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Mechanistic Dissection of The Attentional Modulation of Pain

Valeria Oliva

A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of PhD in the Faculty of Life Sciences

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Abstract

Aims: A shift in attention away from a painful stimulus can cause pain relief. The mechanisms behind this process are thought to either be fully supraspinal or to involve spinal cord modulation by brainstem nuclei. The main object of investigation of the present thesis is to identify the neural pathways engaged during the attentional modulation of pain in healthy subjects and in patients with chronic pain (fibromyalgia) using functional magnetic resonance imaging. The role of endogenous opioids and of noradrenaline is investigated using selective pharmacology.

Methods: In two different studies, healthy volunteers (n=57, Study1) and fibromyalgia patients (n=40, Study2) performed an attentional analgesia experiment during fMRI scanning of brain and brainstem. A third study (n=39, Study3) used an imaging sequence with manual shimming to acquire data simultaneously from brain, brainstem, and spinal cord. The opioid receptor antagonist naltrexone and the noradrenaline re-uptake inhibitor reboxetine are administered to the participants to examine the neurochemical components of attentional analgesia.

Results: The attentional task was successful in inducing analgesia in healthy volunteers in all three studies presented. In Study1, a top-down mechanism where the cortex functionally connects to brainstem nuclei was demonstrated. In Study2, attentional analgesia was also observed in fibromyalgia patients, with involvement of similar brainstem mechanisms. In Study3, spinal cord activity mirrored the perceived pain intensity. A cortico-brainstem pathway was shown to modulate the dorsal horn of the spinal cord. Naltrexone blocked this path as well as the analgesic effect behaviourally. Conversely, reboxetine did not influence attentional analgesia.

Conclusion: During high cognitive load and concomitant painful stimulation, the descending pain modulatory system, including locus coeruleus and rostroventromedial medulla is recruited for spinal cord modulation. This process is dependent on endogenous opioids. Fibromyalgia patients can achieve attentional analgesia by recruitment of brainstem nuclei, similarly to healthy volunteers.

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Author's declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: VALERIA OLIVA DATE: 17/09/2020

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Chapter 1 Introduction

1.1 Overview

Opioids have been a popular treatment for pain since the 1900 and to date they are one of the most effective ways of achieving analgesia (Morgan et al., 2011). However, it is increasingly evident that opioid-based medicaments are highly addictive and lead to drug misuse, which can result in death by overdose (Morone et al., 2013; Van Zee, 2009). In 2015 it has been reported that in the USA alone more than 40 people a day die from opioid misuse (Trang et al., 2015). An opioid crisis has been since declared in the US (Florence et al., 2016), and alternative methods for analgesia are being explored. In 2018 "The Opioid Crisis Response Act" has been issued to address this major problem.

Cognitive modulation of pain is a strategy to achieve pain relief without the need for pharmacological intervention. It can be achieved by shifting focus away from the painful stimulus, it is not associated with side effects and does not cause addiction. A better understanding of the cellular and molecular mechanisms that lead to analgesia induced by distraction from pain is necessary for the development of new therapies for pain relief.

The present thesis is structured as follows. A brief literature review is provided, highlighting the issues that remain unresolved. Methodological challenges and strategies to address them are explained. Following, research toward the understanding of how attention and pain interact in health and disease is presented in the form of three paper chapters. Finally, the overall message of the thesis is discussed in the general conclusions.

1.2 Pain perception and its modulation

1.2.1 *Overview*

Nociception is defined as "The neural process of encoding noxious stimuli" by the International Association for the Study of Pain (IASP). Where a noxious stimulus is "a stimulus that is damaging or threatens damage to normal tissues", typically the skin (Raja et al., 2020). This is a physiological process on which the survival of organisms depends, and is well phylogenetically preserved. Behavioural and cellular responses to noxious stimuli have been extensively studied in all vertebrates, including fish; and the molecular mechanisms behind it have been discovered in crustaceans, molluscs and even some insects (Smith et al., 2009; Walters et al., 2019). Typically, nociception causes withdrawal from the stimulus and gives rise to 'repairing' behaviour of the affected body part. Pain, on the other hand is defined as "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" (Raja et al., 2020). Thus, the sensation of pain is only generated when transmission reaches the brain and enters an individual's awareness. In the brain, the nociceptive stimulus is integrated with contextual and emotional information to generate a complex and highly subjective experience.

1.2.2 The journey of a nociceptive stimulus

Noxious stimuli received below the neck are conveyed to the spinal cord before reaching the brain (Brown, 1982). The spinal cord is a cylindrical structure part of the Central Nervous System (CNS), that runs along the vertebral column. This is the main interface of the CNS with the outside world, being responsible for transferring sensory stimuli from the periphery to the cerebrum. In addition, with extensive innervation of skeletal muscles, the spinal cord is an essential component of motor activity. The grey matter of the spinal cord has a typical "butterfly" shape, that divides it in four horns. The two dorsal horns receive sensory information from the ipsilateral side of the body, while the two ventral horns mediate ipsilateral motor activity.

Inhibitory and excitatory interneurons within the spinal cord can modulate the activity of sensory or motor neurons, as well as connecting the two in spinal reflexes. A typical example of a spinal reflex is the withdrawal response to a painful stimulus, where the affected area is instinctively pulled away from danger (Derderian et al., 2019; Sandrini et al., 2005). This response is outside conscious control and relies on an internal network in the spinal cord composed of a sensory input, integrative interneurons, and a motor output. The latency between the stimulus delivery and the withdrawal reflex is often used in research as a measure of pain perception, for example for defining pain thresholds (Rhudy et al., 2007).

The neurons that connect the skin to the spinal cord are part of the dorsal root ganglion. The cell body of these "pseudo-unipolar" neurons lies just outside the spinal cord and their axon has two branches, one innervating the periphery and one conveying the signal to the spinal cord. Such axons are also called "primary afferent fibres" and are bundled with motor fibres into fascicles that form the spinal nerves (Armett et al., 1961). Primary afferents are categorized by the myelination of their axons, giving rise to two main classes for nociception, A-δ and C fibres (Woolf et al., 2007). Both classes respond specifically to noxious stimuli, for example a very hot temperature (e.g. higher than 43 °C), and are silent during innocuous stimulation. The A-δ fibres are small, myelinated fibres, and are the first to respond to a noxious stimulus. They are specialized, with one fibre only carrying information on one type of stimulus (e.g. thermal). Engagement of A-δ fibres result in a short lasting, sharp painful sensation whose spatial localization is very precise. Conversely, C fibres are non-myelinated and give rise to secondary pain, a slower, dull sensation that is more diffusely localized (Brown, 1982). C fibres typically carry both thermal and mechanical information and are often classified as polymodal. Terminals of primary afferents in the skin expose transduction proteins (TRP) that are sensitive to specific noxious stimuli. These are typically transmembrane ion channels that once activated allow for influx of cation like calcium or sodium, thus

causing membrane depolarization in the primary afferents (Tominaga, 2006).

A-δ and C fibres reach the dorsal horn of the spinal cord, where the nociceptive signal is transferred for the first time. The first synapse is typically between a primary afferent and a projection neuron directly. Projection neurons, or second order neurons, join ascending pathways to convey nociceptive information to supraspinal regions (**Figure 1.1**). The most widely studied is the spinothalamic tract, that connects the spinal cord to the thalamus and is considered the most evolutionarily recent (Kevetter et al., 1984). Second order neurons in this pathway cross the midline and travel through the lateral or medial part of the spinal cord, on the contralateral side in respect to the stimulation. This path is thought to relay information about the type of stimulus (e.g. thermal or mechanical) and the exact bodily location. Collaterals of the spinothalamic tract also project to other supraspinal areas, including brainstem nuclei, both contralaterally and ipsilaterally (Giesler, 2013). Other ascending pathways include for example the spinoreticular and spinomesencephalic tracts, where second order neurons connect the spinal cord to the reticular formation in the medulla and to the mesencephalon respectively (Fields et al., 1975; Hylden et al., 1986). Supraspinal areas like the thalamus and brainstem nuclei then feed into the cortex, where the sensation of pain is generated.

1.2.3 Quantification of pain and cortical response to noxious stimuli

Quantification of pain typically relies on a self-reporting score on a numerical scale. This is a measure used in diagnosis of painful conditions and to decide on the effectiveness of treatments. However, pain reporting is not an objective measure of the pain intensity perceived by an individual. Depending on a variety of factors, including past painful experiences and the unpleasantness of the sensation, pain intensity can be localized differently on a numerical scale. Some patients, for example children, patients with dementia or with disorders of consciousness, might also not be able to quantify their painful sensation on a numerical scale. Additionally, pain

relieving drugs, especially opioids, can cause severe side effects like bradycardia, slowed breathing and nausea. Furthermore, opioidergic medications often lead to addiction, causing vulnerable patients to seek them even when not in pain. Clinical practice as well as pain research need a less subjective measurement of pain intensity to provide better care and further the understanding of how the pain sensation is generated.

The idea that cerebral activity is necessary for the generation of a painful sensation started being accepted in the early 1900. Insights came mainly from patients with lesions in the thalamus and cortical areas, who cannot feel pain. However, localizing the exact source of the painful sensation was challenging. With the advent of advanced brain imaging technologies, researchers tried to resolve the exact brain regions responsible for this process. Different pain modalities have been used to elicit an acute pain sensation, including hot and cold thermal stimuli, mechanical insult, chemical substances, and electrical stimulation. Brain activity was recorded directly, for example through EEG, or via proxies of neural activation in fMRI and PET. Across studies, the cortical and subcortical regions more reliably responding to all types of nociceptive stimuli were the primary and secondary somatosensory cortex, the anterior cingulate cortex, the thalamus, and the dorsal posterior insula (Ingvar, 1999). Depending on the context and on the sensitivity of the brain measurement techniques used, additional regions would show activation, for example the amygdala, the hippocampus, prefrontal and temporal cortices (Tracey, 2005). These regions form an activation map that has been defined the “pain matrix”, a set of cortical and subcortical areas that together generate a painful sensation (Melzack, 1999). This was strikingly different to what has been observed in other sensory modalities, for example vision and smell, where a single cortical area is overall responsible for the sensation. Pain was already starting to be viewed as a much more complex phaenomenon, with distinct characteristics.

It has been proposed that these different brain regions have specialized functions in their contribution to perception. The somatosensory cortices, S1 and S2, receive direct projections from the thalamus, organized in a somatotopic manner. In 1937, it was discovered by Penfield that different subregions of these large cortical areas respond to sensory stimuli delivered to specific parts of the body, thereby forming a map (Penfield et al., 1937). These cortical areas are thought to specifically code sensory discrimination and the intensity of pain perception, forming the so-called "lateral pain system" (Bushnell et al., 1999; Kanda et al., 2000; Kenshalo et al., 1988). On the other hand, affective states associated with pain were thought to be represented in regions like the anterior cingulate cortex and the amygdala, part of the "medial pain system" (Kulkarni et al., 2005; Sewards et al., 2002; Vogt et al., 2000). The medial pain system would therefore mediate motivational responses to painful stimuli, for example aversion (Sewards et al., 2002). This definition however seems rather simplistic and does not consider the heterogeneous characteristics that many cortical areas present. For example, this classification fails to categorise the insular cortex, a region with a role in both affective pain processing (Craig, 2003) and sensory discrimination (Coghill et al., 1999; Craig et al., 2000), that would sit in between the two systems. The same is true for the anterior cingulate cortex, a region that was also found to directly correlate with the intensity of perceived pain (Brooks et al., 2017), in addition to mediate motivational aspects of behaviour. A more recent vision proposes that the personal experience of pain emerges from the net effect of transmission of information within this whole network (Mano et al., 2015; Tracey, 2005). This would mean that the strength of the interaction between regions, more than the response of the individual regions themselves, is responsible for the subjective and context-dependent experience of pain. This is in striking contrast with the direct correlation between the pain intensity and activity in the dorsal posterior insula resolved with human imaging (Segerdahl et al., 2015).

Not all researchers in the field accept the notion of a "pain matrix" as a unique and specific response to nociceptive stimuli. Discussions mainly stem from the absence of nociceptive-specific neural population in somatosensory cortices (Iannetti et al., 2010). While different sensory modalities, for example touch, are associated with specific cortical columns that are spatially segregated form each other, the same is not true for nociceptive stimulation. Cortical columns in the primary somatosensory cortex that responds to nociceptive stimuli also encode non-noxious stimulation (Kenshalo et al., 2000). In addition, neurons in frontal cortices that do seem to specifically respond to nociceptive stimuli and not to lower intensity touch or temperatures, were also found to encode threat (Dong et al., 1994; Hutchison et al., 1999). This finding lead to the hypothesis that these neurons do not necessarily encode a physical sensation of tissue damage, but more generically respond to aversive events (Iannetti et al., 2010). Furthermore, it is thought that studies that identified reliable brain activation to painful stimulation, are not able to discriminate whether the regions resolved are just encoding stimulus salience (Iannetti et al., 2010). Saliency of a stimulus is defined as "its ability to stand out relative to the background" (Itti et al., 2001) that typically re-orients the individual attention (Nardo et al., 2011). Painful stimulation has all these characteristics, being intrinsically salient, subjective, and able to capture attention (Seminowicz et al., 2007; Van Damme et al., 2010). Additionally, regions thought to be pain matrix specific are found to respond to a variety of salient sensory events (Downar et al., 2000, 2003). Even the direct correlation between brain activity and the intensity of nociceptive stimulation or of pain perception could be explained in terms of saliency: events that more vigorously emerge from the background trigger a stronger alerting response in the brain (Iannetti et al., 2010).

Following discussion on the specificity of the "Pain Matrix", researchers worked to address the issues. For example, a recent fMRI study that controlled for stimulus saliency demonstrated that activity parietal

operculum was specific for pain (Horing et al., 2019). In addition, the advent of high-performance computing and the increased use of computational modelling in neuroscience, allowed Wager and colleagues to resolve a "neurologic pain signature" (Wager et al., 2013). In this study, a machine learning algorithm identified a pattern of brain activity in more than 100 volunteers that specifically responded to pain versus nonpainful stimulation. This pattern, or signature, reliably predicted whether the participants were in pain or not. Furthermore, its intensity of activation consistently lowered after administration of a potent analgesic. Once again, the brain regions resolved in this study were consistent with the pain matrix, including thalamus, the insula, the anterior cingulate cortex, and the secondary somatosensory cortex. Importantly, the net activity of all these areas was a much better predictor of the individual painful sensation than any individual region.

1.2.4 Brainstem response to noxious stimulation

Identification of brainstem nuclei in the perception of pain is even more challenging in humans. Its deep subcortical location prevents it being measured with electrophysiological techniques like EEG, as the signal coming from brainstem nuclei is confounded by activity in the cortex. Due to the small size of brainstem nuclei, the heterogeneity, movement with breathing, closeness to air filled spaces, and proximity to the Circle of Willis, measuring brainstem activity with functional imaging has not been much easier (see section 1.4.3 for details). For these reasons, human pain research mainly focused on large cortical structures, with only occasional studies able to examine subcortical areas.

Many brainstem nuclei receive direct projections from second order neurons in the spinal cord and are of special interest because they can modulate cord interneurons with direct descending pathways. These nuclei include the periaqueductal grey (PAG) and the nucleus cuneiformis in the mesencephalon (Blomqvist et al., 1991; Keay et al., 1997), the pontine nuclei locus coeruleus (LC) and parabrachial nucleus, and the rostroventromedial

medulla (RVM) (Cedarbaum et al., 1978, 1978; Doleys, 2014; Sugiyama et al., 2012; Voisin et al., 2005). The PAG is a crucial region in mediating the escape response to potential danger, as signalled by a noxious stimulus (Bandler et al., 1996). In animals, this region is found to be strongly involved with fight or flight behaviour (Henderson et al., 2018). It is typically divided in subregions that are involved in different behavioural strategies to threat (Lumb, 2002; Watson et al., 2016). Dorsolateral columns of the PAG are engaged during active coping, a "confrontational defensive reaction" to a threatening stimulus; ventrolateral columns are involved in passive coping, where the animal escapes from the stressor or freezes (Keay et al., 2001, 1997; Koutsikou et al., 2014). Interestingly, the latter strategy is dependent on endogenous opioids, which might trigger bradycardia and analgesia (Keay et al., 1997; Waters et al., 1997).

The advent of stronger MRI magnets and of advanced signal denoising techniques, allowed researcher to work toward resolving a brainstem signature of nociception. Early studies were able to identify in humans midbrain nuclei like the PAG and the nucleus cuneiformis, that with the ventral tegmental area showed activation in response to electrical pain stimulation (Dunckley et al., 2005). Researchers have also tried to investigate whether the functional organization of the PAG is similar in humans and in animals. This was challenging not only because of technical difficulties, but also because of the limitations caused by the experimental settings. Acute pain, that in an ecological environment would be escapable, is typically made non-escapable for the participants of a human pain experiment, for example by fastening a pain device on the arm. This can lead to confusion for functional mapping of subregions of the PAG. However, findings from anatomical and functional connectivity studies in human volunteers tried to resolve a similar parcellation of the PAG. Functional connections to executive functions regions like the medial prefrontal cortex were predominantly identified in the dorsolateral PAG, whereas connections with areas of the descending pain modulatory system were, mainly localised

in the ventrolateral PAG (Coulombe et al., 2016). Interestingly, anatomical connectivity indicated the opposite pattern, with the ventrolateral PAG connecting mainly with the prefrontal cortex and the dorsolateral area with the brainstem (Ezra et al., 2015). Further studies, perhaps combining anatomical with functional connectivity analysis, are needed to conclusively identify the subdivisions of the PAG in humans.

Recent brainstem-optimised fMRI acquisitions were able to resolve a pain signature also in the lower brainstem areas of the medulla, including the RVM, the LC, and pontine nuclei (Brooks et al., 2017; Napadow et al., 2019; Sclocco et al., 2016). The RVM was additionally found to correlate directly with the perception of pain, linking for the first time medullary activity not only to a role in relaying nociceptive information to the cortex, but to also encoding the subjective cognitive experience of pain perception (Brooks et al., 2017).

1.2.5 Modulation of pain – descending modulatory pathways

Pain is a subjective experience that can greatly vary between individuals and within an individual in different contexts (Beecher, 1946). For many years, it was thought that different pain experiences are only the result of cortical integration of context and emotional states, giving rise to an overall complex sensation. It is now known that interneurons in the dorsal horn of the spinal receive bidirectional modulation from descending control pathways originating in the brain.

Top-down modulation is mainly achieved via direct spinal cord projections that originate in brainstem nuclei. The PAG is the most widely studied nucleus to endogenous pain modulation, being able to generate very powerful analgesia in rodents when stimulated (Koutsikou et al., 2007; Leith et al., 2010; Reynolds, 1969). Attempts of reproducing this result in human using deep brain stimulation were initially successful: PAG stimulation could indeed cause pain relief. However, a number of side effects, like anxiety or even migraine were also triggered (Hosobuchi et al., 1977; Raskin et al.,

1987; Richardson et al., 1977). The analgesia generated by stimulating the PAG was reversed by the opioid antagonist naloxone (Hosobuchi et al., 1977), suggesting a crucial role for endogenous opioids in this region. Consistent with these animals findings, a recent PET study in humans demonstrated the PAG to release endogenous opioids when electrically stimulated (Sims-Williams et al., 2017).

While the PAG has some direct projections to the spinal cord (Bernard et al., 1998; Blomqvist et al., 1991; Mouton et al., 2000; Yezierski, 1991) these are relatively sparse, analgesia achieved by stimulating this region seems to be mediated by another nucleus. Injection of lidocaine, a local anaesthetic used in research to block a specific area, in the RVM can greatly diminish PAG-dependent analgesia (Sandkühler et al., 1984). This finding for the first time highlighted the importance of the interaction between these two regions in achieving endogenous analgesia, and opened for follow up work on the RVM. Typical studies on the functioning of this region used electrophysiological recording while observing rodent's reflexes to noxious stimuli, for example tail-flick and paw withdrawal. In 1986, Fields and colleagues were able to identify two separate populations of cells within the RVM: the 'on-' and 'off-' cells (Cheng et al., 1986). Population of neurons that showed an increased firing rate immediately before the rodent initiated a tail-flick, were labelled 'on-cells'. Population of neurons that decreased their firing rate at the same time, where labelled 'off-cells' (Fields et al., 1983; Heinricher et al., 1989; Potrebic et al., 1994). Interestingly, both populations project to the dorsal horn of the spinal cord, providing a strong indication for this region to bidirectionally modulate the cord response to noxious insult (Fields et al., 1995). Subsequent studies validated this theory, demonstrating that on-cells exhibited an enhanced firing rate in a hyperalgesic condition induced by, for example, opioid abstinence (Kaplan et al., 1991). Lidocaine injection in the RVM was able to abolish the increased nociceptive response, suggesting a causal role of this cell population for the exaggerated perception (Morgan et al., 1994). Conversely, systemic

administration of a mu opioid receptor agonist increased the firing rate of off-cells, leading to the hypothesis that this population has a crucial role in opioid-induced analgesia (Fang et al., 1989). It was also found that opioidergic injections in the PAG could trigger off-cells activity in the RVM, providing insights on how the interaction between these regions leads to analgesia (Cheng et al., 1986). It is worth noting that PAG-dependent modulation of spinal cord is specific for noxious stimuli (Leith et al., 2010).

The PAG-RVM system is not the only brainstem mechanism that can generate analgesia, the spinal cord is densely innervated by noradrenergic pathways. The locus coeruleus, the main source of noradrenaline in the brain, is one of the main nuclei with direct descending projections. Noradrenergic alpha2 adrenoceptors are strongly expressed in the spinal cord interneurons (Olave et al., 2003) as well as in primary afferent terminals (Stone et al., 1998). They are thought to be the main receptors to mediate noradrenergic analgesia via an inhibitory effect on second order neurons and primary afferents. These receptors are indeed especially engaged during persistent injury, as part of a compensatory response to hypersensitivity (Malmberg et al., 2001; Mansikka et al., 2004). Additionally, activating alpha2 receptors through agonists was found to be effective for analgesia, especially in injured animals (Mansikka et al., 1996; Stanfa et al., 1994; Xu et al., 1992; Yaksh et al., 1995). Alpha1 receptors are also expressed widely in spinal cord and could suppress nociceptive activity by acting on inhibitory interneurons (Nalepa et al., 2005), providing an additional mechanism to analgesia induced by noradrenaline. The pharmacological/therapeutic potential of noradrenaline has been thus explored in chronic pain states. For example, systemic delivery of reboxetine, a noradrenaline reuptake inhibitor, prevented the developing of exaggerated response to an innocuous mechanical stimulus in a chronic pain model in rats (Hughes et al., 2015). This effect was dependent on alpha2 receptors. Noradrenaline is therefore increasingly viewed as a powerful alternative to opioids in the treatment of chronic pain pathologies. Indeed, drugs that increase the

availability of this neurotransmitter, like duloxetine and amitriptyline, are increasingly used in clinical practice with promising results (Kremer et al., 2016, 2018).

Descending pathways are thought to be recruited by cortical areas (**Figure 1.1**, Quintero, 2013; Xie et al., 2009). The ACC, an area involved in pain perception, projects directly to the PAG and is thought to be one of the main cortical centres to trigger endogenous pain modulation. This region is typically associated with the emotional modulation of pain with its activity being consistently correlated to pain unpleasantness (Rainville et al., 1997; Vogt et al., 2000). Direct evidence for the involvement of the ACC in the modulation of pain comes once again from analysing the behaviour of rodents. Researchers have demonstrated that electrical stimulation of the ACC shortens the latency of tail flick or paw withdrawal in response to a hot painful stimulus, thereby indicating enhanced nociception (Zhang et al., 2005). Interestingly, this pro-nociceptive process seems to be dependent on RVM activity (Calejesan et al., 2000). Just like the PAG-RVM system, the ACC modulates nociception bidirectionally, being also involved in analgesia. Endogenous opioids have an important effect on the ability of the ACC to modulate pain. For example, a PET study used a broad spectrum opioid ligand to demonstrate high availability of opiate receptors in this region (Baumgärtner et al., 2006). In addition, activation of mu opioid receptors in the ACC have been linked to pain relief (Zubieta et al., 2001). Intriguingly, endogenous opioids in the ACC have also found to be necessary to mediate pain relief triggered by non-opioid drugs (Navratilova et al., 2015). Thus, the ACC is thought to sit on top of pain modulatory nuclei in the brainstem, via connections to the PAG and the LC (Fig 1, De Felice et al., 2016).

Somatosensory cortices are usually thought to passively encode the nociceptive stimulus, with a major role in sensory discrimination and identification of the site being stimulated (see section 1.2.3). However, evidence is suggestive of an active role for these structures in pain modulation. A study found that stimulation of the secondary somatosensory

cortex results in analgesia and in a decreased number of dorsal horn neurons active in response to noxious stimulation (Kuroda et al., 2001). This is suggestive of an active role of this region in recruiting subcortical/brainstem nuclei to modulate the spinal cord. Indeed, the antinociceptive activity in SII was found to be dependent on the RVM (Sagalajev et al., 2017), perhaps recruited indirectly by the ACC (Xie et al., 2009). Although the mechanism through which SII can achieve pain modulation is yet to be resolved, endogenous opioids are likely involved as receptors are highly expressed in this region (Baumgärtner et al., 2006).

Human studies on pain modulation have also identified the dorsolateral prefrontal cortex as an important region for top-down control. This area is implicated in executive control through signalling from adjacent cortical areas, for example the medial prefrontal cortex, and in emotional regulation through connections with limbic structures, such as the amygdala (Ong et al., 2019). It is thus suggested that the dorsolateral prefrontal cortex has an important role in the cognitive aspects of pain perception and its modulation (Seminowicz et al., 2007). Human neuroimaging studies found activity in this area to negatively correlate with pain scores (Lorenz et al., 2003) and to be tonically active during pain inhibition (Freund et al., 2009). In addition, a study showed that ACC-midbrain and midbrain-thalamus correlations were stronger during low dorsolateral prefrontal cortex activity (Lorenz et al., 2003). This area has been consistently implicated in placebo analgesia (Eippert et al., 2009; Geuter et al., 2013; Petrovic et al., 2010; Wager et al., 2004), mainly through functional connections to the PAG, anatomically mediated by the ACC (Eippert et al., 2009; Sevel et al., 2015). Together, these findings suggest that this area is able to exert an inhibitory effect on descending facilitatory pathways.

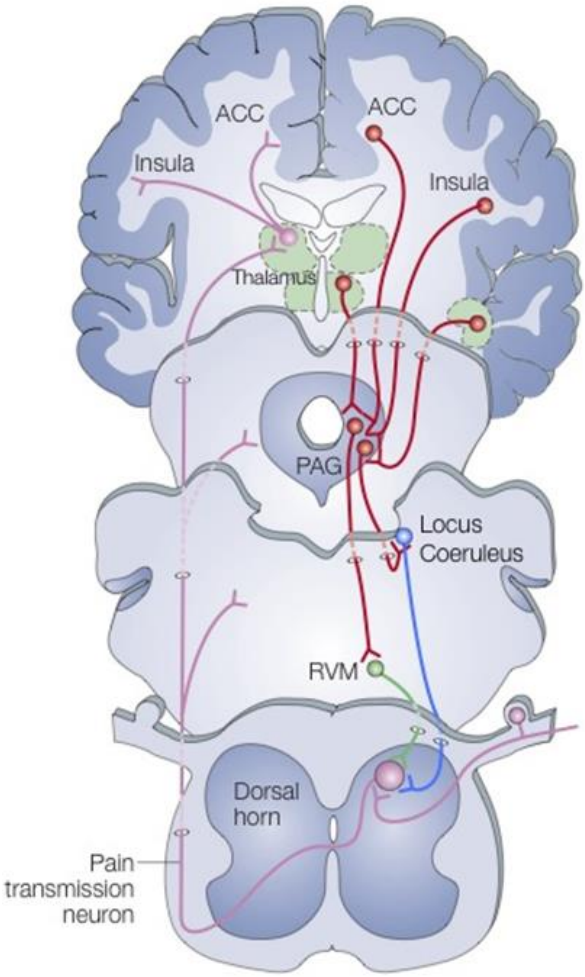


Figure 1.1 *Ascending and descending modulatory pathways. Modified from Fields et al., 2004. The pink arrows represent a bottom-up pathway of pain perception, including projections from the dorsal horn to the periaqueductal grey (PAG), thalamus, insula, and anterior cingulate cortex (ACC). A top-down cortico-brainstem pathway involved in the bidirectional modulation of pain is shown in red, where ACC, Insula, and thalamus recruit PAG, LC and RVM. The noradrenergic projection from the LC to the dorsal horn is shown in blue, the glutamatergic and GABAergic projection from the RVM to the dorsal horn is shown in green.*

1.2.6 Cognitive modulation of pain and attentional analgesia

Pain perception is a complex experience that depends on context, expectations and on emotional and cognitive states. An identical noxious stimulus can elicit radically different perceptions in different people, but also in the same person in different moments. One of the most famous types of cognitive modulation of pain is probably the placebo effect. When a

physician prescribes a medication for pain relief, the patient builds strong expectation on its effect. Even if the drug completely lacks active analgesic components, the patient will likely experience pain relief (Colloca, 2019; Colloca et al., 2005). Interestingly, negative expectation can also have an enhancing effect on the perception of pain, in a phenomenon called nocebo (Colloca, 2017). When the placebo and nocebo effects started to attract more clinical interest, researchers also investigated other forms of cognitive modulation of pain, such as music analgesia, mindfulness-based pain reduction and attentional analgesia. The latter is the focus of this thesis and is especially fascinating as it can regularly be observed outside experimental settings. Just by shifting focus on something different from a painful stimulus, analgesia can be achieved. Like expectation-induced analgesia, the attentional modulation of pain is a bidirectional process, where focus on pain causes an increase in pain perception (Moore et al., 2017). The analgesic effect of attention can be induced by a variety of distractors, for example an attentional task (Bantick et al., 2002; Brooks et al., 2017, 2002; Petrovic et al., 2000; Sprenger et al., 2012; Valet et al., 2004), or even by mind wandering (Kucyi et al., 2013). In a typical experiment, healthy volunteers receive an identical painful stimulation under at least two conditions. In one condition, they are instructed to rest; in the other, to focus on something different, ignoring the sensation. Typically, the intensity of pain reported by the participants is lower when participants do not attend pain.

Early studies on attentional analgesia found that diminished pain perception was observed in conjunction with a reduced response in somatosensory cortex and insula, consistent with these two regions coding sensation (Bushnell et al., 1999; Fairhurst et al., 2007; Markus et al., 2011). Interestingly, the ACC also showed a diminished BOLD signal during distraction from pain (Bantick et al., 2002). This was surprising as this region, supposedly part of the medial pain system, was thought to only code pain affect and not the intensity of the sensation. However, the attentional

modulation of pain does not only involve coding of pain intensity in the cortex but is likely to involve conflict resolution processes. As discussed above (section 1.2.3), pain inherently causes re-orienting of an individual's attention, leading to selection of the appropriate behavioural response (e.g. fight or flight). A second stimulus competes for the subject's attention, thereby inducing a conflict. In line with this idea, frontal cortices implicated in executive functions, such as the orbitofrontal cortex and the ACC, were found to be actively engaged in the attentional modulation of pain (Petrovic et al., 2000). In following studies, it soon became apparent that this process does not only involve cortical areas. Several experiments suggested that the pain modulatory system is involved in attentional analgesia. For example, an early study implicated PAG activity in endogenous pain modulation (Tracey et al., 2002). A significant correlation between the BOLD signal in this area and the analgesic effect, defined as the decrease in pain scores during distraction from pain, was revealed. This provided direct support for an active role of this region in the attentional modulation of pain. A following study demonstrated that the PAG is recruited by the ACC during an attentional analgesia paradigm, showing for the first time a possible cortico-brainstem pathway to analgesia induced by an attentional shift (Valet et al., 2004). These findings lead to the hypothesis of a top-down control system, where the cortex potentially resolves the attentional conflict and triggers analgesia by recruiting brainstem nuclei. Conclusive proof came with a spinal cord fMRI study, where the dorsal horn showed dampened activity during distraction from a painful stimulus (Sprenger et al., 2012). In line with these findings, a correlation between neural activity and analgesia was shown in the RVM (Brooks et al., 2017), which, as described above, can bidirectionally modulate the spinal cord via descending projections. Finally, the analgesic effect of attention was reversed by delivery of the opioid antagonist naloxone, an additional suggestion for the interaction between ACC-PAG-RVM to be responsible for attentional analgesia (Sprenger et al., 2012).

However, not the entire scientific literature is in agreement with the involvement of this descending pathway in attentional analgesia. It has been proposed that separate pathways are recruited in the emotional and attentional modulation of pain (Bushnell et al., 2013). According to Bushnell and colleagues, the ACC-PAG-RVM system is mainly implicated in the emotional modulation of pain perception, for example mood. The reason why this pathway is consistently resolved in attentional analgesia experiments would be the concurrent recruitment of affective and emotional processes. The superior parietal cortex, on the other hand, was implicated in distraction from pain in an experiment that controlled for emotional components (Villemure et al., 2009). This area, part of the attentional network, could thus modulate pain perception not by recruiting brainstem nuclei to modulate the spinal cord, but by cortico-cortical connections with the insula and somatosensory cortices. Additional investigation is needed to reproduce previous findings and clarify whether the network involved in attentional analgesia is restricted to cortical interaction, or it extends to the brainstem.

Cognitive modulation of pain has also been studied in rodents. While it is difficult to impose a shift in attention in animals, especially without introducing additional confounds like reward processing, environment enrichment has been used by researchers to distract rodents from pain. Enrichment is usually achieved by adding toys, running wheels, or peer animals in the same cage and was consistently found to improve neurogenesis (van Praag et al., 2000). In an experiment where rats received nerve injury, the resulting hypersensitivity significantly reduced when rats could run on a treadmill (Stagg et al., 2011). This process was reversed by an opioid antagonist, which was shown to act on PAG and RVM. While this may not be considered a strict equivalent of an attentional analgesia paradigm in humans, it indicates that cognitive mechanisms of pain modulation are phylogenetically preserved and the mechanisms behind it could be similar across species.

1.3 When pain becomes a pathology

1.3.1 Overview

Pain is a physiological mechanism crucial for survival, but it can turn into a pathology. Chronic pain has been defined as “pain that persists past normal healing time” (Treede et al., 2015) and “that typically persists or recurs for more than three months” (Treede et al., 2019). This is a disabling pathology that affects between 13 and 50% of the entire UK population, predominantly females (Fayaz et al., 2016). Currently, a predominant therapeutic approach for chronic pain is prescription of opioidergic drugs, which can lead to dangerous side effects as well as to opioid addiction. Thus, in addition to different pharmacological targets, non-pharmacological approaches to pain relief are being increasingly tested such as Cognitive Behavioural Therapy, exercise interventions and pain management programs.

1.3.2 Molecular and cellular mechanisms in the chronification of pain

Chronic pain is conventionally thought to arise from long-lasting tissue damage and/or inflammatory states. It can initiate with a persistent injury, for example a surgery, where after tissue healing has occurred, pain is never fully resolved. Additionally, nerve injury caused by, for example, cancer or diabetes can lead to the neurochemical and neuroplasticity changes responsible for neuropathic pain. However, the molecular and functional mechanisms leading to the chronification of pain are not yet fully understood. Rodent models, where neuropathic pain is induced by spinal nerve ligation, are extensively used to investigate this issue (Honore et al., 2000). Repeated engagement of nociceptors caused by tissue damage triggers the production of inflammatory molecules, such as prostaglandins, substance P, and cytokines by cells of the immune system or by primary afferents. These can act on G-protein coupled receptors that can sensitize and upregulate ion channels on nociceptors, leading to peripheral

sensitisation (Voscopoulos et al., 2010). Inflammatory molecules and endogenous peptides can also activate a 'silent' class of nociceptors. These are mainly located on C-fibres and do not respond to thermal or mechanical stimuli but are sensitive to neuroinflammation, contributing to primary afferent excitation during sustained nociception (Gold et al., 2010). Peripheral sensitization can lead to hyperalgesia, a physiological mechanism where mild noxious stimuli are perceived as extremely painful. These peripheral changes are normally localized to a site of injury and are resolved after healing. Transient modulations of nociception can however turn into longer lasting modifications in their genetic expression, in a mechanism that contributes to the transition from physiology to pathology (Doleys, 2014; Prato et al., 2017).

Long-lasting modifications to nociception mechanisms in the central nervous system are directly involved in the chronification of pain. For example, it is thought that persistent noxious inputs to the spinal cord can cause neuronal death in dorsal horn interneurons (Moore et al., 2002), responsible for pain modulation. This leads to central sensitization of second order neurons that become more efficient in transmitting noxious stimuli to the brain (Voscopoulos et al., 2010). However, it should be noted that this effect is not seen in all chronic pain models (Polgár et al., 2004). Additionally, central neurons in the spinal cord can feedback to the periphery and contribute to inflammation through axonal reflex (Hagains et al., 2010). Central neuroplasticity mechanisms are also thought to be dependent on NMDA receptors (Woolf et al., 1991), activated by glutamate released by C fibres. These receptors are known to mediate the main mechanism to neural plasticity, long term potentiation, in presence of persistent inputs. Normally, NMDA receptors' channels are blocked by the positive ion magnesium and are not able to allow cations in the neuron after binding glutamate. If the neuron is depolarized by other mechanisms, for example excitation of the AMPA glutamate receptors, the magnesium is expelled, causing the NMDA channel to open. This allows calcium influx, leading to the activation of

signalling transduction cascades that can cause genetic up- or down-regulation and protein phosphorylation (Lüscher et al., 2012). These mechanisms were shown to be crucial for the transition from physiological to pathological nociceptive transmission (Price et al., 1994).

1.3.3 Imbalance in descending pain inhibition in chronic pain

Sustained alterations in the balance of descending modulatory pathways is also thought to be an important mechanism in the development of chronic pain pathologies (Tracey et al., 2007). In support of this view, it has been demonstrated that inflammation can enhance the activity of the pro-nociceptive on cells in the RVM (Burgess et al., 2002). Inhibition of the interaction of these cells with the spinal cord was able to reverse the exaggerated behavioural response to noxious stimuli following nerve injury. This finding indicated an active role of the RVM in the development of a chronic pain phenotype (Bee et al., 2008). Inhibition of the RVM had a similar effect in neuropathic pain models in rodents (King et al., 2009). The noradrenergic system is also thought to play an important role in pain chronification, as it was found to be augmented after nerve injury (Ma et al., 2003). On the other hand, an intact noradrenergic system can delay the onset of enhanced pain perception in injured animals (Hughes et al., 2013). Imbalances between inhibition and facilitation in the descending pain modulatory system are therefore also thought to play a crucial role in maintaining chronic pain states (Burgess et al., 2002; Lee et al., 2008).

Human studies corroborated these hypotheses with numerous findings of dysfunctional endogenous pain inhibition in a variety of chronic pain pathologies (Ossipov et al., 2014) . The most widely used paradigm in the study of endogenous analgesia is conditional pain modulation. Here, a painful stimulus is presented alone or alongside a second noxious stimulus, typically of a different modality, in a second area of the body. In healthy subjects, pain perception to the first stimulus is higher when it is presented alone, in a process defined “pain inhibits pain”, that involves pain modulatory pathways (Knudsen et al., 2011). Studies in different chronic

pain states, including irritable bowel syndrome, chronic back pain and fibromyalgia, revealed this mechanism to be malfunctioning in pathological pain states (Lewis et al., 2012). Interestingly, the efficiency of conditioned pain modulation is also able to predict whether a patient is at risk of developing post-operative pain, with an inverse relationship (Yarnitsky et al., 2008), as well as the efficiency of the noradrenergic drug duloxetine (Yarnitsky et al., 2012).

The cerebral signature of this process is still under investigation. The dorsolateral prefrontal cortex has been implicated in chronic pain maintenance when a PET study resolved abnormal neurotransmission in this region (Grachev et al., 2000). The same area also shows diminished grey matter density in chronic back pain (Apkarian et al., 2004; Seminowicz et al., 2011), which was reversed after pharmacological treatment. Malfunctioning in this area might trigger a series of cascade events that result in imbalances in the pain descending modulatory system. However, it is equally possible that abnormal ascending signalling from the spinal cord and brainstem relays influences the activity of DLPFC and other cortical regions. On the other hand, a recent study used a machine learning approach to categorize healthy volunteers and chronic pain patients on the basis of their BOLD response to non-painful tactile stimulation (Lopez-Sola et al., 2017). In this study, increased activity was detected in regions of the "pain matrix", including the insula, the prefrontal cortex and the cerebellum in fibromyalgia patients compared to healthy controls. Interestingly, activity in these regions directly correlated with the intensity of clinical pain presented by patients, implicating the extent of dysfunction in these areas in the worsening of the pathology. These findings suggest that chronic pain pathologies are characterised by an enhancement of pain facilitatory activity with concurrent malfunctioning of endogenous pain inhibition.

1.3.4 Chronic pain and attention

Attention and pain share a reciprocal, complex relationship. It has been consistently reported that patients suffering from different forms of chronic pain tend show cognitive, and especially attentional deficits. On one hand, patients show hypervigilance toward their painful sensation; on the other, they typically have trouble focusing on external events. Disruption of cognitive processes in chronic pain states has been investigated in experimental settings when it was found that patients in pain perform consistently worse than healthy controls in attentional tasks (Dick et al., 2002; Eccleston, 1994; Grace et al., 1999; Grisart et al., 1999; Oosterman et al., 2012; Veldhuijzen et al., 2012). This effect was not due to impairments in motor ability, but was specific to sustained attention, as revealed by worsening of performance in longer task duration (Oosterman et al., 2012). Additionally, higher reported pain intensity seems to directly correlate with attentional deficits (Weiner et al., 2006). It is thought that these arise because the patients cannot disengage from the ongoing painful sensation, perceived as highly salient, to shift their attention on other events (Legrain et al., 2009). Attention management is therefore an important aspect in cognitive behavioural therapy for chronic pain, helping to divert the patients focus away from their painful sensation (Elomaa et al., 2009; Morley et al., 2004). The neural bases for these attentional impairments are however not currently resolved. A better understanding of the interaction between pain and attention in these pathologies can help to better characterize chronic pain and to improve the use of attentional shifting strategies to pain relief.

1.4 Measuring cortical and subcortical brain activity without entering the skull: functional magnetic resonance imaging

1.4.1 Overview

Functional magnetic resonance imaging (fMRI) allows the non-invasive measurement of neural activity in cortical and subcortical structures. This technique is not directly sensitive to action potentials or electrical activity, but relies on changes in cerebral blood flow to make inferences on the

engagement of brain/spinal cord areas. Increased energetic demand in a CNS region causes the need for blood rich in oxygenated haemoglobin, which has different magnetic properties compared to de-oxygenated haemoglobin. This effect gives rise to the blood oxygen level dependent (BOLD) signal measured by fMRI (Ogawa et al., 1990). A four-dimensional image of the brain/spinal cord is built by measuring BOLD from units, or voxels, without the need of using an exogenous contrasting agent or any type of surgery (Logothetis, 2003, 2008).

Typical task fMRI studies infer spatially localized brain response by comparing the BOLD signal to the time-series of the experiment in a General Linear Model, effectively performing a statistical test for each voxel. In this analysis techniques, a haemodynamic response function is convolved with a model of the experiment, to create a realistic model of the measured signal (**Figure 1.2**). A statistical map of the entire functional image is built where at each voxel is attributed the result of a statistical test. Typically, only voxels whose statistical parameters are higher than a given threshold are categorized as 'active' (Woolrich et al., 2004).

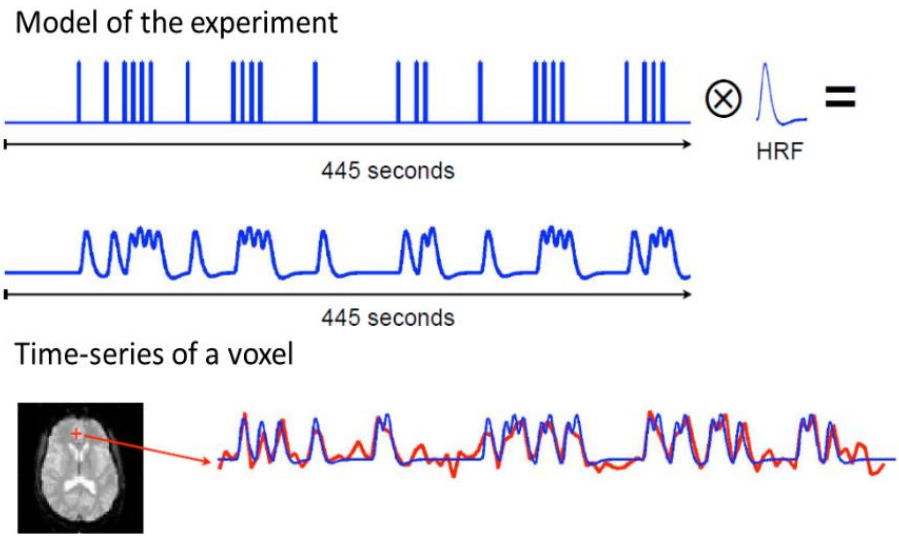


Figure 1.2 *Classical analysis of fMRI data. A model of the experiment is convolved with a hemodynamic response function (HRF) to make it more similar to the measured data. The model is then used for statistical tests in each voxel of the functional image. Reproduced from FSL lectures (2017).*

1.4.2 The problem of multiple comparison and solutions

The most convincing criticism of fMRI research is the famous study of the dead Atlantic salmon (Bennett et al., 2009). Here, a researcher put a dead Atlantic salmon in an fMRI scanner and imaged BOLD in its brain during a visual task. To everyone's surprise, a statistical analysis of the data resulted in quite a few voxels of activation, suggesting residual brain activity in the deceased fish. Unfortunately, since there was no brain activity the resulting "activation" was only a false positive, the result of chance, after a large number of statistical tests in each voxel of the functional image. The multiple comparison problem, caused by too many correlated statistical tests, was already a known problem in the field of statistics, but it was rarely addressed in the fMRI community thus far. In the following years, researchers and software developers began finding solution for the multiple comparison problem. In statistics this is typically solved using a Bonferroni correction, thus by dividing the statistical P value by the number of tests performed. This is however a very stringent method, especially given the very large number of voxels in a brain, and would preclude the detection of subtle signal changes. Two solutions emerged as more appropriate for the analysis of BOLD response in the CNS: cluster thresholding along the usual voxel thresholding and regions of interest (ROI) masking.

Cluster thresholding imposes a specific number of adjacent voxels to respond to the experimental manipulation. This is under the spatial correlation assumption: a given voxel is highly correlated with its immediately neighbouring voxel, and especially so if it they are part of the same brain region. This is often the case when studying large cortical areas. Additionally, an important step of pre-processing of fMRI data is spatial smoothing, where a three-dimensional gaussian kernel is applied to each voxel, averaging its value with the adjacent voxels. This increases spatial correlation even more. A minimum number of voxels reaching a given height threshold is therefore set for a known spatial smoothness (Brett et al., 2003).

Regions of interest masking is normally used when researchers have a strong hypothesis on the CNS region expected to respond to a given stimulus. These hypotheses could derive from previous evidence in animal studies, human lesion studies or even from theoretical models. The use of ROIs greatly improves the multiple comparison problem, depending on the size of the mask used. This approach is however not considered enough to avoid the chance of reporting false positive and it is only typically accepted when used in conjunction with permutation testing. Permutation testing is a non-parametric statistical test that relies on the random shuffling of each data label, for example the group membership in a two-sample t test. This relies on the assumption that if the null hypothesis is true and there are no differences between a group A and a group B for the tested variable, the statistical result will be the same after scrambling the data labels. The resulting P value after permutation testing is therefore the proportion of tests where the difference between A and B is greater or equal to the difference originally observed. A significance level *alpha* is applied as usual. In the case of a one-sample t test, where only one group is tested, the permutation is only applied to the sign of the data, which is be flipped (Nichols et al., 2002; Winkler et al., 2014).

1.4.3 Brainstem imaging

FMRI in the brainstem is presented with additional challenges. Physiological noise, defined as signal of no interest that is derived from physiological processes, is predominant in this area and makes measuring of relevant BOLD signal nontrivial. The brainstem sits in the vicinity to the Circle of Willis, where multiple arteries are joined. Blood flow and CSF pulsatility during the cardiac cycle cause significant signal fluctuation in the BOLD measured in the brainstem. Respiration directly causes apparent movement of this structure and geometric distortions, leading to displacement of single voxels in different phases of the respiratory cycle (Terem et al., 2018). Additionally, the vicinity of the brainstem to air and bone structures, adds distortions to the functional images by causing inhomogeneities in the

magnetic field. The temporal signal to noise ratio is an objective measure of the amount of meaningful signal compared to the random fluctuation (Welvaert et al., 2013). This can be calculated by dividing the mean signal intensity across the time-series by the temporal standard deviation of the functional image (Parrish et al., 2000). The temporal signal to noise ratio is significantly decreased in the brainstem versus the cortex, in a typical functional image (**Figure 1.3**, Brooks et al., 2013). This means that it is much harder to detect a significant change in activity in brainstem nuclei, versus cortical regions. Signal must be recovered to study such areas.

One of the most effective solutions to remove physiological noise influence on functional images is measuring heartbeat and respiration during the scan. This information can then be used in a general linear model in a similar manner to how the experimental model is used during normal fMRI analysis. The variance in the BOLD signal that strongly correlates with one of the two physiological noise sources, or a combination of the two, can be subsequently regressed out, or subtracted, from the rest of the data, leaving meaningful signal (Brooks et al., 2013). Fieldmap unwarping is an additional tool useful for correcting for image distortions caused by the interface between the brainstem and bone/air. In this procedure, an estimate of the field variation is measured before or after the functional images acquisition. The software (FUGUE) will then compute the displacement that would be seen in that area, as well as the necessary correction. Finally, permutation testing aids the identification of a-priori specified brainstem nuclei that would fail to reach the voxel and cluster threshold defined for large cortical areas (Bosma et al., 2016; Brooks et al., 2008).

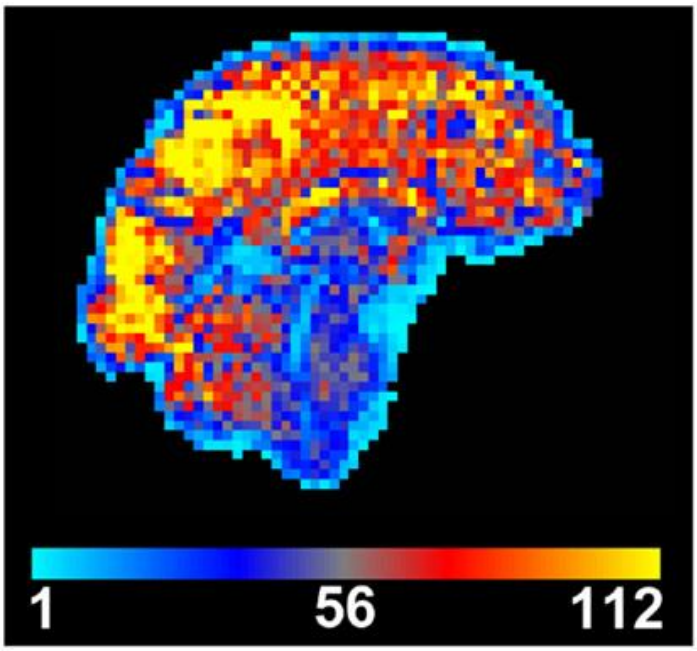


Figure 1.3 *Temporal signal to noise ratio in the brainstem is significantly lower than the cortex. Reproduced from Brooks et al., 2013.*

1.4.4 Spinal cord imaging

When an object (such as a body) is placed within the magnetic field of an MRI scanner, it causes the main magnetic field (B_0) to become non-uniform. This inhomogeneity can give rise to distortion and signal dropout in MRI pulse sequences sensitive to such effects, such as those used to record changes in blood flow, i.e. echo planar imaging (EPI). Typically, the scanner attempts to correct for such inhomogeneity in a process called shimming: the inhomogeneity of the magnetic field is measured, and the necessary correction is computed. Shim coils will apply to the object of investigation the necessary gradient (shim offset) and attempt to improve magnetic field homogeneity. If the magnetic field varies linearly in any of the three axes, for example in a regular brain-only fMRI acquisition, then a combination of linear shim gradients is enough to cancel out this effect. In the spinal cord however, the field presents periodic variations along the cord in the z direction caused by the different magnetic properties of the tissue adjacent to the cord, such as intervertebral discs and vertebrae. The shape

of the body also varies along the z direction with head, neck, and shoulders each introducing different distortions in the field (**Figure 1.4B**). To address this issue, (Finsterbusch et al., 2012) used a technique called z-shimming whereby a specific gradient offset is applied during the acquisition of data from each axial section of the cord. For calibration, a set of reference volumes where the shim offsets are systematically altered in equidistant steps is acquired and visually inspected. The shim offset that gives rise to the highest signal intensity and most appropriate cord shape is chosen for each slice for the subsequent acquisition (**Figure 1.4B**). Thanks to an image acquisition sequence built by Ron Hartley-Davies, Cric's MRI physicist, we were able to use a similar approach on the spinal cord slices in our fMRI acquisition (Chapter 4).

Another complication common to imaging brainstem and spinal cord is caused by cardiac and respiratory processes that cause physiological noise and voxels displacement and consequently a decrease in temporal signal to noise ratio. An effect that is amplified in these regions because of their proximity to blood vessels and air spaces (Brooks et al., 2013; Terem et al., 2018). This problem was again addressed by recording heart and respiratory rate to remove their influence during pre-processing of the images.

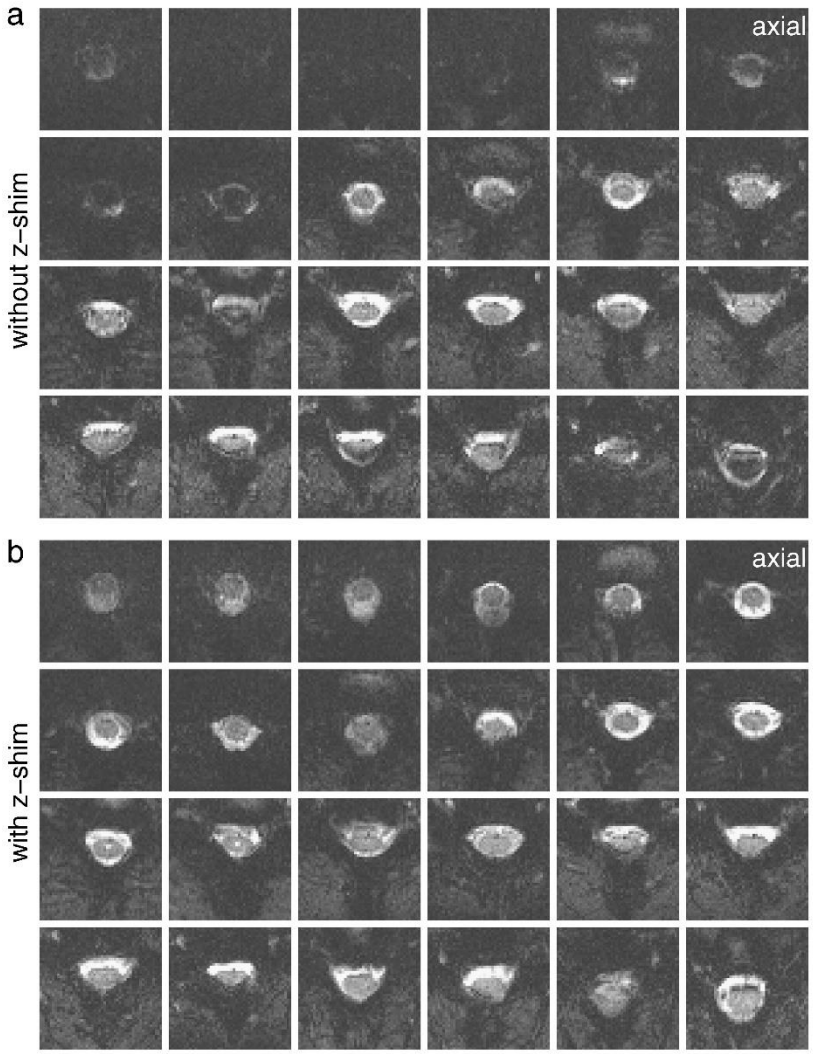


Figure 1.4 *Axial slices in the cervical spinal cord acquired without (a) and with (b) z-shimming. The z-shimming approach recovers most of the signal in the spinal cord. Modified from Finsterbusch et al., 2012.*

1.5 Aims and relevance of the thesis

The aim of the present thesis is furthering the understanding of the mechanisms leading to attentional analgesia in health and pathology.

Prior studies in humans on this topic provide limited evidence for the involvement of the brain and mid-brain in the descending pain modulatory system, whilst the animal literature suggests that the interaction of brain, brainstem and spinal cord is key to understanding this process. However, in humans the involvement of the entire CNS network in attentional analgesia has not been demonstrated in attentional analgesia. There is evidence in the literature for the involvement of endogenous opioids in this process, but the exact site for their action has not been resolved. In addition, noradrenaline has been hypothesized to play a dual role, by modulating attentional demand and spinal analgesia, but its implication remains theoretical.

Understanding more about the brain interaction between pain and attention and their resolution can inform the development of new cognitive strategies to analgesia during acute pain. Examination of this interaction in chronic pain states can improve the understanding of such pathologies, leading to better care. These research questions are also relevant in a broader cognitive neuroscience context for its insights on the re-orienting of attentional resources. The current neuroscience reproducibility crisis problem caused by individual variability and by technical challenges is directly addressed by performing identical analyses on independent datasets. Furthermore, the technical challenges addressed in the present thesis are common to other pain and non-pain imaging research. For example, functional imaging of the spinal cord is also studied in motor learning and in pathologies such as multiple sclerosis.

In study 1, the problem of results reproducibility in fMRI is initially addressed. Data derived from three independent attentional analgesia experiments on healthy volunteers are pooled together to produce a larger dataset (N=57). This is analysed to reproduce and validate previous findings

(Brooks et al., 2017). Subsequently, two effective connectivity analysis techniques, generalised psychophysiological interaction (McLaren et al., 2012), and dynamic causal modelling (Friston et al., 2003), are used to resolve a brain-brainstem network engaged during distraction from pain.

In study 2, fibromyalgia patients were recruited to the imaging centre for an attentional analgesia experiment. This patient population is thought to have deficits in the pain descending modulatory system, but the exact neurobiological mechanism behind these impairments has never been resolved. Additionally, fibromyalgia patients suffer from attentional deficits. Analysis of pain scores combined with brainstem-optimized functional imaging addresses this gap in the literature.

In study 3, an innovative technique for simultaneous functional imaging of the brain, brainstem, and spinal cord (i.e. entire CNS) is used in an attentional analgesia experiment. Effective connectivity analyses aimed at reproducing and expanding the previous results from study 1, by adding the final step of descending pain modulation. The opioid antagonist naltrexone is used to investigate the role of endogenous opioids in attentional analgesia, while the noradrenaline reuptake inhibitor reboxetine examines the role of noradrenaline. Concomitant functional imaging allows to pinpoint the exact location of the effect of these two drugs.

Chapter 2 Parallel cortical-brainstem pathways to attentional analgesia

Work presented in the present chapter refers to the following preprint:

Oliva, V., Gregory, R., Davies, W.-E., Harrison, L., Moran, R., Pickering, A. E., & Brooks, J. C. W. (2020). Parallel cortical-brainstem pathways to attentional analgesia. BioRxiv, 2020.02.20.955161. *https://doi.org/10.1101/2020.02.20.955161*

The paper is currently under review in Neuroimage.

Valeria Oliva conceptualized the paper, performed all the analyses, wrote the paper, revised it, and made the images. LH, RG, and WED acquired all the experimental data. RM was involved in modelling, revised the paper, and supervised the work. AEP conceptualized the paper, revised it, and supervised the work. JCB conceptualized the paper, was involved in the analyses, revised the paper, and supervised the work.

2.1 Introduction

Attentional analgesia is a well-characterised phenomenon whereby increased cognitive load can decrease pain perception (Peyron et al. 2000; Bantick et al. 2002; Brooks et al. 2002; Valet et al. 2004; Brooks et al. 2017; Sprenger et al. 2012). This can be achieved by diverting attention from a painful stimulus to a visual task or simply by active mind-wandering (Bushnell et al., 2013; Kucyi et al., 2013). Central to attentional analgesia is the concept of divided attention, whereby less cognitive resource is available to be allocated to nociception and pain. Since noxious stimuli are inherently salient and therefore attention grabbing (Eccleston et al., 1999), then any concurrent cognitive task must compete for 'attentional' resource. Attention is thus cast both as a key component of pain behaviour (i.e. attending to pain Crombez et al., 2004; Legrain et al., 2009; Roelofs et al., 2002) as well as a putative mechanism for pain relief. The processes regulating attentional focus is of importance in the development, maintenance and potentially resolution of chronic pain states.

The mechanisms that allow attention to regulate pain are currently not well understood and there has been ongoing debate about whether attentional analgesia requires engagement of descending control to attenuate nociception (Brooks et al., 2017; Bushnell et al., 2013; Lorenz, Minoshima, & Casey, 2003; Tracey et al., 2002; Valet et al., 2004). These studies have linked several cortical regions to the attentional analgesic effects, including the anterior cingulate cortex (ACC), dorsolateral prefrontal cortex (DLPFC) and also components of the descending pain control system including periaqueductal grey (PAG), rostroventromedial medulla (RVM) and locus coeruleus (LC). An interaction between cortical and mid-brain structures during distraction from pain has been identified (Lorenz et al., 2003; Valet et al., 2004), but these previous studies were unable to examine interactions between the pontomedullary regions that are known to be important for the descending control of nociception.

The PAG, RVM and LC are all candidates for mediating attentional analgesia given their known anti-nociceptive roles (Millan, 2002). For example, multiple animal studies have demonstrated that interactions between the PAG and RVM produces endogenous analgesia, mediated by spinally projecting neurons in the RVM (Basbaum et al., 1979; Fields et al., 1978; Heinricher et al., 2009). Together with the ACC, these regions form one of the main pain modulatory pathways involved in the bidirectional modulation (i.e. facilitation and inhibition) of nociception in the spinal cord dorsal horn (De Felice et al., 2016; Ossipov et al., 2010; Quintero, 2013).

Similarly, the LC is another potential candidate region that could mediate the interaction between attention and pain because of its projections to the spinal cord which release noradrenaline to produce analgesia (Hirschberg et al., 2017; Llorca-Torralba et al., 2016). Additionally, it has a known role in salience signalling and attention mediated by ascending projections (Aston-Jones et al., 1999; Sales et al., 2019; Sara et al., 2012). Despite it being challenging to resolve with fMRI (Astafiev et al., 2010; Liu et al., 2017), the LC was recently identified as the only region whose activity reflected the interaction between task and temperature in an attentional analgesia paradigm (Brooks et al., 2017). The LC could therefore contribute to attentional analgesia as part of the PAG-RVM system, or as a parallel descending modulatory pathway perhaps receiving inputs directly from ACC (Aston-Jones et al., 1991; Bajic et al., 1999).

Within this framework the ACC is ideally placed to mediate between competing cognitive demands (e.g. between a sustained visual attention task and pain) as it is active during conflict resolution (Braver et al., 2001; Kerns, 2006; Kim et al., 2011), its activity is modulated by attention (Davis et al., 2000) as well as being consistently activated by painful stimuli (Brooks et al., 2017; Garcia-Larrea et al., 2013; Peyron et al., 2000; Wager et al., 2013). The ACC is known to code for pain intensity (Büchel et al., 2002; Coghill et al., 2003) and unpleasantness (Rainville et al., 1997), furthermore, sub-divisions (e.g. dorsal anterior ACC) are involved in high level cognitive

appraisal of pain, including attention (Büchel et al., 2002). Some have proposed a specific role for dorsal ACC (dACC) in pain perception (Lieberman et al., 2015), though this is disputed with other studies suggesting that activity within this structure reflects the multifaceted nature of pain (Wager et al., 2016). Connectivity between the ACC and structures involved in descending pain control e.g. the PAG, has been shown to vary with pain perception due to both attentional modulation of pain and placebo analgesic responses (Bantick et al., 2002; Eippert et al., 2009; Petrovic et al., 2000; Valet et al., 2004) suggesting a potential role in attentional analgesia.

We hypothesised a top-down pathway mediating attentional analgesia where the PAG receives attentional-shift signals from the ACC and/or LC and directs the RVM and/or LC to attenuate nociceptive processing in the spinal cord. Given the multiplicity of possible pathways and interactions by which activity in the brainstem can generate analgesia, we anticipated that effective connectivity analyses could resolve the roles of these regions (identified in our previous investigation (Brooks et al., 2017) during attentional analgesia. To increase the statistical power to undertake this connectivity analysis, additional fMRI datasets were acquired using the same paradigm as per Brooks et al. (2017). Analysis of these additional datasets reproduced our previous regional activation results, and so the three datasets were pooled for the effective connectivity analyses and modelling. We tested for psycho-physiological interactions (PPI, Friston et al., 1997; McLaren et al., 2012; O'Reilly et al., 2012) to explore whether the connectivity between the PAG, RVM, LC and ACC altered during the experimental paradigm. Finally, we used dynamic causal modelling (DCM, Friston et al. 2003) to test the directionality and strength of the connections.

2.2 Methods

2.2.1 Participants

Subjects were recruited using poster and email adverts at the University of Bristol for three different pain imaging studies at the Clinical Research and Imaging Centre (CRiCBristol) that used the same experimental paradigm: an initial study on attentional analgesia (Brooks et al., 2017), a study on sleep disruption and a study on fibromyalgia. The first two studies were approved by the University Bristol, Faculty of Science, Human Research Ethics Committee (reference 280612567 and 291112606 respectively) and the fibromyalgia study was approved by NHS South Central Oxford B Research Ethics Committee (reference 13/SC/0617).

All subjects gave written informed consent after application of standard inclusion/exclusion criteria for participation in MRI studies. The presence of significant medical/psychiatric disorders (including depression) or pregnancy precluded participation. Subjects with a chronic pain condition, or those who were regularly taking analgesics or psychoactive medications, as determined by self-report, were also excluded. All subjects were right-handed, verified with the Edinburgh handedness inventory (Oldfield, 1971).

The discovery cohort were 20 right-handed healthy subjects (median age 25 years, range 18–51 years, 10 females). Subjects attended for two sessions. During the screening visit, written consent was obtained and both task difficulty and temperature of the thermal stimulation were individually calibrated. Subsequently the subjects returned for the test session where they completed the experiment in the MRI scanner (For full details on the discovery cohort see Brooks et al., 2017).

The validation cohort composed of control subjects from two separate studies: Twenty healthy volunteers (median age 23, range 20-33, 10 females) were recruited for a study investigating the effects of sleep disturbance on attentional analgesia. Subjects completed the same experiment protocol on two occasions; after a habitual and a disturbed

night's sleep (at the sleep laboratory at CRiCBristol). For the present study, only data obtained from the control condition was used, wherein subjects experienced their habitual sleep regime the night prior to their scan. A second group of 20 healthy participants (median age 31.5, range 20-59, 18 females) was recruited from the control group of a study analysing attentional analgesia in fibromyalgia patients.

2.2.2 Experiment

Thermal stimuli were delivered to the left volar forearm (approximately C6 dermatome) using a circular contact thermode (CHEPS Pathway, MEDOC) and each lasted 30 seconds. The noxious thermal stimulus was individually titrated to obtain a 6 out of 10 pain rating (42-45˚C plateau). The innocuous stimulus plateau was set at 36˚C. In both cases brief heat spikes of 2, 3 and 4˚C above the plateau temperature were added in a random sequence at a frequency of 1Hz. This heating profile was used to maintain painful perception, whilst avoiding skin sensitisation. The baseline thermode temperature was 32˚C.

For the Rapid Serial Visual Presentation task (RSVP, Potter et al. 1969), subjects identified a visual target (the number "5") among distractors (other letters and numbers), presented using back-projection to a screen visible to subjects lying in the scanner, responding with a button box (Lumina LP-400, Cedrus). Prior to entering the scanner, the speed of character presentation for the hard RSVP task was individually calibrated to obtain a 70% task performance. Task performance was assessed by calculating d', a measure of task performance typically used in behavioural studies calculated by subtracting the z-transformation of the false alarm rate from the z-transformation of the hit rate (Stanislaw et al., 1999). The d' values were generated for a range of trial RSVP speeds for each subject and the data was fitted with a sigmoidal function (commonly used in psychophysics). This best fit model parameters were used to estimate each subject's presentation

speed corresponding to a 70% task performance, which ranged from 32 to 96ms. The speed of presentation for the easy RSVP task was either 192 or 256ms, depending on performance in the hard task (if the "hard" task interval for the subject was <80ms or >80ms, respectively).

2.2.3 Data acquisition

In the scanner, participants received noxious or innocuous thermal stimuli (high/low) while simultaneously performing the RSVP task with two levels of difficulty (easy/hard). Thus, there were four experimental conditions (in a 2x2 factorial experimental design): *easy*|*low, easy*|*high, hard*|*low, hard*|*high*. Each condition was repeated 4 times. Each experimental epoch started with instructions (5s), followed by the 30s experimental condition, followed by a 10s rest period before an 8s rating period where subjects rated the perceived pain intensity from 0 to 10 on a visual analogue scale (VAS) (Brooks et al., 2017). The post-stimulus interval, between the rating period and subsequent instructions, was 17s.

The experiment for the validation cohort (n=38) was essentially identical to that of the discovery cohort. The titrated mean high temperature for the discovery cohort was 44.2˚C and for the validation cohort it was 43°C (range 42–45 °C). The whole imaging session lasted 26 minutes for the discovery cohort and sleep-disruption cohort and was 22 minutes for the fibromyalgia cohort. The difference in experiment duration stemmed from the removal of a superfluous additional control condition, with no distraction during high temperature, in the fibromyalgia study as it was not required as part of the core 2x2 factorial design and had the additional benefit of reducing the number of noxious stimuli overall delivered to these subjects (and more importantly to the patients in the matched study group).

All data was acquired with a 3T Skyra MR system (Siemens Medical Solutions, Erlangen, Germany) and 32-channel receive only-head coil. In addition to blood oxygenation level dependent (BOLD) functional data, T1 weighted structural scans were acquired with an MPRAGE sequence to allow

image registration. Functional imaging data was acquired with TE/TR=30/3000ms, GRAPPA acceleration factor = 2, resolution = 1.5 x 1.5 x 3.5 mm. The slices were angulated perpendicularly to the base of the 4th ventricle to better match the orientation (long axis) of brainstem nuclei. This slice orientation optimised the ability to discriminate between the small brainstem structures in the transverse plane and while allowing the capture of whole brain activity within 3 seconds. Fieldmap data was acquired with a gradient echo sequence (TE1/TE2/TR = 4.92 / 7.38 / 520ms, flip angle 60°, resolution 3 x 3 x 3 mm). During scanning, a pulse oximeter and a respiratory bellows (Expression MRI Monitoring System, InVivo, Gainesville, FL) were used to monitor cardiac pulse waveform and respiratory movement, recorded using an MP150 data logger (Biopac, Goleta, CA, USA) for subsequent physiological noise correction (Brooks et al., 2013).

2.2.4 Behavioural Data analysis

Pain VAS ratings were converted to a 0-100 scale for a repeated measures ANOVA in SPSS software (after Brooks et al., 2017). Following estimation of main effects (task, temperature) and interactions, post-hoc paired t-tests were performed. The presence of attentional analgesia was pre-defined as a significant interaction between task difficulty and high temperature on pain rating assessed with post-hoc paired t-testing ($p < 0.05$). To test for differences between the discovery and validation cohorts; group membership was added as a between subject factor to the two within subject factors (task and temperature). Subsequent analysis is reported on the pooled cohort.

2.2.5 Imaging Data analysis

2.2.5.1 Image Pre-processing

Functional images were corrected for motion using MCFLIRT (Jenkinson et al., 2012) and co-registered to each subject's structural scan using brain boundary-based registration (Greve et al., 2009) and then to the 2mm template ("MNI152") brain using a combination of fieldmap based unwarping using FUGUE (Jenkinson, 2003), linear transformation using FLIRT

(Jenkinson et al., 2001) and non-linear registration using FNIRT (Andersson et al., 2007) with 5mm warp spacing. Functional data was spatially smoothed with a kernel size of 3mm (FWHM) and high pass temporally filtered with a 90s cut-off. Two subjects in the validation cohort and one from the discovery cohort were excluded from the analyses at this stage because of signal dropout (primarily in the brainstem) in the EPI data, leaving 57 subjects.

Physiological data (cardiac and respiratory) were visually inspected and manually corrected as required. All first level models (block design and gPPI) included a basis set of regressors for physiological noise correction, which included 16 cardiac and respiratory terms (sine and cosine terms up to the 4th harmonics), plus 16 terms that attempt to capture the interaction between cardiac and respiratory processes (Brooks et al., 2008; Harvey et al., 2008). It is important to note that the relative phases for each slice (e.g. position in the cardiac cycle at time of acquisition, used to calculate the physiological regressors), were calculated independently and modelled separately in the GLM. Only one out of 57 subjects lacked physiological recordings, due to equipment failure – this subject was not excluded as it was considered unlikely to increase false-positive rate in the final sample. Local autocorrelation correction was performed using FILM (Woolrich et al., 2001) as part of model estimation, which also attempted to correct for physiologically driven signals (originating from cardiac/respiratory processes) using slice-dependent regressors in PNM (FSL). Relative mean motion was extracted from each subject to look for excessive head movement. The average motion across subjects was 0.068mm, ranging from 0.02 to 0.27. Since no subject moved more than half a voxel (i.e. 0.75 mm), no one was excluded on this basis.

2.2.5.2 First level analyses

The four conditions (*easy|high, hard|high, easy|low, hard|low*) and tasks of no interest (cues and rating periods) were modelled using a hemodynamic response function (gamma basis function, σ = 3s, mean lag = 6s) alongside the physiological regressors within the general linear model in

FEAT (Jenkinson et al., 2012). A separate analysis tested for an intra-subject parametric relationship between pain ratings (one per block) and BOLD signal (Büchel et al., 1998). In addition to tasks of no interest and physiological signal regressors, a constant regressor for all blocks (weighting = 1) and a regressor weighting the individual pain ratings for each block were included. None of the regressors were orthogonalized with respect to any other.

2.2.5.3 Second level analyses

Main effects were specified as positive and negative main effect of attention (hard versus easy task, and vice versa) and positive and negative main effect of temperature (high versus low thermal stimulus, and vice versa). A task x temperature interaction contrast was also specified. The parametric data was assessed using a simple group average – to examine whether the linear relationship between pain ratings and brain activity was consistent across the group. Lastly, a paired analysis compared activity during the *easy*|*high* and *hard*|*high* conditions - to examine whether the inter-subject difference in average pain ratings (i.e. *easy*|*high* minus *hard*|*high*) was linearly related to the corresponding difference in BOLD signal (similar to Tracey et al. 2002 and Brooks et al. 2017). To test for differences between the discovery cohort and the validation cohort, we used an unpaired t test with FLAME (height threshold $z > 3.09$, corrected cluster extent threshold $p < 0.05$), in line with guidelines on corrections for familywise error (FWE) (Eklund et al., 2016). Subsequent analyses of the pooled cohort (i.e. all 57 subjects) used the same threshold.

2.2.5.4 Brainstem-specific analyses

Detecting activation in the brainstem is non-trivial due to its susceptibility to physiological noise and artefacts (Brooks et al., 2013), small size of structures of interest and relative distance from signal detectors in the head coil. Consequently, a brainstem focussed analysis was performed at the group level using a series of anatomical masks and statistical inference using permutation testing (Nichols et al., 2002) in RANDOMISE (part of FSL).

Analyses utilised pre-defined regions of interest based on (i) a whole brainstem mask derived from the probabilistic Harvard-Oxford subcortical structural atlas (Desikan et al., 2006) and thresholded at 50% and (ii) previously defined probabilistic masks of the a priori specified brainstem nuclei (RVM, LC, PAG) from Brooks et al. (2017). The number of permutations were set to 10,000 in line with guidelines (Eklund et al. 2016) and results reported using threshold free cluster enhancement (TFCE) corrected $p < 0.05$ (Smith et al., 2009).

2.2.5.5 Psycho-Physiological Interactions (PPI)

Effective connectivity analyses were performed on the pooled cohort. We used generalised PPI (gPPI) to detect changes in interactions between regions during specific experimental conditions (O'Reilly et al. 2012; McLaren et al. 2012; Friston et al. 1997). In this technique a physiological signal (e.g. the time-course extracted from a seed region) is convolved with a modelled psychological variable (i.e. each one of the experimental conditions) to build interaction regressors. All interaction regressors were added to a general linear model (GLM) that also included the non-convolved experimental conditions and tasks of no interest (e.g. the rating period). Contrasts were built to test for connectivity differences that could be explained by the main effects of task and temperature and the task * temperature interaction.

Four regions identified by the main effect analyses (temperature and/or attention) and inter-subject analgesic regression model in the pooled cohort, were selected as seed-regions for the gPPI analysis: PAG, right LC and ACC in the main effect of task and RVM in the main effect of temperature. For each subject, the physiological BOLD time course was extracted from the peak voxel of the pre-processed images (as described in the section 'Image Pre-processing') within each functional mask, and gPPI performed at the first level. Subsequently, group responses were estimated with permutation testing within the same functional masks e.g. effective connectivity between PAG seed region and the other three regions (RVM, right LC, ACC). To aid

interpretation of significant results in the task * temperature interaction contrast, we focussed on the conditions of interest (i.e. *easy|high* and *hard|high*). Parameter estimates were extracted by first defining a sphere of radius 2mm at the voxel of greatest significance in the group gPPI result, then back-transforming this mask to subject space and extracting the signal from the voxel with highest Z-score.

In summary, the procedure for gPPI analysis was:

- Pre-processing of functional data
- Time series extraction from functional masks
- Convolution of time-series with experimental condition
- Contrasts of interest tested using GLM via first level (single subject) analysis
- Group analysis permutation testing with functional masks
- Extraction of parameter estimates from the conditions of interest.

2.2.5.6 Dynamic Causal Modelling (DCM)

Given the inability of gPPI to resolve the directionality of connections, we sought to extend our findings by using DCM (Friston et al., 2003). This technique allows the specification of a hypothetical network model (based on equation 1) fitted to the fMRI data to resolve connection strengths.

The change in activity of each region in a model with j inputs and n brain regions is formalized as follows:

(1)

$$\frac{dx}{dt} = (aA + \sum_{j=1}^{n} u_j bB^j)x + cCu + \omega$$

Where:

x - neuronal state of a region (i.e. BOLD signal convolved with haemodynamic response function)

A - binary vector that defines the connectivity of x is to each of the other regions in the model,

a - vector of parameters that define the strengths of such connections,

u - external input to the model,

B - binary vector that defines whether model connections are modulated by external input,

b - vector of parameters that defines the strength of such modulation,

C - binary vector that defines whether x directly receives the external input,

c - contains parameters that regulate the strength of the received input,

ω - random neuronal noise.

Note since the model is estimated in a Bayesian framework, parameters are not single values but are posterior densities.

Given the results of the PPI analysis, we specified bi-linear, one state, stochastic, input centred DCMs (Daunizeau et al., 2009, 2012) in SPM 12 (Wellcome Trust Centre for Neuroimaging, London, UK). The models were estimated on a computer cluster (BlueCrystal) in the *Advanced Computing*

Research Centre, University of Bristol – http://www.bristol.ac.uk/acrc/. Random effects Bayesian Model Selection (BMS) was used to compare the models and Protected Exceedance Probability, the likelihood of a given model in respect to the others tested, was calculated. Bayesian Omnibus Risk, a measure of the risk of all models having the same frequency within the population, was also computed (Rigoux et al., 2014). Bayesian model averaging (Penny et al., 2010) was used to extract the parameter estimates of interest.

2.3 Results

2.3.1 Comparison of the discovery cohort and validation cohort

The behavioural and imaging datasets from the validation and discovery cohort were quantitatively compared as criteria to justify the decision to pooling the two together for subsequent analyses. A three-way repeated measures ANOVA was carried out on the pain scores using task and temperature as within subject factors and the group (discovery vs validation cohort) as between subject factor. This analysis showed no effect of group on the effects of temperature ($P = 0.481$), nor task ($P = 0.833$), nor on the task * temperature interaction ($P = 0.481$), indicating that the two groups are comparable in terms of the behavioural effect.

An unpaired t-test on the functional image contrasts did not show any statistically significant differences between the discovery and validation cohorts for the main effect of temperature (positive and negative), main effect of task (positive and negative) and interaction contrast (positive and negative). Given the lack of demonstrable statistical differences between the two cohorts, we went ahead with our planned intention to combine the three datasets and all subsequent results relate to the pooled cohort comprising 57 subjects. We also note that the use of strict cluster thresholds for the brain, and of permutation testing for ROI-based analyses in 'noisy' brainstem regions, can produce robust and reproducible results even with a sample size of 20 Brooks et al., 2017).

2.3.2 Behavioural analysis (Pooled cohort)

The average high (noxious) temperature in the pooled cohort was 43.4°C (range 42°C - 45°C). Analysis of the pain ratings showed the expected main effect of temperature on pain scores ($F(1, 56) = 252.799$, $P < 0.0001$, repeated measures ANOVA) but no main effect of task ($F(1,56) = 2.935$, $P = 0.092$). There was a clear task x temperature interaction ($F(1, 56) = 31.969$, $P < 0.0001$, **Figure 2.1**) and post-hoc paired t-test showed performance of the hard task produced a decrease in pain scores in the high temperature

condition (mean *hard|high* = 38.1, SD 17.0 vs *easy|high* = 42.1, SD 16.5, P < 0.0001, Bonferroni corrected), consistent with an attentional analgesic effect (**Figure 2.1**).

Additional exploratory analysis did not detect any evidence of an order effect in the pain ratings (F (3,165) = 0.164, P=0.92 one-way repeated measures ANOVA), meaning that we did not observe a significant sensitisation or habituation in subjects' pain ratings. Similarly, there was no effect of gender on attentional analgesia (F(1,55)=0.091, P=0.764), nor on the main effects of temperature (F(1,55)=1.69, P=0.198) or task on pain ratings (F(1,55)=0.253, P=0.617, all mixed model ANOVAs).

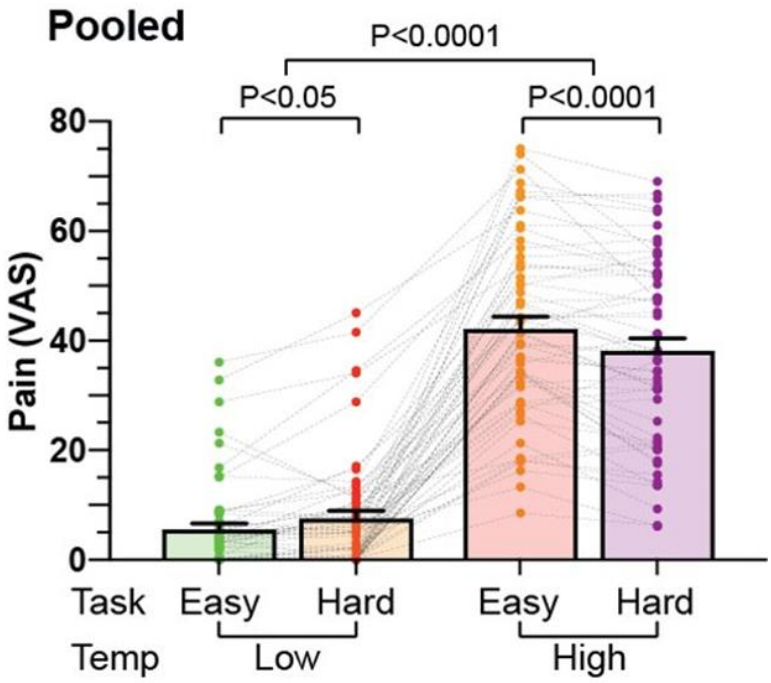


Figure 2.1 *Pain ratings across experimental conditions for the pooled cohort (N=57). A 2-way repeated measures ANOVA on the pain ratings showed the expected main effect of temperature (P < 0.0001) and a task x temperature interaction (P < 0.0001). The attentional analgesic effect was observed as a decrease in pain scores in the high temperature condition during the hard task compared to the easy task (P < 0.0001, post-hoc paired t-test). In contrast there was also a small increase in pain scores in the low temperature condition during the hard task compared to the easy task (P < 0.05). The main effect of task was not significant (P = 0.92). Error bars represent the standard error of the mean.*

2.3.3 Whole Brain & Brainstem-focussed analysis (Pooled cohort)

Activations were found for the positive main effect of temperature in a range of regions including the anterior and posterior cingulate cortices, precuneus, cerebellum, post-central gyrus (S1), dorsal posterior insula and opercular cortex, in the latter three cases with more prominent clusters contralateral to the side of thermal stimulation (**Figure 2.2A**). In the negative main effect of temperature, significant clusters were found in the frontal medial cortex and in the subcallosal cortex (**Figure 2.2A**). We also found a cluster of activation in the RVM at this whole brain level. However, to improve our ability to resolve activity in hindbrain structures, we undertook permutation testing using a whole brainstem mask, which revealed clusters of activation in the positive main effect of temperature in the ventral PAG, LC bilaterally as well as the RVM (**Figure 2.3A**, $p < 0.05$, TFCE corrected).

Analysis of the positive main effect of task, showed extensive areas of activation within the lateral occipital cortex, superior parietal lobule, anterior cingulate cortex and anterior insula, as well as the PAG (**Figure 2.3A**). In the negative main effect of task, clusters were located in the posterior cingulate cortex, frontal medial cortex and in the lateral occipital cortex (**Figure 2.2B**). Permutation tests within the whole brainstem mask showed multiple clusters of activation, including in the LC bilaterally, RVM and PAG (**Figure 2.3B**, $p < 0.05$, TFCE corrected).

In the interaction contrast between task and temperature no cluster reached significance either at the whole brain level nor when using the whole brainstem masked analysis.

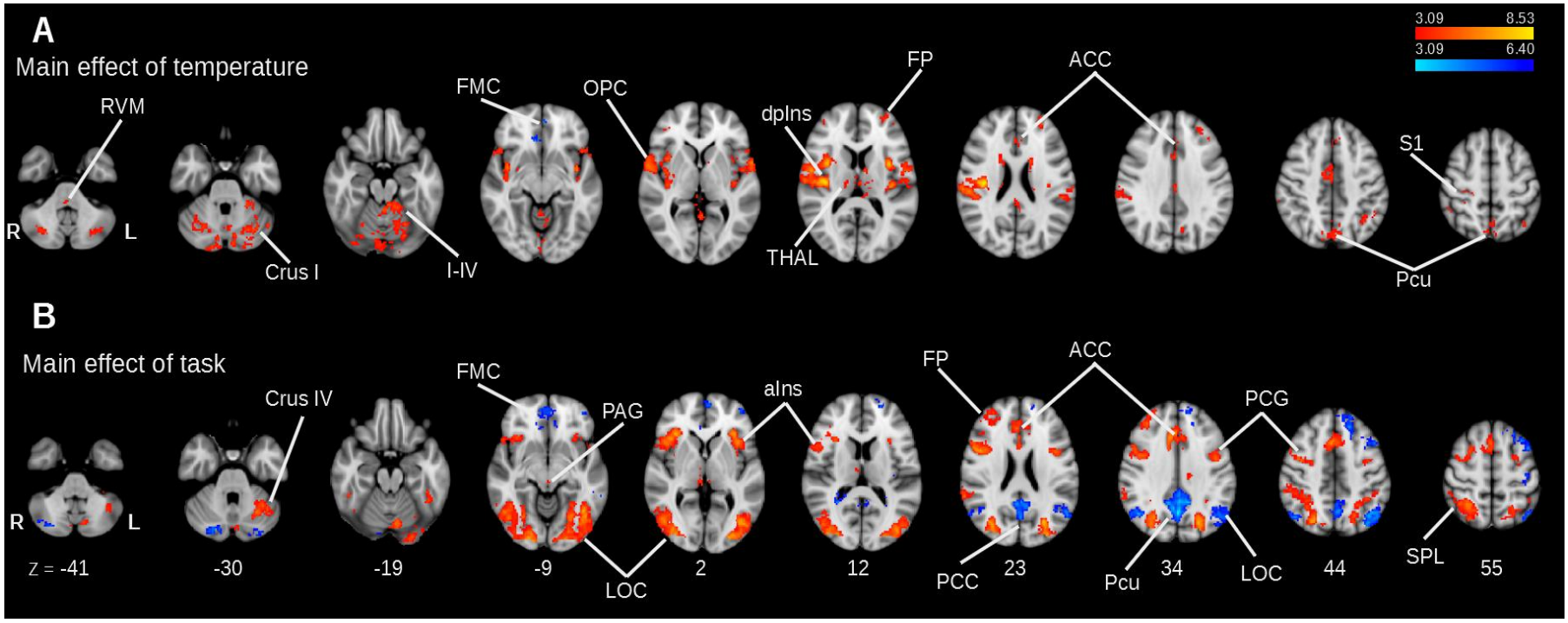


Figure 2.2 *Whole brain main effect analyses in the pooled cohort (N=57). Positive (red/yellow) and negative (blue/light blue). Data was obtained from cluster-based thresholding using an initial threshold of Z > 3.09 and FWE corrected p < 0.05, one-sample t-test. (A) Main effect of temperature. Positive activation in the high temperature conditions was found in anterior cingulate cortex (ACC), thalamus (THAL), dorsal posterior insula (dpIns), precuneus (Pcu), primary somatosensory cortex (S1) and rostroventromedial medulla (RVM). Activation in the negative main effect of temperature (low temperature vs high temperature) was observed in the frontal medial cortex (FMC). (B) Main effect of task. Activity in the positive main effect was found in the anterior insula (aIns), lateral occipital cortex (LOC), ACC, superior parietal lobule (SPL). Activity in the negative main effect was found in the frontal pole (FP), posterior cingulate cortex (PCC) and Pcu.*

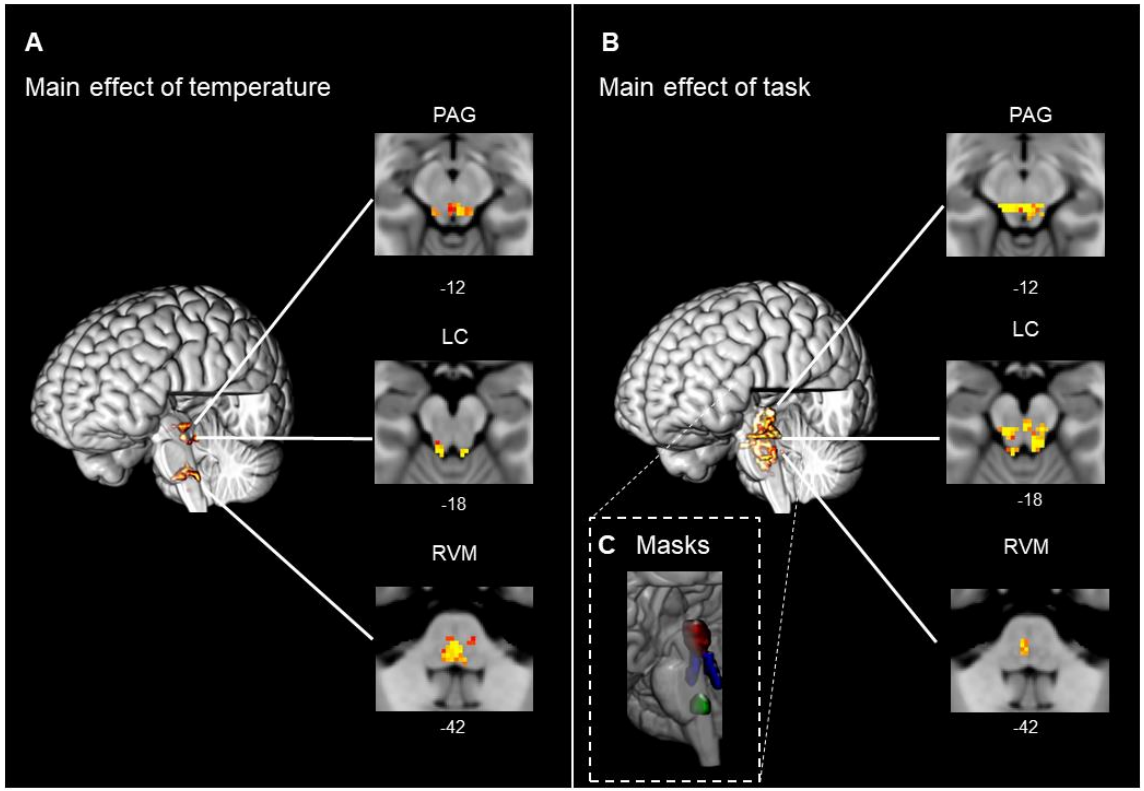


Figure 2.3 *Main effect analyses in the brainstem. Results obtained after permutation testing with a probabilistic whole brainstem mask ($p < 0.05$, TFCE corrected). (A) Clusters of activation in the brainstem corresponding to the main effect of temperature was found in the ventral periaqueductal grey (PAG), rostroventromedial medulla (RVM) and bilateral locus coeruleus (LC). (B) Many areas in the superior brainstem show activity in the main effect of task, including the PAG, RVM and bilateral LC. (C) Shows the position and extent of the anatomical masks defined in (Brooks et al., 2017), identifying the PAG (red), LC (blue), RVM (green).*

These findings from the pooled cohort showed close similarity to those of Brooks et al. (2017) with the same areas found in the main effects analysis (**Supplementary Table 1**). The additional findings at a whole brain level were that both the RVM and the precuneus now appear in the main effect of temperature and the dorsolateral PAG in the main effect of task (the RVM and PAG were only seen in a nucleus specific masked analysis in Brooks et al., 2017). Similarly, activity in the brainstem is now seen in more areas using a whole brainstem mask rather than only in the nucleus specific masks (e.g. main effect of temperature in RVM alone previously versus RVM, LC and PAG in this pooled analysis).

Whilst the patterns of activity within the cerebrum were largely non-overlapping, there were some areas which appeared to be common to both the main effect of task and temperature: ACC, FMC, and cerebellum. To formally test the degree of overlap, we performed a conjunction analysis (Friston et al., 1999) which revealed that of the hypothesised brain regions involved in the task, only the ACC was active in both conditions (cluster forming threshold $Z > 3.09$, FWE corrected $p < 0.05$).

2.3.4 Linear encoding of pain intensity

Brain regions whose activity was linearly related to perceived pain intensity were identified using an intra-subject parametric regression. This revealed a network of positively correlated regions (similar to those seen in the main effect of temperature) including primarily the right (contralateral) dorsal posterior insula and S1, the anterior cingulate cortex, frontal lobe and the precuneus (**Figure 2.4A**). Regions showing a linear decrease in activation with pain ratings were restricted to the occipital cortex bilaterally and ipsilateral primary somatosensory cortex (**Figure 2.4A**). Permutation testing in the brainstem (using RVM, PAG and LC masks) identified only the RVM as showing a positive correlation with pain intensity (**Figure 2.4B**). No brainstem region showed a negative correlation with pain. All these findings were consistent with Brooks et al (2017), with the addition of a cluster identified in the thalamus (**Supplementary Table 2**).

2.3.5 Regions whose activity correlates with analgesic effect

An inter-subject whole-brain mixed effects comparison between the *hard|high* and *easy|high* conditions did not identify any region whose activity linearly correlated with the differences in pain ratings (i.e. analgesia). A parametric regression showed a linear relationship between difference in activity and analgesic effect in only the contralateral (right) LC (i.e. decreased pain ratings were associated with increased BOLD difference), after permutation testing with LC, RVM and PAG masks (**Figure 2.4C**). A positive relationship was noted between the difference ("Delta") in parameter estimates extracted from the rLC and the attentional analgesic effect on pain scores (**Figure 2.4C**).

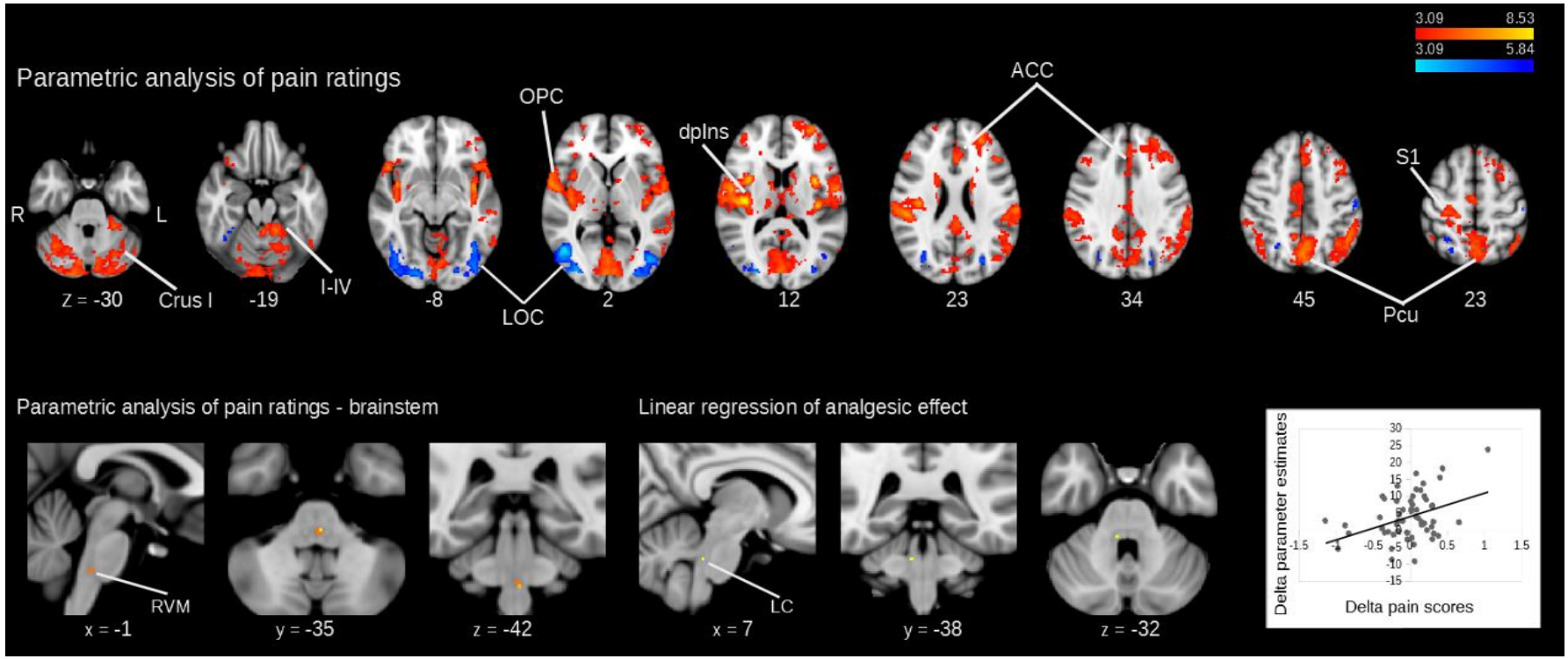


Figure 2.4 *(A) Pain encoding regions were identified by intra-subject parametric regression with pain ratings across all the experimental conditions, in the whole brain analysis. Regions whose activity linearly increased with perceived pain are shown red-yellow and regions whose activity decreases with pain in blue-light blue. (height threshold Z > 3.09, corrected cluster extent threshold p < 0.05) B) Brainstem intra-subject parametric regression with pain ratings, using RVM, LC and PAG masks. Only the RVM showed a linear increase in activity with the pain scores (p < 0.05, TFCE corrected). C) The right (contralateral) LC was the only region whose activity correlated with the analgesic effect (i.e. ratings of easy|high – hard|high, inter-subject parametric regression using a LC mask p < 0.05, TFCE corrected).*

2.3.6 gPPI analysis between neural hubs linked to attentional analgesia

To determine the changes in neuronal communication associated with attentional analgesia, this analysis aimed to identify changes in effective connectivity associated with task difficulty, temperature, and the task x temperature interaction. Results from main effects, conjunction and parametric analgesia analyses provided the motivation for selecting a subset of the activated brain regions, that were subsequently used for connectivity analysis. Time courses were extracted from functional masks for gPPI analyses: RVM for the main effect of temperature, and PAG, rLC and ACC for the main effect of task (see Methods). Permutation testing revealed increased connectivity with the following contrasts (see **Figure 2.5A**):

- RVM seed - increased connectivity to PAG for the interaction contrast
- ACC seed - increased connectivity with the right (contralateral) LC in the interaction contrast and with the PAG in the main effect of task
- PAG seed - did not show any significant change in effective connectivity
- rLC seed - did not show any significant change in effective connectivity.

For all gPPI results, parameter estimates were extracted from the voxel with greatest significance in each individual to explore the nature of these interactions (**Figure 2.5B**). In all cases, the parameter estimates were greater in the *hard|high* compared to the *easy|high* condition, indicating an increase in coupling in the condition associated with attentional analgesia i.e. *hard|high*.

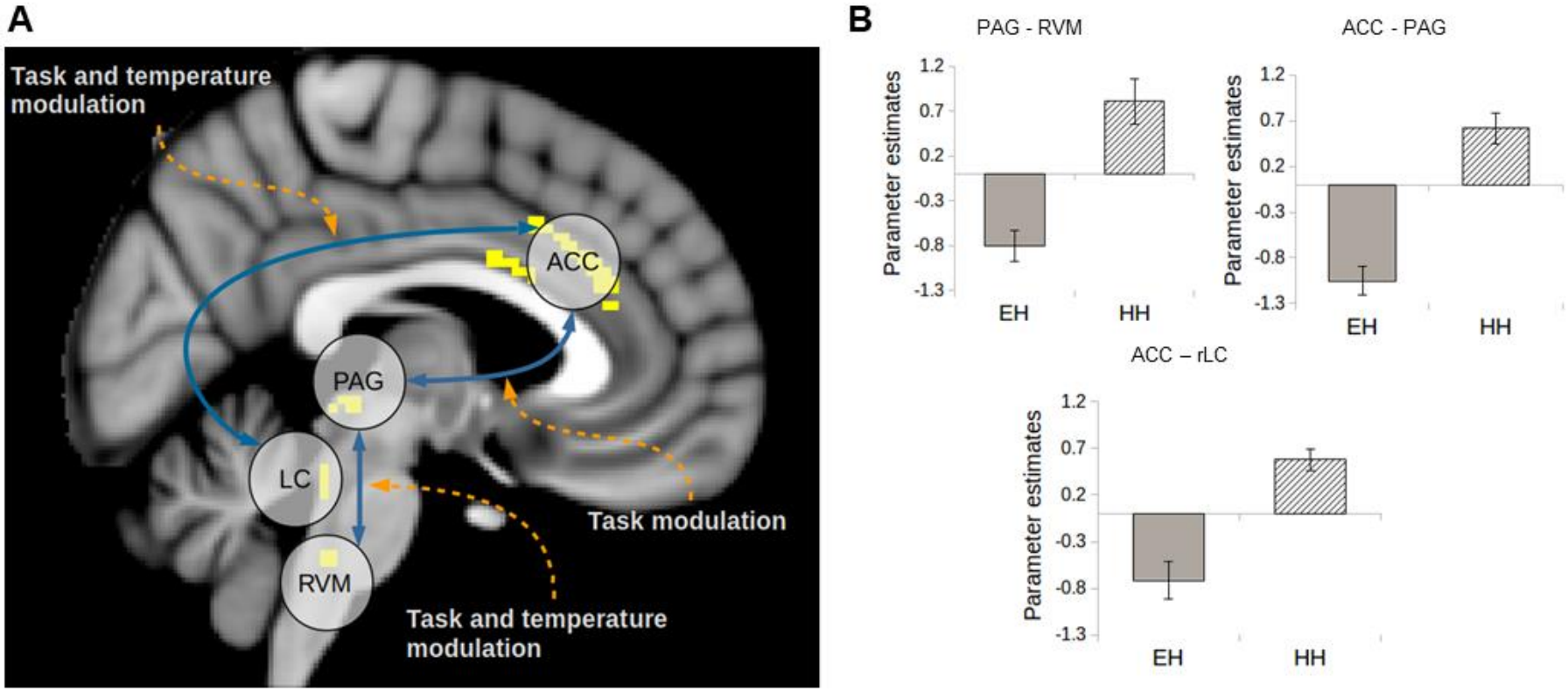


Figure 2.5 *Schematic representation of results of the gPPI analysis. Results were obtained with single-region functional masks and permutation testing (P < 0.05, TFCE corrected). (B) Parameter estimates extracted from the peak destination voxel from the PPI analysis (see text for details), in the easy|high and hard|high conditions. Note that all arrows are double-headed as it is not possible to determine the directionality of connections with gPPI analysis.*

2.3.7 DCM to determine directionality of pathway interactions

Dynamic causal modelling was used to resolve the directionality of the task effect on the connections identified in the gPPI. We systematically varied the location of the task inputs and modulation, while the temperature modulation was kept fixed in all models as a bottom-up effect. External inputs were both hard/easy task and high/low temperature, while modulations were only hard task and high temperature. Five models were specified (**Figure 2.6A**):

- No modulation of connections,
- Task bottom-up on the ACC-PAG-RVM and on the ACC-LC axis.
- Task top-down for both pathways.
- Task top-down in the ACC-PAG-RVM axis and bottom-up in the ACC-LC connection.
- Task bottom-up in ACC-PAG-RVM and top-down in ACC-LC.

Models 3 and 4 were found to best fit the data in BMS, with protected exceedance probability = 0.45 and 0.55 respectively (**Figure 2.6B**), and Bayesian omnibus risk of zero. In both, the task had a top-down influence on ACC-PAG-RVM, while the ACC-LC connection was top-down modulated in one model and bottom-up modulated in the other. Bayesian model averaging was used to extract parameter estimates (**Supplementary Table 3**).

All connections were also tested with an analgesic covariate, to find whether one or more consistently differed in participants that showed an analgesic effect. No connection reached significance in this test.

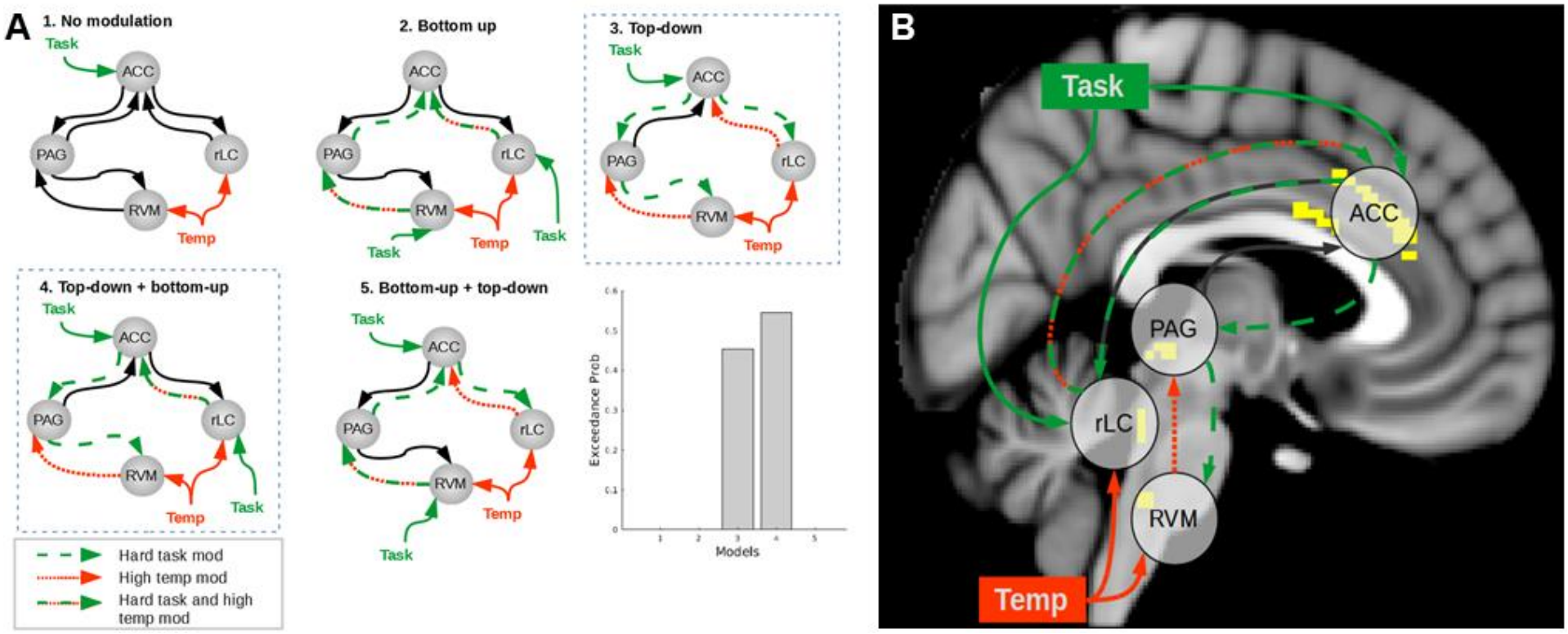


Figure 2.6 *(A) Specified network interactions assessed with Dynamic Causal Modelling and result of Bayesian Model Selection. The effect of temperature is always bottom-up, while the task could have a bottom-up or top-down effect. The inputs are both easy/hard task and low/high temperature, while the modulations of connections are only high temperature and hard task. Model 3 and 4 (outlined with dashed box) have the strongest evidence of reproducing the data, with slightly stronger evidence for model 4. (B) Schematic representation of interactions during attentional analgesia, after PPI and DCM.*

2.4 Discussion

The brainstem involvement in attentional analgesia has been investigated in previous studies, demonstrating a mediating role of the PAG and of its interaction with cortical regions (Brooks et al., 2017; Tracey et al., 2002; Valet et al., 2004). However, possibly because of a lack of statistical power or technical limitations, the neuronal interactions between cortex, PAG and the lower brainstem nuclei in this context have never been fully resolved. Reassuringly, in the context of the reproducibility crisis that is afflicting neuroscience, especially in fMRI experiments (Button et al., 2013; Eklund et al., 2016), we have recapitulated the core findings regarding the brainstem hubs involved in attentional analgesia (Brooks et al., 2017) and have extended our analysis to determine how they interact to produce attentional analgesia by functional network analysis. This identifies a top-down pathway from the PAG to the RVM, engaged by cortical input from the ACC during high cognitive demand. In addition, there is a parallel bidirectional communication between ACC and LC during attentional analgesia.

2.4.1 Identification of brainstem nuclei involved in attentional analgesia

The higher statistical power provided by 57 subjects, some 3-fold greater than in Brooks et al. (2017), yielded stronger findings especially in the brainstem nuclei. In the main effect of temperature, the specificity of the pattern of nociceptive information flow is striking with activations confined to discrete territories including ventral PAG, LC and RVM as well as activations in the region of parabrachial nucleus, nucleus solitarius, sub nucleus reticularis dorsalis and nucleus cuneiformis. This expands our previous result showing only RVM response to high temperature stimulation (Brooks et al., 2017). While it has long been known from animal studies that these brainstem regions receive nociceptive input from the spinal cord (Blomqvist & Craig, 1991; Cedarbaum & Aghajanian, 1978; Keay et al., 1997) this has seldom been clearly demonstrated in human imaging studies. In addition, an intra-subject linear regression analysis with pain scores revealed

that the BOLD signal in the RVM linearly scales with perceived pain, in agreement with recent studies (Brooks et al., 2017; Horing et al., 2019). This clearly demonstrates that this brainstem territory is likely to be playing an important role in coding nociceptive intensity. It is possible that the voxels resolved in this analysis are related to the activity of the pro-nociceptive ON-cells in the RVM. To our knowledge, no other single study has been able to produce such a complete activation map in the human brainstem in response to noxious stimulation (see review by Henderson & Keay, 2018).

In the main effect of task, we again detected activity in PAG, RVM and LC bilaterally, in addition to a more diffuse activation in the brainstem. It is interesting to note that the attentional task recruited the dorsal and ventral PAG whereas the noxious input produced activation in the ventral region of the nucleus, perhaps in line with the known behavioural specialisation of columns within this crucial integrating nucleus (Linnman et al., 2012; Roy et al., 2014).

The magnitude of the analgesic effect showed a correlation with activity in the right LC (a finding that we previously noted in Brooks et al. (2017) but was just below formal statistical significance). This was the only location in the neuroaxis that showed this relationship, and is the reason why connectivity analysis focused on the right LC. One intriguing aspect of this interaction is the lateralised nature of the relationship between the right LC (i.e. contralateral to the stimulus) and the analgesic effect - a finding that has previously been noted in rodent studies where noxious stimuli increase the activity in the contralateral LC to a greater effect (Cedarbaum et al., 1978). The LC is well positioned both anatomically and functionally to mediate a component of attentional analgesia, not only because it is responsive to attentional states and cognitive task performance (Aston-Jones & Cohen, 2005; Sales et al., 2019; Sara, 2009; Yu & Dayan, 2005) and to nociceptive inputs (Cedarbaum & Aghajanian, 1978; Howorth et al., 2009), but also particularly in being able to cause analgesia via its direct spinal cord projections (Hirschberg et al., 2017; Jones et al., 1986).

Intriguingly, the spinal cord-projecting neurons are located in the caudal part of the LC in rodents (Hirschberg et al., 2017), as is the LC region that we found to correlate with the analgesic effect. Previous studies have demonstrated linear relationships between the analgesic effect and activity located in the PAG (Tracey et al., 2002, in 9 subjects) and RVM (Brooks et al., 2017, in 20 subjects). We note that neither of these findings were replicated in our current study of 57 subjects. While all three of these regions have biological plausibility for mediating analgesia, a larger sample size seems necessary to produce robust results with inter-subject regression (especially in small, noisy brainstem nuclei) and this is likely to be complicated by the known interactions between these regions in nociceptive processing (discussed below).

2.4.2 Parallel cortical-brainstem pathways

Given the involvement of PAG, RVM and LC in both aspects of the experiment, and their known involvement in endogenous analgesia (Ossipov et al., 2010), we tested all three nuclei for connectivity changes during the attentional analgesia paradigm.

Cortical regions involved in the endogenous modulation of pain in humans include the anterior cingulate cortex, the dorsolateral prefrontal cortex and the ventrolateral prefrontal cortex (Bushnell et al., 2013). Among these, the ACC was the only frontal cortical area showing activity in the conjunction analysis between main effects of task and temperature, and prior evidence showed its interaction with the PAG to be involved in attentional analgesia (Valet et al., 2004). In light of the recent discussions around compartmentalisation of the cingulate (Van Heukelum et al., 2020), it should be acknowledged within this framework that our results pertain to both MCC (involved in conflict resolution between competing attentional demands) and ACC (nociceptive, affective processing). The location of the ACC region resolved here is indeed on the ACC-MCC border, where activity likely reflects a combination of task demand and pain processing. Intriguingly, inputting the coordinates of the peak attentional activation of

the ACC to Neurosynth (Yarkoni et al., 2011) identified four studies where the same region was involved in response to conflict (Barch et al., 2001; Scholl et al., 2017; van Veen et al., 2005; Wittfoth et al., 2008). In addition, voluntary control over the activation of this area was shown to result in modulation of pain perception in a neurofeedback study (deCharms et al., 2005).

To examine the interplay between the cortical and brainstem structures we hypothesised to be involved in attentional analgesia, we initially performed a generalised PPI, which determines how connectivity changes as a result of experimental manipulation (i.e. effective connectivity). We observed altered connectivity between the ACC and contralateral (right) LC during the interaction between task and temperature. Furthermore, coupling increased between ACC and PAG with task demand, and between PAG and RVM during the task x temperature interaction. Extraction of parameter estimates revealed that all interactions were enhanced in the hard task/high temperature condition.

The identified network interactions lacked directionality and could equally be evidence for an ascending pathway, where the attentional demand modulates how the nociceptive information reaches the brain, or a descending pathway, where the cortex recruits brainstem nuclei to modulate the spinal cord. Therefore, dynamic causal modelling was employed to explore these hypotheses by fitting different models to the data. Bayesian Model Selection validated the results of the gPPI by excluding, for lack of evidence, a model where no connection was modulated by task. In addition, BMS resolved a top-down influence of task on the ACC-PAG and PAG-RVM connections, consistent with a descending pain modulatory system involved in attentional analgesia (Sprenger et al., 2012). The ACC-LC pathway was however not resolved as clearly, with similar evidence in BMS for task modulation of the top-down and bottom-up connection. On examination of the parameter estimates, it was noted that the task modulation had a negative effect on all connections that were also

modulated by temperature. Conversely, the ACC-PAG connection, only modulated by task, has a positive parameter estimate. This effect suggests a disinhibitory effect, or a negative feedback loop in the PAG-RVM and ACC-LC connections. Neurobiological mechanisms that could account for these effects are discussed below.

Effective connectivity changes in these pathways may mediate the process of attentional analgesia. This could be achieved through LC projections to the ACC increasing the signal-to-noise (or salience) of one input over another (Manella et al., 2017; Muller et al., 2019; Sales et al., 2019; Sara, 1985; Vazey et al., 2018) and/or ACC to spinally projecting LC neurons modulating the activity of dorsal horn neurons (i.e. decreasing nociceptive transmission), both actions potentially giving 'precedence' to the task. It is possible that the ACC and the LC work in a reciprocal negative feedback loop during attentional analgesia (Breton-Provencher et al., 2019; Ramos et al., 2007). The reduction in perceived pain could equally be achieved via ACC recruiting the PAG and RVM to produce antinociception at a spinal level during the attention demanding task (Millan, 2002), for example by disinhibition of the RVM “off-cells” (Lau et al., 2014). This conceptually extends previous studies that have identified the ACC-PAG connection as being involved in a distraction from pain (attentional analgesia) paradigm (Valet et al., 2004), as well as in a placebo analgesia paradigm (Petrovic, 2002). The PAG-RVM descending control system has also already been implicated in placebo analgesia (Eippert et al., 2009; Grahl et al., 2018) via an opioid-dependent mechanism. The behavioural component of attentional analgesia has been reported to be impaired by opioid blockade, possibly by disrupting connections between the ACC-PAG-RVM descending control system (Sprenger et al., 2012). It is also quite conceivable that the parallel ACC-LC and ACC-PAG-RVM systems described here work in concert to cause analgesia. Previous animal studies show that electrical stimulation of the PAG triggers noradrenaline release in the cerebrospinal fluid and the analgesic effect of stimulation can be partially

blocked with intrathecal alpha2-antagonists (Cui et al., 1999; Hammond et al., 1985). In addition, it was demonstrated that mice not able to synthesize noradrenaline were less sensitive to the analgesic action of morphine (Jasmin et al., 2002). Thus, it still remains to be demonstrated whether these two pathways are working in a parallel independent fashion, or are dependent upon each other in producing attentional analgesia.

We propose that the ACC acts to resolve the conflict caused by an attention-demanding painful stimulus and the cognitive load of a sustained visual attention task, by sending downstream signals to brainstem structures to facilitate optimal behaviour. This interpretation is in accordance with previous hypotheses on the function of the ACC-LC interaction, implicated in re-orienting attentional processes (Corbetta et al., 2008). In addition, recent evidence from a human fMRI study identified the same connection during conflict resolution in an incongruent Stroop task (Köhler et al., 2016).

We propose that this network could be relevant for mindfulness-based analgesic techniques, especially the "focused attention" type, where focus on an internal signal (e.g. breathing), can distract subjects from pain (Zeidan et al., 2011). While this might be only one of the mechanisms to meditation analgesia, it is worth mentioning that this process is not mediated by endogenous opioids (Zeidan et al., 2016) but relies on the rACC (Zeidan et al., 2012; Zeidan & Vago, 2016), perhaps by exclusively engaging the ACC-LC pathway.

We further postulate that this network may be of importance in chronic pain conditions (e.g. fibromyalgia), where disruption of attention and cognition are co-morbid alongside pain. Pharmacological therapies that target the noradrenergic system have some benefit in chronic pain conditions (Hughes et al., 2015; Kremer et al., 2016, 2018), possibly by acting on the LC system (Hiroki et al., 2017). On the other hand, evidence for malfunction of endogenous pain modulation in such pathologies (Julien et al., 2005; Lannersten et al., 2010; Staud et al., 2005; Vierck et al., 2001),

together with the evidence of low effectiveness of opioid drugs (Goldenberg et al., 2016; Kia et al., 2017), might point toward impairments of the PAG-RVM interaction.

2.4.3 Methodological considerations

Because of the increased sample size, we were able to detect activation in the RVM and PAG in the main effect of temperature and task respectively, without the aid of masking. This experimentally validates the results in Brooks et al., (2017) as well as the use of permutation testing with anatomical masks of *a-priori* specified ROIs. Notwithstanding the difficulty of accurately assigning measured functional activity to specific brainstem nuclei (Betts et al., 2019; Keren et al., 2009; Tona et al., 2017) and the problems faced when trying to image these structures (Brooks et al., 2013), the ability to corroborate our earlier findings should provide confidence for future studies of the brainstem. However, there is still a clear and pressing need for an objectively defined probabilistic brainstem atlas, as exists for other brain structures (Kurth et al., 2010).

We used gPPI analysis, a well-established technique in the neuroimaging field, for network discovery. The strength of gPPI is the ability to detect functional changes in the interaction between two regions, caused by experimental manipulation. This is different from a seed-based analysis that detects functional interactions between regions that remain constant during the whole acquisition period. We then used DCM with the singular purpose of resolving the directionality of the connections (after (Yoshino et al., 2010)), . DCM can be used on its own for network discovery, with a larger model space that tests all possible connections and modulations. However, a large model space is likely to cause a dilution of model evidence, leading to less clear results. In addition, the complexity (e.g. the number of connections) was kept constant across models, to avoid the risk of overfitting.

We employed stochastic DCM, which allows for modelling of random neuronal noise in the system, to improve network resolution in brainstem areas significantly affected by physiological noise (Brooks et al., 2013). This routine was shown to improve the characterization of network structure and parameter inference over deterministic DCM (Daunizeau et al., 2012; Osório et al., 2015) and has been widely used in resting state and task-based fMRI studies since its release (Kahan et al., 2014; Ma et al., 2015, 2014; Ray et al., 2016; Zhang et al., 2015).

2.4.4 Conclusion

In this study we have been able to resolve parallel cortical – brainstem pathways that form a network that is functionally engaged when pain perception is attenuated during attentional analgesia. We note that the spinal cord BOLD response to nociception has previously been shown to be modulated by attention (Sprenger et al., 2012). Whether this spinal modulation of nociception is the product of activation of the ACC-PAG-RVM and/or the ACC-LC system still needs to be demonstrated in humans. It is known that both pathways could involve opioids (Fields, 2004) and so previous studies using naloxone do not discriminate between these possibilities. It would be interesting to explore whether conflict resolution resulting in attentional analgesia is dependent on the ACC-LC interaction or it could be achieved independently via the ACC-PAG-RVM path. A connectivity analysis examining the network activity between cortical territories, brainstem nuclei and dorsal horn *in toto* may help to define the key pathway in attentional analgesia.

2.5 Supplementary material

Supplementary Table 1 *Activation clusters from main effects of temperature and distraction in the pooled cohort obtained with cluster-forming threshold Z>3.09 and cluster-corrected p<0.05. The tables were created with Autoaq (part of FSL), with atlas labels based on the degree of overlap with probabilistic atlases (Harvard Oxford Cortical Structural Atlas, Harvard Oxford Subcortical Structural Atlas, Cerebellar Atlas in MNI152 space after normalization with FNIRT).*

Voxels	MAX	X (mm)	Y (mm)	Z (mm)	Atlas labels
Main effect of temperature in the pooled cohort					
2680	8.53	40	-18	18	46% Central Opercular Cortex, 27% Parietal Operculum Cortex, 5% Insular Cortex
2072	5.08	6	-86	-24	3% Occipital Fusiform Gyrus
839	7.03	-36	4	12	61% Central Opercular Cortex, 10% Insular Cortex
386	4.87	0	-70	48	76% Precuneus Cortex
352	4.84	0	20	28	87% Cingulate Gyrus, anterior division
345	6.58	-38	-18	18	50% Central Opercular Cortex, 20% Insular Cortex, 5% Parietal Operculum Cortex
268	5.26	22	-46	72	54% Superior Parietal Lobule, 12% Postcentral Gyrus
265	5.23	32	-26	62	35% Precentral Gyrus, 28% Postcentral Gyrus
238	4.75	4	-26	8	28.2% Right Thalamus
223	4.87	-28	54	18	85% Frontal Pole
133	4.31	4	-26	30	81% Cingulate Gyrus, posterior division
97	4.09	12	-10	18	55.0% Right Thalamus
95	4.7	-34	-56	42	29% Superior Parietal Lobule, 18% Angular Gyrus, 13% Supramarginal Gyrus, posterior division, 12% Lateral Occipital Cortex, superior division
43	4.25	20	16	18	5.8% Right Caudate
42	4.47	36	46	8	47% Frontal Pole
41	4.34	2	-90	-6	34% Lingual Gyrus, 23% Occipital Pole, 13% Intracalcarine Cortex
39	4.73	-26	-50	-50	57.0% Left VIIIa, 35.0% Left VIIIb
36	3.97	-34	32	40	66% Middle Frontal Gyrus, 7% Frontal Pole
34	4.54	-16	8	22	25.6% Left Caudate
34	4.11	-48	-54	46	44% Angular Gyrus, 25% Supramarginal Gyrus, posterior division, 9% Lateral Occipital Cortex, superior division
32	4.8	36	-82	-22	7% Lateral Occipital Cortex, inferior division
32	4.33	48	-44	50	46% Supramarginal Gyrus, posterior division, 17% Angular Gyrus
29	4.31	-4	-40	-44	100.0% Brain-Stem
26	4.3	-48	-64	-30	99.0% Left Crus I

Negative main effect of temperature in the pooled cohort					
67	4.25	6	30	-6	21% Subcallosal Cortex, 13% Cingulate Gyrus, anterior division
28	4.06	-4	46	-12	63% Frontal Medial Cortex, 28% Paracingulate Gyrus
Main effect of task in the pooled cohort					
4948	7.22	22	-90	-8	29.00% Occipital Fusiform Gyrus, 22.00% Occipital Pole, 10.00% Lateral Occipital Cortex, inferior division
4790	7.33	-28	-76	20	41.00% Lateral Occipital Cortex, superior division
3773	7.13	46	16	0	48.00% Frontal Operculum Cortex, 6.00% Insular Cortex
635	6.81	-34	26	0	38.00% Frontal Orbital Cortex, 20.00% Frontal Operculum Cortex, 13.00% Insular Cortex
540	5.26	36	38	36	56.00% Frontal Pole, 23.00% Middle Frontal Gyrus
433	5.87	-42	6	26	27.00% Precentral Gyrus, 26.00% Inferior Frontal Gyrus, pars opercularis
241	6.33	-8	-74	-38	43.0% Left VIIb, 38.0% Left Crus II
205	5.43	4	-26	-2	12.8% Brain-Stem
58	4.68	-46	-38	38	25.00% Supramarginal Gyrus, anterior division, 10.00% Supramarginal Gyrus, posterior division
46	4.65	44	-8	-10	66.00% Planum Polare
45	4.14	-28	-2	58	28.00% Middle Frontal Gyrus, 16.00% Superior Frontal Gyrus, 6.00% Precentral Gyrus
41	5	-38	-52	-44	63.0% Left Crus II, 13.0% Left VIIb, 6.0% Left Crus I
38	5.71	10	-74	-38	57.0% Right Crus II, 18.0% Right VIIb
27	4.52	-14	-22	36	19.00% Cingulate Gyrus, posterior division
Negative main effect of task in the pooled cohort					
1731	6.31	8	-56	30	38% Precuneus Cortex, 20% Cingulate Gyrus, posterior division
1126	6.6	-38	-72	48	68% Lateral Occipital Cortex, superior division
876	5.43	-34	18	56	63% Middle Frontal Gyrus, 2% Superior Frontal Gyrus
511	5.77	32	-70	-36	44.0% Right Crus I, 24.0% Right Crus II
461	5.95	50	-66	40	83% Lateral Occipital Cortex, superior division
346	4.66	6	28	-4	14% Subcallosal Cortex anterior division
257	5.06	-36	-24	70	32% Postcentral Gyrus, 24% Precentral Gyrus
125	4.97	-38	-72	-36	97.0% Left Crus I
94	5.33	-66	-42	-6	54% Middle Temporal Gyrus, posterior division, 29% Middle Temporal Gyrus, temporooccipital part

92	4.99	-16	64	18	82% Frontal Pole
78	4.47	-42	52	2	88% Frontal Pole
41	4.07	-38	-18	18	50% Central Opercular Cortex, 20% Insular Cortex, 5% Parietal Operculum Cortex
38	4.38	-24	-50	18	1% Precuneus Cortex
34	4.16	-64	-8	-14	44% Middle Temporal Gyrus, anterior division, 24% Middle Temporal Gyrus, posterior division, 8% Superior Temporal Gyrus, posterior division

Supplementary Table 2 *Results from intrasubject parametric regression with pain ratings in the pooled cohort obtained with cluster-forming threshold Z>3.09 and cluster-corrected p<0.05. The tables were created with Autoaq (part of FSL), with atlas labels based on the degree of overlap with probabilistic atlases (Harvard Oxford Cortical Structural Atlas, Harvard Oxford Subcortical Structural Atlas, Cerebellar Atlas in MNI152 space after normalization with FNIRT).*

Voxels	MAX	X (mm)	Y (mm)	Z (mm)	Atlas labels
9467	6.36	32	-28	64	40% Postcentral Gyrus, 26% Precentral Gyrus
3621	8.37	40	-18	18	46% Central Opercular Cortex, 27% Parietal Operculum Cortex, 5% Insular Cortex
3481	7.78	-56	-2	8	45% Central Opercular Cortex, 28% Precentral Gyrus, 5% Planum Polare
1693	5.93	-26	46	26	79% Frontal Pole
287	4.98	-62	-56	-10	54% Middle Temporal Gyrus, temporooccipital part, 21% Inferior Temporal Gyrus, temporooccipital part, 7% Lateral Occipital Cortex, inferior division
285	5.2	4	-26	8	28.2% Right Thalamus
120	4.42	20	-8	28	3.1% Right Caudate
95	4.18	42	48	8	80% Frontal Pole
86	4.71	16	-20	10	099.8% Right Thalamus
71	5.79	-26	-50	-50	57% Left VIIIa, 35% Left VIIIb
59	4.41	46	26	36	65% Middle Frontal Gyrus
46	3.82	-20	-28	68	39% Precentral Gyrus, 23% Postcentral Gyrus
38	4.17	-42	-72	24	72% Lateral Occipital Cortex, superior division
37	4.11	-18	-38	66	51% Postcentral Gyrus
36	3.84	2	-66	-38	69% Vermis VIIIa, 13% Vermis VIIIb
34	3.83	24	54	26	76% Frontal Pole

33	4.48	52	30	22	32% Middle Frontal Gyrus, 30% Inferior Frontal Gyrus, pars triangularis
31	3.78	28	58	0	76% Frontal Pole
1637	6.16	46	-64	2	56% Lateral Occipital Cortex, inferior division, 10% Middle Temporal Gyrus, temporooccipital part
1184	5.36	-42	-74	0	63% Lateral Occipital Cortex, inferior division
176	5.38	-26	-76	30	67% Lateral Occipital Cortex, superior division
126	4.73	26	-52	50	34% Superior Parietal Lobule, 3% Lateral Occipital Cortex, superior division
51	4.32	-54	-16	52	57% Postcentral Gyrus, 8% Precentral Gyrus

Supplementary Table 3 *Summary of mean parameter estimates for connections, and for task and temperature modulation in DCM.*

Parameter	Parameter estimate mean (SD)	Task modulation mean (SD)	Temp modulation mean (SD)
Group Mean			
ACC-PAG connection	0.084 (0.0012)	0.029 (0.0052)	-
ACC-LC connection	0.058 (0.0011)	-0.019 (0.0054)	-
PAG-ACC connection	0.083 (0.0012)	-0.0058 (0.0026)	-
PAG-RVM connection	0.039 (0.0011)	-0.038 (0.0061)	-
LC-ACC connection	0.054 (0.0012)	-0.012 (0.0052)	-0.0404 (0.0065)
RVM-PAG connection	0.034 (0.0012)	-0.0004 (0.0026)	-0.0419 (0.0061)

Chapter 3 Attentional analgesia and its central pain modulatory mechanisms are preserved in Fibromyalgia.

Work presented in the present chapter refers to the following paper:

Oliva, V., Gregory, R., Brooks, J. C. W. & Pickering, A. E. (2020). Attentional analgesia and its central pain modulatory mechanisms are preserved in Fibromyalgia.

The paper is in submission to pain.

Valeria Oliva was involved in the acquisition of the data with RG, conceptualized the paper, performed all the analyses, wrote the paper, revised it, and made the images. RG lead the data acquisition. AEP conceptualized the paper, was involved in the analyses, revised the paper, and supervised the work. JCB conceptualized the paper, was involved in the analyses, revised the paper, and supervised the work.

3.1 Introduction

Fibromyalgia is a common, chronic condition characterised by widespread pain with hyperalgesia in muscles and joints, without any identifiable causative pathology or injury (Borchers et al., 2015; Schmidt-Wilcke et al., 2014; Wolfe et al., 1990). In addition to widespread pain, fibromyalgia is syndromically-linked to fatigue, sleep deficits and difficulties in concentration, an array of symptoms which has been referred to as "fibrofog" (Katz et al., 2004; Vincent et al., 2013). A single underlying pathophysiological cause for fibromyalgia is yet to be fully elucidated (Schmidt-Wilcke et al., 2017) and the diagnostic criteria rely on self-reported measures (Stewart et al., 2019; Wolfe et al., 2010, 2016).

There are a plethora of studies reporting alterations in nociception and pain processing in patients with fibromyalgia. One intriguing line of investigations has described a small fibre deficit and altered function of nociceptive primary afferents (Grayston et al., 2019; Levine et al., 2015; Oaklander et al., 2013; Serra et al., 2014; Üçeyler et al., 2013) which may give rise to hyperalgesia. Fibromyalgia has also been considered as a "centralised" pain condition (Clauw, 2014) characterised by augmented brain responses to noxious stimuli that result in hyperalgesia (Desmeules et al., 2003; Gracely et al., 2002; Price et al., 2002). The putative central aetiology of fibromyalgia has been reported to include impairments in endogenous pain modulatory mechanisms (Brietzke et al., 2019; Julien et al., 2005; Lautenbacher et al., 1997; Vierck et al., 2001). This has, in part, been the justification for the use of treatments to boost monoaminergic signalling in central pain modulatory circuits through the use of re-uptake inhibitors (increasing noradrenaline and serotonin) which are amongst the few medications with evidence of efficacy in fibromyalgia (Clauw, 2014).

Endogenous pain modulation can be engaged by cognitive manipulations, such as expectation of analgesia (i.e. placebo (Benedetti et al., 2019; Eippert et al., 2009) or a shift in attention (Bantick et al., 2002; Valet et al., 2004). In healthy subjects, attentional analgesia has been shown

to involve brainstem structures such as the rostral ventromedial medulla (RVM), locus coeruleus (LC) and periaqueductal grey (PAG) (Brooks et al., 2017; Oliva et al., 2020; Tracey et al., 2002; Valet et al., 2004) that mediate a component of their pain modulatory effects via endogenous monoamines (Ossipov et al., 2010). These brainstem regions are intrinsically challenging to image and have been only sparsely investigated in fibromyalgia despite being implicated as part of the causative central pathology.

We hypothesised that there would be a demonstrable deficiency in attentional analgesia in patients with fibromyalgia, and further that whole brain/brainstem fMRI could help to determine where any deficit originated within the descending pain modulatory system or the attentional network. We find that, in keeping with previous studies, the fibromyalgia group show thermal hypersensitivity and impaired performance on the attention demanding task. Importantly, however, fibromyalgia patients can exhibit attentional analgesia of equivalent magnitude to healthy controls if the task difficulty and intensity of noxious stimulus are individually titrated according to performance and percept. For both groups this analgesic effect correlates with brainstem activity in RVM and PAG suggesting that fibromyalgia patients can engage the descending pain modulatory system.

3.2 Methods

The study had ethical approval from the NHS South Central Oxford B Research Ethics Committee (reference 13/SC/0617). All subjects gave written informed consent for study participation. The study was undertaken in the Clinical Research and Imaging Centre at the University of Bristol (CRiCBristol).

3.2.1 Recruitment

Fibromyalgia patients were recruited from local pain management clinics by clinician referral and poster advertisements. Sex-matched healthy control subjects were recruited using poster and email advertisements at the University of Bristol. All subjects were screened for participation by telephone prior to attending for their single session. To meet inclusion criteria, they required a confirmed clinical diagnosis of fibromyalgia for at least six months prior to entry into the study. They were excluded if they had other diagnosis of chronic painful conditions, were pregnant, or had a history of neurological or major psychiatric illness. Additionally, for control subjects, the presence of significant medical or psychiatric disorder (including depression), or of any chronically painful condition precluded participation. Normal safety inclusion/exclusion criteria for participation in MRI studies were also applied.

A total of 54 subjects (32 patients, 22 controls) were screened for the study, of which 14 failed the screening (3 were left-handed, 9 were unable to attend, 1 was unable to lie flat in the scanner, 1 did not pass the MRI screening). Twenty right-handed fibromyalgia patients (mean age 43, range 25-60, 18 females) were enrolled in the study. Patients were not required to alter their regular medications. Twenty right-handed, healthy subjects (mean age, 35 years, range 20-59 years; 18 females) participated in the study.

3.2.2 Experiment

Written informed consent was taken and MRI safety questionnaires were completed on the day of study. The American College of Rheumatology (ACR) Widespread Pain and Symptom Severity index (Wolfe et al., 2010) was completed with the assistance of clinician experimenters. An Edinburgh Handedness Inventory (Oldfield, 1971), PainDETECT (Freynhagen et al., 2006), the "pain now" and "Pain average" scales from the Brief Pain Inventory (Cleeland et al., 1994), Hospital anxiety and depression scale (HADS, Zigmond et al., 1983) and Pain Anxiety Symptom Scales (PASS, (McCracken et al., 1992)) were also completed. Any medications taken in the 72 hours prior to the session were recorded for all participants. Both groups then underwent thermal Quantitative Sensory Testing (QST) with a circular contact thermode (CHEPS Pathway, MEDOC) applied to the left volar forearm using a standardised protocol and script that included warm detection threshold, heat pain threshold, cold detection threshold and cold pain threshold. Furthermore, study participants were tested for pressure pain thresholds on the thenar eminence using an algometer (Somedic). After a short comfort/snack break, participants returned to CRiCBristol for the fMRI experiment.

The fMRI experiment was identical in structure to the one described in Brooks et al., (2017). Briefly, participants received thermal stimuli to their left forearm for 30s at either 36°C (low temperature) or 42-45°C (high temperature), and a pseudo-random series of 1 second long "spikes" of 2, 3 or 4°C above these temperatures were superimposed to minimise habituation to stimulation. The high temperature was individually adjusted to a level that elicited a verbal pain rating of 6/10 in the absence of task.

Participants also performed a rapid serial visual presentation (RSVP) attentional task (Potter et al., 1969), where they were presented with rapidly changing letters and numbers on a display screen and they were instructed to press a button when spotting the number 5. The task had two possible levels of difficulty (easy or hard). The task was individually titrated such that

its speed of presentation (i.e. inter-stimulus interval, ISI) was performance matched to ability. Each participant's task performance was assayed over a range of ISIs (from 32 to 256ms) and assessed by fitting a sigmoidal function to the data (using d-prime). The speed at which they performed at 70% accuracy was used for the hard task during the experiment. The ISI for the easy task was set to: 192ms if the subject's hard task ISI was < 96ms; 256ms if the hard ISI was ≥ 96 & ≤ 256ms; 384ms if the hard ISI was > 256ms. The experiment therefore had a 2x2 factorial design with four conditions (*easy|high, hard|high, easy|low, and hard|low*). Conditions were presented in pseudo-random blocks within sessions and across participants. Pain ratings and task performance (hits, misses and false alarms) were recorded during the experiment.

3.2.3 MRI data acquisition

Brain images were acquired with a 3T Siemens Skyra whole-body MR system using the same acquisition sequences (as per Brooks et al., 2017; Oliva et al., 2020). Briefly, subjects' heads were positioned within the 32-channel receive only head coil, and memory foam pads placed around the skull to help minimise movement. Following acquisition of localiser images, a sagittal T1-weighted MPRAGE volumetric scan was acquired with TE/TR = 2.28/2200ms, flip angle = 9° and resolution of 0.86 x 0.86 x 0.86mm, phase encoding direction = A-P, GRAPPA acceleration factor = 2. Functional imaging data was acquired with an echo planar imaging (EPI) sequence and GRAPPA acceleration factor = 2, TE/TR = 30/3000ms, flip angle = 90° and a resolution of 1.77 x 1.77 x 3.5mm. Finally, to correct image distortion in EPI data, a gradient echo field map was acquired with TE1/TE2/TR = 4.92 / 7.38 / 520ms, flip angle 60°, resolution 3 x 3 x 3.5mm. During the fMRI experiment, cardiac and respiratory waveforms were recorded using pulse oximeter and respiratory bellows for subsequent physiological noise modelling (Brooks et al., 2013).

3.2.4 Questionnaire, QST and behavioural data analysis

All statistical analyses (questionnaires, QST, pain ratings, task performance) were carried out in SPSS (version 26). Unpaired t-tests were used on questionnaire results to detect differences between patient and control groups.

Hit rate (the proportion of correct responses to targets) and false alarm rate (the proportion of responses to non-targets) were calculated and z transformed. Subsequently, *d'* was calculated as the difference between z transformed hit rate and z transformed false alarm rate. The interstimulus intervals were compared with a Mann-Whitney U-test.

Pain ratings and task performance recorded during the fMRI experiment were analysed with a mixed ANOVA (with two within-subject factors, task, and temperature, and one between-subjects factor, group). Prior to statistical analysis, data was examined for the presence of outliers, normality of distribution and equality of variance.

Results are reported as mean ± standard deviation or median and [range] where appropriate. The indicative significance level was set to $P<0.05$ throughout.

3.2.5 fMRI analysis

Functional images were pre-processed and analysed in FEAT (FSL version 6, Jenkinson et al., 2012). The pre-processing pipeline was consistent with our previous paper (Brooks et al., 2017) and included motion correction with MCFLIRT (Jenkinson et al., 2002), fieldmap unwarping with FUGUE (Jenkinson, 2003), registration to standard MNI template with FNIRT (Andersson et al., 2007) and FLIRT (Jenkinson et al., 2001), 4mm spatial smoothing and high-pass temporal filtering using a 90s cut-off. The general linear model (GLM) in FEAT, part of FSL, was used to assess brain activation to the four experimental conditions (*easy|high, hard|high, easy|low, hard|low*) and nuisance regressors (task instruction, rating periods), which

were convolved with a hemodynamic response function. The design also included temporal derivatives, local autocorrelation correction (FILM, Woolrich et al., 2001) and a set of regressors modelling physiological noise (Brooks et al., 2008; Harvey et al., 2008). Main effect contrasts (positive and negative main effect of task and of temperature, task * temperature interaction) were estimated at the first level, i.e. single subject analysis.

Whole brain group differences were assessed with an unpaired t-test in FEAT using a mixed-effects model (FLAME) and cluster-based correction for multiple comparison ($Z > 3.1$ for height and $p < 0.05$ for cluster extent, in accordance with the latest recommendations (Eklund et al., 2016).

Nonparametric permutation testing (RANDOMISE, Nichols et al., 2002) with 10000 permutations was used to identify signal change in brainstem nuclei. An appropriate two-sample unpaired t-test design was built with GLM (part of FSL) in accordance with the FEAT guidelines. Masks for the PAG, RVM plus left and right LC (defined in Brooks et al., 2017) were used for permutation testing. In all analyses using RANDOMISE, significant activations are reported using a threshold free cluster enhancement (TFCE) corrected $p < 0.05$.

Where interactions were found in the imaging data, further investigation using FEATQUERY was performed to elucidate the nature of these changes. Parameter estimates were extracted from each experimental condition (i.e. *easy*|*low* vs rest, *hard*|*low* vs rest, *easy*|*high* vs rest, *hard*|*high* vs rest) and their relationship to the individual behavioural responses examined.

In a separate FEAT analysis, the whole group mean for healthy controls and fibromyalgia patients was examined in a one-sample t test. RANDOMISE was used for brainstem nuclei.

The magnitude of attention-mediated analgesia was compared to BOLD signal change in the brainstem nuclei (PAG, RVM and LC) specified a priori (as per our earlier study, Brooks et al., 2017). Average pain ratings obtained during high temperature stimulation at the two different task difficulties were subtracted (i.e. *easy|high – hard|high*) and demeaned to obtain a group-level covariate. The difference in the BOLD signal recorded for *hard|high* minus *easy|high* was correlated with the difference in pain ratings in an inter-subject parametric regression model. RANDOMISE was used to assess correlations in PAG, RVM, left and right LC masks. The latter analysis was done on the whole cohort (fibromyalgia patients and healthy controls).

All whole brain results (group means and group comparisons) are reported for $Z > 3.09$, cluster corrected $P < 0.05$. All brainstem results are reported for $P < 0.05$, TFCE corrected.

3.3 Results

3.3.1 Demographics

All patients met the ACR 2010 Diagnostic Criteria for fibromyalgia (Wolfe et al., 2010), scoring 13.5±2.6 on the Widespread Pain Index (WPI) and 10.0±1.5 [7-12] on the Symptom Severity (SS) scale score (WPI ≥7 and SS ≥5, **Table 1**). None of the healthy controls met the ACR 2010 Diagnostic Criteria, scoring 1.0±1.0 [0-3] on the WPI and 2.0±1.1 [1-4] on the SS (**Table 1**). As expected, the fibromyalgia patients had higher scores on the PainDETECT questionnaire compared to controls (15.7± 8.2 vs 2.4±3.3 respectively, $P < 0.0001$), as well as for 'pain now' (5.3±1.6 vs 0.1±0.2 respectively, $P < 0.0001$) and 'average pain' (6.4±1.7 vs 0.7±1.0 respectively, $P < 0.0001$) domains of the BPI (**Table 1**). Fibromyalgia patients had elevated anxiety and depression scores (12.2±3.6 and 10.5±4.7, on HADS respectively) in comparison to healthy controls (4.6±4.0 and 1.3±1.3) on the HADS ($P < 0.0001$ in both cases, **Table 1**). Fibromyalgia patients also had higher scores in the cognitive, avoidance, fear, and anxiety sections of PASS (all $P < 0.0001$, **Table 1**).

Table 1 *Results of questionnaires in fibromyalgia patients and healthy controls.*

Questionnaire	Fibromyalgia patients	Healthy controls	Significance
Widespread Pain Index	13.5±2.6	1.0±1.0	N/A
Symptom Severity	10±1.5	2±1.1	N/A
Hospital Anxiety	12.2±3.6	4.6±4.0	N/A
Hospital Depression	10.5±4.7	1.3±1.3	P < 0.0001
Pain anxiety symptom (cognitive)	18.4±4.3	5.3±6.6	P < 0.0001
Pain anxiety symptom (avoidance)	14.6±5.6	5.8±5	P < 0.0001
Pain anxiety symptom (fear)	11.2±6.8	1.6±1.9	P < 0.0001
Pain anxiety symptom (anxiety)	11.6±5.5	1.5±2.4	P < 0.0001
PainDETECT	15.7± 8.2	2.4±3.3	P < 0.0001
Pain now	5.3±1.6	0.1±0.2	P < 0.0001
Pain average	6.4±1.7	0.7±1.0	P < 0.0001

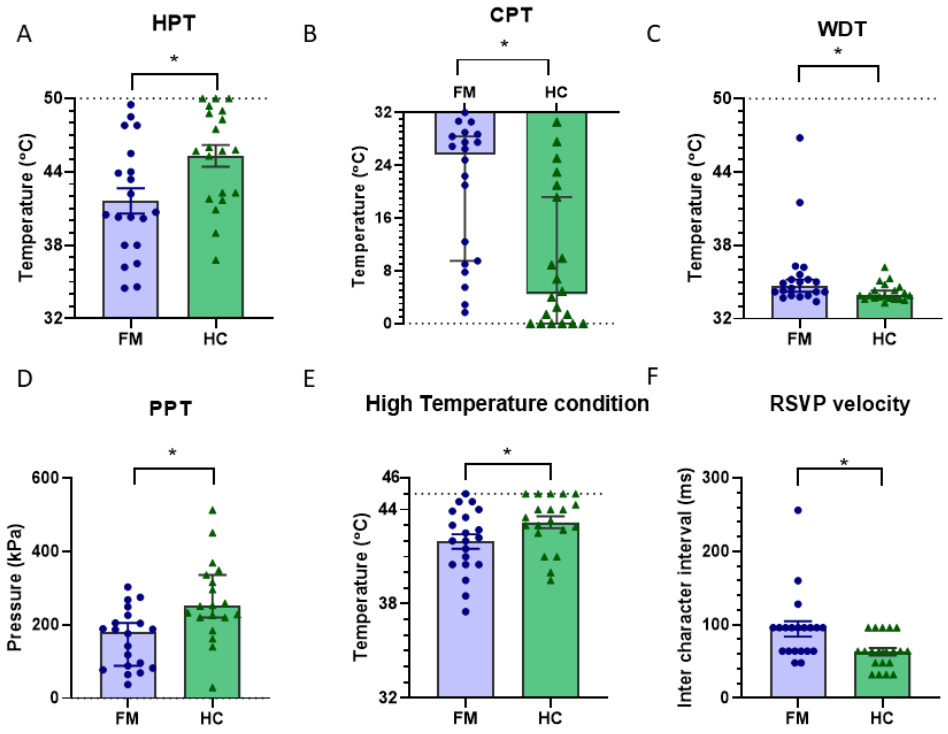
3.3.2 Quantitative sensory testing

Patients with fibromyalgia exhibited hyperalgesia to thermal and deep pressure stimuli when compare to controls, and higher sensitivity to non-noxious thermal stimuli. The heat pain threshold was lower in the fibromyalgia patients (41.6±4.6 fibromyalgia vs 45.3±3.9°C controls, P=0.01, unpaired T-test, **Figure 3.1A**) and the cold pain threshold was at a higher temperature (median 25.65°C, range [1.7 – 32] fibromyalgia vs median 4.450°C, range [0 – 30.6] healthy controls, P = 0.001, Mann-Whitney U test, **Figure 3.1B**). There was an increase in the warm detection threshold in fibromyalgia patients (median 34.70°C, range [33.4 – 46.8] vs 33.90°C, range [33.3 – 36.2] P = 0.016, Mann-Whitney U test, **Figure 3.1C**) but no difference in the cold detection (median 30.60 °C, range [23.7 – 13.2] vs median 30.6°C, range [26.8 – 31.4], P = 0.73, Mann-Whitney U test). Finally, the pressure pain threshold was lower in fibromyalgia patients (fibromyalgia median 181.5kPa, range [37 – 303] vs control median 251kPa, range [29 – 513], P=0.0019, **Figure 3.1D**).

3.3.3 Titration of thermal stimulation and task difficulty

In keeping with thermal hyperalgesia identified by QST, the percept calibrated high (painful) thermal stimulus to be used during fMRI was set at a lower temperature for the fibromyalgia patients. The temperature eliciting a pain intensity rating of 6 out of 10 was 42±2°C for fibromyalgia patients and 43.1±1.7 °C for healthy controls (P=0.047, **Figure 3.1E**). The difficulty of the 'hard' RSVP task to be used during the experiment, was individually calibrated for each participant. Fibromyalgia patients required a longer interstimulus interval in the RSVP task to perform at 70% of optimal (fibromyalgia median 96ms, range [48ms – 256ms] vs control median 64ms, range [32ms – 96ms], P=0.009, Mann-Whitney U, **Figure 3.1F**).

Figure 3.1 *Quantitative Sensory Testing (hot pain threshold (A), cold pain threshold (B), warm detection threshold (C), Pressure pain threshold (D)); high temperature used for the experiment (E), characters presentation speed in the RSVP task (F).*



3.3.4 Pain ratings during the fMRI experiment

The purpose of the experiment was to examine whether pain score evoked by the thermal stimuli (low or high temperature) were affected by the concurrent performance of the RSVP task (easy or hard task). Behavioural data (pain scores) was initially pooled for both groups (**Figure 3.2A**). A mixed ANOVA showed an expected main effect of temperature ($F(1,38) = 174.8$, $P < 0.001$) and a temp*task interaction ($F(1,38) = 13.1$, $P = 0.001$). There was no main effect of task ($F(1,38) = 2.6$, $P = 0.12$). There were no differences between the control and fibromyalgia groups: temp*group ($F(1,38) = 0.2$, $P = 0.65$), task*group ($F(1,38) = 4.7$, $P = 0.66$), or temp*task*group ($F(1,38) = 0.01$, $P=0.97$)). A planned post-hoc paired t-test revealed decreased pain ratings in the *hard|high* (43.8±2.8) versus the *easy|high* (47.9±2.4) condition consistent with an attentional analgesic effect ($P = 0.001$).

To demonstrate the similarity between the controls and the fibromyalgia patients the results are plotted separated by group (**Figure 3.2B-C**). In healthy controls a main effect of temperature and a task * temp interaction was evident ($F(1,19) = 104.2$, $P < 0.0001$ and $F(1,19) = 11.9$, $P = 0.003$ respectively). Likewise, in fibromyalgia patients there was a main effect of temperature and a task * temperature interaction ($F(1,19) = 73.9$, $P < 0.0001$, $F(1,19) = 4.6$, $P = 0.046$, respectively). In both groups, post-hoc paired t-tests revealed that the interaction was due to an attentional analgesic effect with a decrease in pain scores in the *hard|high* versus the *easy|high* condition.

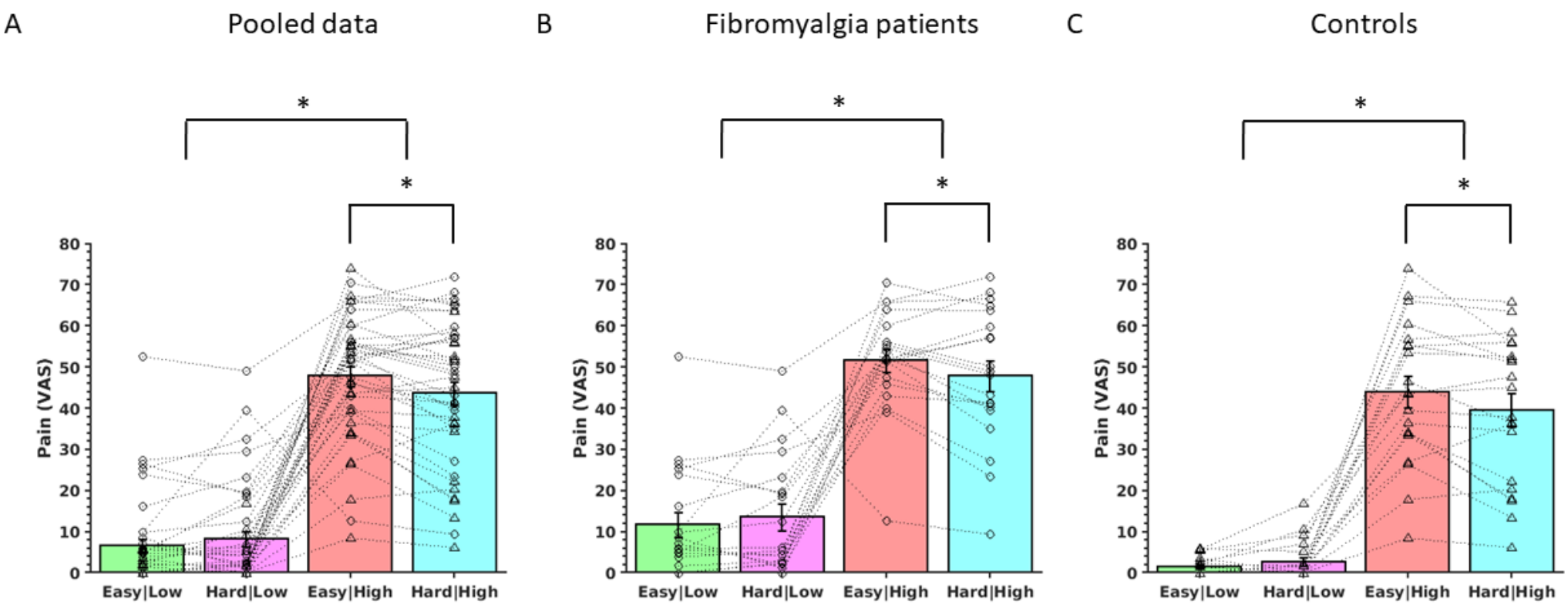


Figure 3.2 *Mean pain ratings after each experimental condition for both fibromyalgia patients and healthy controls (A), fibromyalgia patients only (B), and healthy controls only (C). Error bars represent the standard error mean (SEM).*

3.3.5 Task performance during the fMRI experiment

To see whether the performance of patients and controls on the RSVP task was as expected during the fMRI experiment, button responses were recorded and used to calculate d-prime (d'). Importantly and as intended, the hard task was more challenging than the easy as assessed with a mixed ANOVA revealed a main effect of task ($F(1,38) = 46.0$, $P < 0.0001$, **Figure 3.3**). Patients and controls showed a similar drop in performance when comparing the easy with hard tasks as there was no interaction between task performance and group, ($F(1,38) = 2.7$, $P = 0.11$). We noted that controls performed the task better overall in the scanner as reflected in the between subjects (i.e. group) effect ($F(1,38) = 10.2$, $P=0.003$) indicating that our initial calibration (outside the scanner) had not managed to fully compensate the differences in performance levels between the groups when they were challenged within the scanner.

Further analysis indicated that stimulus temperature had no effect on task performance (main effect of temperature $F(1,38) = 0.2$, $P = 0.63$), and there was no interaction between task and temperature ($F(1,38) = 0.9$, $P = 0.34$), nor between temperature and group ($F(1,38) = 2.6$, $P = 0.12$). However a task * temperature * group interaction was observed ($F(1,38) = 8.3$, $P = 0.007$) and subsequent exploratory post-hoc paired t-tests revealed that the high temperature had a disruptive effect on task performance in fibromyalgia patients when the task was easy (paired t-test, fibromyalgia group only, $P = 0.03$).

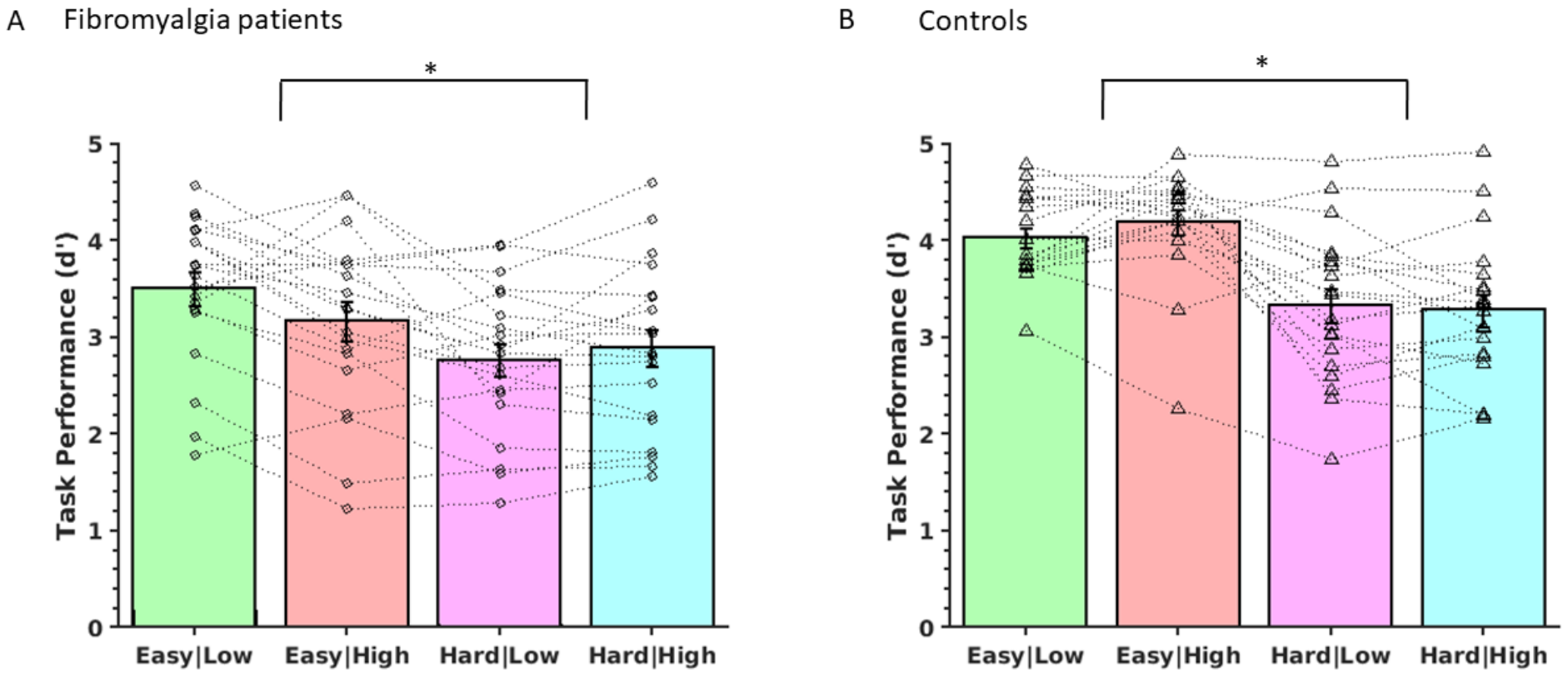


Figure 3.3 *Mean task performance (d') during each experimental condition for fibromyalgia patients (A) and healthy controls (B). Error bars represent the standard error mean (SEM).*

3.3.6 Neuroimaging analysis

The behavioural results indicated that the fibromyalgia patients had thermal hyperalgesia and overall worse performance on the RSVP task, but when these factors were mitigated by adjusting stimulus temperature to percept and task speed to performance (albeit outside the scanner session), they could still produce attentional analgesia. However, it was not clear if they would recruit the same brain networks as healthy controls to produce this analgesic effect. Therefore, the same analysis strategy used for the pain ratings was also applied to the fMRI data. To determine main effects in the patterns of activation in brain and the brainstem both groups were pooled and subsequently differences between groups were assessed.

Whole brain analysis of the main effect of temperature in pooled group data revealed the expected patterns of activity in the cortex including prominent clusters in the contralateral (i.e. right) dorsal posterior insula, primary somatosensory cortex and anterior cingulate cortices among others (**Figure 3.4A**, **Supplementary Table 4**). Brainstem region-masked analyses showed a main effect of temperature in the RVM (**Figure 3.4A**). Analysis of group level differences in the whole brain response to temperature, showed no differences with the singular exception of an enhanced response in healthy controls in the anterior medial frontal pole (Brodmann Area 10, **Figure 3.4B**, **Supplementary Table 4**). Similar analyses in the brainstem only showed a group level difference in the left LC, again with an enhanced response in healthy controls (**Figure 3.4B**).

To explore the possible origins of these differences, we conducted an exploratory analysis based on the observed need to use a hotter high temperature stimulus for the healthy controls than for fibromyalgia patients (**Figure 3.1E**). Therefore, the correlation of BOLD signal change for each area (BA10 and LC) and difference between the high and low

applied temperatures was calculated. The left LC BOLD signal showed a positive correlation with the delta between high and low temperatures (Pearson's R=0.48, P=0.02, **Figure 3.4B**), suggesting that the difference in applied temperature might account for the group level difference. A similar analysis did not reveal any correlation between temperature delta and activity in BA10 (R=0.19, P=0.47).

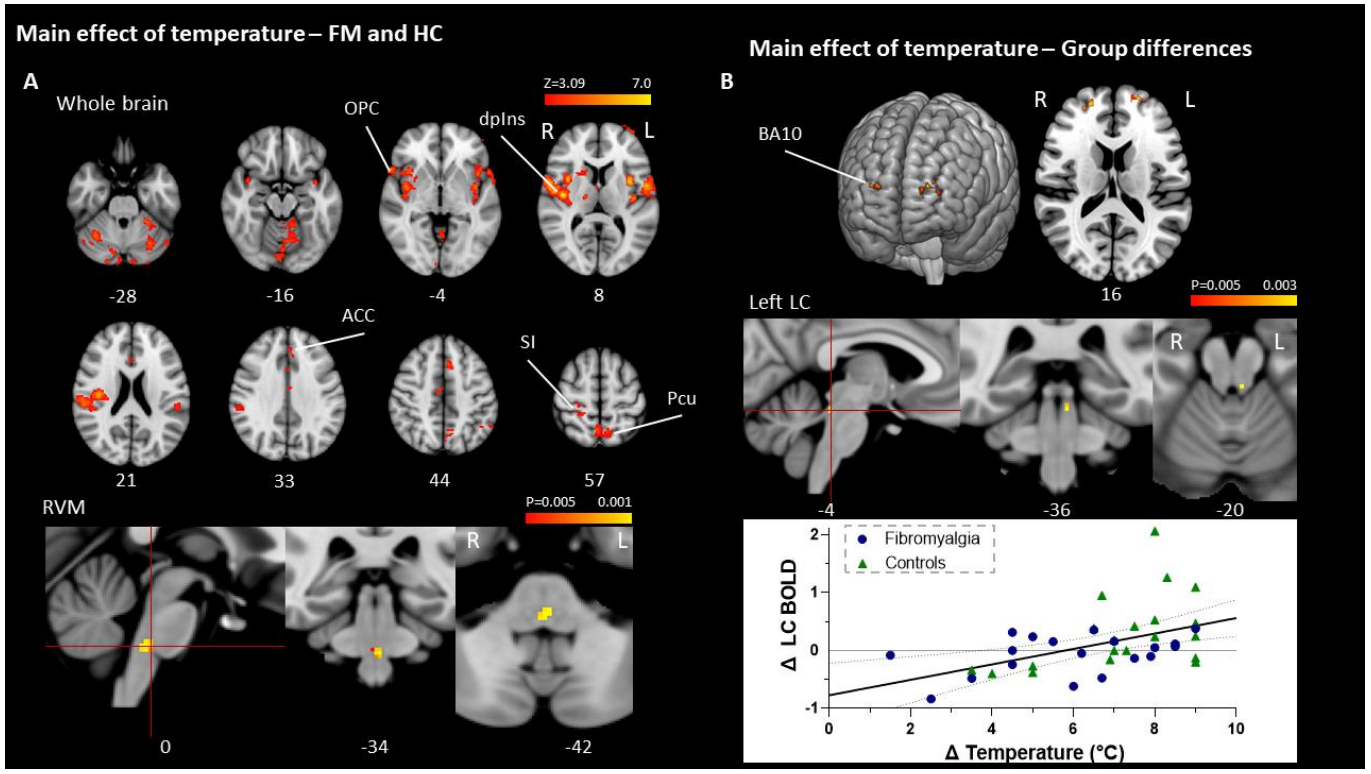


Figure 3.4 *Main effect of temperature in fibromyalgia patients and healthy controls in the whole brain, showing activity in dorsal posterior insula, anterior cingulate cortex and primary somatosensory cortex among the others (Z>3.1 cluster corrected P < 0.05), and in the left LC, showing a stronger response in healthy controls (B, TFCE corrected P < 0.05). Correlation between main effect of temperature in LC and difference in temperatures (R = 0.49, P = 0.002).*

Whole brain analysis of the main effect of task in the pooled data showed a familiar pattern of increased activity in lateral occipital cortex, anterior insula and anterior cingulate cortex amongst others, and a decrease in activity in the precuneus and lateral occipital cortex (**Figure 3.5**, **Supplementary Table 4**). Brainstem region masked analyses showed a main effect of task in the PAG, RVM, and left LC (**Figure 3.5**). No difference between the fibromyalgia and control groups was detected in the main effect of task at whole brain or brainstem level.

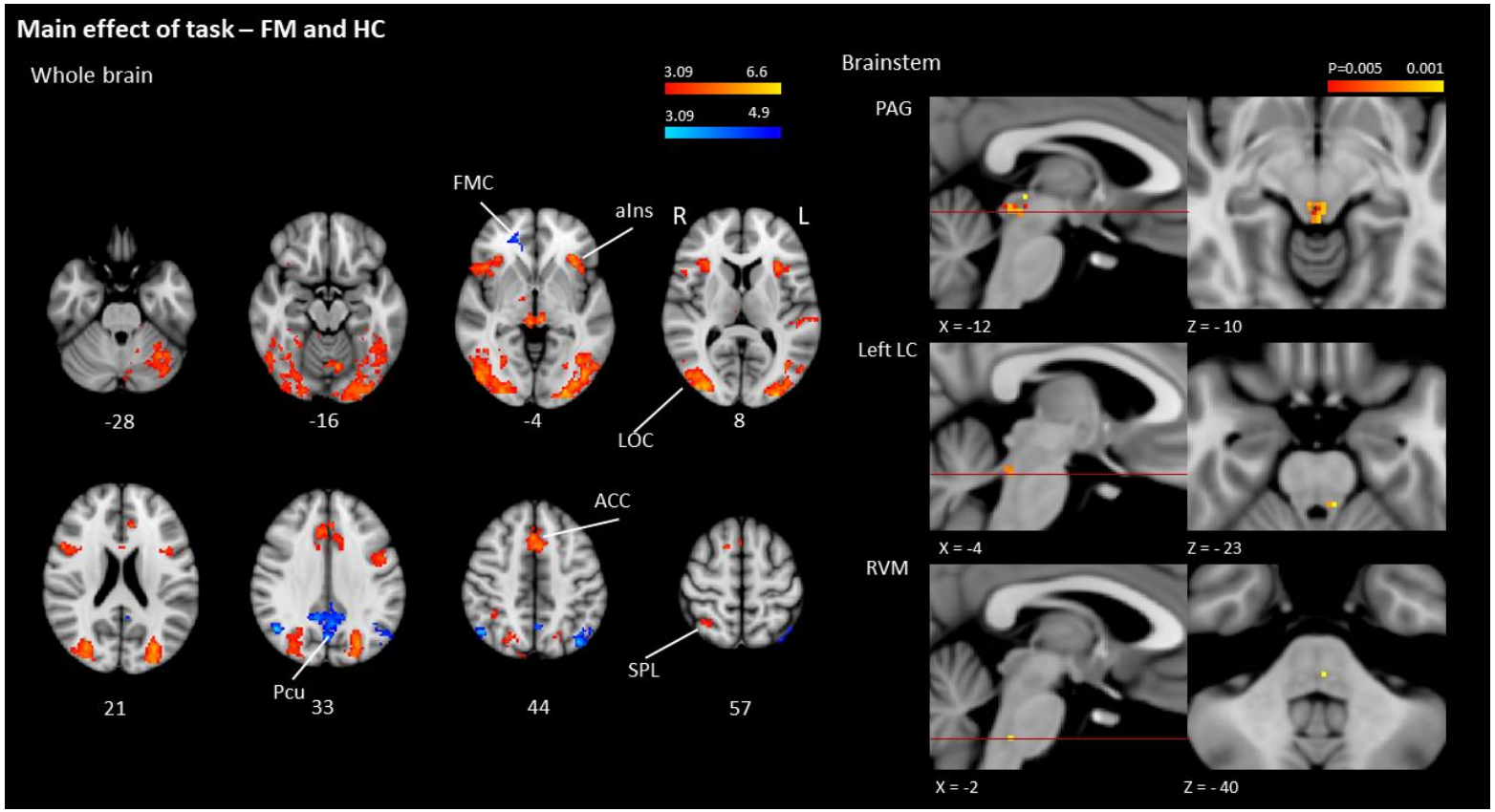


Figure 3.5 *Main effect of task in fibromyalgia patients and healthy controls in the whole brain, showing increase in activity in anterior insula, anterior cingulate cortex and lateral occipital cortex among the others (red-yellow), and a decrease in activity in the precuneus and lateral occipital cortex (Z>3.1 cluster corrected P < 0.05). Main effect of task in the brainstem, in the PAG and RVM (TFCE corrected P < 0.05).*

No task * temperature or task * temperature * group interaction (that could be the neural substrate of the observed behavioral interaction between task and temperature i.e. attentional analgesia) was seen at the whole brain or brainstem level in line with the findings of our previous studies (Brooks et al., 2017; Oliva et al., 2020).

A planned analysis sought correlations between the fMRI data (individual BOLD differences between *hard*|*high* and *easy*|*high* conditions) and the change in pain scores (i.e. analgesic effect, *easy*|*high* minus *hard*|*high*) to improve the power to identify possible neurobiological substrates involved in the analgesic effect (Brooks et al., 2017; Oliva et al., 2020). The whole brain regression analysis (i.e. inter-subject) did not identify any significant regions of activity. However, masked brainstem analyses with the same model showed a positive correlation between analgesic effect and the change in activity in the PAG and the RVM (**Figure 3.6**).

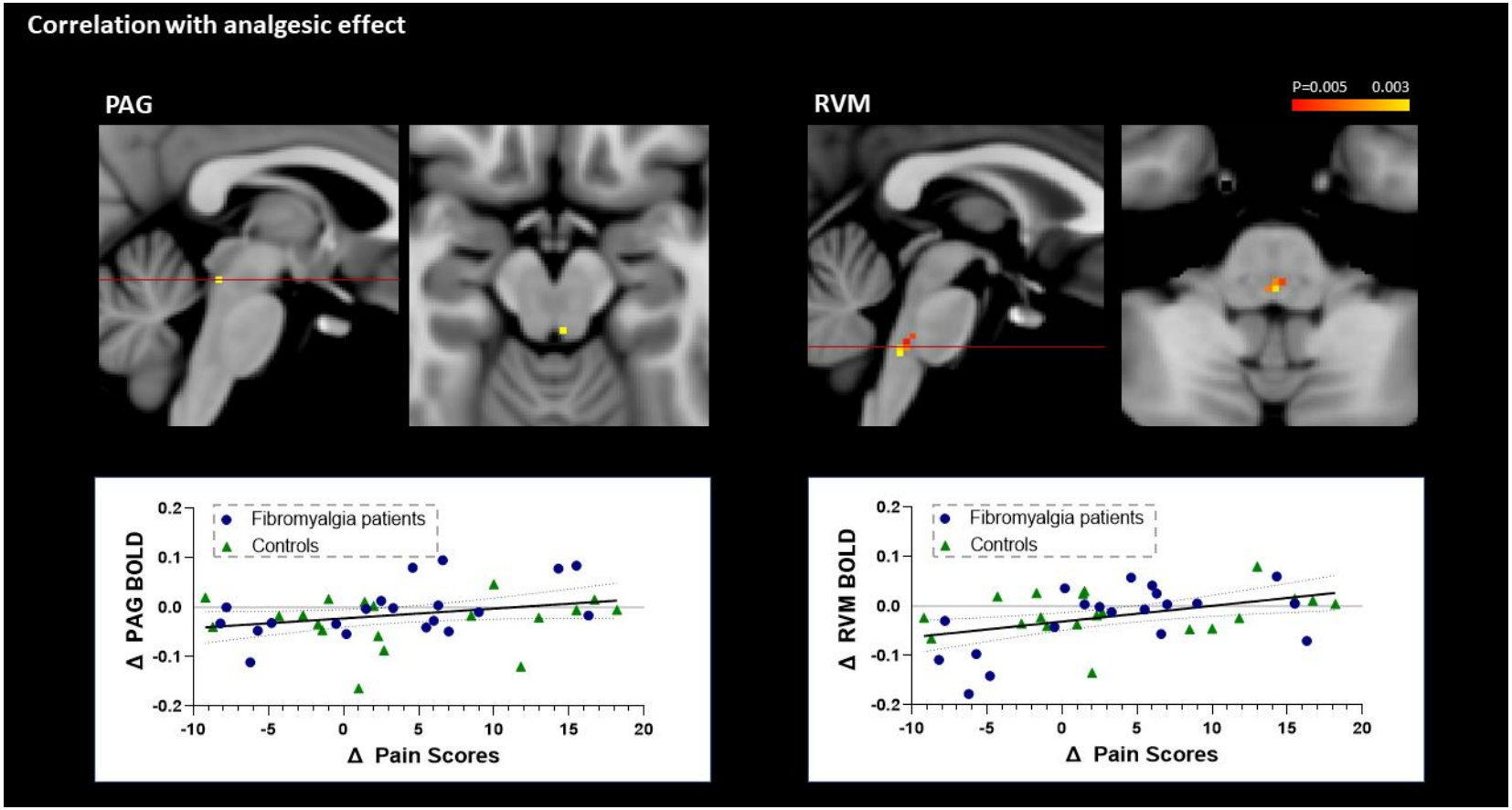


Figure 3.6 *Inter-subject parametric regression with the analgesic effect (i.e. pain ratings of easy|high – hard|high), in PAG and RVM ($p < 0.05$, TFCE corrected).*

3.4 Discussion

We demonstrated that fibromyalgia patients can achieve attentional analgesia with similar central mechanisms to healthy volunteers.

Analysis of pain ratings during the fMRI experiment revealed that high cognitive load was successful in distracting both patients and controls from the painful stimulus. Thus, we revealed that contrary to what was expected, attentional analgesia is preserved in fibromyalgia patients in the context of hot thermal stimulation. This result is in contrast with previous evidence of endogenous pain modulation malfunctioning in this patient population (Julien et al., 2005; Kosek et al., 1996; Lannersten et al., 2010; Staud et al., 2005; Vierck et al., 2001). For example, conditional pain modulation was consistently found to be impaired in fibromyalgia patients (Brietzke et al., 2019; Harper et al., 2018; Paul-Savoie, 2012; Potvin et al., 2016; Schoen et al., 2016), up to the point of becoming a test used for the evaluation of novel pharmaceutical therapies (de Zanette et al., 2014). However, the present is not the only study that suggests that attentional analgesia is preserved in fibromyalgia patients. A Stroop task was successful in causing a decrease of ratings to pressure (Martinsen et al., 2014) and thermal (Ellingson et al., 2018) pain. In addition, expectation of analgesia and music seem to be able to produce some pain relief in this patient population (Goffaux et al., 2009; Häuser et al., 2012; Pando-Naude et al., 2019), although with lower efficacy in patients with a longer disease duration (Kosek et al., 2017). It therefore seems that different modalities of endogenous pain modulation have different efficacy and that if modulation of pain is triggered by cognitive state it has better results. It has been proposed that the lack of analgesia induced by exercise or by a conditioned stimulus in fibromyalgia is caused by the engagement of pain disinhibitory networks, that facilitate instead of attenuating pain (Jensen et al., 2009; Lannersten et al., 2010; Martinsen et al., 2014). Another possibility is that the cortex-brainstem-spinal cord modulatory system is disrupted in this patient population and that they are only able to achieve analgesia by cognitive processes. The latter hypothesis

was motivated by the presence of unchanged spinal withdrawal reflex during placebo analgesia, despite the reduction in pain scores, suggesting that the spinal cord activity was not modulated (Goffaux et al., 2009).

To resolve brain regions crucially involved in attentional analgesia, we used the same strategy as in Brooks et al., (2017) and Oliva et al., (2020): the analgesic effect, defined as the difference in pain ratings between *easy|high* and *hard|high* conditions, was correlated with the BOLD change in the same conditions. This analysis revealed that in both groups PAG and RVM showed a positive linear relationship with the analgesic effect, suggesting that these regions are directly implicated in attentional analgesia. This result, in contrast with previous fMRI evidence showing an impaired relationship between PAG and RVM during conditional pain modulation (Harper et al., 2018), suggests that during attentional analgesia, an endogenous pain modulatory system is recruited in fibromyalgia patients. Interestingly, a resting state fMRI study revealed stronger connectivity between PAG and ACC or Insula in fibromyalgia patients but, notably, not with the RVM (Truini et al., 2016). Conclusive, direct evidence that PAG and RVM modulate the spinal cord during attentional analgesia was not found yet, but it has been extensively suggested (Brooks et al., 2017; Oliva et al., 2020; Sprenger et al., 2012; Tracey et al., 2002). Our result seems therefore in contrast with the suggestion of a supraspinal mechanism to endogenous analgesia in fibromyalgia patients (Goffaux et al., 2009). Functional imaging of brainstem and spinal cord during an endogenous analgesia paradigm would help clarifying this issue by determining whether the downstream communication between brainstem and spinal cord is indeed impaired in fibromyalgia.

Quantitative sensory testing revealed thermal hyperalgesia in fibromyalgia patients in response to both hot and cold stimuli, in line with what previously reported by other research groups (Blumenstiel et al., 2011; Brietzke et al., 2019; Hurtig et al., 2001; Potvin et al., 2016). It has been proposed that this disfunction is due to altered functioning in primary

afferents leading to a latent small fibre neuropathy. A hypothesis supported by the evidence of reduction in small diameter fibres in patients with fibromyalgia (Doppler et al., 2015) and hyperexcitable C-nociceptors (Serra et al., 2014). Indeed, skin biopsies revealed a reduction in unmyelinated fibres in fibromyalgia patients (Üçeyler et al., 2013). On the other hand, recent evidence coming from a retrospective LEP study, failed to reveal the expected abnormal response to the laser stimulation in patients (Van Assche et al., 2020). This adds to the evidence of highly variable phenotype in fibromyalgia.

With the purpose of achieving comparable cognitive load within and between groups, we calibrated the hard version of the attentional task for each participant (Brooks et al., 2017). We found that the inter-character presentation speed was significantly lower in the fibromyalgia group compared to healthy controls. This is in line with previous findings reporting higher reaction time in the patient group in, for example, a Stroop task (Martinsen et al., 2014; Veldhuijzen et al., 2012) and supports the evidence of impaired attentional/cognitive processes in fibromyalgia patients. It has been proposed that such behavioural impairments are reflected by abnormal functioning of the caudate nucleus and hippocampus (Martinsen et al., 2014), a finding that is not reproduced in the present study, possibly because of the different nature of the RSVP task. Because of the calibration, task performance at the target speed was comparable between patients and controls before the attentional analgesia experiment. Interestingly however, during the experimental phase the fibromyalgia patients performed worse than controls. In particular, success rate in patients dropped when they received high temperature stimulation, a phenomenon that was especially observed when the task was easy. This result suggests that painful stimulation has a disruptive impact on the cognitive ability of patients, possibly because of hypervigilance and catastrophizing (Crombez et al., 2004; Ellingson et al., 2018; González et al., 2010; Van Assche et al., 2020). Nevertheless, it is important to note that even during the experiment, a

contrast in performance between easy and hard task was present in fibromyalgia patients, as revealed by a significant main effect of task in the single group ANOVA. Indeed, the perceived difference in difficulty between the hard and easy task was homogeneous between groups, as evidenced by the absence of group difference in the main effect of task: both cohorts engaged the ACC, LOC, and SPL in this contrast.

As mentioned above, there was no group difference in BOLD change in response to task difficulty. In both groups a main effect of task revealed the expected response in brain regions such as lateral occipital cortex, anterior cingulate cortex, anterior insula and PAG, LC and RVM in the brainstem. On the other hand, a group difference was found in the anterior prefrontal cortex (BA10) and in the left LC in the main effect of temperature. Interestingly, on further investigation of this result we revealed that BOLD change in the LC correlated with the temperature used for the attentional analgesia experiment, similarly to what has been seen in a pupillometry study (Eisenach et al., 2017). Therefore, it is possible that the difference in LC activity in this contrast is due to the patients receiving a significantly lower temperature in respect to controls, and therefore weaker spinal inputs. On the other hand, BOLD signal in BA10 does not directly depend on the temperature applied but is possibly related to more cognitive aspects of pain perception (Peng et al., 2018). This region was found to consistently respond to painful stimulus in healthy volunteers in a variety of imaging modalities (e.g. fMRI, NIRS and PET, Peng et al., 2018) and it was reported that patients suffering chronic pain conditions show reduced grey matter density in this and in adjacent cortical regions (Kuchinad et al., 2007). In addition, grey matter density in this area was reported to correlate negatively with the intensity of chronic pain (Fritz et al., 2016; Krause et al., 2014; Moayedi et al., 2011; Obermann et al., 2013). Thus, this region is hypothesized to be important in the chronification of pain, although its role in this context is yet to be fully elucidated (Peng et al., 2018).

In conclusion, the present study demonstrated that fibromyalgia patients are able to produce analgesia when distracted from a painful stimulus. To this end, they engage brainstem nuclei similarly to healthy controls. This new evidence suggests that, contrary to what was believed, at least some of the elements of the pain descending modulatory system are functional in fibromyalgia patients and can be appropriately recruited.

3.5 Supplementary material

Supplementary Table 4 *Results from main effect analyses in the whole brain obtained with cluster-forming threshold Z>3.09 and cluster-corrected p<0.05. The tables were created with Autoaq (part of FSL), with atlas labels based on the degree of overlap with probabilistic atlases (Harvard Oxford Cortical Structural Atlas, Harvard Oxford Subcortical Structural Atlas, Cerebellar Atlas in MNI152 space after normalization with FNIRT). Only those structures to which the cluster had a ≥5% chance of belonging to are presented.*

Voxels	Max	X (mm)	Y (mm)	Z (mm)	Atlas labels
Group differences in main effect of temperature					
124	3.98	-22	60	18	71% Frontal Pole
58	3.87	20	54	16	45% Frontal Pole
Main effect of temperature					
2676	7	42	-12	8	83% Central Opercular Cortex
1605	4.86	0	-74	-14	100% Vermis VI
1292	6.09	-36	4	8	66% Central Opercular Cortex
238	4.58	2	-62	54	69% Precuneus Cortex
166	4.87	24	-40	70	39% Superior Parietal Lobule, 33% Postcentral Gyrus
156	4.19	-20	-84	-38	100% Left Crus II
121	4.48	0	30	28	70% Cingulate Gyrus, anterior division, 13% Paracingulate Gyrus
90	4.23	-54	-30	18	70% Parietal Operculum Cortex, 6% Central Opercular Cortex, 6% Supramarginal Gyrus, anterior division, 5% Planum Temporale
85	4.81	-48	-66	-30	81% Left Crus I
84	4.53	-4	22	44	78% Paracingulate Gyrus, 7% Superior Frontal Gyrus
79	4.73	2	-10	44	73% Cingulate Gyrus, anterior division, 17% Cingulate Gyrus, posterior division
77	4.16	-50	44	-10	83% Frontal Pole
73	3.85	-20	-88	-24	13% Occipital Fusiform Gyrus, 66% Left Crus I
72	4.11	16	-14	6	97% Right Thalamus
65	4.34	30	-26	62	39% Postcentral Gyrus, 27% Precentral Gyrus
62	3.93	4	-6	12	34% Left Thalamus
62	4.71	-28	-50	-48	70% Left VIIIa, 14% Left VIIb
58	3.98	-38	62	8	54% Frontal Pole
56	3.77	-54	-52	48	46% Angular Gyrus, 33% Supramarginal Gyrus, posterior division, 5% Lateral Occipital Cortex
Main effect of task					
4234	6.22	-30	-94	8	5% Lateral Occipital Cortex
3671	6.68	34	-86	4	21% Lateral Occipital Cortex, inferior division
1147	6.27	8	28	30	48% Paracingulate Gyrus, 22% Cingulate Gyrus, anterior division
887	5.47	32	24	2	54% Frontal Operculum Cortex, 11% Inferior Frontal Gyrus, pars opercularis, 5% Inferior Frontal Gyrus, pars triangularis

382	5.53	-30	28	-2	54% Insular Cortex
273	5	-48	0	32	43% Precentral Gyrus, 12% Middle Frontal Gyrus, 11% Inferior Frontal Gyrus, pars opercularis
182	4.03	-4	-42	-20	43% Left I-IV
156	4.26	28	-52	54	43% Superior Parietal Lobule, 12% Angular Gyrus
155	4.96	-8	-70	-16	98% Left VI
140	5.27	4	-30	-4	70.9% Brain-Stem
130	4.59	-54	-20	2	51% Planum Temporale, 10% Heschl's Gyrus (includes H1 and H2)
104	4.25	-8	-74	-38	64% Left Crus II, 31% Left VIIb
54	3.75	-24	-68	-54	92% Left VIIb

Chapter 4 Mechanistic dissection of the attentional modulation of pain

Work presented in the present chapter refers to the following paper:

Oliva, V., Hartley-Davies, R., Pickering, A. E. & Brooks, J. C. W. (2020). Mechanistic dissection of the attentional modulation of pain.

The paper is in submission to Neuron.

Valeria Oliva conceptualized the project, requested ethics approval, screened participants, acquired all the experimental data, conceptualized the paper, performed all the analyses, wrote the paper, revised it, and made the images. AEP conceptualized the paper, was involved in the analyses, revised the paper, and supervised the work. JCB conceptualized the paper, was involved in the analyses, revised the paper, and supervised the work.

4.1 Introduction

Pain is an essential signal that is typically prioritised to enable survival and maintain homeostasis. However, pain is not universal and unmodifiable. A commonly used approach for minimising the amount of perceived pain is the use of active coping mechanisms, such as reappraisal and task engagement (Büssing et al., 2010). Regarding the latter of these, a simple shift in attention away from a noxious stimulus can cause a decrease in pain perception – a phenomenon known as attentional (or distraction) analgesia. Such top-down processes are thought to engage brainstem structures capable of producing anti-nociception (Fields, 2004; Heinricher et al., 2009; Ossipov et al., 2010), through monoaminergic systems targeting the spinal cord (Bushnell et al., 1984; Catherine Bushnell et al., 1985; Duncan et al., 1987; Miron et al., 1989). Understanding how forebrain structures interact with the brainstem, to prioritise or supress incoming information from spinal cord is key to understanding how, in some people, pain may become persistent.

Limited evidence from human fMRI studies demonstrate attentional analgesia to involve direct spinal cord modulation (Sprenger et al., 2012). The authors found that spinal cord response to noxious stimulation was significantly reduced when healthy volunteers performed an attention demanding n-back task during thermal stimulation. In keeping with findings from the animal literature, it has been postulated that modulation of the human spinal cord is achieved by recruitment of brainstem nuclei with direct spinal projections, though this remains to be demonstrated. Recent evidence supports the involvement of periaqueductal grey (PAG), rostroventromedial medulla (RVM) and locus coeruleus (LC) in attentional analgesia (Brooks et al., 2017; Oliva et al., 2020; Tracey et al., 2002). These studies utilised a factorial design, whereby subjects would experience high pain or low pain thermal stimuli, whilst simultaneously performing a hard or easy sustained attention task.

Considering the brainstem circuits likely to be involved in attentional analgesia, the PAG-RVM system achieves analgesia by recruiting ON- and OFF-cells in the RVM, that can bidirectionally modulate spinal cord neurons, enhancing or diminishing their response to noxious stimuli (Fields, 1995). The connection between these two regions, recruited by the anterior cingulate cortex (ACC), has been identified in fMRI studies on pain modulation induced by expectation (i.e. placebo) or by a shift in attentional focus (Eippert et al., 2009; Oliva et al., 2020; Valet et al., 2004), however it remains to be demonstrated whether this is the system responsible for spinal cord modulation in attentional analgesia. This ACC-PAG-RVM pain modulatory network is rich in opioid receptors (Fields, 2004), and their interaction has been shown to be abolished by an opioid antagonist during placebo analgesia (Eippert et al., 2009). It has been suggested that endogenous opioids are involved in attentional analgesia, however the exact location of their action is yet to be resolved (Sprenger et al., 2012).

Similarly, there is evidence for a role of noradrenaline in attentional pain modulation. The locus coeruleus (LC) is the main source of noradrenaline in the brain and has been implicated in cognitive processes like salience signalling or orienting attention, through cortical projections (Aston-Jones et al., 1999; Sara et al., 2012). Additionally, the LC is capable of producing analgesia through spinal projections by an action on alpha2 adrenoceptors (Hirschberg et al., 2017). Thus, this brainstem nucleus sits in a central position in a network mediating attention reorienting and pain modulation. A bidirectional interaction between LC and ACC has been recently implicated in attentional analgesia, possibly operating in concert with the ACC-PAG-RVM system in modulating spinal cord response to painful inputs (Oliva et al., 2020). On the other hand, it is equally possible that the ACC-LC network is responsible for selecting the destination of attentional resources, prioritising a visual task over a noxious stimulus, or vice versa (Corbetta et al., 2008).

To define which of these descending control systems is involved in producing attentional analgesia requires imaging of the spinal cord and brainstem and supratentorial structures. Measuring BOLD changes in the spinal cord has historically been challenging due to its small size, magnetic field distortions and the impact from physiological noise (Brooks et al., 2008; Finsterbusch et al., 2012). This adds to the difficulty of acquiring functional data in a very large field of view required to image the whole neuroaxis. However, significant advances have been made in spinal imaging using a technique called z-shimming, first introduced in 2012 by Finsterbusch et al. The improvement in field homogeneity produced by z-shimming permits measurement of BOLD signal over a larger field of view, including cortex and spinal cord. In practice, functional images are acquired over a range of shim values for calibration, and on a slice-by-slice basis the shim resulting in lowest signal loss is selected for use in the subsequent fMRI experiment. Since its inception, z-shimming has been used for studies on pain perception and motor learning (Islam et al., 2019; Sprenger et al., 2015; Tinnermann et al., 2017; Vahdat et al., 2015), however to our knowledge it has not yet been used in the context of cognitive pain modulation.

We designed a double-blind, three arm, placebo controlled, cross-over experiment in healthy volunteers to investigate the opioidergic and noradrenergic mechanisms of attentional analgesia using whole Central Nervous System (CNS) imaging and an extensively validated experimental paradigm. We took advantage of the improvements in spinal cord fMRI to resolve the involvement of cortex, brainstem, and spinal cord in the attentional modulation of pain, in a single contiguous acquisition. Psycho-physiological interactions analysis (Friston et al., 1997; McLaren et al., 2012) was used to investigate connectivity changes between a-priori specified regions (ACC, PAG, RVM, LC) and the spinal cord, during different contexts of an attentional analgesia paradigm. To test for the involvement of endogenous opioids in attentional analgesia, and to examine the impact on the network caused by their attenuation, the opioid antagonist naltrexone

was delivered to healthy volunteers. Furthermore, the noradrenaline reuptake inhibitor reboxetine was used to define the role of noradrenaline.

4.2 Methods

4.2.1 Experiment overview

The study was approved by the University of Bristol Faculty of Science Human Research Ethics Committee (reference 23111759828) and all participants taking part gave written informed consent. It was conducted within the Clinical Research and Imaging Centre at the University of Bristol (CRiCBristol) and followed the standard operating procedures for imaging studies involving drugs. Healthy volunteers were invited to participate in the study consisting of three imaging sessions, where they were given either naltrexone, reboxetine or an inert placebo. During the experiment, participants received a thermal stimulus (high or low pain intensity) whilst simultaneously performing a sustained attention task (easy or hard). After each block, pain ratings were obtained to track changes due to higher cognitive load (i.e. measure its analgesic effects) and how this was altered by the drugs. At the same time, fMRI data was simultaneously acquired from brain, brainstem, and spinal cord.

4.2.2 Participants

Healthy volunteers were recruited through email and poster advertisement in the University of Bristol and were screened via self-report for their eligibility to participate. Exclusion criteria included any psychiatric disorder (including anxiety/depression), diagnosed chronic pain condition (e.g. fibromyalgia), left handedness, recent use of psychoactive compounds (e.g. recreational drugs or antidepressants) and standard MRI-safety exclusion criteria.

Of fifty-seven subjects screened, two were excluded for claustrophobia, three were excluded for regular or recent drug use (including recreational), and five were excluded due to intolerance of the thermal stimulus. This was defined as high pain score (≥ 8/10) for a temperature that should be non-

nociceptive (<43 °C). In addition, six participants withdrew from the study as they were unable to attend for the full three visits. One participant had an adverse reaction (nausea) to a study drug (naltrexone) and dropped out of the study. One subject was excluded for not performing the task correctly (i.e. pressing the button randomly). Thirty-nine participants completed all three study visits (mean age 23.7 [18 - 45] years, 18 females).

4.2.3 Calibration of temperature and task velocity

In the first screening/calibration visit, the participants were briefed on the experiment and gave written informed consent. The participants were familiarised with thermal stimulation by undergoing a modified version of Quantitative Sensory Testing (QST) based on the DFNS protocol (Rolke et al., 2006). QST was performed using a Pathway device (MEDOC, Haifa, Israel) with a contact ATS thermode of surface area 9cm^2 placed on the subject's left forearm (corresponding to the C6 dermatome). Subsequently, the CHEPS thermode (surface area 5.73cm^2) was used at the same site to deliver a 30 second hot stimulus, to determine the temperature to be used in the experimental visits. Each stimulus consisted of a plateau temperature of 36 to 45°C, with pseudorandomised "heat spikes" of 2, 3, or 4 degrees above the plateau, and each lasting 1s. Participant were asked to rate the sensation they felt during the whole stimulation period, in a scale from 0 (no pain) to 10 (the worst pain imaginable). The temperature corresponding to a 6/10 pain scores was used for the noxious stimulation in the experiment. If the participant only gave pain scores lower than 6, then the maximum programmable plateau temperature of 45°C was used, but with higher temperature spikes of 3, 4 and 5 degrees above, reaching the highest temperature allowed for safety (50˚C maximum).

The session also included a calibration of the Rapid Serial Visual Presentation (RSVP) task (Potter & Levy, 1969), where participants were asked to spot the number 5 among distractor characters. The task was presented 16 times at different velocities (i.e. different inter-character intervals) in pseudorandom order, ranging from 32 to 256ms. To identify the

optimal speed for the hard version of the RSVP task (defined as 70% of each subject's maximum d-prime score), the d' scores for the different velocities were plotted and the curve fit to a sigmoidal function, using a non-linear least squares fitting routine in Excel (Solver). Once parameterised, the target speed for 70% performance was recorded for subsequent use during the imaging session.

4.2.4 Imaging sessions

Following the screening/calibration session, participants returned for three imaging sessions, spaced at least a week apart. Participants underwent drug screening (questionnaire) and pregnancy testing. After eating a light snack, they were given either an inert placebo capsule, naltrexone (50mg) or reboxetine (4mg) according to a randomised schedule. The tablets were encased in identical gelatine capsules and dispensed in numbered bottles prepared by the hospital pharmacy (University Hospitals Bristol healthcare Trust).

One hour after drug dosing, calibration of the RSVP task was repeated (to control for any effect of the drug on performance). The participants were then taken to the MRI suite for the experiment. Before scanning started, participants received the high thermal stimulus at the appropriate temperature, to ensure that the drug had not altered their perception. Participants were then verbally asked for a pain score and if it was 6±1, the temperature was kept the same, otherwise it was adjusted accordingly. Neither reboxetine nor naltrexone caused a significant change in pain perception or task velocity during the calibration, as verified with paired t tests (placebo versus reboxetine and placebo versus naltrexone, see **Supplementary Figure 1**). On average, participants were delivered a temperature of 43.8± 1.25°C. The median velocity for the task was 48ms, range [32-96].

In the MRI scanner, participants received innocuous (low) or noxious (high) thermal stimulus while performing the RSVP task at either difficulty

level (easy or hard). The 4 experimental conditions (*easy|high, hard|high, easy|low, hard|low*), are repeated 4 times each in a random order. The hard version (70% d' performance) of the task and the high (noxious) thermal stimulus were calibrated as described above. In the easy version of the task the inter-character presentation speed was always set at 192ms, except when a participant's hard task velocity of was equal or slower than 96ms, whereby the easy task was set to 256ms. The low (innocuous) thermal stimulus was always set to be a plateau of 36 °C with spikes of 2, 3 and 4˚C above this baseline. Participants performed the task (to identify hits) and gave a pain score immediately after each experimental block on a visual analogue scale (0-100), using a button response (Lumina).

4.2.5 Acquisition of functional images

Functional images were obtained with a 3T Siemens Skyra MRI scanner, and 64 channel receive-only head and neck coil. After acquisition of localiser images, a volumetric T1-weighted structural image of brain, brainstem and spinal cord was acquired using the MPRAGE pulse sequence, (Wang et al., 2014, TR =2000ms, TE = 3.72ms, flip angle = 9°, inversion time = 1000 ms, field of view = 320 mm, GRAPPA acceleration factor = 2). Images a resolution of 1.0 x 1.0 x 1.0mm. Blood oxygenation level dependent (BOLD) functional data of was acquired axially from the top of the brain to the intervertebral disc between C6 and C7, with TR = 3000ms, TE = 39ms, GRAPPA acceleration factor = 2, flip angle = 90°, field of view = 170 mm, phase encoding direction A >> P. Slices were positioned perpendicular to the long axis of the cord for the C5-C6 spinal segments, whilst still maintain whole brain coverage, and had a resolution of 1.77 x 1.77 x 4mm and a 40% gap between slices (increased to 45-50% in taller participants). To determine the optimal shim offset for each slice, reference scans of the entire field of view (FoV) were acquired cycling through 15 shim offsets. The first 20 spinal slices had manual selection of shim offset to identify the optimal one to use for each slice during the experiment. The remaining supraspinal slices were acquired with the scanner default shim. During scanning, cardiac, and respiratory

processes were recorded using a Nonin 7500 pulse oximeter and Lafayette MRI compatible respiratory belt, respectively. These physiological signals and scanner triggers were recorded using an MP150 data acquisition unit (BIOPAC, Goleta, CA), and converted to text files for subsequent use during signal modelling.

4.2.6 Analysis of pain scores

Pain scores recorded during the experiment were investigated collectively for the three visits using a three-way ANOVA in Prism version 8 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Any significant interaction was further investigated with two separate three-way ANOVAs (placebo versus naltrexone and placebo versus reboxetine). Finally, each drug condition was analysed individually with three separate two-way ANOVAs. Post-hoc tests were used to further investigate any interaction.

4.2.7 Pre-processing of functional data and single-subject analysis

Functional images were divided into spinal cord and brain/brainstem, by cropping at the top of the odontoid process (dens) of the 2nd cervical vertebra. The resulting two sets of image data underwent different pre-processing pipelines.

Spinal cord data was motion corrected with AFNI 2dImreg (Cox, 1996), registering all time points to the temporal mean. The extracted motion parameters were used as additional regressor in the subsequent Feat analysis. Data was smoothed with an in-plane Gaussian smoothing kernel of 2mm x 2mm FWHM, using an in-house generated script. The Spinal Cord Toolbox (SCT, v4.1.1) was then used to create a 25mm diameter cylindrical mask around the entire cord to crop the functional data. The SCT was also used to segment the cord from the Cerebrospinal fluid (CSF) and register functional images to the PAM50 template (De Leener et al., 2018). Manual intervention was necessary to ensure that the segmentation of the cord was accurate. The inverse warping fields generated by the registration of spinal

cord fMRI data to the PAM50 template were used to warp a PAM50 CSF mask to subject space. The mask was then used to create a CSF regressor for use during correction for physiological noise during first level FEAT analysis (part of FSL, Jenkinson et al., 2012).

Brain functional data was pre-processed and analysed in FEAT. Pre-processing included smoothing with a 6mm Gaussian kernel, high-pass filtering with a 90s cut-off, and motion correction with MCFLIRT (Jenkinson et al., 2002). Functional data was unwarped with a fieldmap using FUGUE (Jenkinson, 2003), co-registered to the subject's structural (T1) scan and to the 2mm MNI template using a combination of linear (FLIRT, Jenkinson et al., 2001) and non-linear (FNIRT, Andersson et al., 2007) registration with 5mm warp resolution.

Physiological noise correction was conducted for the brain and spinal cord (Brooks et al., 2008; Harvey et al., 2008). Cardiac and respiratory phases were determined using PNM software in FSL, and slice specific regressors determined for the entire CNS coverage. Subsequently these regressors (which are 4D images) were cropped at the level of the odontoid process to be used separately for brain and spinal cord physiological noise correction. For the brain data, the PNM consisted of 32 regressors, with the addition of a CSF regressor for the spinal cord, giving a total of 33 regressors for this region.

All functional images were analysed using a general linear model (GLM) in FEAT. Pre-whitening was performed using FILM (Woolrich et al., 2001). The model included a regressor for each of the experimental conditions (*easy|high*, *hard|high*, *easy|low*, *hard|low*), plus regressors of no interest (task instructions, rating period). Motion parameters and physiological regressors were also added to the model to correct for motion artefacts and physiological noise. The experimental regressors were used to build the following planned statistical contrasts: positive and negative main effect of temperature (high temperature conditions versus low temperature

conditions and vice versa), positive and negative main effect of task (hard task conditions versus easy task conditions and vice versa), and positive and negative interactions.

4.2.8 Analysis Strategy for group analysis

We used a conservative approach to investigate the differences in CNS activity in main effects and interactions caused by administration of reboxetine or naltrexone. An initial analysis examined the brain, brainstem, and spinal cord activation in the planned contrasts (main effects of temperature, task, and their interaction) across all visits. This allowed the generation of functional masks, to use for investigation of differences between drug conditions.

PPI analysis was used to resolve effective connectivity changes between brain, brainstem, and spinal cord during the attentional analgesia experiment. The regions investigated were a-priori specified on the basis of our previous study (Oliva et al., 2020), and included the ACC, PAG, LC and RVM. Following, connectivity changes between the previously mentioned regions and the spinal cord were examined. A first analysis was carried out only on the placebo visit, with the purpose of verifying the reproducibility of our previous results, and of building a functional localizer to be explored for differences after drug administration. Any significant connectivity change identified was thus investigated for a significant effect of the drugs (i.e. causing a stronger or weaker connectivity change).

All first-level analyses and single group averages were performed in blind to the study drug. Following, the experimenter was unblinded to the placebo visit to perform the paired t tests. The experimenter was finally unblinded to all the visits for interpretation of the results.

4.2.8.1 Main effect analysis – spinal cord

In each subject, contrasts of activation from the three visits, derived from the single-subject analysis, were registered to the PAM50 template

with SCT. Following, they were averaged using a within-subject mixed effects OLS model using FLAME (part of FSL) from command line. The resulting average contrasts of activation were merged in time between-subjects. These were then investigated with a one-sample t test in RANDOMISE, using a left C5-6 vertebral mask, derived from SCT. Results are reported TFCE corrected for $P < 0.05$. The significant functional clusters were binarized for use in the paired t tests.

4.2.8.2 Main effect analysis – brainstem

Similarly to what was done in in the spinal cord, in brain and brainstem contrasts of activation from the three visits were averaged with an OLS model in a FEAT analysis. The resulting average was the input of a between-subjects, mixed effects, one-sample t test in FLAME (FEAT). Group activations in each contrast were investigated with permutation testing in RANDOMISE, using a whole brainstem mask. Results are reported TFCE corrected for $P < 0.05$. Functional maps of activation were binarized for later use.

4.2.8.3 Main effect analysis – brain

Brain functional data was averaged and analysed in the same FEAT analyses that investigated the brainstem. Group activations were examined with a whole-brain analysis, with results reported for $Z > 3.1$, cluster corrected $P < 0.05$. This produced functional maps of activation (one per planned contrast) that were then binarized to produce masks that are used later in paired t-tests.

4.2.8.4 Within subject comparison – paired tests

Paired t tests were performed to resolve changes in activity in reboxetine versus placebo and naltrexone versus placebo, separately. Design and contrast files for input in RANDOIMSE were built in FEAT. A group file with appropriately defined exchangeability blocks was additionally defined. Permutation testing in RANDOMISE was finally used to assess group level differences between placebo and the two drugs, separately for brain,

brainstem, and spinal cord. The investigation was restricted to the functional masks derived from the main effect analysis for each contrast.

4.2.8.5 Effective connectivity analysis

For the connectivity analysis, functional images of brain and spinal cord were pre-processed as previously described. Time-series were extracted from the peak responding voxel in the a priori identified regions. In particular, data was extracted from the peak voxel responding to the main effect of temperature in the RVM and spinal cord, the main effect of task in the ACC, PAG and LC, and the task * temperature interaction in the spinal cord. The time-series were included in a GLM that also included the same regressors present in the first level main effects analysis. Interaction regressors were then built by multiplying the time-series by the experimental regressors, and the planned contrasts were specified in the first level analysis. Models for brain and spinal cord seeds were identical, built in separate FEAT analyses. Group responses were obtained with permutation testing in RANDOMISE, using as target the same ROI masks used for time-series extraction. For example, a gPPI analysis that used the RVM as seed, used PAG, LC, ACC, and a left C5-6 vertebral mask to estimate connectivity changes between brain/brainstem and spinal cord.

Paired t tests were used to detect differences between drug visits in the significant connections, as described above.

4.3 Results

4.3.1 Pain scores

A three-way mixed effects ANOVA was used to resolve the main effects of task, temperature, and drug (and their interactions) on pain ratings (**Figure 4.1**). This revealed the expected main effect of temperature on pain scores, with higher ratings associated with high temperature stimuli ($P < 0.001$, $F(1, 38) = 221.6$). A main effect of task also reached significance, with lower pain scores in the hard task conditions ($P = 0.034$, $F(1, 38) = 4.87$), suggestive of a general analgesic effect of the task. However, there was a

significant interaction between task and temperature (P = 0.0025, F (1, 38) = 10.5) implying that the change in pain ratings (due to task) depended on applied temperature. Furthermore, we observed an interaction between drug and temperature on recorded pain ratings (P = 0.04, F (2, 76) = 3.2), suggesting that the effects of drugs on pain perception depended on the applied temperature. The main effect of drug, the interaction between drug and task, and the 3-way drug * temperature * task interaction were all below significance (P = 0.11, F (2, 76) = 2.3; P = 0.3, F (2, 76) = 1.2; P = 0.2, F (2, 76) = 1.6 respectively).

To further investigate drug * temperature interaction, two separate follow-up three-way mixed effects ANOVAs were conducted for placebo versus reboxetine and placebo versus naltrexone. For placebo versus reboxetine, a drug * temperature interaction was revealed (P = 0.0304, F (1, 38) = 5.060, **Figure 4.1**), with lower pain scores in the reboxetine arm, indicating an overall analgesic effect of the drug. Post-hoc tests revealed this interaction to be driven by a decrease in pain scores in the *easy*|*high* (P = 0.0441) and *hard*|*high* (P = 0.0137) conditions. The drug * task and drug * task * temperature interactions were below significance (P = 0.68, F (1, 38) = 0.1738 and P = 0.5578, F (1, 38) = 0.3496).

Comparison of placebo and naltrexone conditions revealed no significant drug interactions: drug * temperature interaction, drug * task interaction and drug * task * temperature interaction were all below significance ($P = 0.39$, $F(1, 38) = 0.7509$, $P = 0.22$, $F(1, 38) = 1.529$, and $P = 0.21$, $F(1, 38) = 1.650$, respectively).

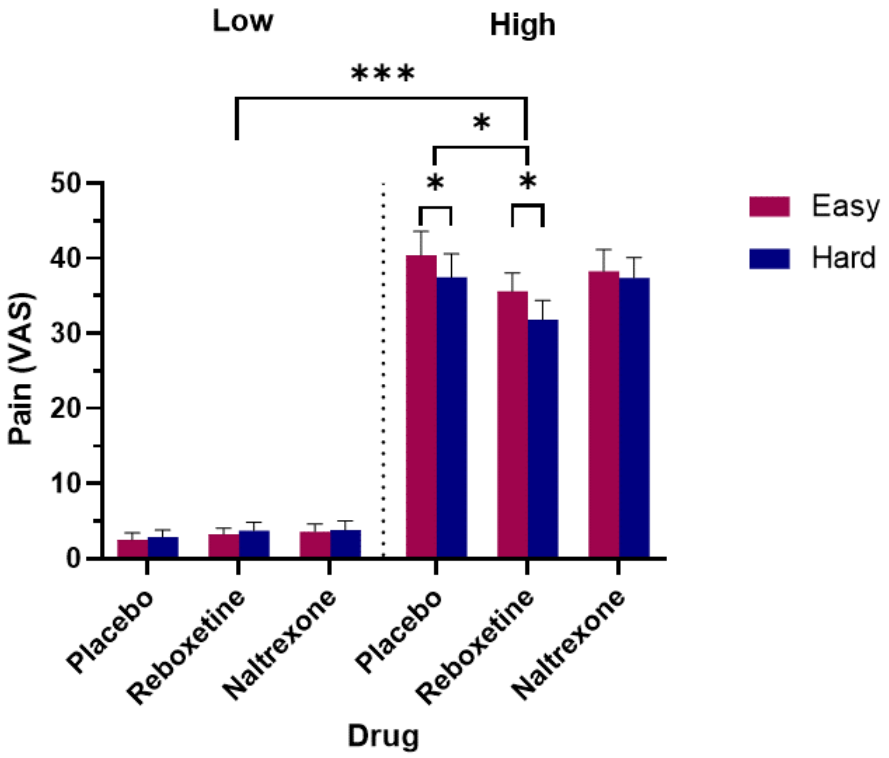


Figure 4.1 *Pain scores across the four experimental conditions (i.e. easy|low, hard|low, easy|high and hard|high), in the three drug visits. A three-way ANOVA revealed a significant main effect of temperature, main effect of task and task * temperature interaction. In addition, a significant drug * temperature interaction was driven by a decrease in pain scores in the reboxetine vs the placebo condition during high temperature stimulation. Error bars are standard error means.*

Finally, two-way mixed effects ANOVAs were used to investigate each drug condition separately to resolve how temperature and task impacted pain scores. In the placebo condition, as expected, the pain scores were higher during high temperature stimulation than during low temperature stimulation, manifesting as a significant main effect of temperature ($P < 0.0001$, $F(1, 38) = 147.7$, **Figure 4.2A**). A main effect of task was also noted, with lower pain scores during the hard task conditions ($P = 0.0078$, $F(1, 38) = 7.884$). There was an interaction between task and temperature ($P =$

0.0019, F (1, 38) = 11.20) and post-hoc testing showed the interaction to be due to a significant lowering in pain scores during the *hard*|*high* (37.5±19.4) versus *easy*|*high* (40.4±19.8, P = 0.0010) condition, indicating an analgesic effect induced by high cognitive load.

Naltrexone dosing appeared to block the analgesic effect of attention as reflected in a loss of the task * temperature interaction (P = 0.5133, F (1, 38) = 0.4355, **Figure 4.2A**), and little difference in pain scores between the *hard*|*high* (37.4±17.1) versus *easy*|*high* (38.3±17.1) conditions, similarly to what has been shown before (Sprenger et al., 2012). There was still a main effect of temperature (P < 0.0001, F (1, 38) = 173.0), but no main effect of task (P = 0.6379, F (1, 38) = 0.2251).

Similar analysis of the effect of reboxetine dosing showed a similar picture to the placebo condition, with a task * temperature interaction (P = 0.0047, F (1, 38) = 9.023, **Figure 4.2A**). A post-hoc test revealed again a decrease in pain scores in *hard*|*high* (31.9 ± 15.84) versus *easy*|*high* (35.6 ± 15.49, P = 0.0034) condition, indicative of reboxetine not having a strong impact on attentional analgesia. There was still a main effect of temperature (P < 0.0001, F (1, 38) = 170.7), but no main effect of task (P = 0.0615, F (1, 38) = 3.713).

4.3.2 Analysis of functional images

Functional images from the three visits were analysed to investigate the biological substrates of attentional analgesia. The analysis of main effects and interactions provided functional masks to use for examination of the impact of the drugs on brain, brainstem, and spinal cord activation.

4.3.2.1 Main effect analysis – Spinal cord

A cluster of activation representing the positive main effect of temperature was located in the left dorsal horn (DH), in the C6 spinal segment (**Figure 4.2B**). This represents a population of neurons that responded more strongly to noxious stimulation versus innocuous

stimulation. The location was remarkably similar to the location of clusters resolved previously in Sprenger et al., (2012), Sprenger et al., (2015), and Eippert et al., (2009). This cluster was anatomically located where expected, given that the thermal stimulus was applied to the left C6 dermatome on the forearm. Since a left cord mask at the C5/C6 level was used to obtain this result, the specificity of the finding was tested with a whole cord mask. This revealed the same main effect of temperature cluster in the left C6 spinal segment, that expanded toward the right side. In addition, a cluster in the bilateral DH in C5 also reached significance (**Supplementary Figure 2**).

Parameter estimates were extracted to investigate the activity of this cluster across the four experimental and three drug conditions. In the placebo condition, parameter estimates from the peak voxel displayed a pattern that was strikingly similar to the pain scores: the BOLD response to a noxious stimulus in the spinal cord was lower in the *hard|high* than *easy|high* condition, suggesting that the spinal cord activity was modulated during attentional analgesia (**Figure 4.2C**). An inter-subject correlation between the delta of parameter estimates (main effect of temperature) and the delta in pain scores (main effect of temperature) was not significant (R = 0.28, P = 0.08, **Supplementary Figure 3A**).

A decrease in parameter estimates in the *hard|high* condition was also revealed after reboxetine administration, again suggesting spinal cord modulation during attentional analgesia (**Figure 4.2C**). The same pattern was *not* observed in the naltrexone condition, where the DH showed a similar response to the *easy|high* and *hard|high* experimental conditions (**Figure 4.2C**), in keeping with the similarity of reported pain ratings for these conditions, indicative of opioid antagonism blocking attentional analgesia.

Within the pooled data, the task * temperature interaction contrast revealed a second discrete and only partially overlapping cluster (**Figure 4.2B**). This was also located on the left side but was slightly caudal and closer to the midline with respect to the main effect of temperature. Extraction of

parameter estimates from this interaction in the placebo condition, revealed it to be driven by increased activity in the *hard|high* condition (**Figure 4.2D**). This suggests an active role of this cluster, possibly composed of spinal interneurons, in modulation of nociception during the analgesic effect. The same pattern was observed in all three drug conditions (**Figure 4.2D**). The delta in parameter estimates (task * temperature interaction) did not significantly correlate with the delta in pain scores across subjects (task * temperature interaction, R = 0.08, P = 0.6, **Supplementary Figure 3B**). No cluster reached significance in the main effect of task.

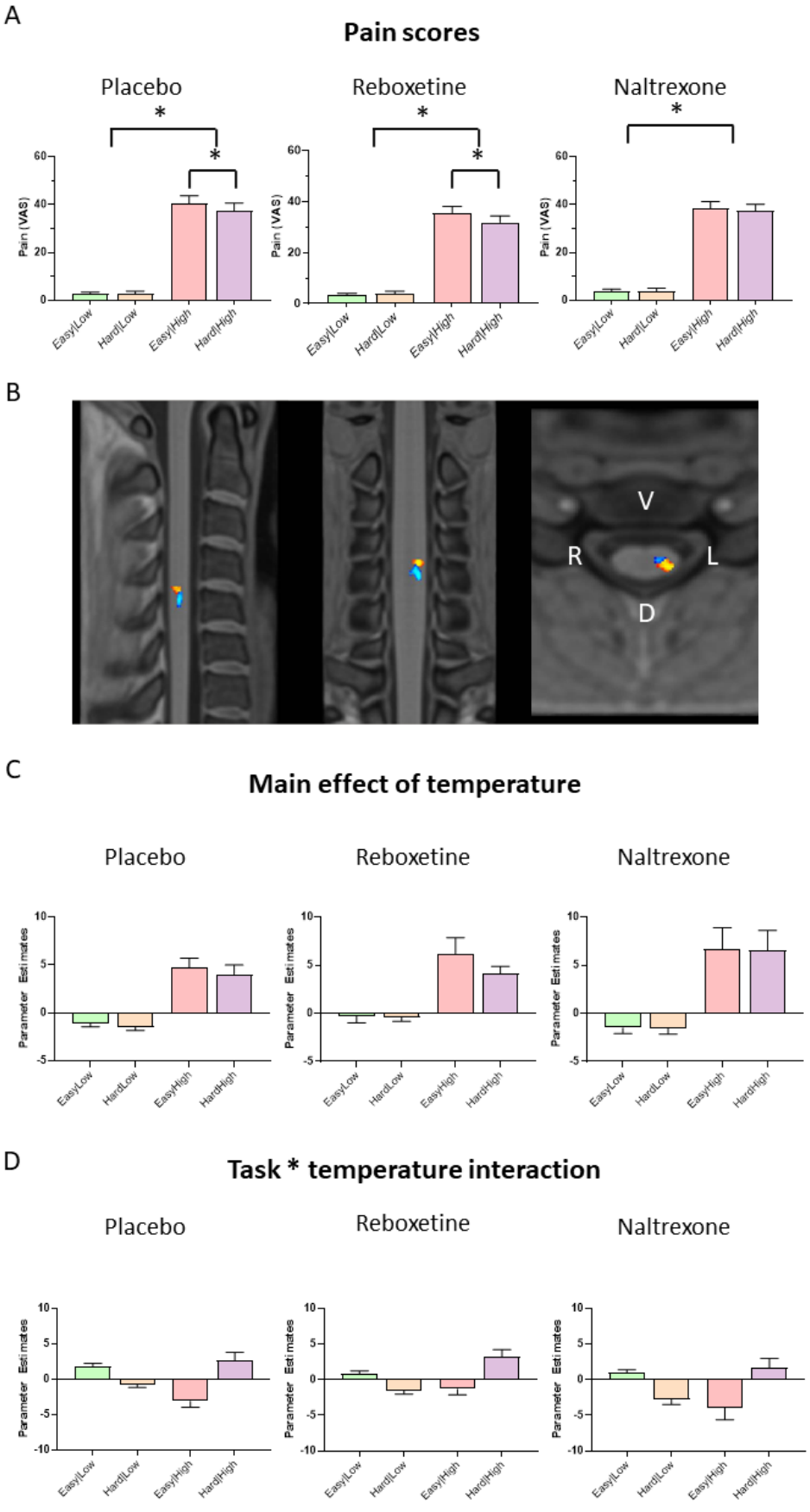


Figure 4.2 *(A) Pain scores across the four experimental conditions (i.e. easy|low, hard|low, easy|high and hard|high), in the three drug visits. (B)*

*Functional clusters showed significance in the main effect of temperature (red-yellow) and in the task * temperature interaction (blue-light blue). (C) Extraction of parameter estimates from the main effect of temperature cluster revealed a decrease in BOLD in the hard|high versus easy|high condition, in placebo and reboxetine but not in naltrexone. (D) Extraction of parameter estimates from the task * temperature interaction cluster revealed an increase in BOLD in the hard|high condition, in all three drug visits. Error bars are standard error means.*

4.3.2.2 Main effect analysis - Brainstem

In the main effect of temperature, activity was detected in the PAG, RVM and bilateral LC (**Figure 4.3A**). Large clusters were found the midbrain (PAG) and lower medulla (RVM), with smaller discrete clusters in the dorsal pons. In the main effect of task, the activity was more diffuse (**Figure 4.3B**), and also included PAG, RVM and bilateral LC. No clusters reached significance in the positive or negative task * temperature interaction, nor in the negative main effects of task and temperature.

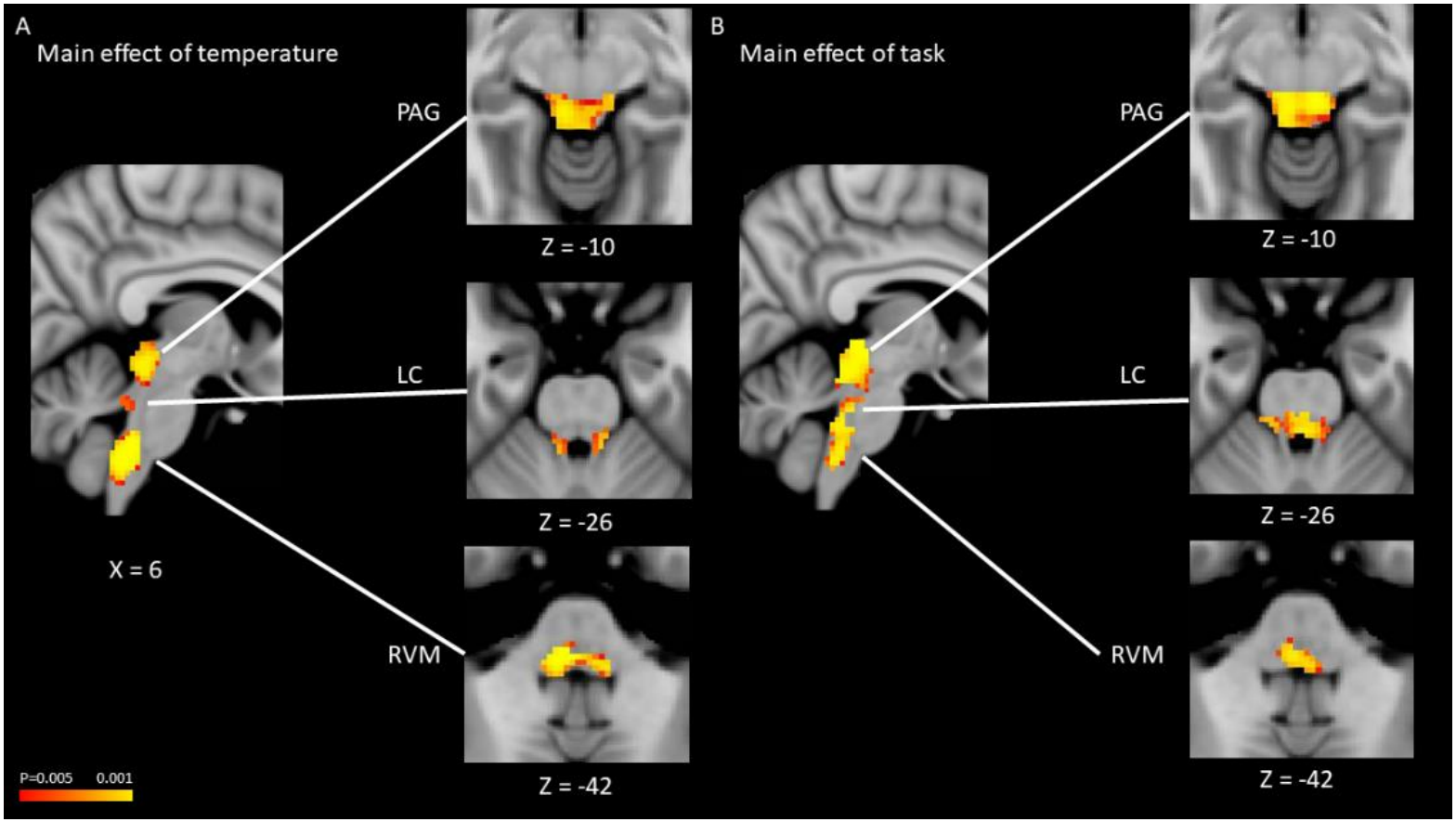


Figure 4.3 *(A)Main effect of temperature activation in the PAG, bilateral LC and RVM, after permutation testing with a whole brainstem mask. (B) Main effect of task activation in PAG, bilateral LC and RVM, after permutation testing with a whole brainstem mask.*

4.3.2.3 Main effect analysis - Brain

Significant activity was observed to the main effect of temperature in a range of cortical territories including the primary somatosensory cortex, the operculum, dorsal posterior insula, and anterior cingulate cortex, with larger clusters contralateral to the side of stimulation (i.e. right side of brain) (**Figure 4.4A**, **Supplementary Table 5**). Only the frontal medial cortex showed deactivation during high temperature stimulation (**Figure 4.4A**, **Supplementary Table 5**). During the main effect of task, activity was recorded in the bilateral occipital cortex, the anterior insula, and the anterior cingulate cortex among the others (**Figure 4.4B**, **Supplementary Table 5**). Clusters active during the negative main effect of task were found in the cerebellum (Crus I) and precuneus among others (**Figure 4.4B**, **Supplementary Table 5**). No cluster reached significance in the positive task * temperature interaction. On the other hand, a cluster in the frontal pole and one in the anterior insula responded to the negative task * temperature interaction (**Supplementary Figure 4A**, **Supplementary Table 5**). These analyses of main effects and interactions provided functional masks which were subsequently interrogated to examine drug effects on brain, brainstem, and spinal cord activity with paired t-tests.

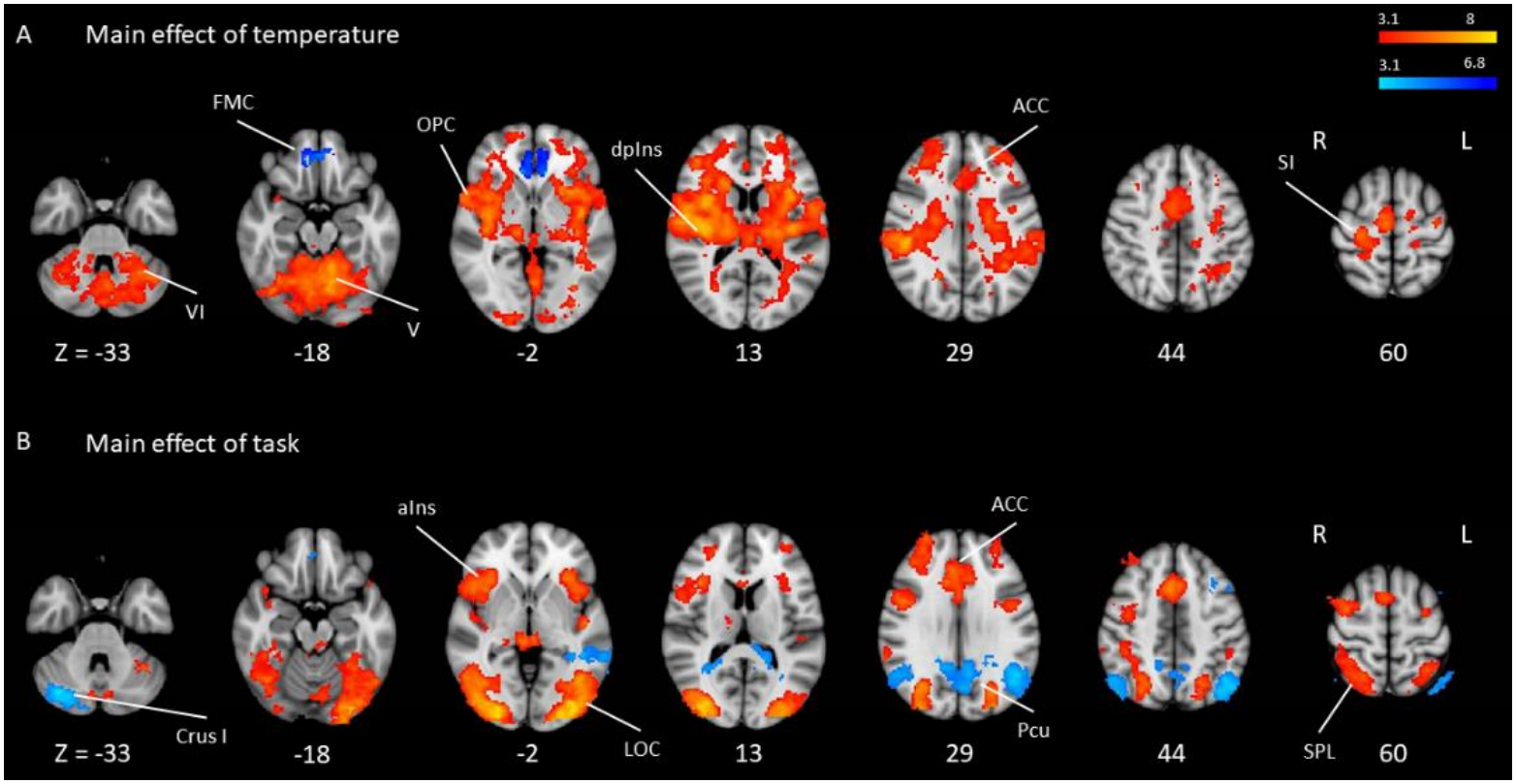


Figure 4.4 *(A) Main effect of temperature after a whole brain analysis. Clusters of activation include the contralateral primary somatosensory cortex, the dorsal posterior insula and the PAG (red-yellow). The frontal medial cortex de-activated during high temperature stimulation (blue-light blue). (B) Main effect of task after a whole brain analysis. Clusters of activation include the superior parietal cortex, the frontal pole, and the anterior cingulate cortex (red-yellow). The posterior cingulate cortex and lateral occipital cortex showed decrease in activation in the hard task conditions (blue-light blue).*

4.3.2.4 Drug effects – paired tests

Differences in functional activity in placebo versus reboxetine and placebo versus naltrexone were investigated using paired t tests, comparing main effects and interactions between drug conditions. No significant differences in spinal cord main effect of temperature or task * temperature interaction were detected, in placebo versus naltrexone nor in placebo versus reboxetine. Thus, the overall spinal cord response to experimental conditions did not significantly change after drug administration. This is in line with the lack of significant drug * task * temperature interaction in the pain scores. However, it does not reflect the significant decrease in pain scores in the reboxetine condition (drug * temperature interaction), suggesting the analgesic effect of this drug might not to be spinally mediated.

In the brainstem, a stronger response to temperature was detected in the lower medulla, in naltrexone versus placebo (**Supplementary Figure 5A**). This is suggestive of a disinhibitory effect of the opioid antagonist on this region. No significant differences were detected in the main effect of task. No significant differences were present in reboxetine vs placebo in any statistical contrast. No brain region responded differently to the main effect of temperature in different drug conditions. On the other hand, in the main effect of task the left anterior insula responded more strongly in the naltrexone condition than in the placebo condition (**Supplementary Figure 5B**).

4.3.2.5 Effective connectivity analysis

To define a network of cortical and brainstem regions whose connectivity was modulated by the experimental conditions, and for previous results of cortico-brainstem interactions in attentional analgesia (Oliva et al., 2020), an initial gPPI analysis was performed on the data acquired under the placebo condition alone. By exploring the connectivity between our a priori identified regions (ACC, PAG, LC and RVM), we created

a “localiser” of effective connectivity for this sample, that was subsequently used to explore changes in the drug conditions in the same subjects.

The following connections reached statistical significance (**Figure 4.5A**):

- RVM seed - increased effective connectivity with PAG and right LC in the task * temperature interaction, and again with the right LC in the main effect of task.

- ACC seed - no significant changes in effective connectivity.

- PAG seed – increased effective connectivity with the ACC in the main effect of task and increased effective connectivity with the LC in the main effect of temperature.

- Right LC seed – increased effective connectivity with the ACC and RVM in the main effect of temperature.

Parameter estimates revealed that the ACC-PAG, PAG-RVM and RVM-LC connections were larger in the *hard|high* versus the *easy|high* condition, consistent with a role in attentional analgesia (**Figure 4.6B**). Reassuringly, these results (obtained in an independent sample) mostly recapitulated those observed in an earlier study (Oliva et al., 2020).

To examine connectivity to the spinal cord (under placebo condition), the same cortical and brainstem seeds were also used to test effective connectivity changes, examining the hypothesis that brainstem nuclei modulate the DH during attentional analgesia. Additionally, time series extracted from the spinal cord was used to investigate functional changes with cortical and brainstem regions of interest.

- RVM seed – increased effective connectivity with the left DH in the main effect of temperature and in the task * temperature interaction.

- ACC seed – no significant changes in effective connectivity.
- PAG seed – no significant changes in effective connectivity.
- LC seed – no significant changes in effective connectivity.
- Spinal cord temperature seed – no significant changes in effective connectivity.
- Spinal cord task * temperature interaction seed – no significant changes in effective connectivity.

Parameter estimates extracted from the spinal cord peak voxel connecting with the RVM in the task * temperature interaction, revealed a strong interaction in the *hard|high* condition, consistent with a role of this path in attentional analgesia (**Figure 4.5B**). No significant correlation with behavioural pain ratings was found.

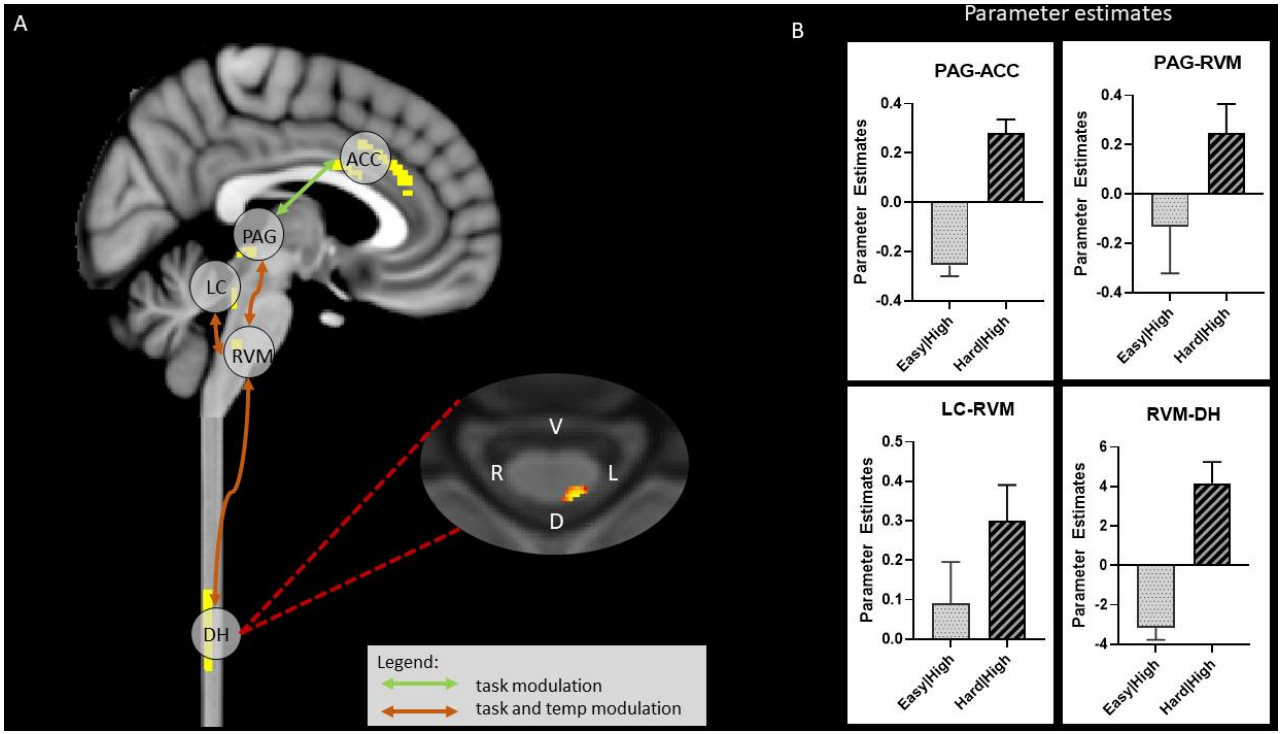


Figure 4.5 *(A) Summary of significant connection changes revealed by the PPI analysis (placebo condition only). The masks used for time-series extraction are shown in yellow. Permutation testing revealed a significant change in connectivity in the main effect of task contrast between ACC and PAG, and in the task * temperature interaction contrast between PAG and RVM, LC and RVM, and finally RVM and DH. Masks used for time-series extraction are shown in the sagittal slices. The spinal cord axial slice shows the voxels significantly connecting to the RVM (threshold at P = 0.1 for visualization purposes). (B) Extraction of parameter estimates revealed an increase in pairing in the analgesic condition (i.e. hard|high). Error bars are standard error means.*

4.3.2.6 Drug effects on connectivity changes – paired tests

Having established the network of brain, brainstem and spinal regions that show effective connectivity changes in the placebo condition, we used paired t tests to explore whether patterns of connectivity altered under either reboxetine or naltrexone conditions compared to placebo (**Figure 4.6A**).

The connection between RVM and DH in the task * temperature interaction was significantly reduced by the administration of naltrexone (**Figure 4.6B**), consistent with opioidergic involvement in attentional analgesia, which behaviourally was also abolished by naltrexone. The strength of the RVM-LC connection in the main effect of temperature was significantly diminished by reboxetine (**Figure 4.6B**). There was also a trend toward the RVM-LC connection in the task * temperature interaction, and the PAG-LC connection in the main effect of temperature being reduced under reboxetine ($P = 0.064$ and $P = 0.052$ respectively). This suggests a negative impact of high noradrenaline availability on the connections between the LC and other brainstem nuclei. The connection between ACC and PAG was significantly weakened during both the reboxetine and naltrexone visits, suggesting this connection to be modulated by both endogenous opioids and noradrenaline (**Figure 4.6B**). None of the other connections altered significantly across drugs.

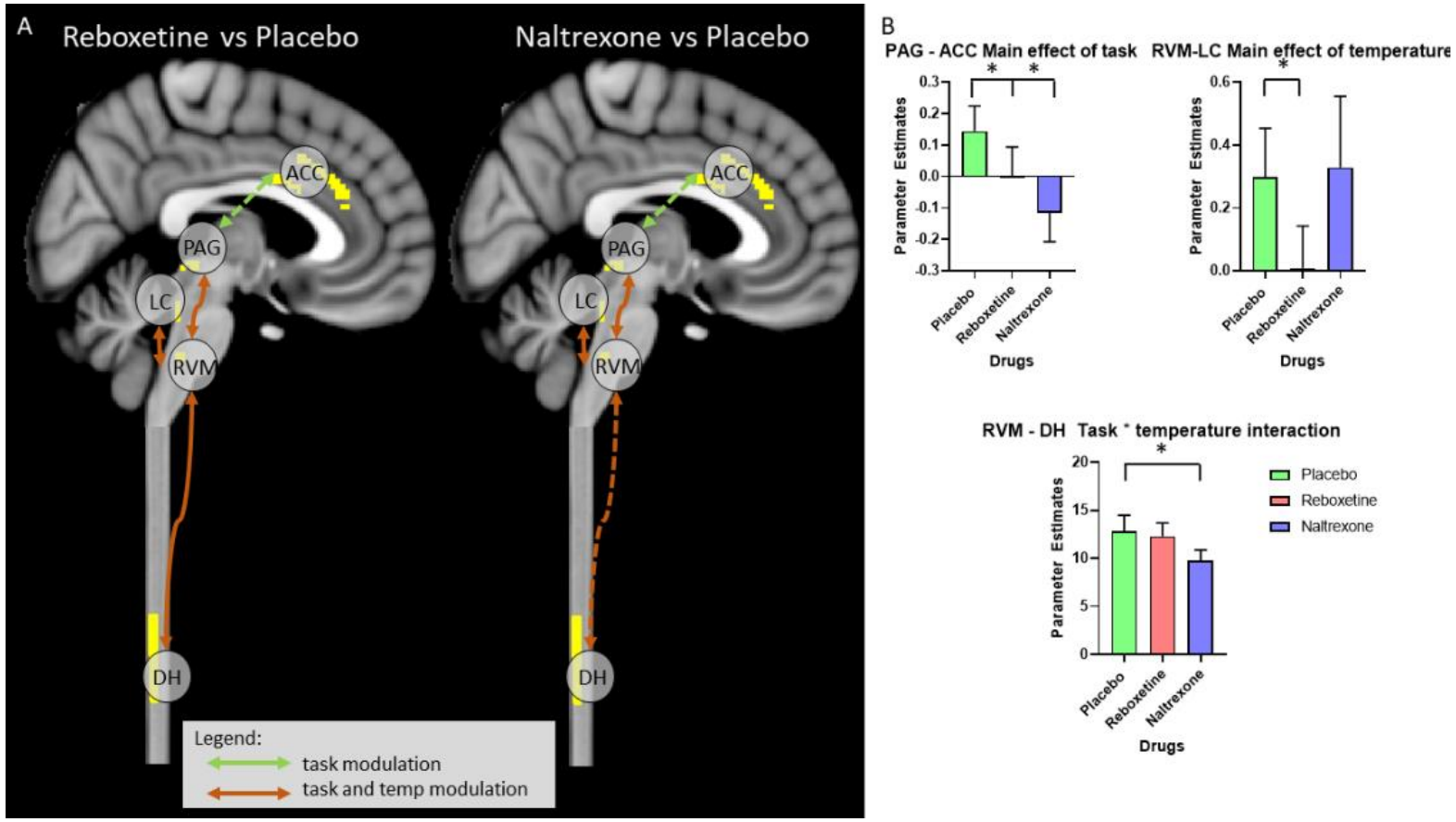


Figure 4.6 *(A) Schematic representation of the connections after in reboxetine or naltrexone. Dashed lines indicate significantly weaker connections after drug administration. (B) Parameter estimates extracted from the PAG-ACC, RVM-LC, RVM-DH connections. Error bars are standard error mean.*

4.4 Discussion

Using simultaneous whole CNS imaging, we demonstrated that the opioid antagonist naltrexone impaired attentional analgesia by acting on ACC-PAG and RVM-DH connectivity, thereby preventing spinal cord modulation. The alteration in connectivity by naltrexone mirrored its behavioural effect of impairing attentional analgesia. We therefore show an opioid-dependent mechanism in the descending pain modulatory pathway implicated in the attentional modulation of pain. Conversely, the noradrenaline re-uptake inhibitor reboxetine did not alter connectivity to the spinal cord, and did not have a behavioural impact on attentional analgesia.

We identified a functional cluster that responded to hot painful stimulation in the ipsilateral DH of the spinal cord. Investigation of this result revealed a decrease in BOLD in the high cognitive load versus the low cognitive load condition, which remarkably mirrored the analgesic effect seen in the pain scores. This is consistent with what has been found before (Sprenger et al., 2012) and indicates modulation of spinal cord activity during distraction from pain. Additionally, we identified a cluster encoding the interaction between pain and attention, located more caudally and more medially in respect to the main effect of temperature cluster. Parameter estimates revealed that the interaction was driven by enhanced activity in the *hard|high* condition, suggesting an active role of this neuronal population in the analgesic effect. The medial location of this cluster is consistent with inhibitory interneurons located in deep laminae (i.e. laminae III-V) receiving inputs from supraspinal regions to inhibit the adjacent main effect of temperature cluster (Bardoni et al., 2013; Hochman, 2007).

Our previous study implicated the ACC-PAG-RVM pathway in attentional analgesia (Oliva et al., 2020), providing further evidence for the long-standing hypotheses that attentional pain relief is achieved by recruitment of the descending pain modulatory system (Sprenger et al.,

2012; Tracey et al., 2002; Valet et al., 2004). Here, we re-produced the same result and expanded it by demonstrating a functional connection between RVM and DH. This for the first time provides evidence for top-down control of spinal cord nociception during distraction from pain, via the ACC-PAG-RVM pathway, known (from non-human animal studies) to be involved in spinal cord modulation (De Felice et al., 2016; Millan, 2002; Ossipov et al., 2010). We therefore propose a system where the ACC signals high cognitive load to the PAG, that recruits spinally-projecting cells in the RVM. It is biologically plausible that analgesia is then achieved through disinhibition of spinally-projecting OFF-cells (Lau et al., 2014), that can inhibit DH neurons directly via GABAergic and opioidergic projections to the primary afferents (Morgan et al., 2008; Zhang et al., 2015).

The RVM is also functionally connected to the LC in the interaction between pain and attention, providing an additional route to analgesia. A possible mechanism for spinal cord modulation is the engagement of spinally-projecting neurons in the LC (Hirschberg et al., 2017) through direct projections from the RVM (Astier et al., 1990; Cedarbaum et al., 1978). Alternatively, the LC could act supraspinally by recruiting the RVM through direct (Fritschy et al., 1990; Kwiat et al., 1992; Tanaka et al., 1996) or indirect projections via the PAG (Kwiat et al., 1992). The latter mechanism was also hypothesised in a spinal cord stimulation study in rodents, where analgesia mediated by the LC did not seem dependent on spinally-projecting neurons but recruited OFF-cells in the RVM (Song et al., 2013).

We next used the opioid antagonist naltrexone to investigate the functional involvement of endogenous opioids in the pain modulatory network implicated in attentional analgesia. A previous study demonstrated that systemic administration of the opioid antagonist naloxone could reduce attentional analgesia behaviourally (Sprenger et al., 2012). We observed a similar result and provide evidence for a functional mechanism behind this effect. Diminished attentional analgesia after naltrexone administration can potentially be explained by the disruption of spinal cord modulation by the

RVM. In this area, endogenous opioids mediate anti-nociception by inhibition of ON-cells, resulting in OFF-cell disinhibition (Fields, 2004; Heinricher et al., 1994; Roychowdhury et al., 1996). During endogenous analgesia, opioidergic stimulation is likely caused by activity in the PAG, a known opioidergic region that was recently shown to release endogenous opioids during deep brain stimulation in humans (Sims-Williams et al., 2017). Indeed, in animal studies opioid antagonists were shown to block PAG-mediated analgesia by acting on the RVM, in line with our result (Kiefel et al., 1993; Roychowdhury et al., 1996). Since the connection between PAG and RVM was not significantly impaired by opioid antagonism, it is possible that this functional interaction is glutamate and GABA-mediated (Aimone et al., 1986; Heinricher et al., 1999; van Praag et al., 1990). According to this hypothesis, the opioid antagonist would specifically prevent the disinhibition of OFF-cells, thereby blocking spinal cord modulation (Heinricher et al., 1994). Naltrexone also had a disruptive effect on the connection between ACC and PAG, recruited during the hard version of the task. The opioid dependence of this connection during endogenous analgesia has already been shown in a placebo study (Eippert et al., 2009), where naloxone abolished the placebo's analgesic effect through inhibition of the ACC-PAG interaction. This effect may be mediated by mu opioid receptors, that were shown to be extensively expressed in the ACC (Baumgärtner et al., 2006), with their recruitment linked to endogenous analgesia in humans (Zubieta et al., 2001). We therefore propose a model where distraction from a painful stimulus triggers release of endogenous opioids in the ACC and RVM, promoting interaction with the PAG and spinal cord, respectively. Naltrexone therefore blocks these mechanisms, preventing modulation of spinal cord, and the consequent decrease in pain perception. This was indeed observed as the spinal cord activity after naltrexone administration was constant during high temperature stimulation, despite the change in cognitive load.

In the present study, naltrexone completely abolished the analgesic effect. However, in a previous attentional analgesia study, an opioid

antagonist only partially attenuated analgesia (Sprenger et al., 2012). This hinted at the existence of a parallel, redundant mechanism to attentional analgesia that does not rely on endogenous opioids. In this context, the functional contribution of the noradrenergic LC has been suggested to play a role in attentional processes (Sara, 2009) and analgesia (De Felice et al., 2016; Hickey et al., 2014; Hirschberg et al., 2017; Millan, 2002). For example, BOLD signal in this region was found to significantly correlate with the behavioural analgesic effect, and its interaction with the anterior cingulate cortex was implicated in attentional analgesia (Brooks et al., 2017; Oliva et al., 2020). However, contrary to expectations, the noradrenaline re-uptake inhibitor reboxetine did not have any effect on attentional analgesia behaviourally. This suggests that either noradrenaline does not play a major role in the attentional modulation of pain, or that higher noradrenergic availability does not have a significant impact on the analgesic mechanisms. On the other hand, reboxetine had an analgesic effect on the pain scores independent of task, a finding in line with the increasingly popular use of noradrenaline-manipulating drugs in treating chronic pain conditions (Bahari et al., 2019; Kremer et al., 2016, 2018). Although reboxetine was previously found to induce analgesia by engagement of spinal cord receptors in a chronic pain model (Hughes et al., 2015), we did not find a similar effect using spinal fMRI. The main effect of temperature cluster in the spinal cord did not differ in the placebo versus reboxetine condition, suggesting that the spinal cord activity was not inhibited by reboxetine. On the other hand, reboxetine diminished the strength of the RVM-LC connection in the main effect of temperature, suggesting a possible functional mechanism for analgesia induced by this drug. It was recently found that the functional connection between RVM and LC during resting state is abnormally strong in chronic pain patients, which, according to the authors, might enhance nociceptive transmission (Mills et al., 2018). It is possible that the higher availability of noradrenaline in the presence of reboxetine acts on inhibitory alpha2 receptors in the LC to reduce its activity, thereby attenuating noradrenergic connectivity to the RVM (Fritschy et al., 1990; Kwiat et al., 1992; Tanaka et al., 1996). Interestingly, reboxetine also had an inhibitory

effect on the connection between ACC and PAG. This suggests that, in addition to endogenous opioids, this interaction is also modulated by noradrenaline, perhaps indirectly via the ACC-LC pathway (Oliva et al., 2020). Further investigation is needed to resolve the exact contribution of noradrenaline signalling in the cognitive modulation of pain.

We used a modified version of an innovative approach for whole CNS imaging, namely z-shimming, that was proposed for the first time by Finsterbusch in 2012 and 2013. By manually choosing the best shim offset for each spinal cord slice, we were able to increase the voxel intensity and decrease signal distortions in the spinal cord, thereby improving the quality of the functional images. Spinal cord-midbrain connections during noxious stimulation have been resolved before (Sprenger et al., 2015), however, to our knowledge, this is the first time the whole CNS has been imaged in the context of the cognitive modulation of pain. Since our goal was of exploring functional connections between brain, brainstem, and spinal cord, we decided to use a single sequence of acquisition, with identical parameters (e.g. orientation of slices, voxel dimensions) for the entire CNS. This differs from other approaches (Finsterbusch et al., 2012, 2013; Islam et al., 2019), and is motivated by the idea that the use of different acquisition parameters for brain and spinal cord could be a confounding factor in connectivity analyses. By taking advantage the z-shimming approach and of the recently developed Spinal Cord Toolbox (De Leener et al., 2017), we have been able to detect significant signal changes in response to experimental manipulations as well as more subtle drug effects on connectivity. On the other hand, we have not been able to reproduce the previous finding of an ACC-LC interaction during attentional analgesia (Oliva et al., 2020). This disparity could be due to the larger voxel size used here to be able to image the entire CNS, which could have penalized LC imaging (Liu et al., 2017). In addition, previous studies resolved inter-subject linear correlations between pain ratings and BOLD signal in PAG (Tracey et al., 2002), RVM (Brooks et al., 2017), LC (Oliva et al., 2020) and DH (Sprenger et al., 2012) during attentional analgesia. None of these results were reproduced here, although a linear

correlation between DH BOLD and pain perception was not far from significance (P = 0.08). A possible explanation is that we did not use identical acquisition parameters across subjects. To be able to image the same anatomical regions, in taller participants we had to increase the gap between slices from 40 to 45-50%. Thus, the small variability in BOLD across individuals could be due to this other confound and not be related to perceived pain intensity.

In summary, we show an opioid-mediated system where, during high cognitive load, the ACC recruits the PAG and RVM to modulate the spinal cord response to a noxious stimulus. Opioid signalling is especially important in the interaction between PAG and ACC and between RVM and DH. LC-mediated noradrenergic modulation of the system occurs in the connection between this nucleus and the RVM.

4.5 Supplementary Material

Supplementary Table 5 *Results from main effect analyses in the whole brain, across the three drug conditions. Obtained with cluster-forming threshold Z>3.09 and cluster-corrected p<0.05. The tables were created with Autoaq (part of FSL), with atlas labels based on the degree of overlap with probabilistic atlases (Harvard Oxford Cortical Structural Atlas, Harvard Oxford Subcortical Structural Atlas, Cerebellar Atlas in MNI152 space after normalization with FNIRT).*

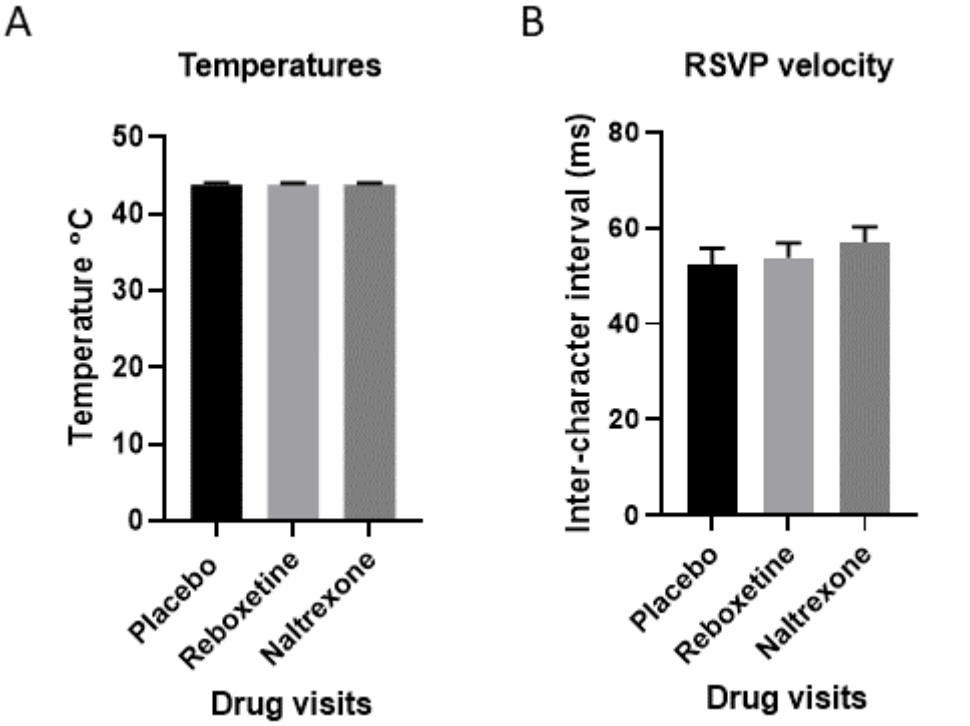
Voxels	MAX	X (mm)	Y (mm)	Z (mm)	Atlas labels
Main effect of temperature					
3295	12.4	36	12	-10	74% Insular Cortex
531	6.56	-32	-14	22	12% Frontal Operculum Cortex, 47% Precentral Gyrus, 26% Central
436	7.53	-34	14	16	Opercular Cortex
246	6.86	-58	0	8	10% Occipital Fusiform Gyrus
131	6.49	38	-66	-22	25% Juxtapositional Lobule Cortex (formerly Supplementary Motor Cortex)
65	6.83	4	-38	-46	100% Brain-Stem
61	6.24	-40	-2	-2	10% Right V
47	5.81	24	-20	64	72% Frontal Pole
43	8.07	-20	52	26	43% Supramarginal Gyrus, anterior division, 20% Parietal Operculum Cortex, 11% Postcentral Gyrus
16	6.11	-4	-68	-40	46% Frontal Operculum Cortex, 15% Insular Cortex
13	5.68	-34	22	6	31% Insular Cortex
11	5.43	-36	-20	-2	11% Frontal Operculum Cortex, 5% Inferior Frontal Gyrus, pars triangularis
10	5.43	36	22	14	29% Middle Frontal Gyrus, 8% Inferior Frontal Gyrus, pars triangularis, 7% Inferior Frontal Gyrus, pars opercularis
10	6.94	40	22	26	10% Supramarginal Gyrus, posterior division, 6% Superior Parietal Lobule
8	5.42	24	-46	-22	45% Frontal Pole, 12% Inferior Frontal Gyrus, pars triangularis, 11% Middle Frontal Gyrus
5	5.87	44	36	14	43% Frontal Pole, 5% Inferior Frontal Gyrus, pars triangularis
4	5.38	18	-12	28	6% Precuneus Cortex
4	5.23	-18	-60	36	11% Postcentral Gyrus
4	5.79	-22	-62	-38	20% Frontal Orbital Cortex
3	5.11	-32	32	-4	45% Frontal Operculum Cortex, 19% Frontal Orbital Cortex, 12% Insular Cortex
3	12.6	0	-36	-6	71% Brain-Stem
2	5.57	20	56	20	5% Temporal Occipital Fusiform Cortex
2	5.03	46	-52	-28	56% Frontal Pole
2	8.8	-40	42	4	34% Frontal Pole
2	5.93	22	50	20	23% Temporal Occipital Fusiform Cortex, 23% Temporal Fusiform Cortex, posterior division
2	5.16	-32	-44	-24	23% Precuneus Cortex, 15% Postcentral Gyrus, 12% Precentral

					Gyrus, 10% Cingulate Gyrus, posterior division
2	8.66	2	-36	52	61% Frontal Pole, 9% Middle Frontal Gyrus, 6% Inferior Frontal Gyrus, pars triangularis
2	5.74	14	-70	-42	83% Frontal Pole
2	5.7	44	40	14	48% Lateral Occipital Cortex, inferior division
2	5.47	-28	60	14	24% Lateral Occipital Cortex, inferior division
2	5.52	52	-70	-16	52% Cingulate Gyrus, anterior division, 16% Paracingulate Gyrus
2	5.03	-34	-80	-2	48% Occipital Pole, 8% Lateral Occipital Cortex, inferior division
2	5.03	24	-22	24	32% Parietal Operculum Cortex, 17% Supramarginal Gyrus, anterior division, 6% Planum Temporale
2	5.09	26	-94	-8	10% Lingual Gyrus, 5% Occipital Fusiform Gyrus
2	5.06	10	-48	-22	10% Frontal Pole, 5% Middle Frontal Gyrus
2	5.68	8	-82	-20	35% Supramarginal Gyrus, anterior division
1	5.02	-22	-4	32	14% Supramarginal Gyrus, posterior division, 7% Planum Temporale, 6% Angular Gyrus
1	5.79	-32	36	18	13% Cingulate Gyrus, anterior division
1	5.15	68	-22	30	35% Supramarginal Gyrus, anterior division
1	5.4	-48	-46	20	14% Supramarginal Gyrus, posterior division, 7% Planum Temporale, 6% Angular Gyrus
1	5.26	12	6	34	13% Cingulate Gyrus, anterior division
1	5.06	22	48	26	,56% Frontal Pole
1	7.82	36	-32	16	37% Planum Temporale, 9% Parietal Operculum Cortex
1	11.5	40	22	44	51% Middle Frontal Gyrus
1	8.47	28	62	20	66% Frontal Pole
1	9.91	-68	-38	36	7% Supramarginal Gyrus, anterior division
1	7.81	-40	-6	36	10% Precentral Gyrus
1	5.93	6	-26	24	9% Cingulate Gyrus, posterior division
1	5.02	-30	40	22	,38% Frontal Pole, 12% Middle Frontal Gyrus
1	5.17	14	-36	-30	20% Brain-Stem
1	6.04	-14	-34	-18	13% Parahippocampal Gyrus, posterior division
1	5.36	-2	-42	-16	10% Brain-Stem
1	5.38	36	12	-14	65% Insular Cortex
1	5.13	-2	-88	-12	48% Lingual Gyrus, 7% Occipital Pole, 4% Intracalcarine Cortex
1	5.69	48	-78	-12	70% Lateral Occipital Cortex, inferior division
1	5.19	-28	16	-12	33% Insular Cortex, 20% Frontal Orbital Cortex
1	5.04	20	-30	64	34% Postcentral Gyrus, 31% Precentral Gyrus
1	5.2	-46	24	-12	73% Frontal Orbital Cortex

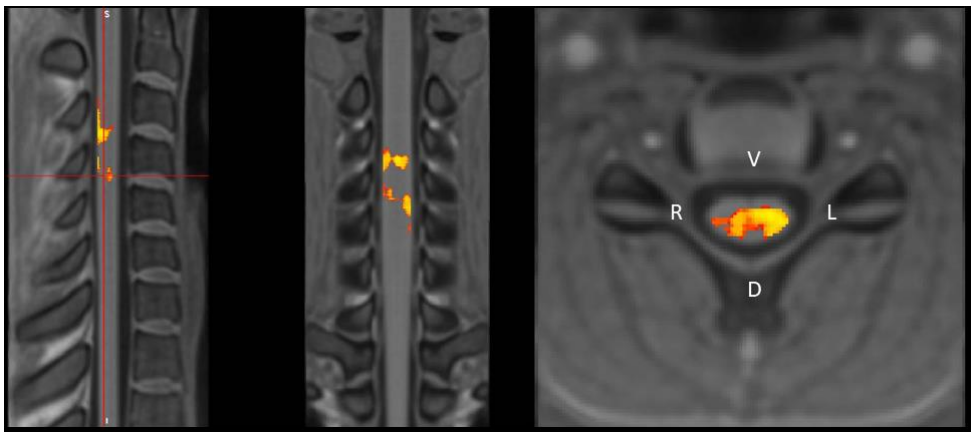
1	5.13	-4	-76	-10	61% Lingual Gyrus
1	5.26	-34	-68	-2	7% Lateral Occipital Cortex, inferior division, 5% Occipital Fusiform Gyrus
1	5.17	-28	-68	2	5% Intracalcarine Cortex
1	5.01	-34	-28	2	3% Insular Cortex
1	5.07	-14	-88	4	22% Intracalcarine Cortex, 8% Occipital Pole, 3% Lingual Gyrus
1	5.15	-44	-18	4	53% Heschl's Gyrus (includes H1 and H2), 6% Planum Polare
1	5.01	-46	-8	6	59% Central Opercular Cortex, 8% Heschl's Gyrus (includes H1 and H2), 7% Planum Polare
1	5.5	-36	50	6	64% Frontal Pole
1	5.38	-34	-76	8	12% Lateral Occipital Cortex, inferior division
1	5.25	-54	-40	14	26% Planum Temporale, 15% Supramarginal Gyrus, posterior division, 11% Superior Temporal Gyrus, posterior division
1	5.09	-34	18	-12	48% Frontal Orbital Cortex, 28% Insular Cortex
Negative main effect of temperature					
25	5.25	-4	46	-14	81% Frontal Medial Cortex, 11% Paracingulate Gyrus
1	6.15	8	30	-12	27% Subcallosal Cortex, 12% Frontal Medial Cortex
Main effect of task					
2585	7.81	46	-46	-8	17% Inferior Temporal Gyrus, temporooccipital part
2535	13.7	-46	-74	-14	59% Lateral Occipital Cortex, inferior division, 17% Occipital Fusiform Gyrus
260	7.23	10	28	36	39% Paracingulate Gyrus, 13% Cingulate Gyrus, anterior division
248	6.05	34	20	6	39% Insular Cortex, 24% Frontal Operculum Cortex
154	6.27	-36	20	6	65% Frontal Operculum Cortex, 10% Insular Cortex
47	5.73	-22	-64	48	60% Lateral Occipital Cortex, superior division, 5% Superior Parietal Lobule
18	5.51	-30	-54	56	44% Superior Parietal Lobule, 7% Lateral Occipital Cortex, superior division
10	5.7	6	-30	-2	65% Brain-Stem
9	5.34	48	6	30	47% Precentral Gyrus, 8% Inferior Frontal Gyrus, pars opercularis
8	5.35	38	-2	58	37% Precentral Gyrus, 29% Middle Frontal Gyrus
8	5.65	-4	-30	-4	73% Brain-Stem
8	5.67	-46	-12	-4	52% Planum Polare, 12% Heschl's Gyrus (includes H1 and H2)
6	5.26	30	48	26	86% Frontal Pole
5	5.43	22	-60	58	2% Lateral Occipital Cortex, superior division, 8% Superior Parietal Lobule
3	5.18	38	-6	50	37% Precentral Gyrus, 10% Middle Frontal Gyrus
3	5.47	-40	16	-8	56% Insular Cortex, 9% Frontal Orbital Cortex
3	5.8	-40	-52	-32	96% Left Crus I

2	5.13	18	-68	56	48% Lateral Occipital Cortex, superior division
2	5.07	-26	-60	-12	42% Temporal Occipital Fusiform Cortex, 16% Occipital Fusiform Gyrus, 9% Lingual Gyrus
2	5.29	30	44	40	77% Frontal Pole
2	11.2	26	-40	-12	42% Lingual Gyrus, 24% Temporal Occipital Fusiform Cortex, 15% Parahippocampal Gyrus, posterior division, 11% Temporal Fusiform Cortex, posterior division
1	5.1	30	-60	62	51% Lateral Occipital Cortex, superior division, 13% Superior Parietal Lobule
1	5.1	-2	-34	-22	99% Brain-Stem
1	5.28	-2	-42	-14	6% Brain-Stem, 75% Left I-IV
1	5.03	40	-2	48	31% Precentral Gyrus, 24% Middle Frontal Gyrus
1	5.03	40	-2	-14	46% Insular Cortex, 11% Planum Polare
1	5.15	42	-38	46	39% Supramarginal Gyrus, posterior division, 18% Superior Parietal Lobule, 7% Postcentral Gyrus
1	5.07	-18	-78	42	57% Lateral Occipital Cortex, superior division, 9% Precuneus Cortex
1	5.03	-42	2	34	38% Precentral Gyrus, 22% Middle Frontal Gyrus
1	5.92	-48	-8	-4	43% Planum Polare, 18% Heschl's Gyrus (includes H1 and H2)
1	5.11	-4	32	26	47% Paracingulate Gyrus, 44% Cingulate Gyrus, anterior division
1	10.1	18	-28	0	88% Right Thalamus
1	6.22	40	22	24	26% Middle Frontal Gyrus, 10% Inferior Frontal Gyrus, pars opercularis, 5% Inferior Frontal Gyrus, pars triangularis
1	5.34	2	36	20	66% Cingulate Gyrus, anterior division, 16% Paracingulate Gyrus
1	5.05	26	48	18	62% Frontal Pole
1	5.11	46	16	12	29% Inferior Frontal Gyrus, pars opercularis
1	5.01	-48	-24	10	59% Heschl's Gyrus (includes H1 and H2), 7% Planum Temporale, 7% Central Opercular Cortex
1	9.19	42	-2	0	75% Insular Cortex
1	11.3	-40	-6	34	14% Precentral Gyrus
Negative main effect of task					
333	6.18	-48	-62	24	40% Lateral Occipital Cortex, superior division, 34% Angular Gyrus
9	5.21	12	-50	38	42% Precuneus Cortex, 19% Cingulate Gyrus, posterior division
8	5.53	-24	-52	22	2% Precuneus Cortex
4	6.56	-10	-34	8	57% Left Thalamus
3	5.49	48	-74	46	1% Lateral Occipital Cortex, superior division
2	5.55	34	-52	4	2% Lingual Gyrus, 1% Precuneus Cortex
1	13	-14	-40	36	14% Cingulate Gyrus, posterior division

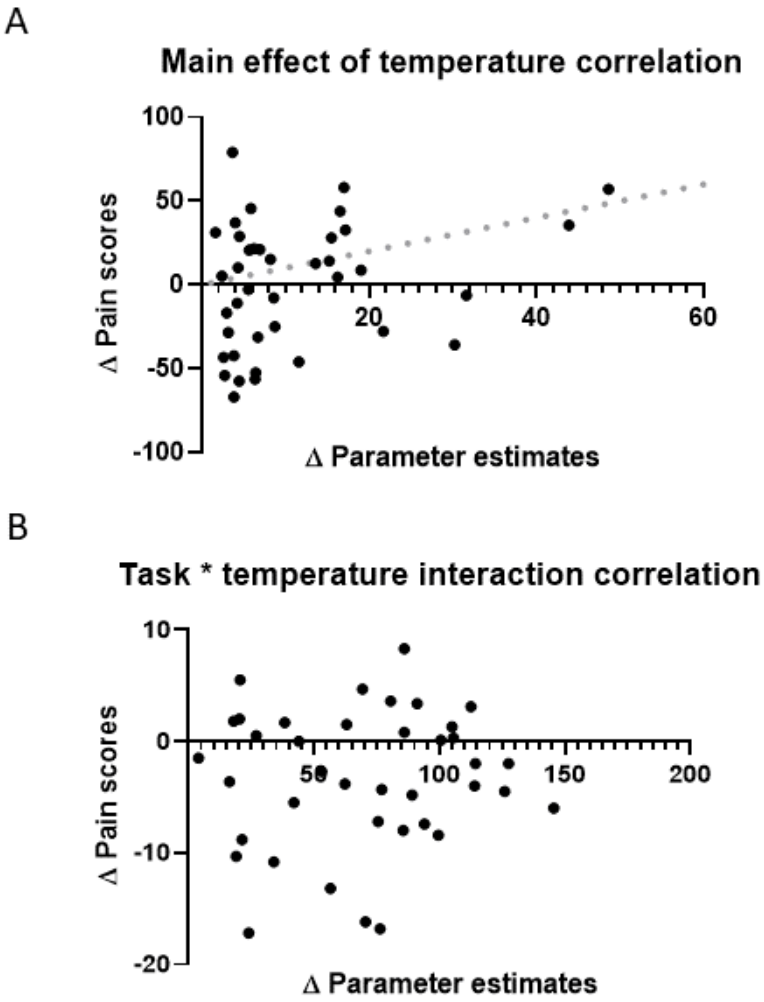
1	6.38	18	-42	30	1% Cingulate Gyrus, posterior division
1	9.05	34	-52	24	4% Angular Gyrus
1	6.78	40	-52	22	18% Angular Gyrus
1	7.07	38	-48	8	1% Angular Gyrus, 1% Supramarginal Gyrus, posterior division
1	7.41	-68	-52	2	22% Middle Temporal Gyrus, temporooccipital part
1	5.48	-4	48	-14	83% Frontal Medial Cortex, 5% Paracingulate Gyrus
1	5.03	22	-80	-36	95% Right Crus II
Negative task * temperature interaction					
5	6.39	46	44	22	80% Frontal Pole
1	5.27	42	20	2	63% Frontal Operculum Cortex, 8% Frontal Orbital Cortex, 5% Insular Cortex
1	5.23	50	40	22	50% Frontal Pole, 13% Middle Frontal Gyrus
1	5.09	56	26	32	19% Middle Frontal Gyrus
1	5.52	52	34	32	12% Middle Frontal Gyrus, 5% Frontal Pole



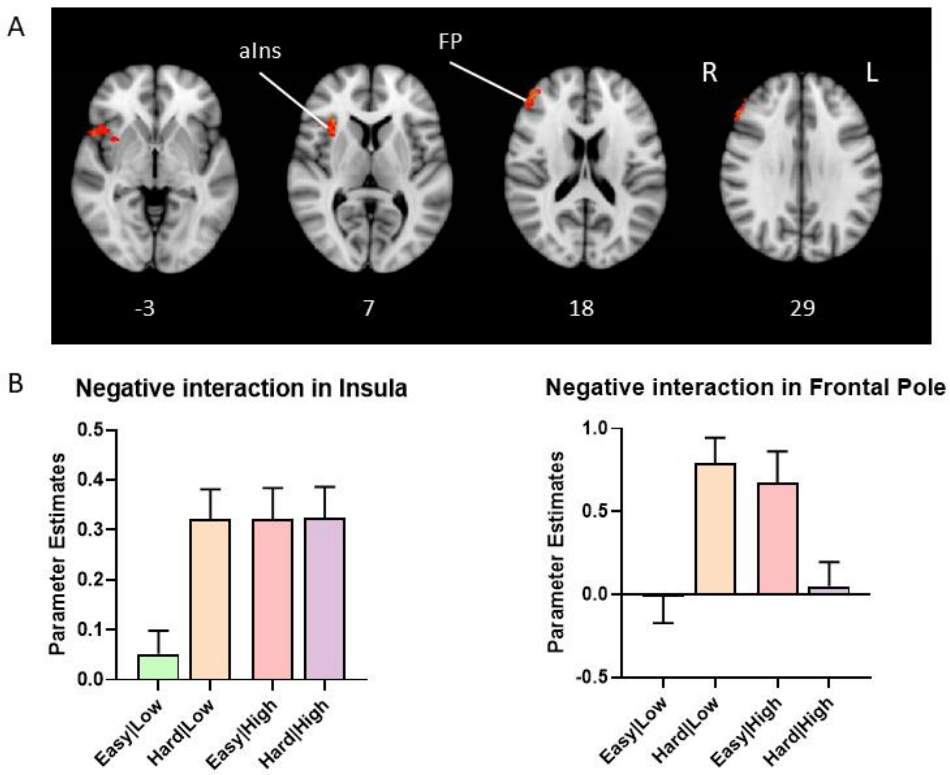
Supplementary Figure 1 *(A) Administration of Reboxetine or Naltrexone did not significantly cause a change in temperature delivered as revealed by paired t tests (Placebo versus Reboxetine P = 0.74; Placebo versus Naltrexone P = 0.57). (B) Task velocity also did not change after Reboxetine nor Naltrexone administration (Placebo versus Reboxetine P = 0.63; Placebo versus Naltrexone P = 0.1).*



Supplementary Figure *2 Main effect of temperature analysis using a whole cord mask revealed a cluster in the C6 bilateral DH, with lower significance in the right side. A bilateral cluster in the C5 dorsal horn was also found.*

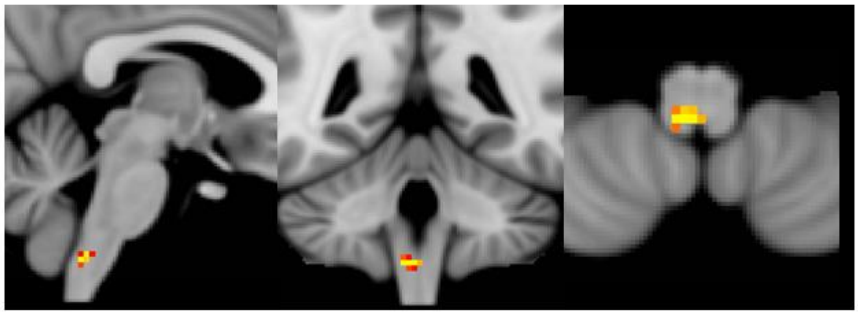


Supplementary Figure 3 *(A) The delta of parameter estimates extracted from the main effect of temperature spinal cord cluster did not significantly correlate with the delta of pain scores (main effect of temperature). (B) Similarly, the delta in parameter estimates extracted from the task * temperature interaction cluster in the spinal cord did not significantly correlate with the delta of pain scores (interaction).*

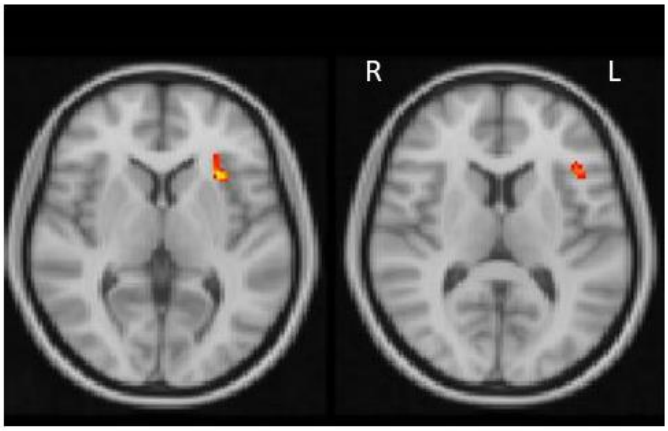


Supplementary Figure 4 *The anterior insula and the frontal pole responded significantly to the negative task * temperature interaction. Investigation of this result through extraction of parameter estimates does not suggest a role for these two regions in attentional analgesia. The anterior insula activates during all the conditions of high cognitive load (e.g. both pain and attention). The frontal pole also seems to activate during the condition of high cognitive load, except than during conflict (e.g. when the task is hard, and the temperature is high).*

A



B



Supplementary Figure 5 *(A) A cluster in the lower medulla responded more strongly in the naltrexone than in the placebo main effect of temperature. Result obtained with permutation testing using a main effect of temperature brainstem mask, obtained from the pooled analysis. (B) The anterior insula responded more strongly in the naltrexone than in the placebo in the main effect of task (obtained with permutation testing with a main effect of task mask, obtained from the pooled analysis).*

Chapter 5 General Conclusions

5.1 Summary

The present collection of studies significantly contributed to the understanding of the attentional modulation of pain in health and disease. An attentional task with two levels of difficulty distracted study participants from a hot painful stimulus delivered on their left forearm. Concurrent fMRI in the entire CNS resolved the neural interactions behind attentional analgesia. Finally, through pharmacological manipulation, the functional contribution of endogenous opioids was resolved. The noradrenergic influence on attentional analgesia requires further investigation.

The experimental set-up was successful in inducing attentional analgesia behaviourally in healthy volunteers, in the three different studies presented. The decrease in pain scores was of approximatively 5% in the three studies, consistent with previous work (Bantick et al., 2002; Tracey et al., 2002).

In agreement with what has been showed before (Sprenger et al., 2012), the opioid receptor antagonist naloxone abolished the analgesic effect, suggesting an opioid-dependent mechanism in attentional analgesia. On the other hand, the analgesic effect was present after administration of the noradrenaline reuptake inhibitor reboxetine, with a similar effect size to the control condition. The noradrenergic contribution to attentional analgesia is therefore less clear.

Analyses of main effects resolved the cerebrum, brainstem, and spinal cord contribution to different aspects of attentional analgesia. Identical analysis techniques and software have been used for the three different studies and were able to reach most of the same findings. Main effect of temperature analysis in the cerebrum resolved a network of regions that

showed increased activity with noxious versus innocuous stimulation. This network included the primary somatosensory cortex, the dorsal posterior insula, and the anterior cingulate cortex, among the others, consistent with the most important regions contributing to the composite neurological pain signature (Wager et al., 2013). Importantly, the same cerebral set of regions was observed in the three different studies, and also after administration of an opioid antagonist or a noradrenaline re-uptake inhibitor. Main effect of task analyses revealed significant activity in the hard version of the task versus the easy version of the task, in regions consistent with the visual attention network (Nieuwenhuis et al., 2011). This included the lateral occipital cortex, the anterior cingulate cortex, and the anterior insula, in a pattern that was seen across the three studies. The drugs used did not have any effect on the attentional network, except for an increase in activity of the anterior insula after naltrexone administration. Notably, the ACC responded significantly to both main effect of task and of temperature, with overlapping clusters. The involvement of this area in both aspects of the attentional analgesia experiment suggested a role in attentional analgesia, and prompted investigation of the interactions of the ACC with the brainstem.

In the brainstem PAG, LC and RVM were involved in both aspects of the experiment, with significant activation in main effect of temperature and main effect of task. Despite the challenges with imaging the brainstem with functional imaging (see section 1.4.3), these results were also consistent across the three different studies. Neither reboxetine nor naltrexone had any impact on the overall activity of the three nuclei during noxious stimulation or attentional demand. The lack of effect of reboxetine on LC activity was especially surprising. Animal data (Szabo et al., 2001) showed that reboxetine causes a dose-dependent inhibition of LC firing, by increasing the noradrenergic effect on alpha2 receptors. The lack of this effect in the study presented could be caused by underdosing of reboxetine. Alternatively, it is possible that the physiological noise in the LC did not allow for detection of such an effect.

On examination of spinal cord activity, a functional cluster responding to noxious stimulation was resolved in the dorsal horn of the spinal cord, in the C6 spinal segment. BOLD signal extracted from this area was lower in the high cognitive load versus the low cognitive load condition during high temperature stimulation, indicating spinal cord modulation during attentional analgesia. This effect was also observed after reboxetine administration, but was abolished by naltrexone, in line with the behavioural findings. A cluster in the spinal cord also reached significance in the task * temperature interaction contrast, with strong activity in the analgesic condition. It is therefore possible that this pool of neurons has an active role in modulating the dorsal horn response to noxious stimulation during distraction from pain. This cluster was not modulated by noradrenaline or endogenous opioids.

Connectivity analyses were used to resolve functional interaction of brainstem nuclei with each other and with the cortex during attentional analgesia. It was found that high cognitive load has a top-down influence on the ACC-PAG-RVM system, during high temperature stimulation. In parallel, the attentional task also modulates the bidirectional interaction between ACC and LC. Both pathways are likely to have a causal involvement in the analgesic effect, with direct modulatory projections to the spinal cord. This was indeed demonstrated for the ACC-PAG-RVM pathway, where evidence for spinal cord modulation by the RVM was found. On the other hand, the role of the LC in this network is less clear. Study 3 failed to re-produce the functional interaction with the ACC, but provided evidence for a LC-RVM pathway to analgesia. The disparity in findings might be due to the larger voxel size used in study 3 to be able to measure functional activation in the whole CNS. A meta-analysis of LC imaging studies indeed found large voxels to dramatically decrease the ability of measuring functional signal form this very small nucleus (Liu et al., 2017). Further studies, perhaps with LC-specific imaging, are needed to finally resolve the functional connections to and from the LC during attentional analgesia.

Contrary to expectations, the same experimental paradigm was also successful in inducing attentional analgesia in fibromyalgia patients. Appropriate calibration of the thermal stimulus and cognitive task might have been crucial for achieving this unexpected result. This suggests that therapeutic approaches that target attentional processes have the potential of being efficient for this patient population. For example, cognitive behavioural therapy strategies that promote attention diversion and mental imagery (Elomaa et al., 2009). Importantly, the brainstem mechanisms behind attentional analgesia also seem to be identical to the ones in healthy volunteers, with engagement of PAG and RVM. This challenges the long-standing hypothesis that these patients have dysfunctional pain modulatory mechanisms (Julien et al., 2005; Kosek et al., 1996; Lannersten et al., 2010; Staud et al., 2005; Vierck et al., 2001).

5.2 Strengths and limitations

The experiment used in the present thesis had two level of task difficulty and two levels of stimulus intensity, giving rise to a 2x2 design. This accurate definition of the CNS regions and connections between regions that responded specifically to high cognitive demand, to high thermal stimulation, and to the interaction between pain and attention. This is more sophisticated than the approaches that have been employed previously, where the only factor with two levels was the cognitive load (Bantick et al., 2002). As a consequence, these early studies could have only resolved regions involved in attentional processing. Regions resolved in the present thesis are confirmatory to the previous findings and are more directly implicated in generating analgesia.

A recent article demonstrated that different choices in the pre-processing or analysis pipelines of the same fMRI dataset can lead to radically different results (Botvinik-Nezer et al., 2020). The problem of between-group reproducibility in human imaging studies is indeed well known in the field and has been pointed out at other times (Eklund et al., 2016, see section 1.4.2). Typically, problems related to inter-individual

variability are addressed with mixed statistical models and the problem of multiple comparison with stringent statistical thresholds. The work presented here uses both strategies and addresses reproducibility issues by replicating identical analyses on independent datasets. Results of main effect analyses in brain and brainstem were indeed consistent across the three studies presented. This is especially important considering that a different head and neck coil, acquisition and pre-processing parameters were used in study 3 (Chapter 4) to be able to image the bigger field of view. In addition, functional imaging results presented here are in good agreement with findings from different research groups, adding robustness to the findings. Examples include spinal cord modulation during distraction from pain (Sprenger et al., 2012), involvement of the PAG in attentional analgesia (Bantick et al., 2002; Tracey et al., 2002; Valet et al., 2004), and functional interaction between ACC and LC in conflict resolution (Köhler et al., 2016).

An external, acute stimulus was used to cause pain in the fibromyalgia Study (Chapter 3). The reasons for it being consistency with the same studies on healthy volunteers, as well as temporal precision and titrability. However, this is an ecologically different sensation from the endogenous pain perceived by these patients. Experimental pain is also likely to not cause as much of the emotional response typically associated with chronic (inescapable) pain. Therefore, care should be taken in suggesting that our study is a definite demonstration that attention diversion can be a viable strategy in treatment of this patient population. Additionally, fibromyalgia patients reported regularly taking analgesic medications to manage their pathology, including noradrenergic drugs. It is possible that such drugs had an impact on the attentional and nociceptive processes.

An obvious limitation of Study 3, common to most human pharma-fMRI experiments, is that the drugs were given systemically. This means that such medications could act on all available receptors in the central and peripheral nervous system, more than in a specific target area. Although through

simultaneous functional imaging it was possible to resolve the specific mechanisms affected, we were unable to target a specific region or neuronal population like is common in animal studies.

5.3 Future directions

This thesis shed light on the functional mechanisms in brainstem and spinal cord in the attentional modulation of pain. However, the issue of how conflict is resolved in the brain during this paradigm remains unresolved. As discussed in section 1.2.3, pain is a highly attention demanding process (Legrain et al., 2009), thus a concurrent attentional task competes for attentional resources. The process that results in one of the two having precedence is likely to require high-level computations and weighting of the two stimuli. Computational models in conflict resolution experiments have been useful in suggesting mechanisms that might underlie this process in the ACC, for example a cost-benefit function (Botvinick et al., 2001, 2004). Attentional analgesia studies would highly benefit from this approach, that would help to clarify how pain and attention interact in the brain, and how one takes precedence over the other. Such a study would also be of interest for general neuroscience research by furthering the understanding of conflict resolution in the brain.

The acquisition sequence used in Chapter 4 can also be used to address unresolved mechanisms behind chronic pain pathologies, for example fibromyalgia. QST demonstrated hyperalgesia to hot and cold stimuli in these patients, consistent with results from other groups (Brietzke et al., 2019; Hurtig et al., 2001). Different mechanisms have been proposed to explain this finding, for example altered central processing of the stimuli, or hyperexcitability of peripheral nociceptors. Altered spinal cord activity during rest has indeed been recently demonstrated in fibromyalgia patients (Martucci et al., 2019). It would be interesting to investigate whether the spinal cord also shows altered response during noxious stimulation. Full CNS imaging would also resolve whether the enhanced perception in these patients is a bottom-up (i.e. malfunctioning processes in the spinal cord

cause enhanced pain perception in the cortex), or top down effect (i.e. alterations in brain and brainstem cause facilitation of spinal cord response).

Reboxetine was used in study 3 to investigate the role of noradrenaline in attentional analgesia. This drug is a noradrenaline reuptake inhibitor and was expected to enhance the analgesic effect of attention. However, no differences with the placebo condition were found behaviourally. This might mean either that noradrenaline does not play a significant role in attentional analgesia, or that higher noradrenaline availability in the system does not have a big impact on this process. Noradrenergic antagonists can cause severe side effects in healthy volunteers, for example sedation and low blood pressure. Thus, an alternative method of investigation needs to be used. Positron emission tomography is able to image cerebral blood flow as well as the dynamics of specific neurotransmitters/receptors. Using a tracer that tracks noradrenaline transporters or receptors (Chen et al., 2020; Sander et al., 2017) during attentional analgesia would be useful for addressing this issue.

Additionally, pupil diameter was consistently found to correlate directly with the activity of the LC. This technique is less invasive than PET as it does not require tracer administration, and can even be used in individuals that could not have an MRI (e.g. because of metal implants). An attentional analgesia study using this method would significantly contribute to the understanding of the contribution of the LC in this process, especially if used in combination with fMRI.

Finally, we have been able to reproduce a similar acquisition protocol to Finsterbusch et al., (2012), where the ideal shim value for each spinal cord slice was manually defined. While efficient, this method is however time-consuming and subjected to human error. Spinal cord imaging would greatly benefit from an automated system, for example using machine learning, for detection of ideal shim offsets.

5.4 Applications

The remarkable similarity of the biological mechanisms to attentional analgesia with the ones involved in placebo analgesia suggests that different strategies can be used to harvest the brain's potential to cause pain relief. While distraction from pain only induced a small decrease in pain perception in the studies presented, it is worth noting that the attentional task used in the studies presented is not particularly engaging. Also, participants are in no way motivated in performing well as the monetary reward they received for taking part to the studies was not variable. This suggests that a more complex and engaging task, for example a videogame, could induce stronger analgesia.

Ideally, in the future no "tricks" (e.g. manipulation of expectation or attention) will be needed to achieve analgesia. A patient would learn strategies to consciously engage specific brain regions and "switch on" the analgesic brain. A similar idea has been explored in a pain neurofeedback fMRI study (deCharms et al., 2005). This technique presents study participants with a live feedback on the activation of a specific region of their own brain, which they learn to actively modulate. While this study was successful in inducing significant pain relief by modulating ACC activity, the lack of extensive application in clinical practice suggests major challenges with this protocol. The present thesis resolved a reproducible functional network for endogenous analgesia. It is possible that direct targeting of the pathways resolved, perhaps through DCM-based neurofeedback (Koush et al., 2013) can be even more successful in inducing pain relief without medication.

5.5 Conclusions

The present collection of studies provided functional and mechanistic insights on the attentional modulation of pain, in health and disease. Pain relief can be reliably achieved by engaging in a visual cognitive task, through recruitment of endogenous opioids. Noradrenaline seems to be involved, but its contribution is not fully resolved.

During high cognitive demand, the ACC recruits PAG and RVM in a top-down network that modulates the spinal cord response to a hot painful stimulus. This pathway is dependent on endogenous opioids, that are especially important in the functional connections between ACC and PAG, and between RVM and spinal cord. This provides strong evidence against the hypothesis that cognitive modulation of pain is a supratentorial process, while emotional/expectation modulation of pain involves spinal cord modulation (Bushnell et al., 2013, see section 1.2.6). The results presented here indeed show overlapping biological mechanisms across different modalities of endogenous analgesia, perhaps recruited by different cortical areas (e.g. ACC in attentional analgesia and DLPFC in placebo analgesia).

Evidence for a role of the LC in attentional analgesia was suggested in two out of three studies presented, in consistency with previous work (Brooks et al., 2017). However, its involvement in the network is less clear, with possible interactions with the ACC and the RVM during attentional analgesia. Further investigation is necessary to resolve the involvement of this nucleus and of noradrenaline.

Interestingly, the pain relief induced by a shift in attention was not long-lasting as would be expected after taking an analgesic tablet. The experimental conditions were randomised, meaning that "analgesic" conditions were interspersed with "painful" conditions. Thus, the biological mechanisms to attentional analgesia were only recruited briefly, as needed when attention was diverted. This is consistent with a continuous bidirectional modulation of spinal cord activity by the brainstem (Stroman et al., 2016), where cortical regions (e.g. the ACC) can shift the downstream influence from facilitatory to inhibitory and vice versa.

The analgesic effect of attention was also shown in fibromyalgia patients, with similar biological substrates. Although the therapeutic effectiveness of this strategy on endogenous pain still needs to be clarified, therapies that address the interaction between pain and attention might be

useful for this pathology. Furthermore, the long-standing idea that brainstem nuclei are malfunctioning and are not efficient in achieving analgesia in fibromyalgia patients, is challenged.

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