex responses in adults whereas its effects were inhibitory in both P10 and P21 rats. The aim of this experiment was to examine whether expression levels of GPR55 receptors are developmentally regulated.

GPR55 mRNA transcript levels were tested in the dPAG, vPAG, RVM, DH and VH. The first observation was there was less GPR55 protein expressed compared to all the other endocannabinoid-related targets (Figure 6.14). The ratio of GPR55/GAPDH mRNA was lower (0-0.8) compared to CB1/GAPDH mRNA (0-4), NAPE-PLD/GAPDH mRNA (0-2) or DAGLα/GAPDH mRNA (0-3). Also, no significant age-related differences were found, except for the RVM. Within the RVM, GPR55 mRNA transcript levels undergo significant

postnatal refinement (n = 4 for P10, 3 for P21 and 4 for adult; $F(2,8)=18.51$, $P<0.001$, one-way ANOVA, Figure 6.14), and Bonferroni post-tests revealed a surge in GPR55 mRNA at P21 (P10 vs. P21, P21 vs. adults; both $P<0.01$). There were no significant differences in the mRNA expression of GPR55 receptors between P10 and adult rats.

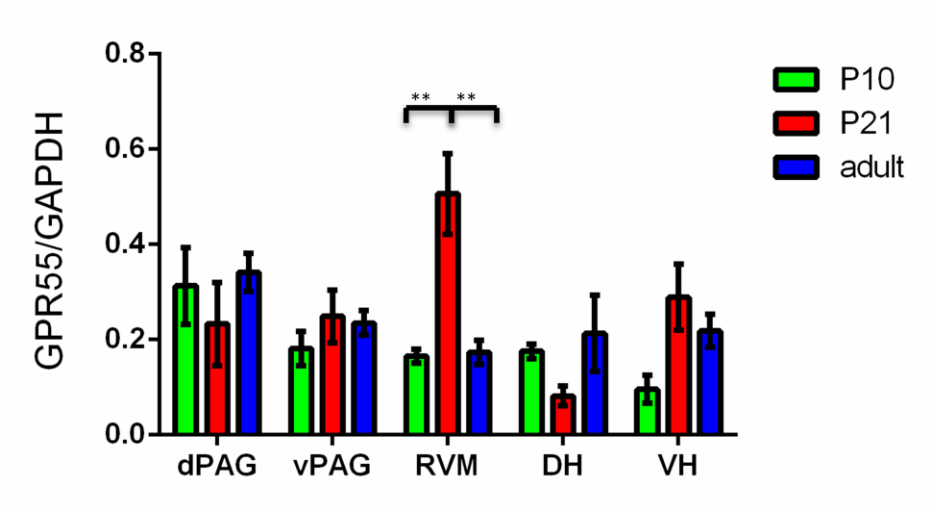


*Figure 6.14. GPR55 mRNA expression within the descending pain modulation circuit in P10, P21 and adult rats. GPR55 receptor expression was normalised to GAPDH. Data are presented as mean ± SEM and analysed by one-way ANOVA with Bonferroni post-tests. Within the RVM, GPR55 mRNA transcript level peaked at P21. **=P<0.01.*

For GPR55 immunohistochemical experiments, efforts were made to optimise the protocol because the antibody used in this study was not commercially available and only one other paper to date cited its use (Li et al., 2013b) and immuno-staining of GPR55 in the CNS was not done previously. Once the optimum conditions were set up (1:1000 primary antibody dilution, with TSA), a small trial was carried out in the PAG of P10, P21 and adult PAG (Figure 6.15). Due to insufficient amount of primary antibody, only one brain per age was processed. In the P10 and adult PAG, there was very little GPR55 immunoreactivity in the vPAG. However, some fibre and cell body staining could be observed in P21 rats.

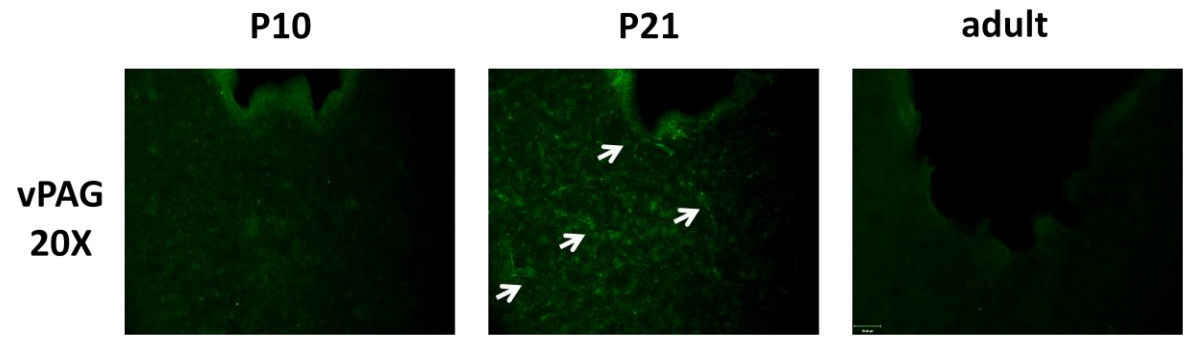


Figure 6.15. GPR55 immunoreactivity in the vPAG of P10, P21 and adult rats. White arrows depict where specific staining was found. In both P10 and adult vPAG, no GPR55 positive staining was observed. However, GPR55 immunoreactive fibres and cell bodies could be observed in P21 vPAG. All images were captured with a 20x objective attached to a confocal head.

Upon the arrival of the second batch of antibody, GPR55 receptor immunohistochemistry was also performed in formaldehyde-fixed adult rat spleen tissue as a positive tissue control, where high levels of GPR55 receptor mRNA were reported (Oka et al., 2010). Negative controls, omitting either the primary or the secondary antibody were also included. Using the conditions previously determined, it was found that GPR55 antibody on its own showed no immunoreactivity (Figure 6.16, GPR55 no 2nd 2.5X), but staining was observed with TSA incubation (Figure 6.16, TSA only 2.5X). Moreover, the staining for GPR55 antibody with TSA (Figure 6.16, GPR55 + TSA 2.5X, 10X) and TSA alone did not look different, hence it was concluded that there might be endogenous peroxidase activity in the spleen tissue.

The next trial was performed with an additional hydrogen peroxide blocking step, in order to quench endogenous peroxidase activity and reduce non-specific background staining. However, the addition of hydrogen peroxide prior to primary antibody incubation did not improve the staining, as non-specific binding was found in both of the negative (no primary or secondary) controls and the background signal became higher after hydrogen peroxide block compared to the first trial (Figure 6.16, bottom four images). The results from this trial suggest that apart from peroxidase activity, there might also be endogenous biotin activity in the spleen tissue. This issue will need to be investigated further in future studies, and the necessary steps will be described in the following *Discussion* section.

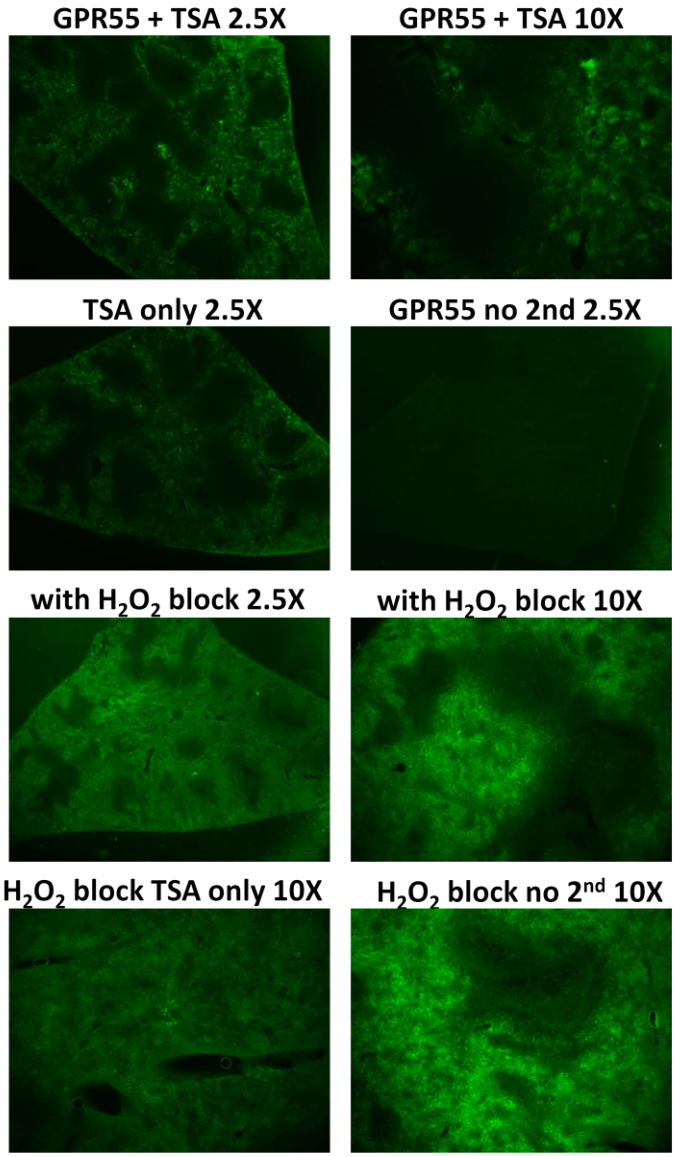


Figure 6.16. GPR55 immunoreactivity in adult rat spleen tissue. The top four images show sections without hydrogen peroxide (H_2O_2) blocking steps, and the bottom four show images with H_2O_2 blocking steps. Non-specific background staining was found in all of the stated conditions, which confounded results. All images were captured with 2.5X or 10X objective fitted on a timelapse fluorescent microscope.

6.5 Summary

The data in this thesis showed that the endocannabinoid system within the descending pain modulation pathway undergoes significant postnatal development. The expression of CB1 receptors changes throughout postnatal development and these changes are site specific: CB1 receptor expression decreased in the vPAG but increased in the RVM and DH. The expression of NAPE-PLD increased in the vPAG, RVM and DH and reached maturity by P21. The expression of DAGLα decreased in the dPAG, RVM and DH and

reached maturity by P21. The expression of GPR55 receptors remained to be fully elucidated, but preliminary data suggest that GPR55 receptors are expressed in the RVM and are most abundant at P21.

6.6 Discussion

The endocannabinoid system is essential for normal brain maturation, its involvement in corticolimbic development and the relevant molecular pathways and mechanisms were described elsewhere (Keimpema et al., 2011). Since the endocannabinoid system is also a major component in pain processing (Kinsey et al., 2009), it was surprising that research in its role in the developing pain system is currently lacking. Based on the findings reported in the previous chapter, cannabinoid receptors are functional in the early postnatal period through to adulthood, but are subject to considerable developmental regulation. Of particular interest is that GPR55 receptors emerged as a possible analgesic target only for neonatal and P21 animals. In this study, the expression of a variety of components within the endocannabinoid signalling system within the major regions of the descending pain signalling pathway was investigated.

The expression of endocannabinoid related proteins and mRNA were included in this study to fully characterise the regulation of the specific targets at both transcriptional and translational levels. The targets (CB1, NAPE-PLD, DAGLα and GPR55) were chosen to give a comprehensive representation of this system. The expression of synthetic enzymes NAPE-PLD and DAGLα could be indirectly correlated to anandamide and 2-AG. It would be beneficial to include the degrading enzymes FAAH and MAGL in future studies, which would provide researchers with further information about anandamide and 2-AG levels in the descending pain pathways.

6.5.1 Alterations in the expression of CB1 receptors during postnatal development

It was shown that CB1 receptor expression undergoes significant postnatal modifications. In line with previous published findings (Berrendero et al., 1999, Fernandez-Ruiz et al., 2004), CB1 immunoreactive fibres (Figure 6.2A) were found closely along the lining of the aqueduct, and staining intensity decreased as the animals aged. The loss of CB1 receptor immunoreactivity may be a natural process resulting from postnatal apoptosis, as there were no significant differences between the functions of CB1 receptor activation between young and older rats (*Chapter 5, section* 5.4.1). On the other hand, CB1

immunoreactivity in the RVM and DH increased as the animals matured. Interestingly, the analgesic effect of intra-RVM and spinally applied CB1/CB2 receptor agonist HU210 decreased as the animals aged (*Chapter 5, section 5.4.5*). Since CB1 receptors are mostly located on GABAergic neurones (Tsou et al., 1998), the difference in analgesic potency may be indirectly linked to an altered GABAergic transmission, as modulated by CB1 receptors. Further exploration of the relationship between CB1 and GABA immunoreactivity in the developing pain pathways would be of great interest.

6.5.2 Alterations in the expression of NAPE-PLD during postnatal development

The expression of NAPE-PLD increased in all regions tested as the animals aged, which supports the view that anandamide levels increase during postnatal maturation (Berrendero et al., 1999). The number of NAPE-PLD immunopositive cells in the vPAG and RVM reached adult levels by P21 (Figure 6.6A, 6.7A). NAPE-PLD mRNA levels also steadily increased throughout postnatal development in the vPAG (Figure 6.9). Interestingly, both TaqMan RT-PCR and immunohistochemistry experiments indicated that NAPE-PLD expression in the DH is highest at P21. As mentioned in previously published studies (Hathway et al., 2012, Kwok et al., 2013), around P21 is the critical period for postnatal maturation of descending pain modulation; the finding that NAPE-PLD reaches adult level by P21 strongly suggest a trophic role for both NAPE-PLD and anandamide.

6.5.3 Alterations in the expression of DAGLα during postnatal development

The synthetic enzyme of 2-AG, contrary to the initial hypothesis, also appears to undergo significant postnatal refinement. No changes were detected in the vPAG, but TaqMan RT-PCR experiments showed that DAGLα mRNA levels decreased in the dPAG as the animals aged (Figure 6.12). In immunohistochemical experiments the number of DAGLα immunoreactive cells decreased in the RVM and DH, and reached adult levels by P21 (Figure 6.10A, 6.11A). These results suggest that 2-AG levels decrease within the descending pain pathway during postnatal development, and reach mature levels by P21. On one hand, since DAGLα expression is mostly neuronal the loss of DAGLα might be related to the loss of neurones during postnatal development (*Chapter 4, section 4.4.1*). On the other hand, the loss of DAGLα might be necessary for the development of appropriate synaptic formation. To test this, future studies could examine the effects of increasing 2-AG levels on nociceptive processing by chronic inhibition of MAGL; a suitable candidate for this would be the MAGL inhibitor JZL184 (Long et al., 2008).

6.5.4 Expression of GPR55 receptors in the descending pain pathways during postnatal development

The expression of GPR55 receptors within the descending pain modulation pathway was investigated. GPR55 mRNA was detected in all the regions tested (Figure 6.13). The expression was lower compared to other endocannabinoid related targets. Within the RVM, GPR55 mRNA peaked at P21, which again echoes the theme that P21 is a critical timepoint when developmentally regulated changes occur. In this study, immunohistochemical localisation of GPR55 receptors was attempted but this particular area of research is still in a very early stage. Using a very small sample size, GPR55 immunopositive cells were found in the vPAG of P21 rats. However, this is only preliminary data and more work needs to be done to verify the precise localisation of GPR55 receptors. It would also be interesting to examine the levels of the endogenous GPR55 ligand LPI throughout postnatal development, as it would provide some indication on endogenous GPR55 mediated activity during maturation. Therefore, it is not possible at this stage to determine whether the functional switch in GPR55 mediated actions between young and older rats corresponds to the expression of GPR55 receptors during postnatal development.

The most difficult issue was overcoming the technical challenges of utilising a non-commercially available antibody, as conventional immunohistochemistry methods were not suitable for this experiment and there were no other successful protocols available. Several problems were encountered, including the failure to reduce background non-specific staining and blocking endogenous peroxidase/biotin activity in the tissue. It is also possible that the detection system (TSA) used in this study is not suitable for the antibody. It may be beneficial to use other systems such as 3,3'-diaminobenzidine (DAB), or other techniques such as Western blotting and *in situ* hybridization. The rationale behind immunofluorescence was that it enables the precise anatomical localisation of GPR55 proteins, as methods such as Western blotting utilise brain homogenates and will, therefore, be less precise.

6.5.5 Conclusion

All the endocannabinoid-related components tested in this study showed significant expressional changes during postnatal development. The anandamide synthesising enzyme NAPE-PLD became more prominently expressed as the animals aged, whereas the 2-AG synthesising enzyme DAGLα decreased. Both of these events occurred at around P21, which provided further evidence in suggesting that this age is the critical period for nociceptive processing. CB1 receptors in the RVM and DH also became more prominently expressed in the older rats, which is fitting with the observation that as

animals mature, the output of descending modulation becomes more inhibitory (please see review (Fitzgerald, 2005)). It would be beneficial to include CB2 receptors in future studies, in order to fully elucidate the underlying molecular circuitry behind the differential cannabinoid-mediated functions between the ages. GPR55 experiments showed some promising results; future work into the precise localisation of GPR55 receptors in the developing nociceptive circuit will strengthen some of the findings reported in this thesis.

Chapter 7 General Discussion

7.1 Introduction

Historically neonatal pain was clinically under-treated due to the misguided belief that neonates do not experience pain to the same extent as adults at a cortical level, and therefore are incapable of appreciating pain as harmful (Hatch, 1987). Clinicians were also reluctant to prescribe analgesics over concerns of the potential harmful effects analgesic drugs would have on developmental processes (Hatch, 1987, Anand and Soriano, 2004). Recent advances in developmental neurobiology have provided powerful evidence in disputing these misguided believes (Grunau and Craig, 1987, Fitzgerald and Jennings, 1999, Fitzgerald and Walker, 2009). It is now known that neonatal nociceptive responses are misdirected and exaggerated (Grunau and Craig, 1987, Andrews and Fitzgerald, 1994, 1999, Granmo et al., 2008), and patients who received adequate analgesics have improved behavioural and cognitive outcomes compared to those that did not (Anand et al., 1999b, van Lingen et al., 2002). As demonstrated by previous studies, the fine-tuning of central nociceptive processing depends on the intrinsic activity of neurotransmitter systems (Fitzgerald, 1987b, Beggs et al., 2002, Granmo et al., 2008, Beggs et al., 2012). In this thesis I have investigated the anatomical distribution of the opioid and endocannabinoid signalling systems, and used hindlimb flexion responses as a measure of spinal excitability to examine the functions of these two systems within descending pain pathway (PAG, RVM and DH) on spinal reflex excitability during postnatal development of the rat (P10, P21 and adult).

By studying flexion responses to noxious thermal and mechanical stimuli, infants were found to often move towards the source of stimulus rather than pulling away from it (Andrews and Fitzgerald, 1994). Moreover, animal studies have shown that in neonatal rats, thermal nociceptive thresholds are considerably lowered in comparison to adults, and hypersensitivity to suprathreshold noxious stimuli is seen in neonates up to P15 (*Falcon et al., 1996*). Generalised movements in all limbs were observed after low intensity skin stimulation on the hindpaw in P8 rats (*Fitzgerald and Jennings, 1999*). The inappropriate and exaggerated flexion responses observed in early life are underpinned by increased excitability and decreased inhibition at the level of the DH. During the first few postnatal weeks the DH is dominated by A-fibre mediated input (Fitzgerald, 1985); C-fibres although functional and mature from birth, penetrate the central grey later than A-fibres (Baccei et al., 2003). Consequently, the DH in the first few postnatal weeks is disorganised: specifically the superficial DH during this period is dominated by A-fibre inputs (Beggs et al., 2002, Granmo et al., 2008), and low threshold stimuli can activate spinal circuits and contribute to hyperexcitability within the DH. In addition, neurones in the neonatal DH fire spontaneously (Li et al., 2009, Li and Baccei, 2011), and GABAergic

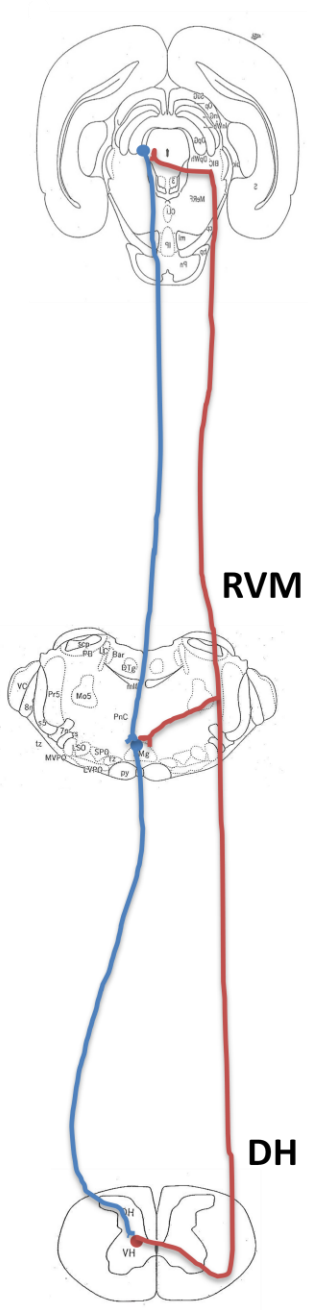
processing in neonatal animals mediates excitatory effects rather than inhibitory (Hathway et al., 2006).

Excitability in the DH is partly governed by descending input from supraspinal sites (PAG and RVM). The PAG controls DH activity indirectly via the RVM, and the overall output of this pathway can be both facilitatory and inhibitory. This can be demonstrated by SPA, where low intensity electrical stimulation in the PAG and the RVM is pronociceptive, and high intensity stimulation mediated inhibitory effects on pain-related behaviours (Behbehani and Fields, 1979). It was shown that at the third postnatal week, focal electrical stimulation in the RVM of the rat produced pronociceptive behaviours. Regardless of the stimulation intensity applied, at P21 flexion responses and firing activity in the DH are never inhibited by RVM stimulation (Hathway et al., 2009). Moreover, a recent study revealed that opioidergic signalling in the RVM plays a significant role in the maturation of supraspinal nociceptive circuits (Hathway et al., 2012). Firstly, it was observed that intra-RVM microinjection of MOR agonist DAMGO facilitates EMG responses to mechanical stimulation in P21 rats, whereas DAMGO in adult RVM substantially reduces EMG activity. Secondly, mature animals that received systemic naloxone between P21-28 do not develop the normal biphasic responses to mechanical stimuli with electrical RVM stimulation: the nociceptive circuits remains hyperexcitable, as inhibition of nociceptive behaviours are absent in these rats (Hathway et al., 2012).

The results from these studies raised a few interesting questions, which this thesis aimed to address: the first one being the influence of immature PAG projections over spinal excitability. There had been few attempts to address this issue: it is known that SPA in the PAG does not occur until P21 onwards in rats (Van Praag and Frenk, 1991). Another study, which injected opioidergic ligands into the PAG both dorsally and ventrally in neonatal rats reported analgesia, however, there were also some confounding issues within the design of the study: i) behavioural tests might not be the most suitable because of long period of maternal separation, ii) recovery time in this study was short, so neonatal animals were subjected to multiple painful procedures with little recovery time (Craig et al., 1993, Grunau et al., 2006a, Holsti and Grunau, 2010). Secondly, if the opioidergic signalling system undergoes significant postnatal refinement, and plays a crucial role in mediating the maturation of the descending pain modulation system, to what extent is the endocannabinoid system involved, given i) it is known to have a role in neurogenesis and synaptogenesis, processes that are essential in the development of the CNS and ii) contributes to pain modulation in the normal matured animals.

7.2 Summary of findings

The aim of this thesis ass to further our understanding on how the descending pain pathway matures, and demonstrate plasticity by showing how neurotransmitters adapt during the postnatal period to achieve fine-tuned nociceptive responses. A summary of all the major findings described in this thesis is included in *Figure 7.1*. Both opioid and endocannabinoid signalling systems within the descending pain pathway undergo significant postnatal refinement. Some parallel changes between these two neurotransmitter systems were observed; spinally applied opioids and cannabinoids were most antinociceptive in P10 animals, and protein expression of MOR and CB1 receptors in the DH increased as animals aged. In addition, expression of endogenous opioids and endocannabinoid-synthesising enzymes within the regions of the descending pain pathway increased throughout postnatal development. On the other hand, some functional and anatomical differences between these two systems were found. Activation of endocannabinoid signalling via CB1 or CB2 receptors was antinociceptive in all ages and all regions investigated, whereas activation of opioidergic signalling in the PAG before P21, and in the RVM at P21 are pronociceptive. Moreover, there were no changes in the expression of MOR in the supraspinal sites, but expression of CB1 receptors decreased in the PAG and increased in the RVM. In general, although data in this thesis suggest that the opioid and endocannabinoid signalling system mature at a slightly different rate from each other throughout postnatal development, as animals mature, endogenous ligands become more readily available. Consequently, neurochemical transmission within the descending pain pathway develops to be more efficient and balanced pain modulation is achieved. It is also important to note that results from the thesis highlighted P21 as a critical timepoint for postnatal development of pain modulation. Most of the functional and anatomical changes in the opioid and endocannabinoid signalling systems occurred at around P21: for example, 1) activation of MOR in the PAG of adult rats produced analgesic effects whereas it was pronociceptive in P21, concurrently the expression of POMC dramatically increased and reached adult level at P21; 2) activation of GPR55 receptors in the RVM before P21 was antinociceptive whereas in adults the effects were pronociceptive. P21 rats are classified as pre-adolescent and they are often weaned at this age (Ferdman et al., 2007). It is known that other physiological and behavioural changes, such as social interaction and feeding patterns (Kennedy, 1957, Herbst and Sunshine, 1969) occur during the weaning stages. The effects of weaning were not addressed in this thesis, as all P10 and P21 rats used in this thesis were taken before weaning occurs, but future work investigating the relationship between weaning and pain modulation will be beneficial.



PAG

	P10	P21	Adult
Opioids	• Agonism and antagonism of MOR has no effect on nociceptive responses • Expression of endogenous opiates is lowest	• Agonism of MOR is pronociceptive • Endogenous opiate level increase dramatically and reach maturity	• Agonism of MOR is antinociceptive whereas antagonism of MOR is pronociceptive •High levels of endogenous opiates
Cannabinoids	• Activation of CB1 and CB2 receptors is antinociceptive • Activation of GPR55 receptors is also antinociceptive • Expression of CB1 receptor is high, but low for NAPE-PLD	• Activation of CB1 and CB2 receptors is antinociceptive • Activation of GPR55 receptors is also antinociceptive • Expression of CB1 receptors decrease whereas NAPE-PLD increase and reach adult levels	• Activation of CB1 and CB2 receptors is antinociceptive • Activation of GPR55 receptors has no effect on nociceptive responses • High levels of endocannabinoid-synthesising enzymes

RVM

	P10	P21	Adult
Opioids	• Agonism of MOR is antinociceptive, but antagonism of MOR has no effect on nociceptive responses • concentration of endogenous opiates is lowest • MOR mRNA level is also lowest	• Agonism of MOR is pronociceptive (Hathway, Vega-Avelaira et al. 2012) • Endogenous opiate level is the highest	• Agonism of MOR is antinociceptive (Hathway, Vega-Avelaira et al. 2012) • High levels of endogenous opiates, but are decreased compared to levels seen at P21
Cannabinoids	• Activation of CB1 and CB2 receptors is antinociceptive • Activation of GPR55 receptors is also antinociceptive • Expression of CB1 receptor and NAPE-PLD are low	• Activation of CB1 and CB2 receptors is antinociceptive • Activation of GPR55 receptors is also antinociceptive • Expression of CB1 receptors and NAPE-PLD increase, and reach adult levels	• Activation of CB1 and CB2 receptors is antinociceptive • Activation of GPR55 is pronociceptive • High levels of endocannabinoid-synthesising enzymes

DH

	P10	P21	Adult
Opioids	• Agonism of MOR is antinociceptive and most efficacious • Expression of POMC is highest, but lowest for MOR and Enkephalin	• Agonism of MOR is antinociceptive • Expression of POMC dramatically decrease, but MOR and enkephalin level increase and reach maturity	• Agonism of MOR is antinociceptive but less efficacious compared to neonatal animals • High levels of endogenous enkephalin, MOR expression in the DH is prominent
Cannabinoids	• Activation of CB1 and CB2 receptors is antinociceptive and most efficacious • Expression of CB1 receptors and NAPE-PLD is low, but high for DAGLα	• Activation of CB1 and CB2 receptors is antinociceptive • Expression of CB1 receptors and NAPE-PLD increase, and reach adult levels by this age • Expression of DAGLα decrease and reach adult levels	• Activation of CB1 and CB2 receptors is antinociceptive • High levels of CB1 receptors and NAPE-PLD on nociceptive neurones in the DH

Figure 7.1 The panel on the left shows a simplified version of the spino-bulbar pathway for pain modulation: descending pain pathway is in blue whereas the ascending pain pathway is in red. Age-related changes in the opioid and cannabinoid signalling systems are illustrated in the box on the right, both functional and anatomical differences between the ages within the regions (PAG, RVM and DH) are described.

7.3 Experimental considerations

7.3.1 Animals

The ages chosen for this thesis were P10, P21 and adult (older than P40, body weight 180-220g). All in vivo electrophysiological experiments were performed in rats that were ± 2 days from the age, and anatomical experiments were performed in rats that were precisely at those stated ages. Other studies have used rats in the intermediate age range, such as P3, P7 and P14 (Barr and Wang, 2012, Walker, 2013). It was hypothesised that most changes, particularly within supraspinal sites, occur at around P21, the pre-adolescent period of the rat (Hathway et al., 2009, Hathway et al., 2012). Therefore, the ages chosen represent a full range of developmental timepoints during postnatal development. Moreover, animals were all tested prior to weaning; the effect of stress, particularly from maternal separation, could thus be limited.

It is strongly argued that sex differences in pain exist (Greenspan et al., 2007), which are mediated by gonadal steroid hormones (Craft et al., 2004). In humans, the mean threshold, tolerance and perceived unpleasantness to noxious thermal stimuli are lower in women (Wiesenfeld-Hallin, 2005). Similar findings are reported in animal models: durations of licking and flexing after subcutaneous formalin (10%, 50μL) injection into the hindpaws were longer in female compared to male rats (Aloisi et al., 1994). On the other hand, the effect of sex differences on pain perception in the early postnatal period is less well understood. Some studies have shown that tail flick latencies in the hot plate test were significantly shorter in young female rats (Bhutta et al., 2001). However, using the incision pain model, it was reported that mechanical hindpaw sensitivity did not differ between the two sexes in adult mice (Banik et al., 2006) and neonatal (P3 to P14) rats (Beggs et al., 2012). After careful consideration, it was decided that experiments in this thesis should be performed in littermates, including both male and female pups. The advantages of this are effects of between litter differences can be eliminated and the number of animals used is reduced.

7.3.2 Intra-PAG or intra-RVM injection volumes

The primary concern when performing intra-cerebral injections in animals of different postnatal ages was the variation in the size of brains. Previous study have shown that brains from P21 and adult rats are of comparable sizes and do not differ in either rostrocaudal or mediolateral dimensions (Hathway et al., 2009). The injection volume for P10 rats were determined by previously published studies (Szaflarski et al., 1995). Taking into account the effect of diffusion into other brain sites, solubility of drugs and previous published studies, it was decided that 1μL was a suitable injection volume. A

small needle (26 gauge) was selected and the same procedure was used at each postnatal age to insure a localised delivery of drug without systemic spread. Great care was taken to each injection and all injection sites were examined post-mortem, only the ones that reach the target sites were included in the analysis.

7.3.3 Choice of drugs

In this thesis mu opioid receptors were targeted because firstly, morphine (primarily a mu opioid receptor agonist) is the gold standard analgesic agent. Secondly, this thesis is a natural extension of a previous study that reported functional differences in mu opioid receptor activation in the RVM between P21 and adult rats (Hathway et al., 2012). Considering the endocannabinoid signalling system plays an important role in CNS development, effects of cannabinoid receptor activation at the different postnatal ages were also studied. However, due to the complexity of their pharmacology, it was not possible to target only one type of cannabinoid receptor. The cannabinoid drugs chosen were ones that are known to affect nociceptive processing and frequently documented in other literature (Finn et al., 2003, Rea et al., 2007). The development of selective cannabinoid ligands will be beneficial to this type of study.

Moreover, the doses of drugs used in this thesis are all determined from previously published studies. Only one dose per drug was administered per animal so dose-response data is not available. To further elucidate the functions of both opioidergic and cannabinoid signalling system during postnatal development, dose-response experiments must be carried out to characterise the efficacies of the different opioid and cannabinoid receptor ligands.

7.3.4 Anaesthesia

Anaesthesia can affect sensory processing, it can alter the firing patterns of neurones, which is correlated to physiological functions such as motor abilities, behavioural responses and cognitive processing (Devonshire et al., 2010). It was also postulated that anaesthesia has detrimental effects on neurodevelopment; early exposure of anaesthetics in the neonatal period induced rapid apoptosis in primate brains (Sanders et al., 2013). Studies on rodents on the other hand, found that prior neonatal anaesthesia does not affect responses to repeated injury (Beggs et al., 2012). In this thesis all animals were only exposed to one type of inhalation anaesthetic (isoflurane), with no recovery procedures involved. Equilibration time after induction of anaesthesia varied a little due to size of animals but the final anaesthetic depth was held at around 1.3% for all ages. At this depth of anaesthesia stable EMG responses can be evoked, as

described previously elsewhere (Kwok et al., 2013). The level of anaesthesia between experiments was kept constant.

Whilst other studies have used decerebration to eliminate the effects of anaesthesia on withdrawal reflex responses (Dobson and Harris, 2012), this type of surgery typically results in severe cranial bleeding and high mortality rate. Moreover, it is difficult to relate to this model clinically, hence it was decided that decerebration was not suitable for the type of studies described in this thesis.

7.3.5 Electromyographic (EMG) recordings

As with any other *in vivo* preparations, it comes with a few considerations. The advantages of EMG recordings are 1) it is a whole animal response, providing an overview of both sensory and motor processing, 2) descending influences can be investigated together, which is not possible in in vitro slice preparations, 3) the withdrawal reflex is a highly stable and well characterised method for assessing the excitability of spinal nociceptive circuits (Sherrington, 1910, Lim et al., 2011, Hathway et al., 2012, Kwok et al., 2013) and 4) the EMG electrode is constructed with a modified 27 gauge hypodermic needle, which ensures that recordings are restricted to local muscle activity in small animals.

The disadvantages of this type of experiments are 1) electrophysiological preparations in neonatal animals are challenging; it is difficult to obtain stable recordings and n-numbers in some experiments are small; 2) EMG recordings are limited to the muscle (bicep femoris of the hindlimb) studied, withdrawal reflexes in the other limbs are also under the influences of postnatal development (Tive and Barr, 1992) and needs to be taken into account, 3) the damage to the skin resulted from the insertion of EMG electrode is not avoidable, 4) this preparation is performed under anaesthesia and 5) it does not allow for more detailed characterisation of specific inputs to the spinal nociceptive circuits and is susceptible to changes in the motor output. The aforementioned disadvantages were overcome by 1) using a small needle to limit the damage to the skin, and making the incision before all recordings to keep the effect of the damage constant and 2) maintaining the anaesthetic depth at 1.3% throughout and in between experiments (see previous paragraph *Anaesthesia*). Future studies could segregate the sensory and motor components of the withdrawal reflex by combining EMG recordings with single unit recordings in the brain and spinal cord, which will give a more detailed analysis of cutaneous tactile and nociceptive processing of the individual neurones (Cleary and Heinricher, 2013, Koch and Fitzgerald, 2014).

7.3.6 Mechanical stimulation by von Frey hairs (vFh)

The reflex response evoked by vFh is a widely used tool in pain research, in both animal models and human subjects (Cornelissen et al., 2013). The calibration of each individual hair provides a standardised method and allows researchers to evaluate and compare the extent of hypersensitivity and allodynia between experimental preparations (Chaplan et al., 1994). By combining withdrawal responses evoked by vFH with EMG recordings, the subjectivity of this assay can be eliminated because a reliable measurement of muscle activity corresponding to the mechanical stimulation was obtained. However, in this thesis, some 'ceiling effects' were observed, particularly in the age groups where it was not possible to go beyond the vFh strength of 300g, and the maximum strength vFh failed to elicit a withdrawal response. The time of application of vFh was not recorded; therefore the duration of EMG response cannot be specified. Moreover, other modalities of noxious stimulation, such as pressure, chemical and thermal were not considered in this thesis.

7.3.7 Immunohistochemistry

Modern immunohistochemistry on paraformaldehyde-fixed tissue sections allows for fast and accurate histological localisation of antigens compared to other molecular biology techniques. The tissue requires minimal amount of handling once fixed as they don't need to be stored frozen, and the cryoarchitecture is generally well preserved (Rehg et al., 2012). Immunohistochemical protocols are easy to follow; there are various secondary detection systems to choose from and trouble-shooting resources are readily available (e.g. IHC world). Nonetheless, the quality of results depends on the fixatives and antibodies used. All antibodies featured in this thesis, except for the one raised against the GPR55 antigen, were utilised in previously published studies and had standardised protocols. It was not possible to devise a protocol for GPR55 immunohistochemistry in this thesis; inevitably the optimisation of immunohistochemical protocols for a newly synthesised antibody can be time and material consuming. Future studies should perhaps use a different primary antibody (available from Caymen Chemicals item number 10224, abcam item number ab174700). These antibodies react with human GPR55 receptor antigens and reactivity with the rat will depend on the conservation of the GPR55 receptor antigen between species. Alternatively, a different secondary detection system such as DAB could be paired with the GPR55 antibody (gift from prof. K Mackie) used in this thesis.

7.3.8 TaqMan RT-PCR

Apart from immunohistochemistry, TaqMan RT-PCR was an alternative method to quantify expressional changes of endogenous opioid and cannabinoid targets. The

fluorophore conjugated probe was designed to span the intron and exon boundary, which allows for better detection of target genes compared to other traditional RT-PCR assays such as SYBR Green (Hathway et al., 2012). It is also fast throughput, as reaction times are set at 40 minutes, and multiple genes/samples can be run concurrently to avoid time differences and other confounding factors. The experiments are also unaffected by differences in brain sizes because a set amount of RNA material was required, the amount of starting DNA material per region per age would be identical. Ideally, the results obtained from TaqMan RT-PCR experiments would reflect the results obtained from immunohistochemistry; in this thesis, POMC and NAPE-PLD were consistently shown to be upregulated throughout postnatal development, whereas there are some discrepancies between these two methods when it comes to the results of other targets. It is to be remembered that most genetic material is stored inside the nucleus of cell bodies, and proteins localised along the axons of neurones will not be taken into account by TaqMan RT-PCR techniques.

7.4 Wider discussion of work presented in this thesis

7.4.1 The influence of other neurotransmitter systems on postnatal maturation of nociceptive processing

It is without question that both endogenous opioid and cannabinoid signalling systems are crucial to the developing CNS and pain processing. This thesis has outlined some of the functional and anatomical refinements mediated by these two neurotransmitter systems within the descending pain modulation pathway at the different developmental timepoints. The trophic actions of endocannabinoids have been described elsewhere (Fernández-Ruiz et al., 1999, Berghuis et al., 2007, Harkany et al., 2007, Oudin et al., 2011b), and it would be appropriate to suggest that opioids play a significant role in mediating the maturation of the pain signalling system. It was demonstrated in primary cultured cells from the rat hippocampus that activity at opioid receptors increases the number of neurones but decreases the number of astrocytes and oligodendrocytes in culture (Persson et al., 2003). It would be interesting to examine whether the same effect occurs in cells cultured from the neonatal rat brainstem.

The MOR was specifically targeted due to its prominent expression within the pathway both functionally and anatomically. Other opioid receptors, in particular DORs are also implicated during postnatal development and in pain. The role of DOR in neurogenesis and neuroprotection via activation of Trk-dependent tyrosine kinases was observed in

the embryonic mouse forebrain (Narita et al., 2006). Similar to MOR, DOR within the descending pain pathway is subjected to developmental regulation; it is downregulated in large diameter sensory neurones (Beland and Fitzgerald, 2001), and competitive ligand binding at DOR in the brain is absent until P12 onwards (Wohltmann et al., 1982). It would therefore be interesting to investigate whether the functions of DOR changes during postnatal maturation.

Other neurotransmitters involved in the descending pain pathway may also mediate the maturation of nociceptive processing during the postnatal period. GABA is a likely candidate; both the opioid and cannabinoid signalling systems act on GABAergic terminals; MOR and $GABA_B$ receptors are colocalised in the dorsal reticular nucleus of the RVM (Pinto et al., 2008), and CB1 binding sites can be found on GABAergic neurones in almost all brain regions (Rea et al., 2007). Moreover, it was shown previously that GABA mediated signalling undergo a switch from excitatory to inhibitory within nociceptive circuits during postnatal development (Baccei, 2007). Subcutaneous injection of the $GABA_A$ receptor agonist midazolam potentiates EMG responses upon vFh stimulation at P3 but not P21 rats (Koch et al., 2008a), this finding suggests that $GABA_A$ receptor activation is pronociceptive during the neonatal period. Interestingly, the antinociceptive effect of $GABA_A$ receptor antagonism by gabazine and bicuculline in P3 rats that were spinalised at the upper thoracic level is reversed. These findings indicate that spinal GABAergic transmission is influenced by descending inputs from supraspinal sites (Hathway et al., 2006). The effect of immature GABAergic processing within supraspinal sites on the differential behavioural responses to noxious stimulation in the early postnatal period warrants further investigation.

Other likely candidates include serotonin; its involvement in pain transmission and reflex modulation was described elsewhere (Schmidt and Jordan, 2000). Alterations in serotonergic transmission in the early postnatal period lead to abnormal sleep patterns and mood disruptions (Popa et al., 2008). More importantly, the expression of the different subtypes of serotonin receptors in brainstem motor regions undergoes significant changes in the postnatal period, and serotonergic activity within this region evolves from being inhibitory to excitatory (Volgin et al., 2003). Serotonin is also implicated in trophic processes such as regulation of cell division, differentiation and control of growth cones (Whitakerazmitia, 1991). The role of serotonin in postnatal maturation of nociceptive processing is an interesting area for investigation.

7.4.2 Postnatal maturation of other supraspinal sites that input onto the descending pain pathway

It is known that stress is directly correlated to nociceptive processing; stress-induced analgesia (SIA) has been demonstrated in a variety of pain models (Terkelsen et al., 2004, Watanabe et al., 2005), but prolonged exposure to stressors causes chronic pain in diseases such as migraine and tension-type headaches (Leistad et al., 2006). The neural substrates of stress are the hypothalamus, thalamus, pituitary gland, amygdala and the cingulate cortex (Dedovic et al., 2009). These regions all have descending inputs into the PAG-RVM axis (Gauriau and Bernard, 2002), and play a significant role in formulating an appropriate response to noxious stimuli (Bernard et al., 1996).

Stress has significant impact on postnatal maturation. Early exposure to environmental noise (E10-18) leads to impairment in emotive and learning abilities in neonatal mice, and these effects can still be observed when the mice are fully matured (Nishio et al., 2001). Rat pups of mothers that were subjected to prenatal restraint stress display a delayed growth pattern, their body weights are lowered when compared to age-matched controls and the appearance of certain developmental landmarks such as ear opening, auditory startle and cliff avoidance responses are hindered (Barlow et al., 1978). Early environmental stress can alter the responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis to stress; hypothalamic corticotropin-releasing factor (CRF) mRNA levels were upregulated in rat pups that were subjected to maternal separation daily for 180 minutes from P2 to P14, and restraint stress in adult rats that had early postnatal stress provoked a higher increase in corticosterone level when compared to controls (Plotsky and Meaney, 1993). In addition, neonatal maternal deprivation, neonatal colonic irritation and pain are associated with the development of irritable bowel syndrome (IBS) in later life (Barreau et al., 2007). Brain imaging studies have shown that adult IBS patients show increased hypothalamic gray matter and cortical thinning in the anterior cingulate cortex, these changes are correlated to their susceptibility to chronic visceral pain (Blankstein et al., 2010), which suggests that supraspinal mechanisms involved in stress also play a role in adult pain processing.

Several brain regions, including the dentate gyrus of the hippocampal formation and various brainstem nuclei develop during an extended period that begins during gestation and continues well into the postnatal period; the late development means that these regions are particularly sensitive to environmental and experience-induced structural changes (Gould and Tanapat, 1999). Early embryonic stress induces release of adrenal

steroids that can inhibit the proliferation of granule cell precursors in the rat dentate gyrus (Gould and Tanapat, 1999), which in turn can disrupt the structure and the functions of the hippocampal formation, such as memory. Similarly in the brainstem, early acute stress (forced-swim paradigm and restraint stress) induced neuronal activation and expression of immediately early genes such as c-fos, fos-b, c-jun and jun-b (Cullinan et al., 1995). Since CNS development is driven by neuronal activity, the effect of stress on maturation of pain signalling pathways warrants further investigation.

It is also worth reiterating that POMC expression peaked at around P21 (see *Chapter 4*). POMC-positive neurones are likely to originate from the hypothalamus (Meister et al., 2006), and are involved in endocrine functions (Veening et al., 1991). Together these findings suggest regions that are concerned with stress processing, specifically the hypothalamus, may play a role in mediating the maturation of pain pathways. The functions of hypothalamic stimulation upon noxious stimulation at the different ages remain to be elucidated.

7.4.3 Plasticity within nociceptive pathways during postnatal development

The development of the CNS is driven by neural activity. Early work on the mammalian visual cortex by Hubel and Wiesel confirmed that neural activity can modulate development by either stimulating the formation of new synapses, or by destabilising existing connections (Hubel and Wiesel, 1970). For example, glutamatergic transmission in rat brain primary cultured cells promoted synapse maturation and stability (Hua and Smith, 2004). Another study reported that the postnatal maturation period is characterised by intense apoptotic pruning of neurones and synapses within the CNS, which allows the appropriate connectivity within the CNS to be enhanced (Yuan and Yankner, 2000).

Similarly, normal maturation of nociceptive circuits can be prevented by blocking endogenous glutamatergic transmission with intrathecal application of the non-competitive NMDA receptor antagonist MK801 (Beggs et al., 2002). Early injury increases neural activity, and this alteration in the pattern of activity during postnatal maturation has significant functional consequences on nociceptive processing in later life (Walker, 2013). Newborn rats that underwent surgical removal of a small patch of skin (2mm x 2mm) in the hindpaw have significantly enlarged DH receptive fields at 3 and 6 weeks after birth (Torsney and Fitzgerald, 2003). This prolonged behavioural hypersensitivity indicates that the normal pattern of spinal nociceptive circuit is altered by early injury induced activity. Furthermore, neonatal injury can lead to long-term

hypoalgesia at basal condition and hyperalgesia upon re-inflammation at the original site of injury (Ren et al., 2004). This suggests that neonatal injury has a 'global' effect on nociceptive processing in adulthood; a reorganisation of neuronal networks responsible for pain transmission is implied.

Children that require regular hospitalisation are likely to undergo painful surgical procedures; these experiences are likely to affect the maturation of endogenous pain signalling system and represents a puzzling clinical challenge (Taddio and Katz, 2005, Grunau et al., 2006a). The molecular footprint of early injury remains to be elucidated, opioids are likely to be involved because they have been shown to be up-regulated in early injury models (LaPrairie and Murphy, 2009). Using immunohistochemistry it was reported that β-endorphin and enkephalin protein levels were upregulated in the PAG of rats after intraplantar injection of carrageenan (1%, 5 µl) on the day of birth (LaPrairie and Murphy, 2009). This increase in endogenous opioid tone is at least in part, responsible for the global hypoalgesia observed after the initial carrageenan induced injury.

On the other hand, the endocannabinoid system is implicated in neuroprotection after neonatal ischaemic injuries. In a model of neonatal-hypoxic-ischaemic encephalopathy, administration of WIN55212 (0.1mg/kg) at P7 reduced the final volume of necrosis in the brain (Fernández-López et al., 2007). The authors, using HPLC and enzyme-linked immunosorbent assays, found that WIN55212 offers neuroprotection by counteracting glutamate-induced excitotoxicity; in brain slices from P7 Wistar rats that were exposed to oxygen-glucose deprivation for 30 min, glutamate release was inhibited by administration of WIN55212 (50 µM) (Fernández-López et al., 2007). These findings suggest that cannabinoids play a role in restoring CNS development after injury in the neonatal period, more studies targeting the pain signalling pathway will be hugely beneficial.

7.5 Implications of findings

Animals included in this thesis were all naive; they were not an injury or pain model, and were only subjected to one type of nociceptive testing. All findings reported here, therefore, represent the events that occur during normal postnatal development.

The nociceptive pathways undergo a prolonged period of maturation postnatally; noxious processing is immature in the first 3 postnatal weeks and both the endogenous opioid and cannabinoid signalling systems play a crucial role in mediating the maturation of

descending pain modulation pathway. Apart from spinally mediated mechanisms, supraspinal activity also contributes to the maturation of nociceptive processing, as the effects of focal pharmacological manipulation of the PAG and the RVM were different between young and mature rats.

The findings of this thesis have significant implications both scientifically and clinically, as any changes in the normal pattern of maturation results in long term alterations in nociceptive processing that may be irreversible. The quality of pain treatment and management in the neonatal units can be improved by clinicians being aware of the changes that occur during this sensitive period, and not disrupt the normal endogenous activity where possible. On the other hand, scientists should be informed that rodent brains do not reach maturity within the first 3 weeks of life. This may have significant impact on studies that utilises young brain, such as in vitro patch clamping electrophysiology, as appropriate neuronal connections cannot be assumed at that age.

7.6 General conclusions

This thesis demonstrated that both the endogenous opioid and cannabinoid signalling systems undergo relatively long and significant periods of postnatal maturation.

In this period opioid sensitivity is both region and age dependent; spinal MOR activation produces profound analgesia in neonatal rats and analgesic efficacy decreases as the animals aged. Supraspinal MOR activation results in pronociceptive effects in younger rats; a switch in function occurs at around P21 and in matured rats only inhibitory effects can be observed. The changes in MOR-mediated functions throughout postnatal development are reflected by alterations in the expression of MOR and related peptides within the descending pain pathway. The most important anatomical finding is the increase in POMC protein and mRNA at P21, which coincides with the timing of the postnatal switch from excitation to inhibition.

Cannabinoid signalling is antinociceptive in all ages tested. However the orphan GPR55 receptor may be an antinociceptive target exclusively in young animals. The expression of endocannabinoid targets within the nociceptive pathways suggests that anandamide and 2-AG levels are developmentally regulated during postnatal development. The expression of GPR55 mRNA in the RVM also peaks at P21, but the role of this novel receptor in the maturation of pain transmission will need to be further investigated.

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