THE NEUROPHYSIOLOGICAL BASIS OF THE DIVERGENT SYMPATHETIC RESPONSES TO LONG–LASTING EXPERIMENTAL MUSCLE PAIN IN HUMANS

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Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

Signature

Publications arising from this thesis

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List of abbreviations

˚C Degree Celsius

ACC Anterior cingulate cortex

ACh Acetylcholine

AVS Audio-visual stimulus

BMI Body mass index

BOLD Blood oxygen level dependent

BP Blood pressure

BRS Baroreflex sensitivity

cm Centimetres

CNS Central nervous system

CVLM Caudal ventrolateral medulla

DAP Diastolic arterial pressure

dlPAG Dorsolateral periaqueductal grey

dlPFC Dorsolateral prefrontal cortex

dlpons Dorsolateral pons

DMH Dorsomedial hypothalamus

DMN Default mode network

dmPAG Dorsomedial periaqueductal grey

dmPFC Dorsomedial prefrontal cortex

ECG Electrocardiography

fMRI Functional magnetic resonance imaging

FWHM Full-width at half maximum

G Gauge

GABA Gamma-amino butyric acid

h hour

HF High frequency

HR Heart rate

HRV Heart rate variability

Hz Hertz

kHz kilohertz

L2 Second lumbar vertebrae

LF Low frequency

lPAG Lateral periaqueductal grey

M1 primary motor cortex

mA Milliamps

MAP Mean arterial pressure

MCC Mid-cingulate cortex

min Minute

ml Millilitres

MNI Montreal neurological institute

mPFC Medial prefrontal cortex

MRI Magnetic resonance imaging

Ms millisecond

MSNA Muscle sympathetic nerve activity

NA Noradrenaline

NAc Nucleus accumbens

NOS Nitric oxide synthase

NRS Numerical rating scale

NTS Nucleus tractus solitarius

OFC Orbitofrontal cortex

PAG Periaqueductal grey

PASS Pain anxiety symptoms scale

PBN Parabrachial nucleus

PCC Posterior cingulate cortex

PCS Pain catastrophizing scale

PNS Peripheral nervous system

PVAQ Pain vigilance and awareness questionnaire

PVN Paraventricular nucleus

RMS Root-mean-square

RMSSD Root mean square successive difference

RVLM Rostroventrolateral medulla

S state anxiety

S1 Primary somatosensory cortex

S2 Secondary somatosensory cortex

S2 Second sacral spinal segment

S4 Fourth sacral spinal segment

SAP systolic arterial pressure

SEM Standard error mean

sgACC Subgenual anterior cingulate cortex

SPM Statistical parametric mapping

SSNA Skin sympathetic nerve activity

STAI State and trait-anxiety inventory

SUIT Spatial unbiased infratentorial template

T trait anxiety

T1 First thoracic vertebrae

TE Echo time

TR Repetition time

VAS Visual analogue scale

vlPAG Ventrolateral periaqueductal grey

VMH Ventromedial hypothalamus

Abstract

It is known that some individuals with chronic pain go on to develop high blood pressure. Indeed, patients with post-surgical chronic pain have nearly twice the prevalence of clinical hypertension than medical patients without pain (Bruehl, Chung, Jirjis, & Biridepalli, 2005). Accordingly, we could postulate that a person who consistently exhibited increases in muscle sympathetic nerve activity (MSNA), blood pressure and heart rate during experimental muscle pain may – if he or she developed chronic pain from an injury in the future – go on to develop hypertension (Bruehl et al., 2005). Interestingly, long-lasting experimental muscle pain induced by hypertonic saline solution in humans causes a sustained and consistent increase in muscle vasoconstrictor drive and blood pressure in some subjects, and a sustained decrease in others (Fazalbhoy, Birznieks, & Macefield, 2012, 2014).

To further our understanding of the complex physiological changes that bring about these divergent responses, this thesis has explored the association of baseline physiological and psychological levels with the direction of the sympathetic response during tonic muscle pain. Furthermore, this project included combined microneurography and neuroimaging techniques to identify areas of the brain involved in generating sustained increases or decreases in sympathetic nerve activity to muscle, as well as changes in the brain associated with the generation of MSNA bursts during experimental muscle pain. The final chapter explored the effects of an audio-visual stimulus on the direction of the response.

The results reported in this thesis highlight the fact that the muscle sympathetic responses to experimental muscle pain are not based on baseline physiological or psychological parameters but are associated with different signal intensity changes in

important autonomic brain regions. Furthermore, distraction from the painful stimulus through audio-visual distraction does not influence the direction of the response. While this series of experiments has shed light on the neurophysiological mechanism through which the divergent sympathetic response to experimental muscle pain arises, many questions remain to be answered. For instance, it is unknown *why* such divergent responses would occur in humans. Furthermore, whether these responses remain sustained over a longer period of time needs to be determined.

CHAPTER 1 Introduction

1.1. Autonomic nervous system

The nervous system is divided into the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS comprises the brain, the brainstem, the cerebellum, and the spinal cord. The PNS is composed of the cranial nerves III through XII, which supply the head, and the spinal nerves, which derive from the spinal cord and innervate the torso and limbs. The PNS is further subdivided into somatic and autonomic. The somatic PNS works under conscious control and is comprised of two types of fibres (axons): the efferent (motor) fibres innervate skeletal muscles via the ventral roots and spinal nerves, while the afferent (sensory) axons convey sensory information to the CNS via the spinal nerves and dorsal roots (Catala & Kubis, 2013). The autonomic nervous system acts without conscious control – although it can be accessed by higher-order cognitive or emotional functions - and is accountable for motor functions of the visceral organs, which are critical for the maintenance of homeostasis (Loewy & Spyer, 1990). It targets the smooth muscles in the organs throughout the body, as well as the blood vessels and glands (Loewy & Spyer, 1990). It is subdivided into the sympathetic, parasympathetic, and enteric branches (Darby, 2014). The enteric branch is located within the gastrointestinal tract and is exclusively involved in control of gastrointestinal secretions and motility; it shall not be considered further here. The sympathetic and parasympathetic branches each work as a two-motor-neuron system: a pre-ganglionic neuron, and a post-ganglionic neuron, and for those organs that receive both sympathetic and parasympathetic innervation, the two branches operate antagonistically (Chase & Clemente, 1968).

1.1.1. Sympathetic nervous system

The sympathetic nervous system is the main player during periods of stress and adversity. Also called 'the fight or flight system,' it is responsible for increased cardiac and respiratory rates, as well as increases in blood pressure (Guyenet, 2006). The preganglionic neurons are located in the lateral grey column (or intermediolateral cell column) of the spinal cord between the first thoracic (T1) and second lumbar (L2) vertebrae (Loewy & Spyer, 1990). It is therefore often called the thoracolumbar outflow. The preganglionic neurons receive inputs from premotor neurons in the brainstem via the dorsolateral funiculus, in addition to input from spinal and propriospinal interneurons (Charkoudian & Wallin, 2014). Preganglionic sympathetic neurons are thinly myelinated and conduct at velocities of up to 15m/s (Darby, 2014). Once they leave the spinal cord via the anterior roots they synapse with ganglia located in the sympathetic chain or with prevertebral ganglia that are located close to large blood vessels (Darby, 2014). The primary neurotransmitter they release onto these post-ganglionic sympathetic neurons is acetylcholine (ACh; Loewy & Spyer, 1990). The post-ganglionic sympathetic neurons lie within the ganglia comprising the sympathetic chain (the paravertebral ganglia), located parallel to the spinal cord on either side, as well as within the prevertebral ganglia (Chase & Clemente, 1968). In contrast to the preganglionic neurons, these are unmyelinated and therefore conduct at a much slower rate (~0.5 to 1m/s). With the exception of post-ganglionic sudomotor neurons, which use acetylcholine as the neurotransmitter (Jänig, Krauspe, & Wiedersatz, 1982), all post-ganglionic sympathetic neurons release noradrenaline (NA) onto their target organs; the chromaffin cells of the adrenal medulla are considered to be modified post-ganglionic cells and release adrenaline and noradrenaline (Loewy & Spyer, 1990). These include the salivary glands, as well as

the heart and blood vessels. Sympathetic innervation to the smooth muscles surrounding blood vessels causes vasoconstriction (Charkoudian & Wallin, 2014). The sympathetic division of the autonomic nervous system is illustrated in *Figure 1.1*.

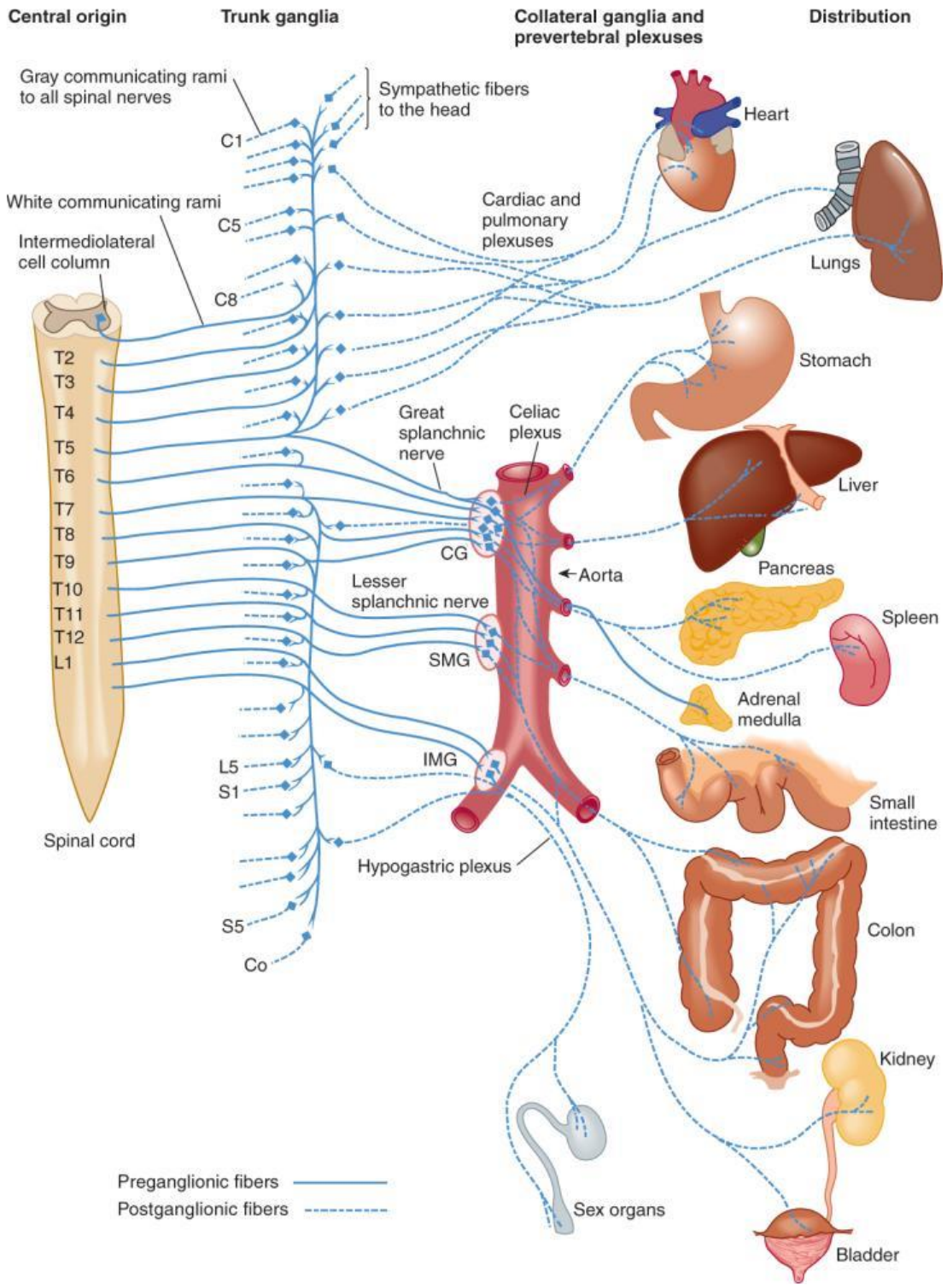

Figure 1.1: Sympathetic division of the autonomic nervous system (Buijs & Swaab, 2013).

1.1.2. Parasympathetic nervous system

The parasympathetic nervous system differs from the sympathetic nervous system in its arrangement, as well as in its function. It plays a major role during periods of rest and digestion. It is responsible for slowing down the heart, lowering the blood pressure, and promoting digestion. The first neuron of the parasympathetic nervous system originates from the brainstem nuclei of cranial nerves III, VII, IX and X, as well as from the intermediolateral cell column of the second, third and fourth sacral spinal segments (S2-S4; Chase & Clemente, 1968). From these craniosacral sites these neurons synapse with postganglionic parasympathetic neurons located within ganglia close to or within the walls of the viscera; as such their preganglionic axons are longer, and postganglionic axons shorter, than those of the sympathetic nervous system (Loewy & Spyer, 1990). All parasympathetic postganglionic neurons use ACh as the dominant neurotransmitter (Darby, 2014). The parasympathetic division of the autonomic nervous system is illustrated in *Figure 1.2*.

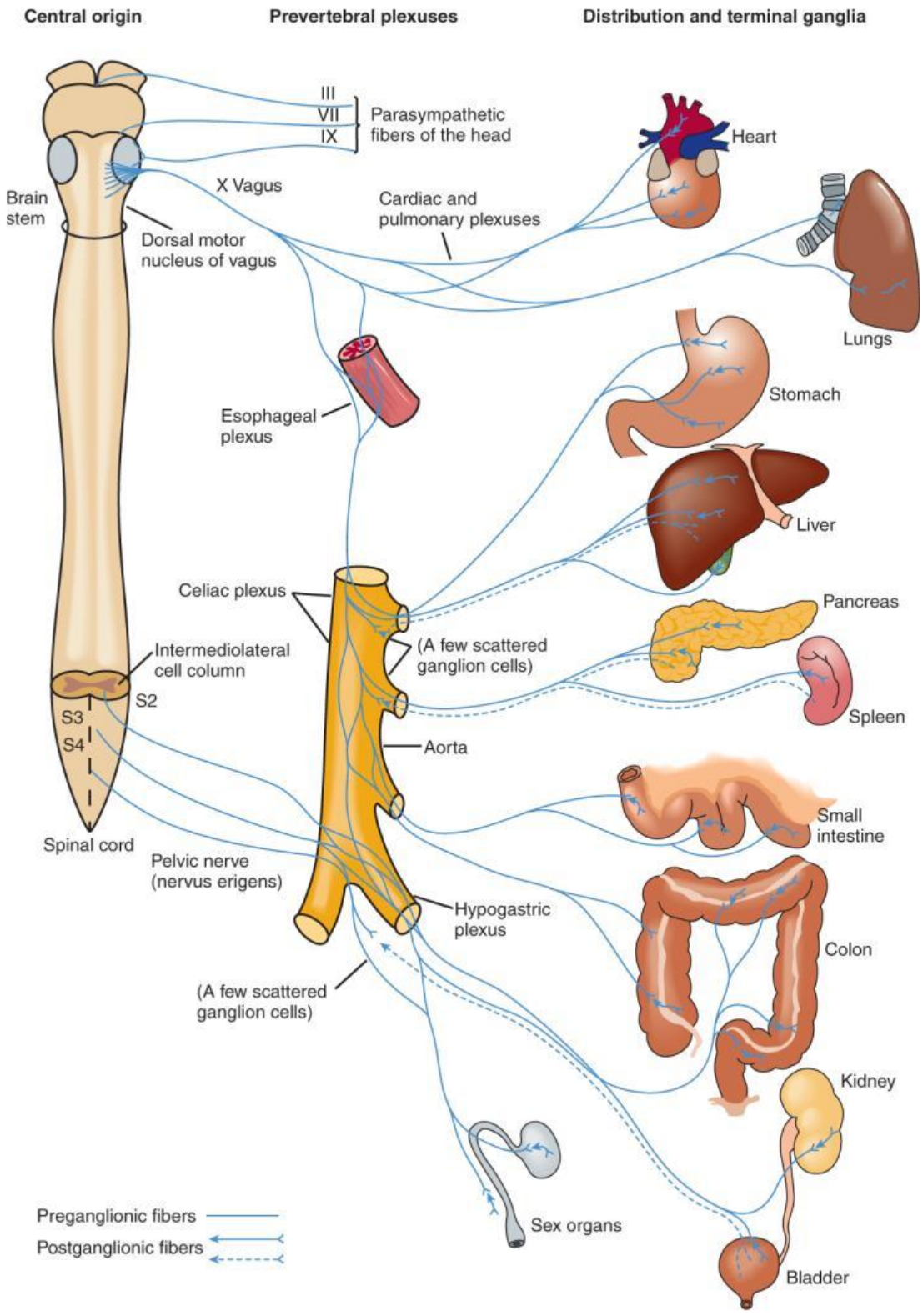

Figure 1.2: Parasympathetic division of the autonomic nervous system (Buijs & Swaab, 2013).

Although most visceral and cranial structures are innervated by both the sympathetic and parasympathetic nervous system, certain structures such as smooth muscles in systemic blood vessels, as well as sweat glands and hair follicles, do not follow this rule (Chase & Clemente, 1968); they receive sympathetic innervation exclusively. Together, the sympathetic and parasympathetic nervous systems work in balance to ensure homeostasis (Darby, 2014).

1.2. The arterial baroreflex

The arterial baroreceptor reflex is the main mechanism through which blood pressure is controlled on a beat-to-beat basis. The afferent limb of the reflex involves the arterial baroreceptors, which are stretch receptors found in the distensible walls of the aortic arch and carotid sinus, at the base of the internal carotid artery, just distal to the bifurcation (Guyenet, 2006). These two sites are of particular importance as they reflect whole-body perfusion pressure and the perfusion pressure of the brain, respectively. A rise in arterial blood pressure increases the radial distending pressure and increases the firing rate of these dynamically sensitive stretch receptors (Guyenet, 2006). The action potentials generated by the baroreceptor afferents are then conducted to the central nervous system via different routes: those originating in the aortic arch travel in the vagus nerve, while those arising from the carotid sinus travel in the glossopharyngeal nerve (Saper, 2002). Both routes eventually synapse in the nucleus tractus solitarius (NTS) in the dorsomedial medulla (Dampney et al., 2003). Neurons in the NTS send a direct excitatory (glutamatergic) projection to the nucleus ambiguus and the dorsal motor nucleus of the vagus, sites where parasympathetic cardiac preganglionic neurons originate (Saper, 2002). An increase in activity within these second-order neurons engages the parasympathetic limb of the baroreflex, bringing

about an increase in cardiac vagal outflow and the release of acetylcholine at the sinoatrial node of the heart, and hence causes a decrease in heart rate and stroke volume (Dampney et al., 2003).

The sympathetic limb of the baroreflex involves three synapses within the medulla. After synapsing onto barosensitive neurons in the NTS, excitatory projections from these neurons synapse onto neurons in the caudal ventrolateral medulla (CVLM), the terminals of which contain the inhibitory neurotransmitter gamma-aminobutyric acid (GABA; Benarroch, 1998). The GABAergic neurons in the CVLM in turn synapse onto sympathoexcitatory premotor neurons in the rostral ventrolateral medulla (RVLM) that innervate sympathetic vasomotor preganglionic neurons in the spinal cord (Dampney, 1981). The RVLM is the primary output nucleus for sympathetic vasoconstrictor drive to the muscle, splanchnic, and renal vascular beds, and as such plays an important role in the ongoing regulation of total peripheral resistance and hence blood pressure (Guyenet, 2006).

In summary, the activation of the baroreceptors at the level of the aortic arch and carotid sinus cause increased parasympathetic activity to the heart, via the vagus nerve, and simultaneously cause the withdrawal of sympathetic excitation to the heart and blood vessels. The baroreflex pathway is illustrated below (see *Figure 1.3*).

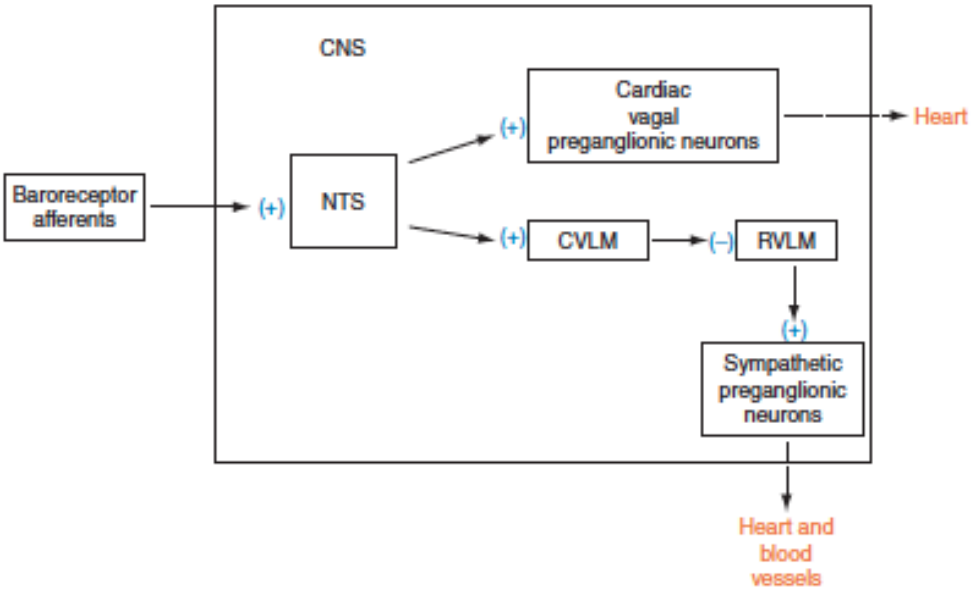

Figure 1.3: Central nervous system baroreceptor pathway linking baroreceptor afferents to sympathetic and parasympathetic outflow.

Plus (+) and minus (-) symbols refer to excitatory (glutamatergic) synapses and inhibitory (GABAergic) synapses, respectively (from Sved, 2009).

This baroreflex circuitry has been extensively investigated in animals, where experiments in anesthetized cats have shown that excitation of RVLM neurons evoked increases in muscle sympathetic nerve activity and blood pressure (Dampney et al., 2003). The majority of sympathetic premotor vasoconstrictor neurons originate in the RVLM and electrolytic destruction of the RVLM in rabbits results in precipitous, life-threatening falls in blood pressure (Dampney & Moon, 1980). Because blood pressure is primarily influenced by changes in heart rate, stroke volume, and the degree of sympathetically-mediated vasoconstriction in skeletal muscles, muscle sympathetic nerve activity (MSNA) is understandably a major contributor to the control of total peripheral vascular resistance (Hart & Charkoudian, 2014).

1.3. Muscle sympathetic nerve activity

Muscle sympathetic nerve activity (MSNA), which can be recorded via microelectrodes inserted directly into a peripheral nerve in awake human subjects, reflects the activity of postganglionic sympathetic neurons supplying the skeletal muscle vascular beds; an increase in MSNA causes vasoconstriction and thereby increases blood pressure (Macefield, 2013). The main role of MSNA in healthy individuals is to regulate acute falls in blood pressure, via the arterial baroreflex, and thereby maintain a constant perfusion pressure to skeletal muscle. MSNA is greatly influenced by the arterial (high-pressure) and cardiopulmonary (low-pressure) baroreceptors, as well as cardiac rhythmicity (Fagius & Wallin, 1980). When blood pressure rises, baroreceptors are excited and sympathetic output to the heart and blood vessels is inhibited, and parasympathetic outflow to the heart increased, leading to a decrease in heart rate and stroke volume, and a decrease in sympathetically-mediated vasoconstriction (Guyenet, 2006). Through the baroreflex, MSNA exhibits strong cardiac rhythmicity; it is specifically the carotid arterial baroreceptors that seem to provide the dominant temporal coupling of MSNA to the cardiac cycle (Jänig & Häbler, 2003).

MSNA is modulated by various factors such as respiration, age, and body weight. For example, MSNA decreases during inspiration and increases in expiration (Eckberg et al., 1985; Fatouleh & Macefield, 2013; Hagbarth & Vallbo, 1968; Macefield & Wallin, 1995; Seals et al., 1990). Increased age and body weight also raise resting levels of MSNA (Hart et al., 2009; Hart & Charkoudian, 2014; Joyner, Barnes, Hart, Wallin, & Charkoudian, 2015; Lambert et al., 2007; Sverrisdóttir, Johannsson, Jungersten,

Wallin, & Elam, 2001). However, MSNA remains constant in a given individual over many years (Fagius & Wallin, 1993). Moreover, there are inter-individual differences: some individuals have high levels of MSNA at rest while others have low levels; identical twins have similar levels, whereas the burst incidence in fraternal twins differs widely (Wallin, Kunimoto, & Sellgren, 1993). The effects of mental stress on MSNA are also well documented. Indeed, metabolic syndrome patients with anxiety and mood disorders have greater MSNA burst frequency at rest, compared to metabolic syndrome patients and controls without these psychological symptoms (Toschi-Dias et al., 2013). Furthermore, single unit recordings from muscle vasoconstrictor neurons in metabolic syndrome patients with high levels of anxiety, as well as in patients with panic disorder and major depressive disorder, revealed greater incidence of multiple firing during a burst (Lambert et al., 2006, 2008, 2010). *Table 1.1* summarizes some of the influences on MSNA.

Table 1.1: Physiological and clinical influences on resting MSNA (Charkoudian & Wallin, 2014).

Factor	**Influence**
Female sex (and / or female sex hormones)	Decrease (most / not all studies)
Race	Difference Pima Indian vs. white (men)
	Difference African-American vs. white (men)
Altitude (acute and long-term)	Increase
Pregnancy	Increase
Hypertension	Increase (most / not all studies)
Heart failure	Dramatic increase
Obstructive sleep apnoea	Increase
Chronic renal failure	Increase

Since MSNA causes vasoconstriction and is the main contributor to increasing blood pressure once it has dropped, it would be reasonable to think that higher levels of MSNA would be associated with higher blood pressure. Indeed, it is known that elevated levels of MSNA are associated with essential hypertension (Esler & Kaye, 2000), renovascular hypertension (Johansson et al., 1999), and the high blood pressure that results from untreated obstructive sleep apnoea (Takeuchi et al., 1994; Trombetta et al., 2010). However, there is no relationship between MSNA and blood pressure among young healthy individuals. That is, a person with high MSNA can have normal blood pressure and vice versa (Charkoudian & Wallin, 2014). Although this does not hold true for people older than age 50 (Hart & Charkoudian, 2014), it should not undermine the importance of the baroreflex, which is important not only for transient blood pressure compensations, but also for setting the level of blood pressure over the long term (Hart & Charkoudian, 2014).

The role of sympathetic activity in long-term blood pressure control is highly debated. Although early animal studies failed to show any alterations in blood pressure control upon sino-aortic denervation (Cowley et al., 1973), more recent studies have shown the importance of the sympathetic nervous system in long-term blood pressure control (Lohmeier, 2001). Indeed, stimulation of the carotid baroreflex in dogs causes sustained decreases in systemic blood pressure (Lohmeier et al., 2004, 2010). In humans, there is evidence that afferent carotid baroreceptor stimulation leads to sustained decreases in blood pressure, decreased central sympathetic outflow and increased parasympathetic tone (Heusser et al., 2010). Moreover, people with baroreflex failure tend to become hypertensive in the future (Heusser et al., 2005). Since baroreceptor afferents are conducted to the central nervous system, in particular

the medullary nuclei, it is vital to understand the central neural sites responsible for the control of MSNA.

1.4. Supraspinal regulation of MSNA

1.4.1. Animal studies

Experimental animal studies have revealed that there are four classes of neurons that constitute the central cardiovascular pathway (Dampney, 1994). First, there are preganglionic autonomic motor neurons, such as vagal (Nosaka et al., 1979) and sympathetic (Jänig, 1985) preganglionic neurons, which control the heart, blood vessels, and adrenal medulla. Second, there are autonomic premotor neurons that project to and control the activity of these first preganglionic neurons (Dampney, 1994). Cardiovascular sympathetic premotor neurons have their nuclei in the brainstem and hypothalamus (Strack et al., 1989a,b). Indeed, five specific cell groups – in the rostral ventrolateral medulla, rostral ventromedial medulla, caudal raphe nuclei, A5 noradrenergic cell group in the caudal ventrolateral pons, and the paraventricular nucleus (PVN) in the hypothalamus – all innervate preganglionic outflow to the adrenal medulla and all major sympathetic ganglia (Strack et al., 1989a,b). Third, there are primary afferent neurons that transmit signals from peripheral receptors, which influence cardiovascular function (Loewy & Spyer, 1990). Finally, there are interneurons that link primary afferent inputs of higher brain centres controlling cardiovascular function to autonomic premotor neurons, including the CVLM (Feldberg & Guertzenstein, 1976), the parabrachial complex (Herbert et al., 1990), locus ceruleus (Elam, et al., 1984, 1986), the midbrain periaqueductal grey (PAG;

Carrive & Bandler, 1991), and cortical regions such as the insula (Cechetto & Chen, 1990).

1.4.2. Human studies

In 2010, Macefield and Henderson were the first to record muscle sympathetic nerve activity in awake human subjects while performing functional Magnetic Resonance Imaging (fMRI) of the brainstem, in order to identify the baroreflex circuitry. Although it remains unclear whether fMRI signal intensity changes represent synaptic transmission or neuronal firing (Logothetis, Pauls, Augath, Trinath, & Oeltermann, 2001; Mukamel et al., 2005; Viswanathan & Freeman, 2007), Macefield and Henderson showed that when bursts of MSNA were present, Blood Oxygen Level Dependent (BOLD) signal intensity was high in the RVLM, while signal intensity within the NTS and CVLM was low; the reverse was true when bursts were absent (Macefield & Henderson, 2010). These results confirmed the role of the brainstem in human cardiovascular control and specifically muscle sympathetic outflow.

Further studies allowed the group to identify cortical structures involved in central cardiovascular control (James, Macefield, & Henderson, 2013). They found that signal intensity in certain cortical structures - such as the dorsomedial and ventromedial hypothalamus, the precuneus, insula, and dorsolateral prefrontal cortex - varied with the intensity of the concurrently recorded bursts of MSNA, which correlated with signal intensity of the left and right RVLM. Since the RVLM is the final output nucleus for MSNA, this work suggests that higher cortical areas may be involved in the regulation of MSNA in awake human subjects (James et al., 2013). Indeed, experiments involving animals have shown direct projections from the sensorimotor

cortex to the NTS and the RVLM (M'hamed, Sequeira, Poulain, Bennis, & Roy, 1993; Verberne & Owens, 1998) so that central command could potentially cause increases in muscle sympathetic nerve activity. Along those lines, a number of brain regions, including the insular cortex, amygdala, hypothalamus, PAG, parabrachial nucleus, NTS, and ventrolateral medulla have been proposed to make up the "central autonomic network" (Benarroch, 1993). These findings were more recently confirmed, with consistently activated regions such as the left amygdala, right anterior and left posterior insula, as well as the midcingulate cortices, forming the core of the central autonomic network (Beissner, Meissner, Bär, & Napadow, 2013). Furthermore, several studies have shown that damage to certain areas of the brain can lead to disruption of cardiovascular control. For example, reduced cardiovascular arousal following a stroke isolated to the anterior cingulate cortex has been reported (Critchley et al., 2003). Neurocardiogenic dysfunction has also been hypothesized to be the cause of sudden unexplained death following stroke and epileptic seizures (Scorza et al., 2009).

The role of the autonomic nervous system is vital for the homeostatic and physiological adjustments crucial in life (Darby, 2014). Sympathetic outflow to the muscle vascular bed can be altered through physiological challenges, but can also be increased in pathological conditions, such as heart failure or obstructive sleep apnoea (Charkoudian & Wallin, 2014). Little is known however, about the effects of pain on muscle sympathetic nerve outflow in humans, and the interaction between the sympathetic nervous system and pain is complex.

1.5. Noxious stimulation

Intramuscular injection of hypertonic saline has been used in the study of experimental pain in humans for many years. It was first used in humans in 1938 by Kellgren to study referred pain, and shown to induce a deep and dull ache that often referred to distal structures (Kellgren, 1938). For example, injection of hypertonic saline solution in the tibialis anterior causes referred pain in the ankle (Graven-Nielsen et al., 1997a,b). Hypertonic saline induces pain, as it is a specific stimulus for nociceptors (Graven-Nielsen & Mense, 2001). In 2009, Burton and colleagues used intramuscular or subcutaneous injections of hypertonic saline to examine the effects of deep and superficial pain on muscle sympathetic nerve activity (Burton, Birznieks, Bolton, Henderson, & Macefield, 2009) Capra and Ro (2004) induced tonic muscle pain for 20 minutes to simulate longer-lasting musculoskeletal pain. More recently, Fazalbhoy and colleagues used intramuscular infusion of hypertonic saline to cause a sustained, steady level of pain for one hour (Fazalbhoy et al., 2012, 2014). This method of inducing pain offers the advantage of allowing a controlled investigation into how long-lasting tonic pain may modulate the cardiovascular responses. As these sensations closely resemble the description of pain in patients with actual chronic pain conditions, it may shed light on the initial mechanisms underlying the complex chain of physiological changes that lead to the establishment of long-lasting pain conditions.

Important for survival, pain helps to avoid tissue damage, mobilizing all relevant homeostatic systems for a fight-and-flight response or, alternatively, promoting conservation of energy and thus promoting healing (Craig, 2002). In the short term, these physiological responses serve as a protective mechanism, preventing the

establishment of prolonged damage. Conversely, chronic pain, which is thought to arise from the activation of nociceptors initially (but can be maintained without the ongoing noxious stimulus) is maladaptive and can have detrimental psychological and physiological consequences that affect many systems (Blyth et al., 2001). One in five people in Australia suffers from chronic pain (Blyth et al., 2001), and there is a significant increase in psychological distress in people with chronic pain which interferes with their daily activities (Blyth et al., 2001). Furthermore, individuals with chronic pain are more likely to rate their health as poor compared with individuals without pain (Mäntyselkä et al., 2003). In terms of the cardiovascular system, chronic pain is associated with an increased risk of developing hypertension (Bruehl et al., 2005).

1.6. Pain and the sympathetic nervous system

While the role of sustained activation of the sympathetic nervous system and its association with the development and maintenance of chronic pain (such as in chronic regional pain syndromes) is highly debated (Ali et al., 2000; Benarroch, 2006; Elam & Macefield, 2004; Macefield, 2010), the effects of pain on the sympathetic nervous system are well-documented, with changes in blood pressure, heart rate, sweat release, and blood flow to muscle and skin being reported in both animals and humans (Burton et al., 2009; Burton, Birznieks, Spaak, Henderson, & Macefield, 2009; Fazalbhoy et al., 2012; Horeyseck & Jänig, 1974; Kobuch, Fazalbhoy, Brown, & Macefield, 2015; Lewis, 1942).

1.6.1. Animal studies

In animals, the effects of pain on the cardiovascular system have been extensively investigated. In general, the cardiovascular response seems to be dependent on the origin of the pain. The first experiments exploring this relationship date back to 1866 when Lovén showed that electrical stimulation of cutaneous afferent fibres in rabbits decreased the activity in vasomotor neurons innervating the same area of skin as the afferent fibres, and increased the activity in other vasomotor neurons, leading to an increase in arterial blood pressure (Lovén, 1866). Further experiments have shown that superficial pain causes an increase in the activity of sympathetic postganglionic muscle fibres, in anesthetized and spinalised cats (Boczek-Funcke et al., 1992; Horeyseck & Jänig, 1974a, 1974b), as well as anesthetized rats (Häbler, Jänig, Krummel, & Peters, 1994). However, noxious stimulation of skeletal muscle (but not noxious stimulation of the skin) in anesthetized rats has shown to inhibit muscle vasoconstrictor neurons (Kirillova-Woytke, Baron, & Jänig, 2014). In 1984, Sato and colleagues showed that manipulation of an inflamed knee joint (causing deep pain) in anesthetized cats caused increases in blood pressure and heart rate (Sato, Sato, & Schmidt, 1984). Finally, activation of group III afferent fibres through sciatic nerve stimulation is capable of causing increases in blood pressure and heart rate in spontaneously hypertensive rats (Yao, Andersson, & Thorén, 1982).

Thus, the animal literature (with the exception of the study conducted by Sato and colleagues) suggests that generally superficial pain evokes an increase in the cardiovascular activity, which is probably driven by increases in MSNA. In contrast, pain originating from deeper structures evokes decreases in blood pressure and heart rate (Bandler, Carrive, & Zhang, 1991). Bandler and colleagues (1991) attribute these

conflicting responses to the ability of the organism to deal with the noxious stimulus. Indeed, the coping mechanisms to certain stressors, including pain, differ depending on whether that stressor is "escapable" or "inescapable" (Keay & Bandler, 2002). When the situation is escapable, humans and animals use a more active coping strategy, which evokes an increase in the fight-or-flight response. Conversely, an inescapable stressor is met with passive coping mechanisms, which are associated with sympathetic depression (Bandler et al., 1991).

1.6.2. Human studies

Early studies in humans suggested that cardiovascular responses to noxious inputs also vary according to the tissue of origin (Lewis, 1942). Lewis (1942) proposed that superficial pain (that arose from skin) resulted in an increase in pulse rate, which reflects an increase in sympathetic activity. In contrast, pain that arose from deeper structures showed a decrease in pulse rate and blood pressure, which is attributable to a decrease in sympathetic activity (Lewis, 1942). These findings were confirmed by Feinstein and colleagues in 1954, who found that injection of 6% hypertonic saline solution into the paravertebral muscles of the thorax - and rarely neck and back - was associated with pallor, sweating, a fall in blood pressure, and bradycardia in awake human subjects (Feinstein, Langton, Jameson, & Schiller, 1954). Furthermore, research has shown that acute noxious stimuli, such as pressure to the nail-bed and the trigeminal region, as well as instillation of soap solution in the eye, and mechanical pressure on the muscles, evoke a generalized increase in MSNA (Nordin & Fagius, 1995; Schobel et al., 1996). Similarly, the painful physical stress during immersion of the hand into ice-cold water triggers increases in MSNA with parallel increases in blood pressure (Fagius, Karhuvaara, & Sundlöf, 1989; Kregel, Seals, & Callister,

1992). Burton and colleagues (2009) used intramuscular and subcutaneous injections of hypertonic saline to examine the effects of deep and superficial pain on muscle sympathetic nerve activity. They showed that both types of pain – which lasted between 6 and 8 minutes - increased MSNA amplitude, blood pressure, and heart rate in almost all subjects (Burton et al., 2009a). Therefore, in humans, the majority of acute painful stimuli produce an increase in MSNA. Notable exceptions have been shown during painful electrical stimulation to the skin, which causes a short-lasting decrease in MSNA (Delius, Hagbarth, Hongell, & Wallin, 1972a).

Chronic pain is associated with an increased risk of developing hypertension, a phenomenon that is thought to originate from the alterations in the relationship between the cardiovascular and pain regulatory systems (Bruehl et al., 2005). However, little is known about the early physiological changes that accompany the establishment of chronic pain. Indeed, there are few studies that have investigated the changes in sympathetic nerve activity during chronic pain: in a single patient with chronic regional pain syndrome (CRPS), suspected to be sympathetically maintained because of the marked cutaneous vasoconstriction, there was no difference in sympathetic outflow to the painful limb compared to the contralateral non-painful limb (Casale & Elam, 1992). It may be that both limbs were affected equally - in most instances sympathetic outflow is distributed symmetrically to the left and right limbs. In a larger study involving 25 fibromyalgia patients, pain intensity was positively correlated with MSNA burst frequency (Zamunér et al., 2015).

In order to investigate the consequences of long-lasting pain on the cardiovascular system, Fazalbhoy and colleagues (2012) infused hypertonic saline solution into the

tibialis anterior muscle of healthy subjects for ~45 minutes. They found that the physiological effects of longer-lasting pain produced two types of responses: one group of subjects showed a constant increase in burst amplitude of MSNA, blood pressure and heart rate, and another group showed a decrease in these parameters (Fazalbhoy et al., 2012). Interestingly, the subjects in that study all showed an increase in blood pressure and a decrease in heart rate in the first few minutes after the onset of pain. It was hypothesized that this initial response may represent the urge to respond to a noxious stimulus, and that the changes in blood pressure that followed may be attributed to the different coping mechanisms utilized by each individual. A subsequent study demonstrated that the changes in MSNA observed during long-lasting muscle pain were consistent over time in the majority of individuals (Fazalbhoy et al., 2014).

1.6.3. Supraspinal regulation

In 1996, Schobel and colleagues observed that subjects with borderline hypertension had increased tolerance to pain compared to normotensive subjects. They further demonstrated that these changes did not correlate with changes in MSNA, or the baroreflex, and therefore may be attributable to central changes (Schobel et al., 1996).

More recently, Burton and colleagues showed that a bolus injection of hypertonic saline in the leg muscles in people with spinal cord injury does not cause reflex increases in blood pressure or heart rate, or any cutaneous vasoconstriction or sweat release below the lesion (Burton, Brown, & Macefield, 2008). These findings therefore suggest that cardiovascular changes to pain in humans appear to be mediated by supraspinal sites.

It has recently been shown that supraspinal centres regulating cardiovascular activity overlap substantially with those receiving nociceptive input (Benarroch, 2006; Bruehl & Chung, 2004). These same regions, including the insula, the anterior cingulate cortex (ACC), the amygdala, the hypothalamus, as well as the PAG, parabrachial nucleus (PBN), and NTS, also contain groups of neurons that initiate autonomic, antinociceptive and behavioural responses to these stimuli (Benarroch, 1993; Saper, 2002). Furthermore, it is interesting that there appears to be a relationship between the cardiovascular and descending pain modulation systems, since the greater the magnitude of cardiovascular response during the cold pressor test, the greater an individual's endogenous analgesic ability (Chalaye et al., 2013).

In fact, the insula receives pain and temperature information (Craig, 2003) and is connected to the hypothalamus, as well as brainstem autonomic regions such as the NTS, parabrachial nucleus, and ventrolateral medulla (Saper, 2002). Similarly, the ACC also receives nociceptive inputs, is involved in the affective and motivational components of pain sensation (Vogt, Berger, & Derbyshire, 2003), and extensively interacts with areas of the central autonomic network (Gabbott, Warner, Jays, Salway, & Busby, 2005). The amygdala provides emotional significance to the painful stimulus, and via its projections to the hypothalamus and brainstem, it can initiate autonomic responses (Davis & Whalen, 2001). At the level of the diencephalon, the lateral and posterior areas of the hypothalamus are involved in autonomic control as well as pain modulation (Abrahamson & Moore, 2001). In the brainstem, the NTS and the PAG are two important supraspinal structures that also receive both cardiovascular information as well as nociceptive input (Bruehl & Chung, 2004). As noted above, the NTS receives information from baroreceptor inputs and, through negative feedback,

provides an excitatory signal to cardiac parasympathetic neurons via the vagus nerve, and also reduces the excitatory drive to the RVLM (Sved, 2009). The NTS also receives projection neurons from spinal laminae involved in nociceptive processing (Bruehl & Chung, 2004). Finally the PAG is involved in the processing of noxious stimuli (Carrive & Bandler, 1991), and stimulation of the lateral columns (lPAG) preferentially evokes a fight-or-flight response, with increases in blood pressure and heart rate, similar to the response evoked by cutaneous pain. Conversely, stimulation of the ventrolateral columns of the PAG (vlPAG) promotes rest and digest, with a parallel decrease in blood pressure and heart rate – as seen during experimental deep pain in animals (Depaulis et al., 1992).

1.7. Microneurography

Microneurography is a method that allows the direct recording of axonal activity via a tungsten microelectrode inserted percutaneously into a peripheral nerve in awake human subjects (Vallbo, Hagbarth, & Wallin, 2004). The technique was developed in Sweden in the 1960s to record activity from low-threshold mechanoreceptors in muscle and skin, but is most often used to record activity in unmyelinated postganglionic sympathetic axons (Vallbo et al., 2004). Due to their accessibility, the common peroneal nerve or the tibial nerve in the popliteal fossa are the most commonly used nerves in the lower limb (Macefield, 2013). These peripheral nerves are particularly useful as they contain several fascicles, each separated from one another via the perineurium, a high impedance fibrous barrier, which prevents cross-talk between neighbouring fascicles. Generally, close to the fascicular innervation zone all nerve fibres in one fascicle supply one type of tissue: either muscle or skin (Vallbo et al., 2004).

1.7.1. Sympathetic microneurography

Sympathetic microneurography is used to record multiunit sympathetic nerve activity, which is characterized by bursts of impulses separated by silent periods. However, microneurography can also be used to record the activity from individual postganglionic sympathetic axons, using highly selective microelectrodes (Macefield, 2013). An example of a multi-unit recording is shown in *Figure 1.4*.

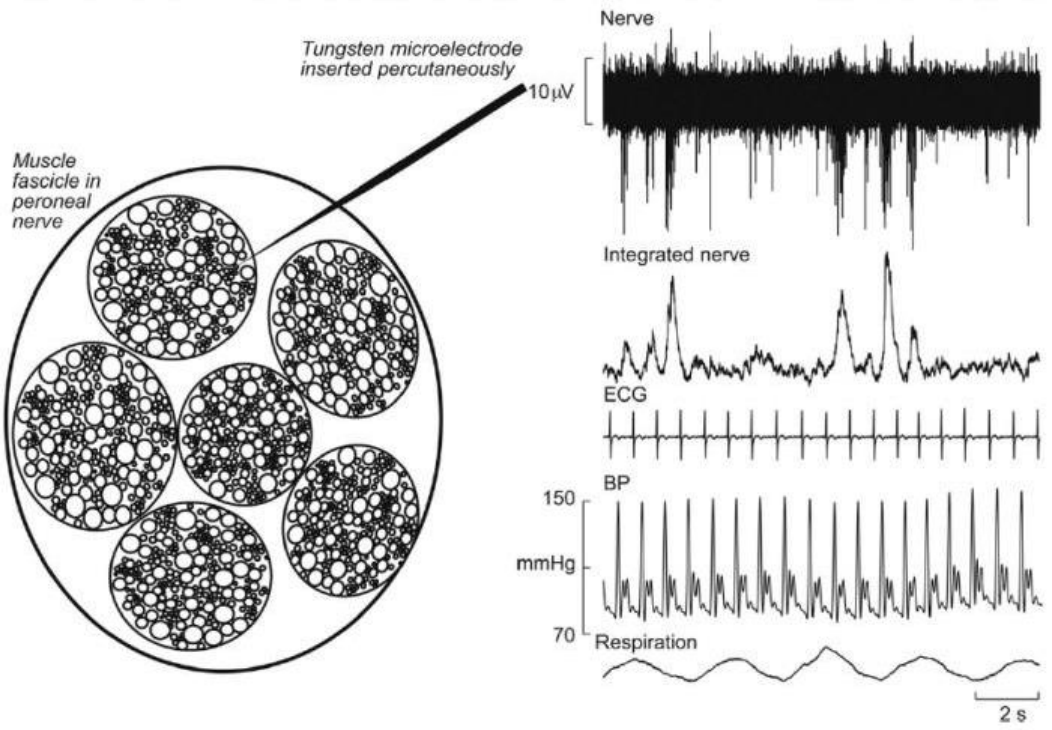

Figure 1.4: Spontaneous bursts of MSNA recorded via a tungsten microelectrode inserted into a muscle fascicle of the common peroneal nerve (from Macefield, 2013).

Bursts of MSNA exhibit a tight coupling to the cardiac rhythm via the baroreflex (Jänig & Häbler, 2003; Macefield, Elam, & Wallin, 2002). The sympathetic nervous system, however, is not only involved in the baroreflex or blood vessel constriction, but also provides cutaneous innervation to sweat glands, hairs, and adipose tissue via sympathetic axons travelling in cutaneous fascicles (Chase & Clemente, 1968).

Therefore, it is important to distinguish the nerve fascicle carrying MSNA from the one carrying skin sympathetic nerve activity (SSNA; Macefield, 2013). Cutaneous fascicles are defined as such because intraneural stimulation through the microelectrode evokes radiating paraesthesia but no muscle twitches. Moreover, stroking over the fascicular innervation zone activates low-threshold mechanoreceptors in the skin.

Several features differentiate bursts of MSNA from bursts of SSNA. In contrast to MSNA, bursts of SSNA have variable shapes and durations and do not display overt cardiac rhythmicity. SSNA does not exhibit a sustained increase during an inspiratory-capacity apnoea, only generating a single burst during the inflation phase. Single bursts of SSNA are also evoked by a brisk sniff, as well as with arousal: a loud clap, or an unexpected tap on the nose will generate a burst of SSNA. Such manoeuvres have no effect on MSNA (Delius et al., 1972a,b).

1.7.2. Analysis

The standard analysis of sympathetic nerve activity involves counting the number of visible bursts occurring per minute (burst frequency) or per 100 heart beats (burst incidence), or measuring the total neural activity (the cumulative sum of burst amplitudes in 1 minute; Macefield, 2013). Based on single-unit recordings of sympathetic post-ganglionic axons, it is known that individual neurons fire primarily only once per burst, indicating that increases in burst amplitude are brought about largely through recruitment of additional neurons (Macefield et al., 2002). Burst amplitude, frequency, and incidence reveal slightly different features about MSNA. Burst amplitude reveals the strength of the signal and is dependent on the number of

action potentials generated during the burst and the distance between the electrode tip and the active fibres. Burst frequency and burst incidence, by contrast, are dependent on the number of bursts and cardiac cycles. Because MSNA is tightly coupled with cardiac rhythmicity, the higher the heart rate, the greater the potential for a burst to occur; this therefore influences burst frequency but not burst incidence (Charkoudian & Wallin, 2014).

It is known that variability in the amplitude of the bursts and the number of bursts does occur, and that this is possibly due to two different sites in the central nervous system being involved in a feedback loop interacting with the arterial baroreceptors: one being responsible for determining the occurrence (incidence) of bursts and the other for the strength (amplitude) of the bursts (Kienbaum et al., 2001). According to Kienbaum's model (2001), one synapse is regulated by the strength of the input from the baroreceptors (as well as other afferent inputs), such that a stronger withdrawal of baroreceptor input will lead to a stronger sympathetic burst, while the other synapse acts as a gate control and determines whether the burst can be passed on to the spinal cord and the target vasculature (Kienbaum et al., 2001).

1.8. Imaging of central cardiovascular control

The central circuitry underlying the baroreflex mechanism has been thoroughly studied in experimental animals. Few studies, however, have investigated the circuitry in human subjects. The first neuroimaging studies of autonomic function in humans involved the functional magnetic resonance imaging during respiratory challenges (Gozal et al., 1995, 1996; Harper et al., 1998; King, Menon, Hachinski, & Cechetto, 1999). Further investigations of the central neural sites responsible for controlling the

autonomic nervous system in humans involved manoeuvres that evoke increases in MSNA, such as static hand grip exercise (Critchley, Corfield, Chandler, Mathias, & Dolan, 2000; Goswami, Frances, & Shoemaker, 2011; Macey et al., 2012; Macey, Kumar, Ogren, Woo, & Harper, 2014; Norton et al., 2015; Nowak et al., 1999), the Valsalva manoeuvre (Henderson et al., 2002, 2003; King et al., 1999; Macey et al., 2012; Ogren et al., 2012; Wu, Bandettini, Harper, & Handwerker, 2015), immersion of the hand (or forehead) into ice cold water (Harper et al., 1998, 2003; Harper, Bandler, Spriggs, & Alger, 2000; Macey, Macey, Woo, Keens, & Harper, 2005), or lower body negative pressure (Goswami, Frances, Steinback, & Shoemaker, 2012; Kimmerly, O'Leary, Menon, Gati, & Shoemaker, 2005). For example, the Valsalva manoeuvre evokes a sustained increase in MSNA and arterial blood pressure, and is associated with increased signal intensity within multiple brain regions, including the dorsal pons and medulla (Harper et al., 1998; Henderson et al., 2002). Lower body negative pressure unloads the low-pressure baroreceptors, leading to a sustained increase in MSNA, and is associated with functional changes within the insula, anterior cingulate cortex, and orbitofrontal cortex, as well as the midbrain and thalamus (Kimmerly et al., 2005). Similarly, a maximal inspiratory breath-hold also increases MSNA through the unloading of low-pressure baroreceptors (Macefield, 1998), and causes significant signal intensity changes in the NTS, RVLM, and CVLM (Macefield et al., 2006). Although these studies have provided great insight into the central circuitry responsible for producing changes in MSNA, none have measured MSNA changes and functional brain imaging synchronously.

1.8.1. MSNA-coupled fMRI

In 2010, Macefield and colleagues managed to record MSNA while simultaneously measuring brain activity using functional magnetic resonance imaging (fMRI). In order to do so, the protocol involved a scan repetition time of 8 sec, including 4 sec image collection period, followed by 4 sec of rest, during which MSNA was recorded (Macefield & Henderson, 2010). This procedure allowed one to take advantage of the temporal delays inherent in blood oxygen level dependent (BOLD) imaging, where microvascular responses to an increase in neuronal activity lag by about 5 sec (Logothetis et al., 2001). One second was removed in order to account for the time taken for a volley of MSNA to travel from the brainstem to the peripheral recording site at the level of the fibular head (Fagius & Wallin, 1980). Thereby, changes in BOLD signal intensity that are temporally coupled to MSNA reflect changes in neural activity with the production of the bursts of MSNA recorded 4 seconds previously.

As noted in section 1.4, Macefield and Henderson were able to define the human baroreflex circuitry by measuring regional brainstem activity changes during spontaneous changes in MSNA in awake subjects (Macefield & Henderson, 2010). The group then explored the cortical brain regions that regulate MSNA, by extending the imaging to the whole brain, and identified suprabulbar regions that regulate resting MSNA by projecting to the RVLM (James et al., 2013).

1.9. Aims & Hypotheses

Aim 1: Investigating whether baseline physiological parameters predict the sympathetic responses to muscle pain.

In this study, we set out to determine whether baseline physiological levels, including baseline blood pressure, heart rate, and muscle sympathetic nerve activity (MSNA), as well as age, sex, and BMI may be predictors of the direction of the muscle sympathetic response to experimental muscle pain. This material, which has been published, extends the work undertaken in my Bachelor of Medical Research project and continued during my PhD. Because of this overlap, I present this work in Appendix A as: Baseline physiological parameters do not determine whether muscle sympathetic nerve activity increases or decreases during pain. *Front Neurosci* 9:471.

Aim 2: Investigating whether psychological parameters determine the cardiovascular responses to muscle pain.

The aim of this second study was to investigate whether anxiety levels and attitudes to pain could account for the divergent sympathetic responses to muscle pain. We tested the hypothesis that subjects with higher levels of pain anxiety, and pain catastrophizing and vigilance, as well as state and trait anxiety scores would show increases in MSNA during tonic muscle pain. This published work is presented in Chapter 3 as: Inter-individual responses to experimental muscle pain: Baseline anxiety ratings and attitudes to pain do not determine the direction of the sympathetic response to tonic muscle pain in humans. *Int J Psychophysiol* 104:17–23.

Aim 3: Understanding the processes in the brain that determine how long-lasting muscle pain produces sustained increases or decreases in muscle sympathetic nerve activity.

In this study, we used concurrent microneurography and functional brain imaging to determine if known autonomic brain regions respond differently in individuals in whom MSNA increases to those in whom MSNA decreases. We tested the hypothesis that changes in MSNA during muscle pain are associated with a sustained increase or decrease in blood oxygen level dependent signal intensity in the dorsomedial hypothalamus, the periaqueductal grey and the rostroventrolateral medulla. This work has been published and is presented in Chapter 4 as: Central circuitry responsible for the divergent sympathetic responses to tonic muscle pain in humans. *Hum. Brain Mapp.* 38(2):869-881.

Aim 4: To identify brain regions that are recruited during MSNA bursts during tonic muscle pain.

The aim of this study was to identify brain regions that are coupled to MSNA bursts during rest and during tonic muscle pain. We hypothesized that in addition to areas known to be coupled to MSNA bursting at rest, additional regions such as the cingulate cortex and midbrain would be recruited during MSNA bursts during pain.

This work has been accepted for publication and is presented in Chapter 5 as: Muscle sympathetic nerve activity-coupled changes in brain activity during sustained muscle pain. *Brain & Behaviour* 8: e00888

Aim 5: To examine the muscle sympathetic responses to experimental muscle pain during audio-visual distraction.

The aim of this study was to investigate whether distraction via engagement in an affectively neutral stimulus could bring about changes in the muscle sympathetic and cardiovascular responses to experimental muscle pain. This work has been accepted for publication and is presented in Chapter 6 as: The effects of audio-visual distraction on the muscle sympathetic responses to experimental muscle pain. *Experimental Brain Research.* 236: 1919-1925

CHAPTER 2 General Methods

2.1. General procedures

Experiments were performed on healthy university subjects. For all studies in the thesis, subjects responded to an advertisement that outlined the procedures to be undertaken, including the nerve recording, muscle pain and fMRI; all procedures were approved by the Human Research Ethics Committees of Western Sydney University and the University of New South Wales. Written consent was obtained from all subjects in accordance with the Declaration of Helsinki. Exclusion criteria included a history of cardiovascular disease or chronic musculoskeletal pain. The subjects who volunteered for the experiment did so knowing that they were going to have a microelectrode inserted into a nerve, another needle inserted under the skin, and a cannula inserted into a muscle. Moreover, they knew they were going to experience strong muscle pain during the intramuscular infusion of hypertonic saline for up to an hour.

For experiments conducted in Chapters 3 and 6, the subjects were either seated in a comfortable reclined position with the legs supported in an extended position. For the chapters involving fMRI recordings, the subjects were laying on an MRI bed with the legs supported by a foam cushion. For either of the protocols, the room was kept quiet and at a constant temperature of 22◦C.

2.1.1. MSNA recording procedures

For Chapters 3 and 6, the subjects were seated in a comfortable reclined position with the legs supported in an extended position. The course of the common peroneal nerve was identified via external stimulation (2–10 mA) using a 1 mm surface probe which

delivered 0.2 ms pulses at 1Hz from an isolated stimulator (Stimulus Isolator; ADInstruments, Sydney, Australia). Spontaneous bursts of muscle sympathetic nerve activity (MSNA) were recorded from muscle fascicles of the common peroneal nerve supplying the ankle or toe extensor or foot everter muscles via tungsten microelectrodes (FHC, Bowdoin, ME, USA) inserted percutaneously at the level of the fibular head. Multi-unit neural activity was amplified (gain 20 000, bandpass 0.3–5.0 kHz) using an isolated amplifier (NeuroAmp EX, ADInstruments, Sydney, Australia) and stored on computer (10-kHz sampling) using a computer-based data acquisition and analysis system (PowerLab 16SP hardware and LabChart 7 software; ADInstruments, Sydney, Australia). ECG (0.3–1.0kHz) was recorded with Ag–AgCl surface electrodes on the chest and sampled at 2 kHz. Blood pressure was recorded continuously using finger pulse plethysmography (Finometer Pro, Finapres Medical Systems, The Netherlands) and sampled at 400 Hz. Respiration (DC-100Hz) was recorded using a strain-gauge transducer (Pneumotrace, UFI, Morro Bay CA, USA) wrapped around the chest.

Chapters 4 and 5 involved concurrent MSNA and functional magnetic resonance imaging recordings. Therefore, the protocol was slightly adjusted to accommodate this procedure. Instead of being seated, subjects lay supine on an MRI bed in a comfortable position with the legs supported by a foam cushion. The external and internal stimulation processes were carried out in the exact same manner as in Chapters 3 and 6. Once a stable recording was attained, MSNA was recorded continuously for 5 minutes of rest, prior to the subjects being wheeled from the laboratory to the scanner with the microelectrode remaining in situ.

2.1.2. Noxious stimulation protocol

For all chapters, the noxious stimulation comprised a 7% hypertonic saline solution which was prepared by diluting sterile, 20% hypertonic saline with sterile water. Two syringes of 10 ml each were filled with the 7% hypertonic saline, placed in an infusion pump (Harvard Instruments, USA), and connected to a three-way tap via a 75cm extension tubing primed with hypertonic saline. A 23-gauge butterfly needle was then attached to the three-way tap via a cannula, primed, and inserted 1.5 cm deep into the belly of the ipsilateral tibialis anterior muscle, about 5 cm lateral and 10 cm inferior to the tibial tuberosity. The cannula was inserted as soon as a stable recording of spontaneous MSNA was achieved. Infusion of the 7% hypertonic saline solution was started at a time unknown to the subject and was maintained for 45 min; the pain lasted for ~60min. The initial rate of infusion was set at 0.25ml/min and was constantly adjusted to maintain a pain level of 5–6/10 on a Numerical Rating Scale (NRS). Subjects were asked to rate their pain continuously by sliding a linear potentiometer (Response Meter, ADInstruments, Sydney, Australia) that was calibrated to the NRS, with a rating of "0" meaning "no pain/discomfort" at all, and a rating of "10" being equivalent to the "worst pain the subject ever had experienced." For the imaging studies, the subjects' pain ratings were monitored continuously and adjusted according to each subject pressing four colour-coded buttons, each colour associated with either "pain onset," "pain at 5/10," "pain below 5/10," or "pain above 5/10." When the pain level dropped below 4/10 or rose above 6/10, the infusion rate was changed by 0.02 ml/min accordingly. After the infusion was completed, the recording was continued until the pain stopped.

2.1.3. MSNA-coupled fMRI

For the studies that included imaging, the brain was scanned using a 3 Tesla MRI scanner (Philips Achieva, 32-channel SENSE head coil). The head was immobilized in the head coil, and padding was added to prevent head movement. Two scans encompassing the whole brain were collected: a high-resolution 3D T1-weighted anatomical image (200 axial slices, echo time (TE)=2.5 ms, repetition time (TR)=5600 ms, raw voxel size = 0.87 mm^3), followed by a series of 250 gradient echo echo-planar sensitive to blood oxygen level dependent contrast (BOLD) fMRI images (46 axial slices, (TE)=40 ms, (TR)=8 s, raw voxel size = 1.5 x 1.5 x 3.25 mm^3). During the fMRI scan, all axial slices were collected during the first 4 seconds of the 8 s TR.

Functional magnetic resonance imaging (fMRI) measures changes is blood oxygen levels, in order to infer neuronal activity (Logothetis et al., 2001). It can be used both for resting state or responses to stimulation, such as noxious inputs. As mentioned above, the protocol for simultaneously measuring MSNA and functional magnetic resonance imaging, a scan repetition time of 8 sec, including 4 sec image collection period, followed by 4 sec of rest, during which MSNA is recorded. This procedure allows one to take advantage of the temporal delays inherent in blood oxygen level dependent (BOLD) imaging, where microvascular responses to an increase in neuronal activity lag by about 5 sec (Logothetis et al., 2001). One second is removed in order to account for the time taken for a volley of MSNA to travel from the brainstem to the peripheral recording site at the level of the fibular head (Fagius & Wallin, 1980). Thereby, changes in BOLD signal intensity that are temporally coupled to MSNA reflect changes in neural activity with the production of the bursts of MSNA recorded 4 seconds previously.

2.2. Analytical procedures

2.2.1. MSNA and cardiorespiratory parameters

LabChart 7 Pro software (ADInstruments, Sydney, Australia) was used to record the following parameters: muscle sympathetic nerve activity (burst amplitude and frequency), heart rate, blood pressure, respiration, pulse pressure, heart rate variability (HRV), and pain ratings. Individual bursts of MSNA were displayed as a mean-voltage neurogram, computed as the root-mean-square (RMS) processed signal with a moving time average window of 200 ms. This signal was then analysed using the "Peak Analysis" module of the LabChart 7 Pro software to calculate the amplitude of each burst. The absolute values were averaged into 5-min blocks and reported as percentages from the "baseline" values. An average of all blocks was taken to determine the direction of the response. Subjects with overall average MSNA amplitude 10% lower than baseline were arbitrarily assigned to the decreasing group; averages 10% higher than baseline were considered as increasing. Baseline MSNA amplitude was compared to the 5-min block with the mean value calculated over the entire infusion period, and to the highest average for the increasing group and to the lowest average value for the decreasing group. Changes in mean heart rate and mean blood pressure were also measured in 5 min epochs, normalized to the baseline value prior to the infusion of hypertonic saline.

2.2.2. fMRI

For Chapters 4 and 5, using Statistical Parametric Mapping 12 (Wellcome Trust Centre for Neuroimaging, University College London, UK) the fMRI images were realigned and spatially normalized to the Montreal Neurological Institute (MNI) template. The

VBM8 toolbox DARTEL template was used, which was derived from 550 healthy control subjects of the IXI database (http://brain-development.org/) and is in MNI152 space. The images were then intensity normalized to eliminate any slow drift in signal intensity, and bias corrected. Once realigned and spatially normalized, the images were then smoothed using a 6mm full-width at half-maximum Gaussian filter. A brainstem-only analysis was also performed: it was isolated and spatially normalised using the Spatial Unbiased Infratentorial Template (SUIT) using the SUIT toolbox (Diedrichsen et al., 2011), intensity normalised, bias corrected, and smoothed using a 4mm FWHM Gaussian filter. Signal intensity changes were measured during the subsequent 4s (ON), taking into account the 5 second neurovascular coupling delay between a neuronal event and the peak BOLD signal (Logothetis et al., 2001) and the 1 second required for the burst of MSNA to travel from the brain to the peripheral recording site (Fagius & Wallin, 1980). Because the images were collected in a caudal to rostral direction, the region of the brainstem from the caudal medulla to the rostral pons was scanned in the 1st second, the rostral pons to the diencephalon in the 2nd second, the diencephalon and surrounding cortex in the 3rd second, and the remainder of the cortex in the 4th second.

For Chapter 4, signal intensity changes were searched for using a box-car design, with a baseline period of 60 volumes (scans 16–75) and a pain period of 175 volumes (scans 76–250). A one-sample random effects analysis was performed with all subjects in a single group to determine signal intensity changes associated with the pain period ($p < .05$, family wise error corrected). To determine regions in which signal intensity changes were different in the increasing versus decreasing MSNA group, a two-sample random effects analysis was performed using both the whole-brain and brainstem only images ($p < .001$, uncorrected).

For Chapter 5, the analysis methods differed from Chapter 4 as the aim was to identify brain regions that were functionally coupled to the generation of bursts of MSNA at rest and during pain. In each subject, and for each of the 4 s periods, the brain volumes during periods in which there were no bursts were averaged to create a mean "no MSNA burst" image. Similarly, brain volumes during periods in which there were MSNA bursts were averaged to create a mean "MSNA burst" image. These two images were created for the baseline and tonic pain periods separately. To determine brain areas in which signal intensity was greater during MSNA bursts compared to periods of no MSNA bursts, we entered these two images for the baseline period into a second level, random effects paired t-test for each of the 4-second periods. To determine changes in MSNA-coupled BOLD signal intensity evoked by tonic pain, we subtracted the mean "MSNA no burst" image from the mean "MSNA burst" image during the baseline and tonic pain periods. This resulted in a single baseline and a single tonic pain image for each subject, of which each voxel's signal intensity value reflected the difference between periods of no bursts compared to periods of MSNA bursts. The percentage difference between these two images was then calculated for each voxel, resulting in a brain map in which each voxel's value was the percentage change in MSNA-coupled signal intensity during tonic pain compared with baseline.

CHAPTER 3 Inter-individual responses to experimental muscle pain: baseline anxiety ratings and attitudes to pain do not determine the direction of the sympathetic response to tonic muscle pain in humans

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Kobuch, S., Fazalbhoy, A., Brown, R., Macefield, V.G. (2016): Inter-individual responses to experimental muscle pain: Baseline anxiety ratings and attitudes to pain do not determine the direction of the sympathetic response to tonic muscle pain in humans. *Int J Psychophysiol* 104:17–23.

3.1. Abstract

We have recently shown that intramuscular infusion of hypertonic saline, causing pain lasting ~ 60 min, increases muscle sympathetic nerve activity (MSNA) in one group of subjects, yet decreases it in another. Across subjects these divergent sympathetic responses to long-lasting muscle pain are consistent over time and cannot be foreseen on the basis of baseline MSNA, blood pressure, heart rate or sex. We predicted that differences in anxiety or attitudes to pain may account for these differences. Psychometric measures were assessed prior to the induction of pain using the State and Trait Anxiety Inventory (STAI), Pain Vigilance and Awareness Questionnaire (PVAQ), Pain Anxiety Symptoms Scale (PASS) and Pain Catastrophizing Scale (PCS); PCS was also administered after the experiment. MSNA was recorded from the common peroneal nerve, before and during a 45-minute intramuscular infusion of hypertonic saline solution into the tibialis anterior muscle of 66 awake human subjects. Forty-one subjects showed an increase in mean burst amplitude of MSNA (172.8 ± 10.6%) while 25 showed a decrease (69.9 ± 3.8%). None of the measured psychological parameters showed significant differences between the increasing and the decreasing groups. We conclude that inter-individual anxiety or pain attitudes do not determine whether MSNA increases or decreases during long-lasting experimental muscle pain in healthy human subjects.

3.2. Introduction

Similar to the sensory aspects of pain – superficial pain being perceived as sharp or burning, and deep pain as dull and aching (Henderson, Bandler, Gandevia, & Macefield, 2006) – the cardiovascular responses to noxious inputs vary according to

the tissue of origin. In 1942, it was shown that superficial pain (arising from skin) resulted in an increased pulse rate, whereas pain that arose from deeper structures (muscle) showed a decrease in pulse rate and blood pressure (Lewis, 1942). Feinstein and colleagues confirmed these findings in 1954, when they found that muscle pain was associated with a fall in blood pressure, as well as bradycardia, in awake human subjects (Feinstein et al., 1954).

3.2.1. Pain and sympathetic nerve activity

Over the past few years we have been using intramuscular as well as subcutaneous injections of hypertonic saline to examine the effects of deep and superficial pain on muscle sympathetic nerve activity (MSNA) in awake human subjects (Burton et al., 2009a). Interestingly, it was shown that both types of pain – when hypertonic saline was injected as a bolus – caused an increase in MSNA (Burton et al., 2009a) and a transient increase in skin sympathetic nerve activity (SSNA; Burton et al., 2009b). These results support previous findings of increased cardiovascular responses to deep and superficial pain in animals (Boczek-Funcke et al., 1992; Horeyseck & Jänig, 1974b; Sato et al., 1984).

In more recent studies we have used intramuscular infusion of hypertonic saline to produce a sustained, steady-state level of pain (Fazalbhoy et al., 2012, 2014; Hall et al., 2012). This continuous nociceptive input has been shown to produce a transient increase in skin sympathetic nerve activity (SSNA) followed by a sustained decrease in SSNA in all subjects (Hall et al., 2012), yet divergent muscle sympathetic responses, such that half of the subjects experienced a sustained increase in MSNA, blood pressure and heart rate during tonic muscle pain, while the other half showed sustained

decreases (Fazalbhoy et al., 2012, 2014; Kobuch et al., 2015). Moreover, the direction of the sympathetic response to tonic muscle pain was reproducible (Fazalbhoy et al., 2014). That is, subjects who had an increase in MSNA, blood pressure, and heart rate during the first session showed a similar response in a subsequent session, conducted weeks apart. This consistency was also true for the subjects experiencing a fall in MSNA. We recently showed that the divergent muscle sympathetic responses to muscle pain cannot be explained by sex or by differences in resting blood pressure, heart rate, heart rate variability or MSNA (Kobuch et al., 2015).

So, we are left with trying to understand why these two divergent patterns of sympathetic response to long-lasting muscle pain come about. Here, we posit that psychological differences may account for the different physiological responses. In particular, given that increases in sympathetic nerve activity are features of the fight or flight response to a threatening stimulus, we speculate that subjects showing higher anxiety levels, or greater negative attitudes to pain, will show increases in MSNA during tonic muscle pain, while those who are better able to cope do not.

3.2.2. Negative emotions and pain

Catastrophizing – "an exaggerated negative mental set brought to bear during actual or anticipated painful experience" (Sullivan et al., 2001)– has been associated with increased distress and higher pain ratings during painful interventions (Chaves & Brown, 1987; Spanos, Radtke-Bodorik, Ferguson, & Jones, 1979; Sullivan, Adams, Rhodenizer, & Stanish, 2006). Parallel increases in catastrophic thinking and intensity of pain (Carter et al., 2002; Sullivan et al., 2006), as well as severity of depression and anxiety (Keefe, Brown, Wallston, & Caldwell, 1989; Martin et al., 1996), have also

been observed. Accordingly, one might expect that subjects with high catastrophizing scores would exhibit greater sympathetic responses to pain.

3.2.3. Anxiety and MSNA

It is well known that patients with anxiety have an elevated cardiovascular risk (Musselman, Evans, & Nemeroff, 1998; Rosengren et al., 2004). Metabolic syndrome patients with anxiety and mood disorders have greater MSNA burst frequency at rest, compared to metabolic syndrome patients and controls without these psychological symptoms (Toschi-Dias et al., 2013). Moreover, single unit recordings from muscle vasoconstrictor neurones in metabolic syndrome patients with high blood pressure revealed a higher incidence of multiple firing during a burst when levels of anxiety were high (Lambert et al., 2010). This disturbed sympathetic firing pattern was also found in patients with panic disorder and major depressive disorder (Lambert et al., 2006, 2008). Other indirect measures of sympathetic activity, such as pulse transit time (Richards & Bertram, 2000) and catecholamine levels (Villacres, Hollifield, Katon, Wilkinson, & Veith, 1987), have also been shown to reflect higher sympathetic nerve activity - with higher levels of anxiety in healthy participants and panic disorder patients. Therefore, we might expect that anxious subjects would show an increase in sympathetic activity during pain.

Given the above, in the current study we administered questionnaires to subjects before and after an intramuscular infusion of hypertonic saline to test the hypothesis that elevated levels of anxiety, and/or negative attitudes to pain, leads to a higher prevalence of increases than decreases in MSNA during tonic muscle pain.

3.3. Methods

Experiments were carried out on 66 healthy subjects (27 females, 39 males), aged 18 to 39 years. All subjects provided written informed consent to the experimental procedures, which were conducted under the approval of the Human Research Ethics Committee of Western Sydney University and satisfied the requirements of the Declaration of Helsinki. No subject had any history of cardiovascular disease, chronic musculoskeletal pain, or mental health disorders.

3.3.1. Psychometric measures

Prior to commencing the experiment, the following psychological questionnaires were administered: The State and Trait-Anxiety Inventory (STAI), Pain Vigilance and Awareness Questionnaire (PVAQ), Pain Catastrophizing Scale (PCS), and Pain Anxiety Symptoms Scale (PASS). The PCS was also administered after the experiment to assess the subject's attitudes to the pain experience during the experiment. The STAI is commonly used to measure state (S) and trait (T) anxiety (Spielberger et al., 1983). The inventory contains 20 items for assessing state anxiety, which gauges a person's feelings of anxiety at a particular time, and 20 items for assessing trait anxiety, which evaluates a person's feelings of anxiety in general. Each item is rated on a 4-point scale from “almost never” to “almost always,” with scores ranging from 20 to 80 for each subtest. Higher scores indicate greater anxiety, with a score of 39 – 40 suggesting clinically significant symptoms (Knight, Waal-Manning, & Spears, 1983; Spielberger, et al., 1983). The PVAQ (McCracken, 1997) is a 16-item questionnaire that measures attention to pain. The PCS encompasses three dimensions of catastrophizing: rumination (“I can't stop thinking about how much it hurts”), magnification (“I worry that something serious may happen”), and helplessness (“it's awful and I feel that it

overwhelms me"; Sullivan et al., 1995). The subject is asked to reflect on previous painful experiences, and to indicate on a 5-point scale (0 being "not at all" and 4 being "all the time") the degree to which they experienced each of the 13 feelings during pain. Summing responses of all 13 items gives a total PCS score, ranging from 0 to 52, and a score $\geq$ 30 represents a clinically significant level of catastrophizing attitude towards pain (Sullivan et al., 1995). The PASS (McCracken et al., 1992) is a tool to measure fear of pain across "cognitive, overt behavioural, and physiological domains" (McCracken et al., 1992). This study incorporated the PASS-20, a short version of the PASS, as an instrument to assess fear avoidance behaviour of pain, which has a significant correlation with the suffering and disability of chronic pain (McCracken & Dhingra, 2002).

3.3.2. Experimental procedures

Once the questionnaires were completed, the subjects were seated in a comfortable reclined position with the legs supported in an extended position. The protocol for MSNA and cardiorespiratory parameters measurement, as well as noxious stimulation procedures were described in the general methods section. At the conclusion of the experiment, each subject completed a McGill Pain Questionnaire, in which the quality of the pain was described using a standardised set of descriptors.

3.3.3. Statistical analysis

Statistical analysis consisted of non-paired two tailed t-tests, paired t-tests to assess pain-related changes in MSNA amplitude, and linear regression. Correlation coefficients between psychometric measures as well as between the psychometric measures and MSNA were also calculated. Statistical analyses were performed using

Prism version 6 for Mac OS X (GraphPad software, San Diego, California, USA). All values are expressed as means and standard error. Probability levels of $p < .05$ were deemed significant.

3.4. Results

3.4.1. Subjective experience of tonic muscle pain

Intramuscular infusion of hypertonic saline solution into the tibialis anterior muscle caused a sustained dull ache in the muscle belly that often extended into the ankle. The level of pain was kept constant, around 5 out of 10 on the Numeric Rating Scale (NRS), by adjusting the rate of the infusion according to the subject's feedback.

3.4.2. Muscle sympathetic nerve activity during tonic muscle pain

As reported previously, infusion of hypertonic saline evoked two divergent patterns of MSNA response: 41 subjects showed a significant increase in burst amplitude (172.8 ± 10.6%, $p < .001$, t-test) during the steady-state of pain, while 25 showed a significant decrease relative to baseline (69.9 ± 3.8%, $p < .0001$, t-test). This is illustrated graphically in *Figure 3.1A*. There were no significant differences between the groups in baseline MSNA burst frequency: 13.3 ± 1.3 bursts/min in the increasing group and 17.1 ± 1.5 bursts/min in the decreasing group ($p = .07$, t-test). The average burst frequency during pain was 15.8 ± 1.3 bursts/min in the increasing group and 12.4 ± 1.4 bursts/min in the decreasing group ($p = .09$, t-test). The difference between baseline and pain was not significantly different between the groups (2.5 ± 1.8 bursts/min in the increasing group and −4.7 ± 2.1 bursts/min in the decreasing group).

3.4.3. Psychometric measures

Table 3.1 shows the correlation matrix coefficients between each psychometric measure in the top part, and between the psychometric measures against MSNA in the lower part. While correlations between State Anxiety and other psychometric measures were low – with the exception of Trait Anxiety – it is clear that correlations were generally higher (indicated by bold type) between the psychometric measures themselves, with no strong coupling between these measures and MSNA.

When the psychological parameters were compared between the group of subjects who showed an increase in MSNA during tonic muscle pain and the group who showed a decrease, there were no differences in state anxiety scores (34.0 ± 1.7 vs 33.8 ± 2.2, p = .92, t-test; *Figure 3.1B*) or trait anxiety scores (34.2 ± 1.4 vs 35.6 ± 1.7, p = .60, t-test; *Figure 3.1C*). The state anxiety scores ranged from 20 to 52 in both the increasing and decreasing MSNA groups. The trait anxiety scores ranged from 22 to 55 in the increasing group, and from 21 to 49 in the decreasing group. There were no differences in pain vigilance and awareness scores (34.7 ± 2.0 vs 33.7 ± 2.1, p = .75, t-test; *Figure 3.1E*), the scores ranging from 0 to 60 in the increasing group, and from 15 to 50 in the decreasing group. Pain anxiety symptoms scores were also similar between the two groups (29.8 ± 2.7 vs 28.3 ± 3.2, p = .74, t-test; *Figure 3.1D*); scores ranged between 3 and 72 in the increasing group, and between 2 and 51 in the decreasing group. Finally, there were no differences between the groups in pain catastrophizing scores measured either before (increasing group: 12.41 ± 1.2 vs decreasing group: 13.7 ± 1.7, p = .53, t-test; *Figure 3.1F*) or after the experiment (16.65 ± 1.7 vs 18.9 ± 2.1, p = .42, t-test; *Figure 3.1G*). PCS scores before the experiment ranged between 0 and 33 in the

increasing group, and between 0 and 25 in the decreasing group. PCS scores after the experiment ranged between 1 and 39 in the increasing group and 3 and 35 in the decreasing group.

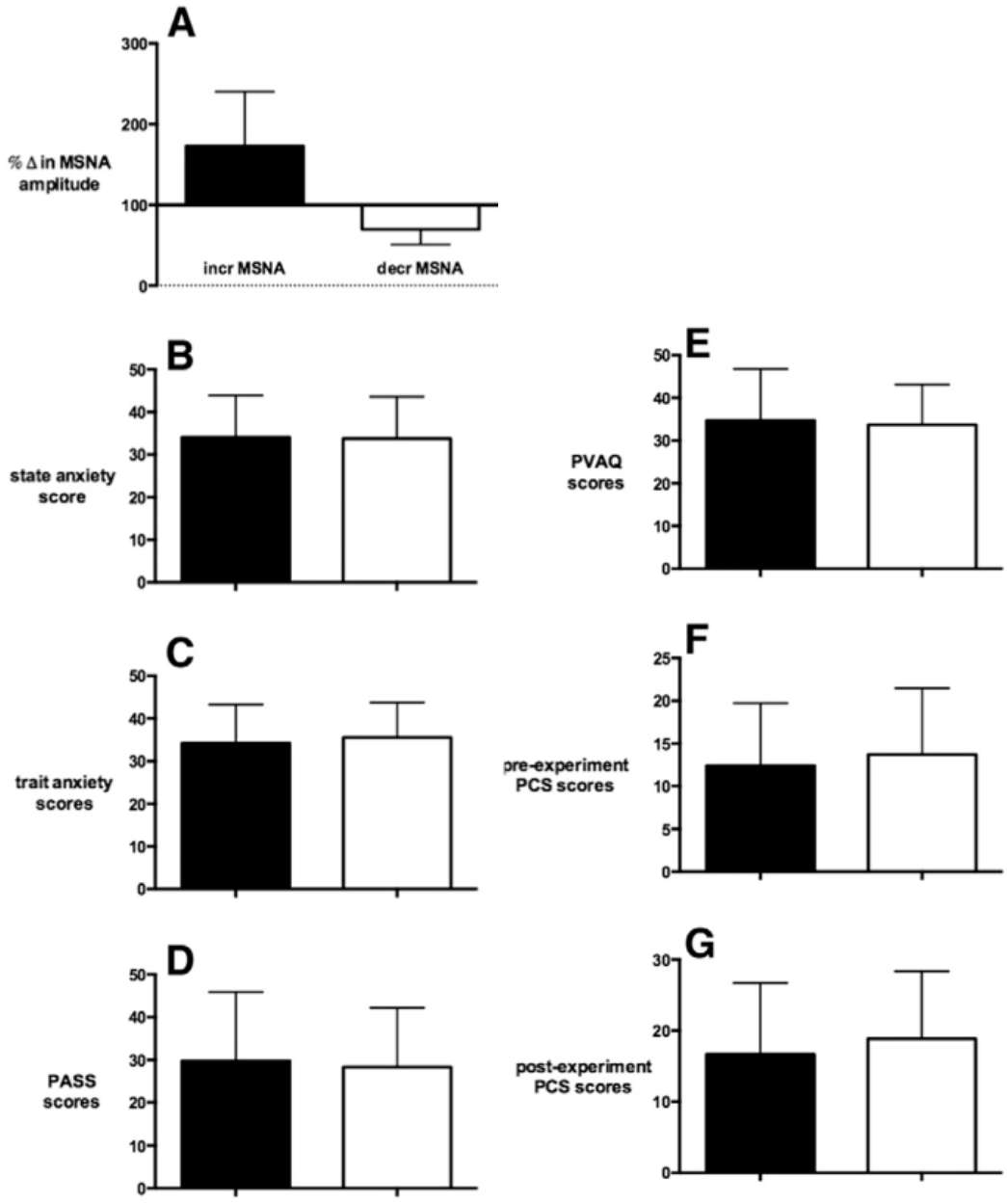

Figure 3.1: Anxiety and attitudes to pain scores for the increasing and decreasing MSNA groups.

Changes in MSNA amplitude (A), state anxiety scores (B), trait anxiety scores (C), PASS scores (D), PVAQ scores (E), pre-experiment PCS scores (F), and post-experiment PCS scores (G) for each group.

Table 3.1: Correlation coefficients between psychometric measures and between psychometric measures and MSNA.

Significant correlations are shown in bold.

	State anxiety	Trait anxiety	PVAQ	PASS	PCS pre	PCS post
State anxiety	1	**0,44**	0,07	0,09	**0,21**	0,01
Train anxiety	**0,44**	1	0,04	0,11	0,05	0,00
PVQA	0,07	0,04	1	**0,53**	**0,40**	0,08
PASS	0,09	0,11	**0,53**	1	**0,28**	0,16
PCS pre	**0,21**	0,05	**0,40**	**0,28**	1	0,19
PCS post	0,01	0,00	0,08	0,16	0,19	1
Change in MSNA	0,00	0,01	0,00	0,00	0,05	0,02

3.4.4. Anxiety and MSNA

As depicted in *Figure 3.2* there was no significant relationship between state anxiety levels and baseline MSNA burst frequency (R^2 = 0.025; *Figure 3.2A*) or changes in MSNA burst amplitude (*Figure 3.2B*; *Table 3.1*). Similarly, neither trait anxiety levels and baseline MSNA burst frequency (R^2 = 0.002; *Figure 3.2C*), nor trait anxiety and changes in MSNA amplitude (*Figure 3.2D*; *Table 3.1*) showed any correlation.

3.4.5. Attitudes to pain vs MSNA

There was no significant relationship between the scores recorded for the Pain Anxiety Symptoms Scale (PASS) and baseline MSNA frequency (R^2 = 0.099), although the line deviated significantly from zero (*Figure 3.3E*), or changes in MSNA amplitude (*Figure 3.3F*; *Table 3.1*). Likewise, although the line deviated significantly from zero, there was no statistically significant relationship between the scores recorded for the

Pain Vigilance Anxiety Questionnaire (PVAQ) and baseline MSNA frequency (R^2 = 0.083; *Figure 3.3G*), and changes in MSNA amplitude (*Figure 3.3H*; *Table 3.1*).

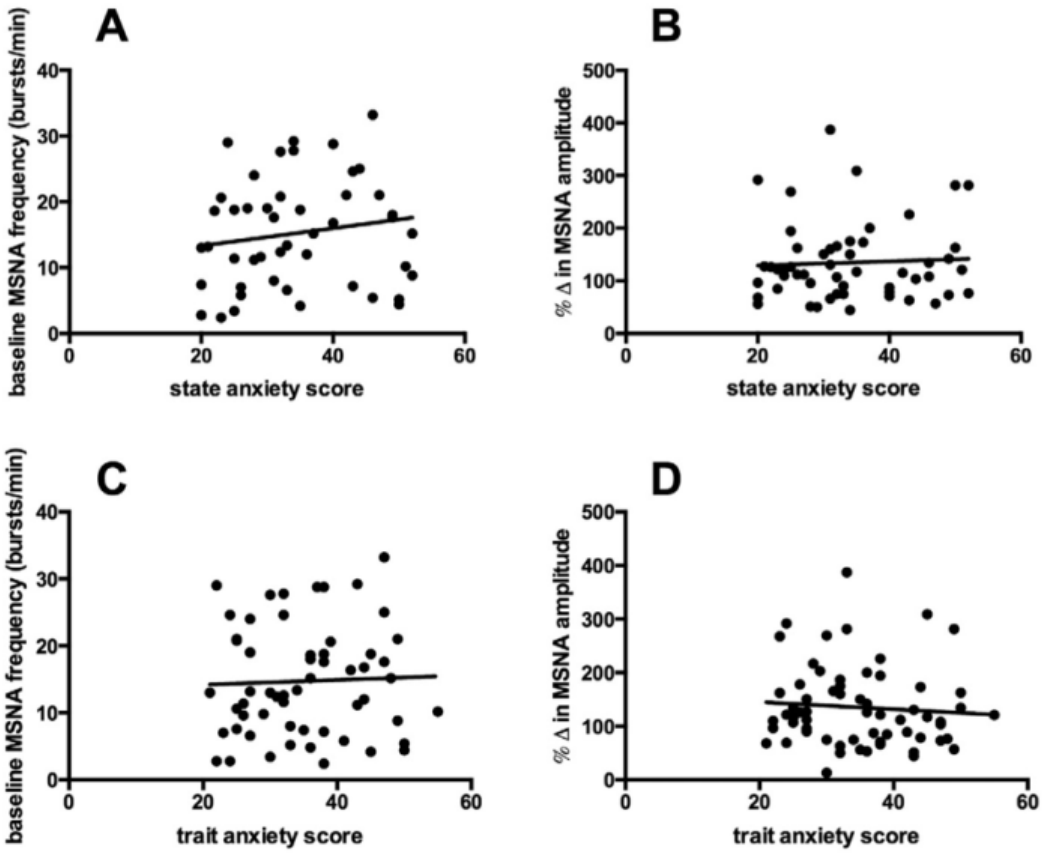

Figure 3.2: Relationship between state anxiety scores and MSNA.

State anxiety scores plotted against baseline MSNA frequency (A), and against changes in MSNA amplitude (B). Trait anxiety scores plotted against baseline MSNA frequency (C), and against changes in MSNA amplitude (D).

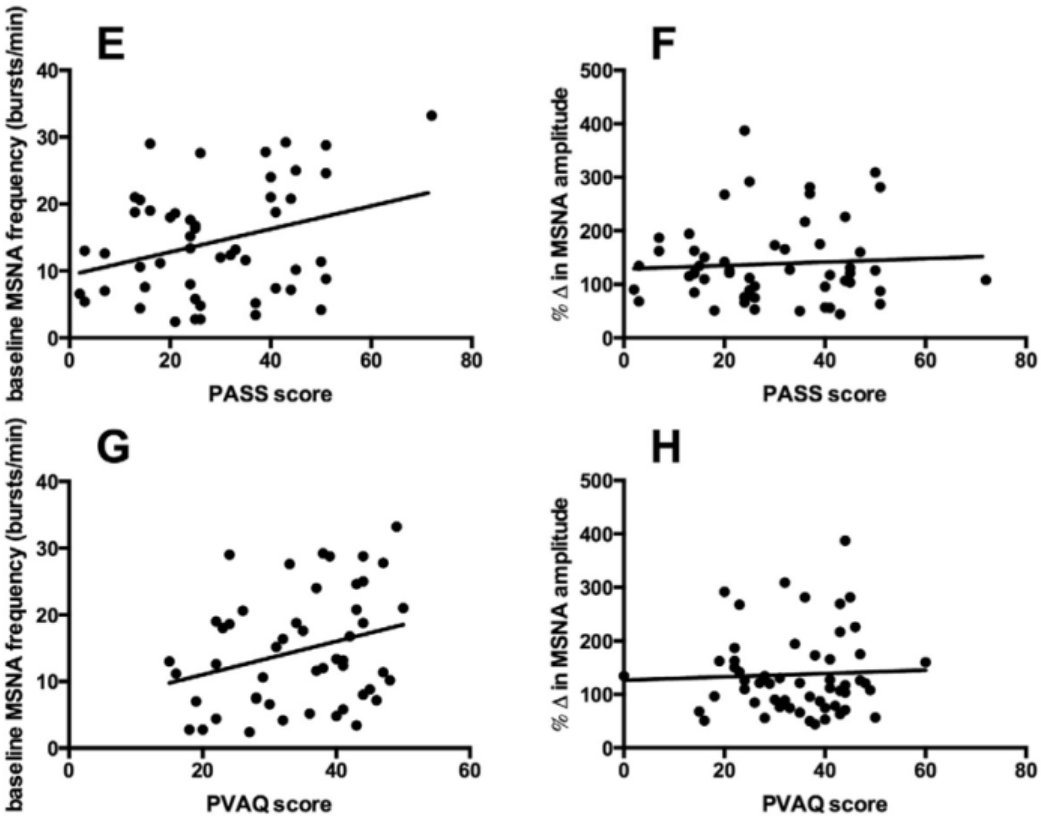

Figure 3.3: PASS and PVAQ scores plotted against MSNA.

PASS scores plotted against baseline MSNA frequency (E), and against changes in MSNA amplitude (F). PVAQ scores plotted against baseline MSNA frequency (G), and against changes in MSNA amplitude (H).

3.4.6. PCS vs MSNA

The Pain Catastrophizing Scale (PCS) was administered before and after the experiment. The scores before the experiment pertained to the subject's pain experience in general, the values recorded after referred to the subject's pain experience during the experiment reveal that there was no significant relationship between the PCS scores before the experiment (R^2 = 0.05; *Figure 3.4A*) and baseline MSNA burst frequency, and changes in MSNA burst amplitude (*Figure 3.4B*; *Table 3.1*). Finally, there was no statistically significant correlation between the PCS scores

obtained after the experiment and baseline MSNA levels (R^2 = 2.922e- 006; Figure *3.4C*), or changes in MSNA amplitude (*Figure 3.4D*; *Table 3.1*).

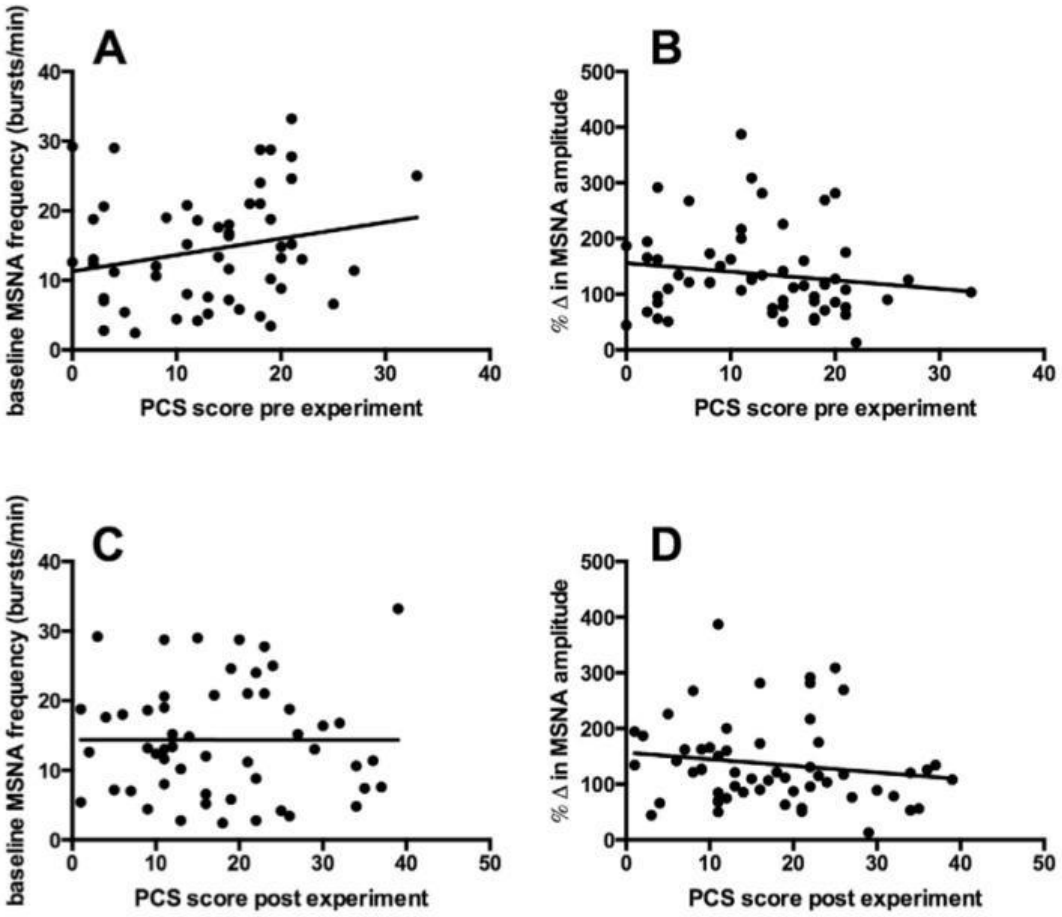

Figure 3.4: Relationship between PCS and MSNA.

PCS pre-experiment scores plotted against baseline MSNA frequency (A), and against changes in MSNA amplitude (B). PCS post-experiment scores plotted against baseline MSNA frequency (C), and against changes in MSNA amplitude (D).

3.5. Discussion

This study adds to our body of work investigating the effects of experimental muscle pain on the sympathetic nervous system (Burton et al., 2009a,b; Burton et al., 2008; Fazalbhoy et al., 2012, 2014; Hall et al., 2012; Kobuch et al., 2015). We have shown

that, across subjects, the divergent cardiovascular responses to long-lasting experimental muscle pain are not dependent on anxiety levels. Neither did different attitudes to pain provide a significant prediction as to whether a subject would show an increase or a decrease in MSNA during muscle pain. Both the increasing and decreasing MSNA groups had similar scores in all psychological parameters measured in this study. Moreover, higher scores did not correlate with higher MSNA levels at rest, or with the degree of change in MSNA burst amplitude during tonic muscle pain.

As stated in the Introduction, we had good reasons to think that anxiety levels or attitudes to pain may determine whether MSNA increases or decreases during tonic muscle pain. Chaves and Brown (1987) noticed that patients who were excessively negative during a dental procedure were more likely to experience distress at the time of the intervention. Spanos et al. (1979) interviewed subjects on the level of pain following a cold-pressor test and observed that individuals who engaged in catastrophic thinking reported the highest levels of pain. Moreover, Keefe et al. (1989) showed that "catastrophizers" with chronic pain had increased physical and emotional suffering associated with their pain condition. Higher scores have also been shown to predict the persistence of pain and development of chronic pain (Pavlin, Sullivan, Freund, & Roesen, 2005; Picavet, Vlaeyen, & Schouten, 2002), and have been linked to increased levels of depression, anxiety, and fear (Börsbo, Peolsson, & Gerdle, 2008; Drahovzal, Stewart, & Sullivan, 2006; Edwards, Smith, Kudel, & Haythornthwaite, 2006; Leeuw et al., 2007). Furthermore, there are significant relationships between measures of depression, anxiety, fear, and anger, and increased pain experience (Leeuw et al., 2007; Sullivan & N. Neish, 1999; Rudy, Kerns, & Turk, 1988), with Carroll et al. (2004) suggesting that symptoms of depression might increase the chances of musculoskeletal pain exacerbation. Similarly, fear and avoidance of pain

have been shown to exhibit a tight correlation with the suffering and disability associated with chronic pain (McCracken & Dhingra, 2002).

The influences of anxiety, panic disorder, and depression on the sympathetic nervous system have recently gained much attention, in particular with respect to their contributions to increased cardiovascular risk (Lambert et al., 2008; Musselman et al., 1998; Rosengren et al., 2004). Altered sympathetic activity, either through distorted baroreflex function (Lambert et al., 2002), elevated resting MSNA (Toschi-Dias et al., 2013), disturbed firing patterns of individual sympathetic neurons (Lambert et al., 2006, 2010), or increased catecholamine levels (Villacres et al., 1987) have been reported. Nevertheless, despite the known effects of certain psychological parameters on MSNA and pain experience, we found no differences in these psychometric measures between the increasing and decreasing MSNA groups. Accordingly, we reject the hypothesis we set out to test. So, given that neither baseline physiological or psychological differences exist between the two groups of subjects, we are left with trying to explain why there are two divergent muscle sympathetic responses to long-lasting muscle pain.

Based on studies in experimental animals it is entertaining to speculate that differences may exist in the brain to account for the two patterns of response. In the brainstem the nucleus tractus solitarius (NTS) and the periaqueductal grey (PAG) may be two key players in this response, as they are two important supraspinal structures that receive both cardiovascular information as well as nociceptive input (Bruehl & Chung, 2004). While the NTS is the first synapse in the baroreflex and also receives projections from spinal laminae involved in nociception, the PAG processes noxious stimuli (Carrive &

Bandler, 1991), and mediates different patterns of autonomic activity depending on the stressor (Bandler et al., 2000). Indeed, stimulation of the lateral columns of the PAG (lPAG) preferentially induces a fight-or-flight response, with increases in blood pressure and heart rate, similar to the response evoked by cutaneous pain (Depaulis et al., 1992). Conversely, stimulation of the ventrolateral columns of the PAG (vlPAG) promotes rest, with a parallel decrease in blood pressure and heart rate – as seen during experimental deep pain in animals (Depaulis et al., 1992). Therefore, it is plausible that when the NTS and PAG receive information about painful stimuli from higher centres, parallel changes in blood pressure, heart rate, and muscle sympathetic nerve activity are also seen. However, in the absence of direct evidence, this remains pure speculation.

3.6. Limitations

It should be emphasized that the participants included in the study were all healthy volunteers willing to be exposed to 1 h of strong pain. While all participants were informed that they could stop the infusion at any time and withdraw from the experiment, no one did so. In this way, we can see that all participants coped well with the pain, which may well differ if we targeted a population who were recruited on the basis of high anxiety or pain anxiety scores. Nevertheless, the ranges of scores in each of the psychometric measures were sufficiently wide in the 66 participants to indicate that we were recruiting a representative sample of healthy subjects, some of whom exhibited high anxiety and some low.

3.7. Conclusions

Contrary to our prediction, baseline anxiety levels or attitudes towards pain do not explain the divergent sympathetic responses we observe, nor do they appropriately predict whether a given individual shows an increase or a decrease in muscle sympathetic nerve activity during long-lasting muscle pain. These findings add to our earlier conclusions that the divergent sympathetic responses cannot be explained by baseline MSNA, blood pressure, heart rate or sex. Whether differences exist in the cortical and subcortical processing of muscle pain in the two groups remains to be seen.

CHAPTER 4 Central circuitry responsible for the divergent sympathetic responses to tonic muscle pain in humans

Chapter 4 is published as:

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4.1. Abstract

Experimentally induced tonic muscle pain evokes divergent muscle vasoconstrictor responses, with some individuals exhibiting a sustained increase in muscle sympathetic nerve activity (MSNA), and others a sustained decrease. These patterns cannot be predicted from an individual's baseline physiological or psychological measures. The aim of this study was to investigate whether the different muscle sympathetic responses to tonic muscle pain were associated with differential changes in regional brain activity. Functional magnetic resonance imaging (fMRI) of the brain was performed concurrently with microelectrode recording of MSNA from the peroneal nerve during a 40-min infusion of hypertonic saline into the ipsilateral tibialis anterior muscle. MSNA increased in 26 and decreased in 11 of 37 subjects during tonic muscle pain. Within the prefrontal and cingulate cortices, precuneus, nucleus accumbens, caudate nucleus, and dorsomedial hypothalamus, blood oxygen level dependent (BOLD) signal intensity increased in the increasing-MSNA group and remained at baseline or decreased in the decreasing-MSNA group. Similar responses occurred in the dorsolateral pons and in the region of the rostral ventrolateral medulla. By contrast, within the region of the dorsolateral periaqueductal grey (dlPAG) signal intensity initially increased in both groups but returned to baseline levels only in the increasing-MSNA group. These results suggest that the divergent sympathetic responses to muscle pain result from activation of a neural pathway that includes the dlPAG, an area thought to be responsible for the behavioural and cardiovascular responses to psychological rather than physical stressors.

4.2. Introduction

Research in experimental animals has revealed that noxious stimulation of different body tissues evokes different behavioural and cardiovascular responses. That is, cutaneous pain elicits fight/flight behaviours, coupled with increases in heart rate, blood pressure and sympathetic vasoconstrictor drive, whereas muscle and visceral pain bring about quiescence and decreases in heart rate, blood pressure and vasoconstrictor drive (Keay & Bandler, 2001). Although some clinical observations in humans suggested similar divergent responses (Lewis, 1942; Feinstein et al., 1954), we found that acute cutaneous or muscle pain, induced by bolus subcutaneous or intramuscular injection of hypertonic saline, both evoked transient (□30 s) increases in skin sympathetic nerve activity (SSNA) and heart rate, with small sustained increases in blood pressure and muscle sympathetic nerve activity (MSNA) that matched the duration of pain (Burton et al., 2009a,b; Hall et al., 2012; for review see Burton et al., 2016). These data demonstrate that, in humans, differential sympathetic responses to acute cutaneous and muscle pain do not occur. Moreover, we had shown that these sympathetic responses are psychogenic rather than of spinal reflex origin, as they do not occur when noxious stimuli are delivered to the legs in individuals with complete spinal cord injury (Brown & Macefield, 2008).

We recently found that prolonged muscle pain, induced by intramuscular infusion of hypertonic saline, is accompanied by two patterns of change in MSNA, with subjects showing either a sustained increase or a sustained decrease in MSNA (Burton et al., 2016; Fazalbhoy et al., 2012, 2014; Kobuch et al., 2015; Kobuch, Fazalbhoy, Brown, & Macefield, 2016). These divergent patterns of MSNA are reproducible over time

(Fazalbhoy et al., 2014), and not based on sex, or differences in resting MSNA, blood pressure, or heart rate (Kobuch et al., 2015). Moreover, anxiety and attitudes to pain do not account for these divergent sympathetic responses to muscle pain (Kobuch et al., 2016). Interestingly, chronic pain is associated with a significantly greater risk of hypertension, particularly in females, and it has been proposed that this comorbidity reflects fundamental alterations in the relationship between the cardiovascular and pain regulatory systems (Bruehl et al., 2005). Although the underlying brain circuitry responsible for hypertension in individuals with chronic pain is not known, it is possible that the divergent responses we previously found to occur in healthy individuals during tonic muscle pain also reflect differences in interactions between pain and cardiovascular circuits even in healthy subjects.

As noted above, given that noxious stimuli below the lesion in patients with complete spinal cord injury do not evoke any changes in blood pressure, heart rate, skin blood flow or sweat release, the circuitry responsible for pain-related changes in MSNA must lie within supraspinal sites (Burton et al., 2008). Studies in the cat suggest that the autonomic responses to pain form part of a behavioural response mediated by neurons in the midbrain periaqueductal grey matter (PAG; Bandler et al., 1991, 2000). Of course, numerous human brain imaging studies have also revealed a "central autonomic network" consisting of higher brain regions such as the insular, cingulate, and prefrontal cortices, as well as precuneus and amygdala (Benarroch, 1993; (Macefield & Henderson, 2016). Furthermore, recent con- current functional magnetic resonance imaging (fMRI) and microneurography studies from our laboratory have confirmed the function of the well-described autonomic-related regions of the brainstem, such as the rostral and caudal ventrolateral medulla, nucleus tractus

solitarius and dorsolateral pons (Lundblad et al., 2014; Macefield & Henderson, 2010, 2016). The aim of the current study was to use concurrent fMRI and microelectrode recordings of MSNA to determine if these autonomic regions respond differentially in individuals who displayed increases versus those who displayed decreases in MSNA during tonic muscle pain. Given that the dorsomedial hypothalamus (DMH) is part of the classical hypothalamic defence area, evoking increases in blood pressure and heart rate in the cat (Coote, Hilton, & Perez-Gonzalez, 1979), we tested the hypothesis that a sustained increase in MSNA during long-lasting muscle pain is associated with a sustained increase in BOLD signal intensity of the DMH, while a sustained decrease in MSNA is associated with a sustained decrease in DMH activity. And given that the DMH has connections with the PAG and RVLM, we tested the hypothesis that a sustained increase or decrease in MSNA during tonic muscle pain is associated with parallel increases or decreases in signal intensity of the PAG and RVLM.

4.3. Methods

4.3.1. Participants

Experiments were performed on 37 healthy university subjects (11 females; mean ± SEM age: 21.9 ± 0.5 years), each of whom had contributed psychometric data to our recent study in which we examined differences in anxiety and attitudes to pain in 66 subjects (Kobuch et al., 2016). Subjects responded to an advertisement that outlined the procedures to be undertaken, including the nerve recording, muscle pain and fMRI; all procedures were approved by the Human Research Ethics Committees of Western Sydney University and the University of New South Wales. Written consent was obtained from all subjects in accordance with the Declaration of Helsinki.

4.3.2. Microneurography

The protocol for MSNA measurement, as well as noxious stimulation procedures were described in the general methods section. MSNA was recorded continuously for 5 minutes of rest, prior to the subjects being wheeled from the laboratory to the scanner with the microelectrode remaining in situ.

4.3.3. Cardiovascular monitoring

Before the scanning period, 5 minutes of continuous blood pressure were recorded using radial arterial tonometry (Colin 7000 NIBP; Colin Corp., Aichi, Japan). Respiration was measured using a strain-gauge transducer (Pneumotrace, UFI, Morro Bay, CA, USA), and heart rate was monitored with a piezoelectric pulse transducer placed on the big toe. The blood pressure monitor was removed before entering the scanner.

4.3.4. MRI

Using a 3 Tesla MRI scanner (Philips Achieva, 32-channel SENSE head coil), two scans encompassing the whole brain were collected: a high-resolution 3D T1-weighted anatomical image (200 axial slices, echo time (TE)=2.5 ms, repetition time (TR)=5600 ms, raw voxel size = 0.87 mm^3), followed by a series of 250 gradient echo echo-planar sensitive to blood oxygen level dependent contrast (BOLD) fMRI images (46 axial slices, (TE)=40 ms, (TR)=8 s, raw voxel size = 1.5 x 1.5 x 3.25 mm).

During the fMRI scan, all axial slices were collected during the first 4 seconds of the 8 s TR. Two 10 ml syringes filled with hypertonic saline were placed in an infusion

pump (Harvard Instruments, USA), and connected to a three-way tap via a 30 cm length of extension tubing primed with hypertonic saline. A 23 G butterfly cannula was then attached to the three-way tap, primed, and inserted 1.5 cm deep into the belly of the right tibialis anterior muscle, approximately 5 cm lateral and 10 cm inferior to the tibial tuberosity. An infusion of hypertonic saline solution (7%) was commenced during the 50^{th} scan (volume 50) for all subjects and was sustained throughout the duration of the entire scanning period. Each subject was unaware of when the hypertonic saline infusion was to begin. The initial rate of infusion was set at 0.25 ml/min and was constantly adjusted to maintain a pain level of 5 out of 10 on a Numerical Rating Scale (NRS; 0 = no pain, 10 = most intense pain imaginable). The subjects' pain ratings were monitored continuously and adjusted according to each subject pressing four colour-coded buttons, each colour associated with either "pain onset," "pain at 5/10," "pain below 5/10," or "pain above 5/10." At the completion of the scanning session, each subject was asked to complete a McGill Pain Questionnaire (Melzack, 1975) and draw the area of perceived pain on a standard diagram of the leg.

4.4. Analysis

4.4.1. Pain psychophysics

Using the points at which each subject indicated the onset of pain as well as when the pain reached 5/10, we plotted an estimate of the average pain intensity over time for all subjects. We found that, on average, pain began approximately 18 volumes after the start of the infusion, i.e., at volume 68, and first reached a 5/10 rating at approximately volume 72, after which it was sustained for the remaining scanning

period (*Figure 4.1*). These pain intensity changes were similar in all subjects, whether they were subsequently categorized as displaying an increase or a decrease in MSNA.

4.4.2. MSNA

Individual bursts of MSNA were displayed as a mean-voltage neurogram, computed as the root-mean-square (RMS) processed signal with a moving time average window of 200 ms. The 4 s inter-scan (OFF) period (see below) allowed the measurement of MSNA bursts. Bursts were manually counted and the amplitude was measured from the RMS-processed nerve signal. Scans 16 to 75 were selected as the baseline period and scans 76 to 250 as the tonic pain period. In each subject, the absolute values for MSNA amplitude were averaged for the pain period and reported as percentage change relative to the baseline period.

4.4.3. Functional MRI

Using Statistical Parametric Mapping 12 (Wellcome Trust Centre for Neuroimaging, University College London, UK) and custom software, all fMRI images were realigned, spatially normalized to the Montreal Neurological Institute (MNI) template, intensity normalized to eliminate any slow drift in signal intensity, and bias corrected. Scans were then smoothed using a 6mm full-width at half-maximum (FWHM) Gaussian filter. In addition, we performed a brainstem-only analysis for the subjects for whom the acquired images extended to the bottom of the caudal medulla (n = 30). The brainstem was isolated and spatially normalized to the Spatial Unbiased Infratentorial Template (SUIT) using the SUIT toolbox (Diedrichsen et al., 2011), intensity normalized, bias corrected, and smoothed using a 4mm FWHM Gaussian filter.

Signal intensity changes were measured during the subsequent 4 s (ON period), taking into account the (1) 5 s neurovascular coupling delay between a neuronal event and the peak BOLD signal (Logothetis et al., 2001) and the (2) 1 s required for the burst of MSNA to travel from the brain to the peripheral recording site (Fagius & Wallin, 1980). The first 15 scans were excluded to allow for global signal equilibration. We searched for signal intensity changes using a box-car design, with a baseline period of 60 volumes (scans 16–75) and a pain period of 175 volumes (scans 76–250). A hemodynamic delay function was not included, given that we accounted for this delay in our analysis design. A one-sample random effects analysis was performed with all subjects in a single group to determine signal intensity changes associated with the pain period ($p < .05$, family wise error corrected). To determine regions in which signal intensity changes were different in the increasing versus decreasing MSNA group, a two-sample random effects analysis was performed using both the whole-brain and brainstem only images ($p < .001$, uncorrected). Significant clusters were overlaid onto a standard whole-brain and brainstem template in MNI space. For each significant cluster, the percentage changes in signal intensity were extracted by comparing the signal intensity of the pain period to baseline. We also performed non-paired two sample t-tests between baseline and pain for the signal intensity changes in each region that was significantly different between the increasing and decreasing MSNA groups. In addition, we performed correlation analyses in each subject to determine whether there were significant linear relationships between the peak or trough in MSNA amplitude against the corresponding BOLD signal intensity changes (Pearson's correlation, $p < .05$); for each subject, the average change in MSNA or

BOLD signal intensity was calculated over 10 volumes. All results were expressed as mean ± SEM.

4.5. Results

4.5.1. Subjective experience of tonic muscle pain

Intramuscular infusion of hypertonic saline solution induced moderately intense pain that began approximately 18 volumes (144 s) following the start of the infusion and quickly reached a pain rating of ~5/10 within approximately 4 volumes (32 s). The pain intensity remained at approximately this level for the remainder of the MRI session. A two-sample t-test revealed that there was no significant difference in the mean pain intensity ratings in the increasing MSNA compared with the decreasing MSNA group (increasing: 5.7 ± 0.2; decreasing: 6.0 ± 0.2; $p = .28$). In addition, there was no significant group difference between the total volume of hypertonic saline infused in either group (increasing: 18.8 ± 2.6 ml; decreasing: 18.1 ± 0.9 ml; $p = .82$) or the area of perceived pain spread (increasing: 1054 ± 102 pixels; decreasing: 1009 ± 131 pixels; $p = .80$; *Figure 4.2*). There was also no difference in the words chosen from the McGill pain questionnaire to describe the ongoing pain; both groups chose "dull," "aching," and "throbbing" to describe the pain (*Figure 4.3*).

4.5.2. MSNA during tonic muscle pain

Consistent with our previous findings, tonic muscle pain induced two distinct sympathetic responses: 26 subjects exhibited a significant increase in MSNA burst amplitude (71.8 ± 26.7%), while 11 subjects showed a significant decrease (-32.5 ± 18.6%) throughout the pain period ($p < .0001$, t-test; *Figure 4.1*). The changes in MSNA

began at approximately the same time as the subjects first perceived pain and were sustained for the duration of the infusion.

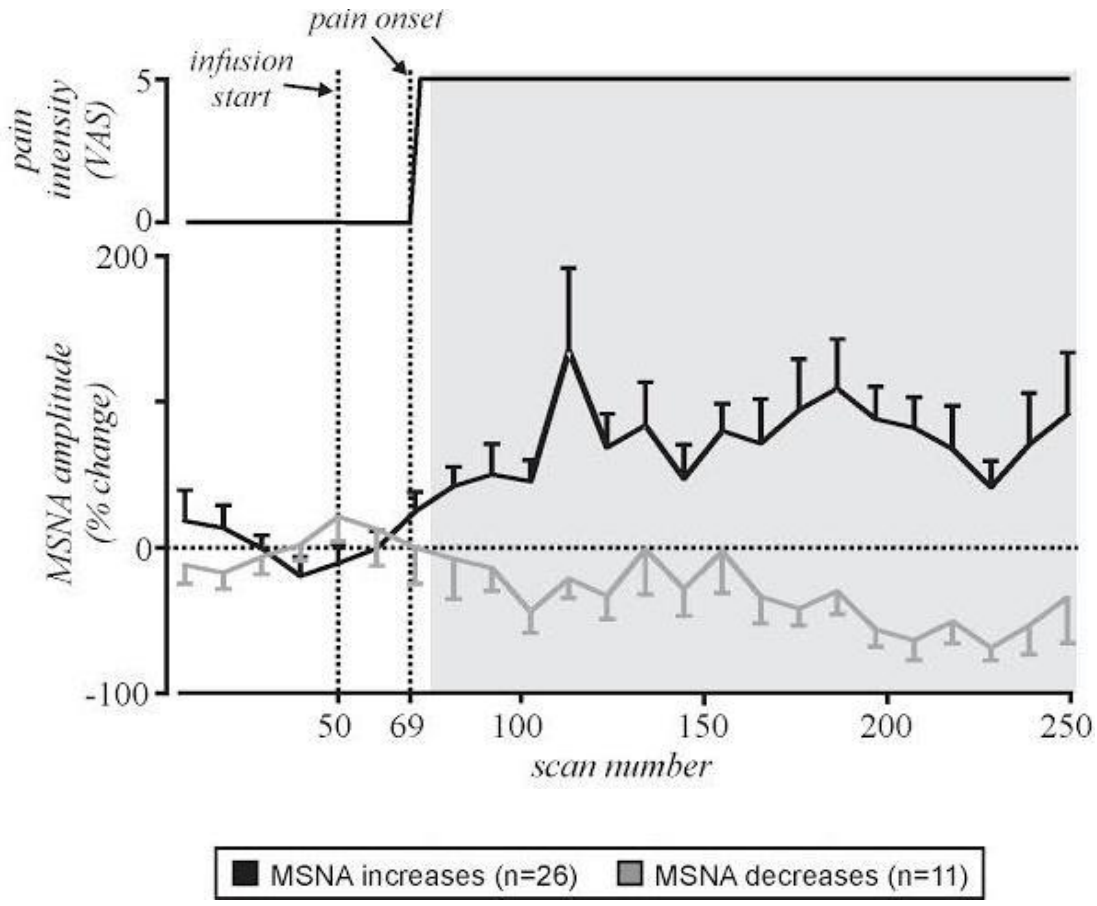

Figure 4.1: Plots of perceived pain intensity and MSNA amplitude changes during pain.

Plots of perceived pain intensity and MSNA amplitude changes during continuous hypertonic saline solution infusion into the right tibialis anterior muscle of 37 subjects. The MSNA plot indicates mean (±SEM) percentage change in MSNA amplitude during pain relative to the baseline period in the increasing (black, n=26) and decreasing groups (grey, n = 11). The vertical dashed line indicates the start of the hypertonic saline infusion. The grey shading indicates the volumes during which all subjects were in moderate pain.

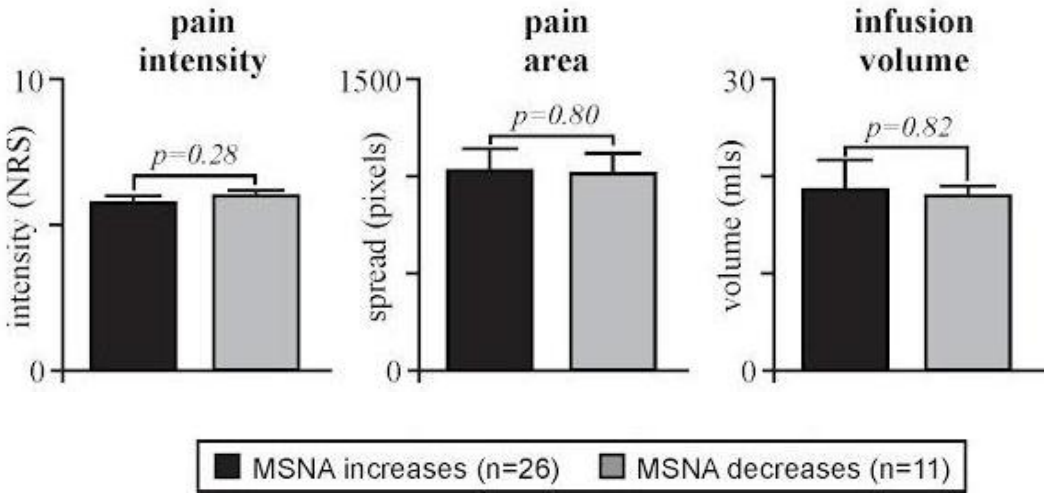

Figure 4.2: Pain intensity, pain area, and infusion volume in subjects who displayed increases and decreases in MSNA.

Plots of mean (±SEM) pain intensity, pain area, and infusion volume in subjects that displayed increases and decreases in MSNA.

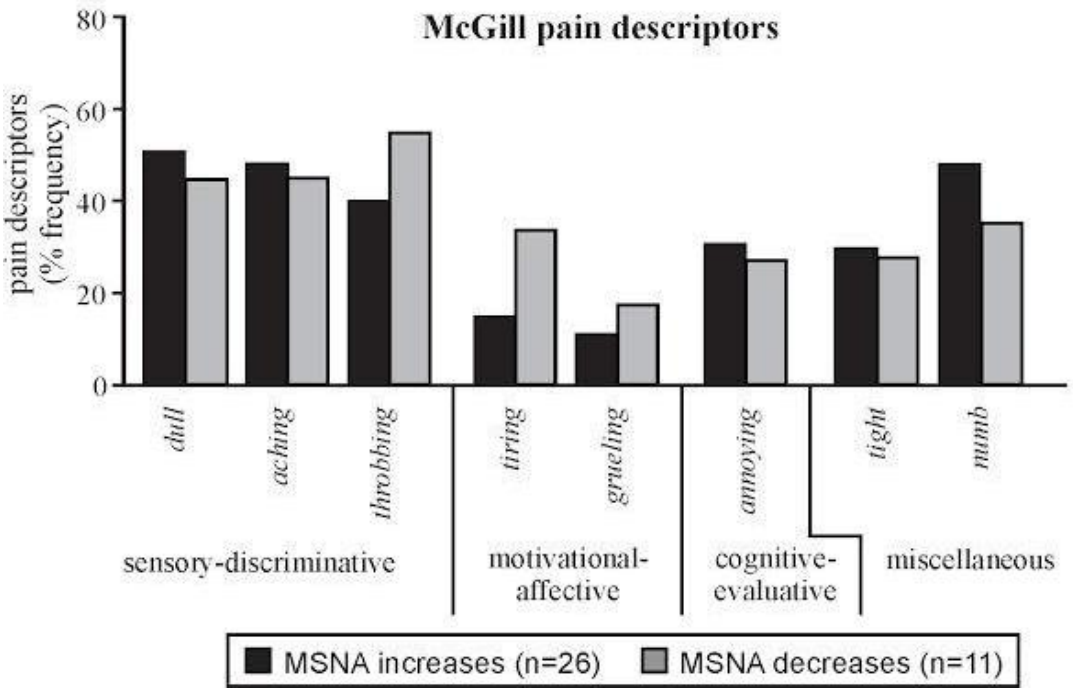

Figure 4.3: Frequency of words most often chosen from the McGill pain questionnaire.

Plots of the frequency of words most often chosen by subjects from the McGill pain questionnaire to describe the ongoing muscle pain. Note there is no difference in any of the pain parameters between the increasing and decreasing MSNA groups.

4.5.3. Signal intensity changes

Pain was associated with significant increases in BOLD signal intensity in a number of brain regions that have been previously shown to be activated during acute muscle pain. In all 37 subjects BOLD signal intensity increased in the left (contralateral to the noxious stimulus) primary somatosensory cortex and right primary motor cortex in the region representing the leg and foot, contralateral secondary somatosensory cortex, contralateral anterior and dorsal posterior insular cortex, as well as bilaterally in the posterior cingulate cortex and the thalamus (*Figure 4.5, Table 4.1*).

In none of these regions (*Figure 4.4, Table 4.1*) were there any significant differences in signal intensity between subjects in whom MSNA increased compared with those in whom MSNA decreased. However, a two-sample analysis revealed significant differences in other regions (*Figure 4.5, Table 4.2*). Signal intensity increased in the increasing MSNA group and remained unchanged in the decreasing MSNA group in the right (ipsilateral) dorsolateral prefrontal cortex (dlPFC: % change over entire pain period: increasing 1.13 ± 0.08 vs. decreasing 20.29 ± 0.07), left nucleus accumbens (NAc: increasing 1.46 ± 0.10 vs. decreasing -0.27 ± 0.09), right subgenual anterior cingulate cortex (sgACC: increasing 2.71 ± 0.18 vs. decreasing -0.46 ± 0.19) and left dorsomedial hypothalamus (DMH: increasing 1.52 ± 0.09 vs. decreasing 0.27 ± 0.09). By contrast, signal intensity increased during the pain period in the increasing MSNA group and decreased in the decreasing MSNA group in the left orbitofrontal cortex (OFC: increasing 1.76 ± 0.11 vs. decreasing -0.26 ± 0.09), left caudate nucleus (increasing 1.08 ± 0.09 vs. decreasing -0.99 ± 0.08) and left precuneus (increasing 0.61 ± 0.10 vs. decreasing -1.01 ± 0.10).

In addition, in the increasing MSNA group, the signal intensity changes were significantly different from baseline in the following cortical regions: dlPFC ($p < .001$), left OFC ($p < .001$), left hypothalamus ($p < .001$), left caudate ($p < .001$), subgenual ACC ($p < .001$), precuneus ($p = .002$). In the decreasing group, the signal intensity changes were significantly different from baseline in the following cortical regions: dlPFC ($p < .001$), left OFC ($p < .001$), left hypothalamus ($p = .02$), subgenual ACC ($p < .001$), precuneus ($p < .001$), and left accumbens ($p < .001$).

In no region was signal intensity lower in the MSNA increasing group relative to the MSNA decreasing group. In the brainstem-specific analysis (n=30), we found significantly different signal intensity changes in the increasing compared with the decreasing MSNA group (*Figure 4.6*, *Table 4.1*). Signal intensity increased in the increasing MSNA group and remained at baseline levels in the MSNA decreasing group in the right lateral medulla, within the region of the rostral ventrolateral medulla (RVLM: increasing 1.08 ± 0.10 vs. decreasing 0.32 ± 0.09), right dorsolateral pons (dlPons: increasing 1.53 ± 0.10 vs. decreasing 0.27 ± 0.08) and the left dlPons (increasing 1.60 ± 0.10 vs. decreasing 0.39 ± 0.07). In direct contrast, BOLD signal intensity was significantly greater in the MSNA decreasing group compared with the increasing group in the region of the midbrain encompassing the left periaqueductal grey matter (PAG: increasing 0.61 ± 0.17 vs. decreasing 1.45 ± 0.17). This was the only region in the entire brain where signal intensity was greater in the MSNA decreasing group.

Furthermore, the following regions showed significant signal intensity changes during pain compared to baseline in the increasing MSNA group: left PAG ($p < .001$), right

RVLM ($p < .001$), left dlPons ($p < .001$), right dlPons ($p < .001$). In the decreasing group the signal intensity changes during pain were significantly different from baseline in the left PAG ($p < .001$) and right RVLM ($p = .04$).

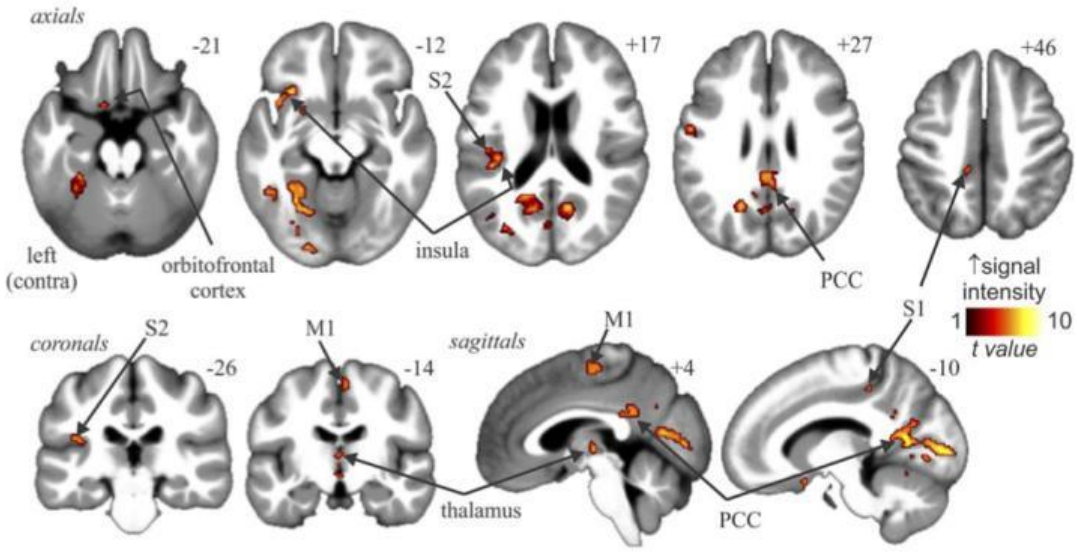

Figure 4.4: Signal intensity increases during pain.

Signal intensity increases (hot colour scale) during continuous hypertonic saline solution infusion into the right tibialis anterior muscle in all 37 subjects. Significant clusters were overlaid onto a mean T1-weighted anatomical image set created from all 37 subjects. Slice locations in MNI space are indicated in the upper left of each image. The left side of the image is the side contra- lateral to the noxious stimulus. M1: primary motor cortex; PCC: posterior cingulate cortex; S1: primary somatosensory cortex; S2: secondary somatosensory cortex.

Table 4.1: Signal intensity increases in all subjects during muscle pain.

Location in Montreal Neurological Institute space, t values, and cluster sizes of regions in which signal intensity increased in all subjects during tonic muscle pain.

Signal intensity increases	**x**	**y**	**z**	**t value**	**Cluster size**
Orbitofrontal cortex					
Left (contralateral)	-10	10	-20	6.85	9
Insular cortex					
Left	-30	18	-16	8.10	74
	-38	10	-14	7.12	
	-36	18	-10	6.87	
Primary somatosensory cortex					
Left	-10	-36	46	5.95	6
Secondary somatosensory cortex					
Left	-38	-30	18	7.08	55
Primary motor cortex					
Right (ipsilateral)	4	-14	60	6.39	28
	4	-20	58	6.28	
	4	-14	54	6.17	
Posterior cingulate cortex					
Right	2	-40	26	7.02	78
	6	-42	24	6.98	
Left	-4	-42	26	6.62	

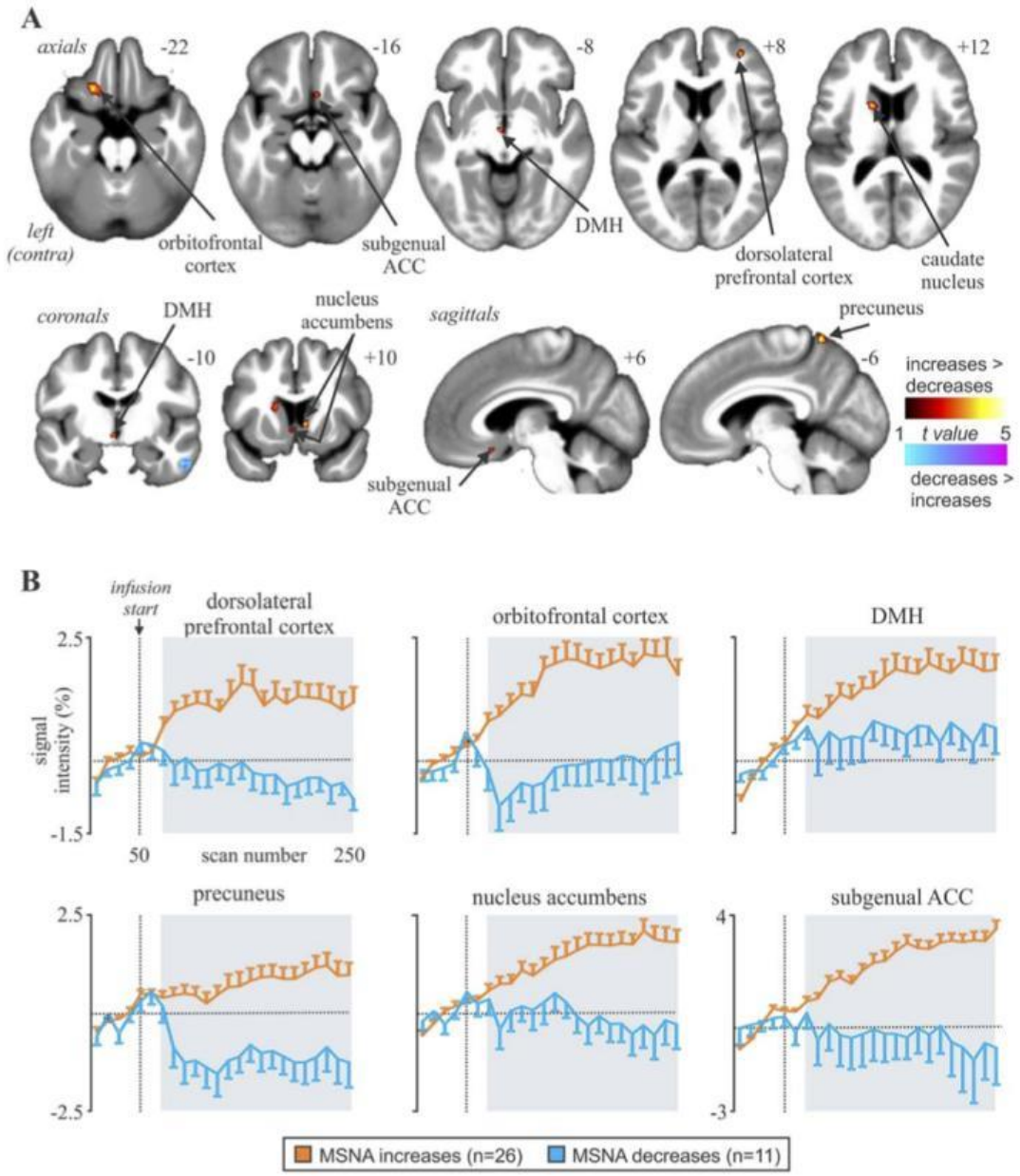

Figure 4.5: Regions above the brainstem showing signal intensity differences between subjects with increasing versus decreasing MSNA.

A: Brain regions above the brainstem in which signal intensity increases were greater (hot colour scale) or lower (cool colour scale) in the increasing MSNA than in the decreasing MSNA group during tonic muscle pain. Significant clusters were overlaid onto a mean T1-weighted anatomical image set created from all 37 subjects. Slice locations in MNI space are indicated in the upper right of each image. The left side of the image is the side contralateral to the noxious stimulus. B: Plots of mean (±SEM) percentage signal intensity changes during pain relative to the baseline period for significant clusters in the increasing MSNA (orange) and decreasing MSNA (blue) groups. The vertical dashed line indicates the start of the

hypertonic saline infusion and the grey shading the pain period. ACC: anterior cingulate cortex; DMH: dorsomedial hypothalamus.

Table 4.2: Regions above the brainstem showing signal intensity differences between subjects with increasing versus decreasing MSNA.

Locations in Montreal Neurological Institute space, t values and cluster sizes of regions in which signal intensity was significantly different in subjects that displayed increased muscle sympathetic nerve activity (MSNA) compared with those that showed decreased MSNA during tonic muscle pain. Only regions above the brainstem are included.

Signal intensity increases	**x**	**y**	**z**	**t value**	**Cluster size**
Orbitofrontal cortex					
Left (contralateral)	-20	24	-22	3.76	24
Subgenual anterior cingulate cortex					
Right (ipsilateral)	6	18	-16	3.25	6
Dorsomedial hypothalamus					
Left	-2	-10	-8	3.51	7
Nucleus accumbens					
Right	10	-10	0	3.67	18
Left	-4	10	-6	3.18	5
Dorsolateral prefrontal cortex					
Right	32	48	6	3.78	14
Caudate nucleus	-14	8	14	3.69	22
Left					
Precuneus					
Left	-6	-52	66	4.40	22

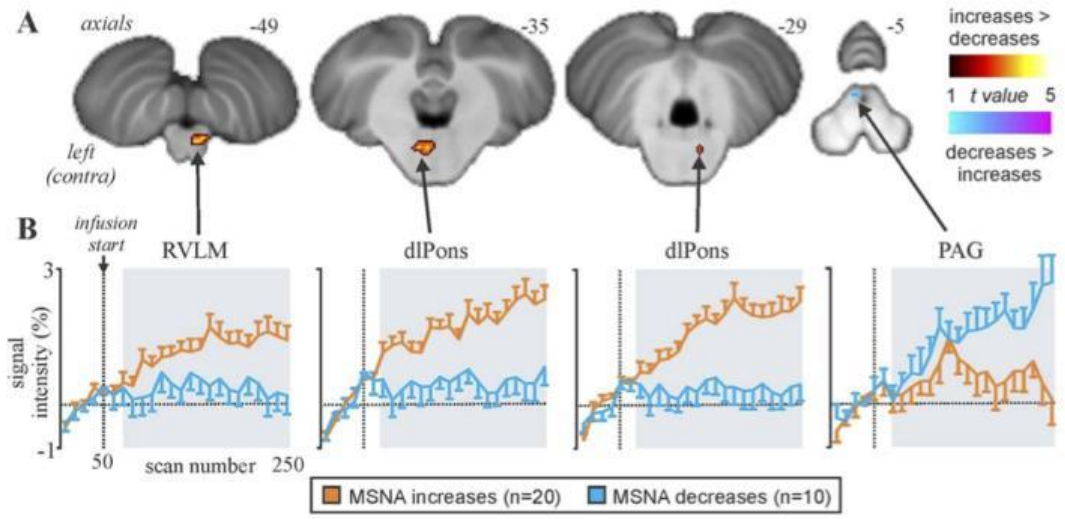

Figure 4.6: Brainstem regions showing signal intensity differences between the increasing and decreasing MSNA groups.

A: Brainstem regions in which signal intensity increases were greater (hot colour scale) or lower (cool colour scale) in the increasing MSNA (n = 20) than in the decreasing MSNA (n = 10) group during tonic muscle pain. Significant clusters were overlaid onto a mean SUIT T1-weighted anatomical image set created from 30 subjects. Slice locations in MNI space are indicated in the upper right of each image. The left side of the image is the side contralateral to the noxious stimulus. B: Plots of mean (±SEM) percentage signal intensity changes during pain relative to the baseline period for significant clusters in the increasing MSNA (orange) and decreasing MSNA groups (blue). The vertical dashed line indicates the start of the hypertonic saline infusion and the grey shading the pain period. RVLM: rostroventrolateral medulla; dlPons: dorsolateral pons; PAG: midbrain periaqueductal grey.

Table 4.3: Brainstem regions showing signal intensity differences between subjects with increasing versus decreasing MSNA.

Locations in Montreal Neurological Institute space, t values and cluster sizes of regions in which signal intensity was significantly different in subjects that displayed increased muscle sympathetic nerve activity (MSNA) compared with those that showed decreased MSNA during tonic muscle pain. Only brainstem regions are included.

Signal intensity increases	**x**	**y**	**z**	**t value**	**Cluster size**
Rostroventrolateral medulla					
Right (ipsilateral)	8	-40	-49	4.30	12
Dorsolateral pons					
Right	8	-30	-29	3.28	3
Left (contralateral)	-6	-32	-35	3.60	13
Midbrain periaqueductal grey					
Left	-4	-34	-5	3.20	2

4.5.4. Correlation with MSNA

In addition to differences in cortical and subcortical signal intensity changes between the increasing and decreasing MSNA groups, we found that changes in signal intensity in most of these regions were correlated with the peak/trough (maximum change) in MSNA amplitude (*Figure 4.7*). Positive correlations occurred in the left OFC (Pearson's, r = 0.41, p = .02), right dlPFC (r = 0.43, p = 0.01), left precuneus (r = 0.50, p = .002), left dlPons (r = 0.45, p = .01), and right dlPons (r = 0.51, p = .004). In contrast, a negative correlation between increasing MSNA and signal intensity increases occurred in the left dorsolateral PAG (dlPAG; r = -0.43, p = .02). We found no significant linear relationships in the right RVLM (r = 0.33, p = .09), left NAc (r =

0.32, p = .07), left caudate nucleus (r = 0.23, p = .21), right sgACC (r = 0.27, p = .14), or left DMH (r = 0.25, p = .15).

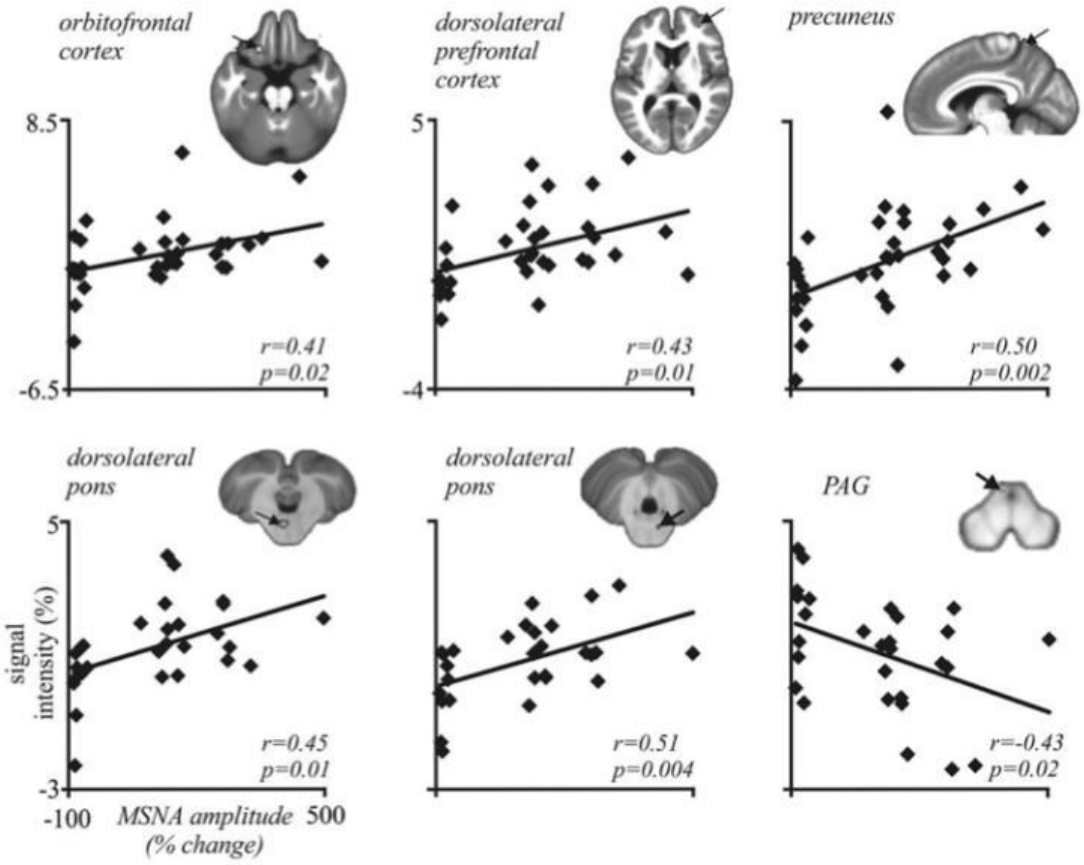

Figure 4.7: Brain regions in which peak or trough MSNA amplitude during pain were significantly correlated with signal intensity changes.

4.6. Discussion

In this investigation, we revealed brain responses associated with divergent changes in MSNA during tonic muscle pain. Although all subjects reported similar pain intensity ratings, the long-lasting pain evoked robust increases in MSNA in some individuals and decreases in others. These divergent MSNA responses were associated with different signal intensity changes in a number of brain regions, namely the prefrontal

and cingulate cortices, precuneus and DMH, and brainstem regions such as the PAG and RVLM. These data suggest that, during tonic muscle pain, descending modulation of the brainstem circuits, which are thought to control the cardiovascular responses to pain, can evoke different MSNA responses in different individuals.

Consistent with our previous investigations, we found that tonic muscle pain resulted in divergent muscle sympathetic responses (Fazalbhoy et al., 2012, 2014; Kobuch et al., 2015, 2016). Since these opposite MSNA responses were associated with different signal intensity changes in cortical regions involved in higher-order emotional and cognitive function, such as the prefrontal and cingulate cortices, one might expect that psychological differences underlie the different MSNA responses. However, we previously reported that pain catastrophizing, pain anxiety, and pain vigilance, as well as state and trait anxiety levels, did not differ between groups (Kobuch et al., 2016). In addition, here we report no group difference in pain parameters such as the intensity, spread, or quality of the pain. Although there is a well-documented relationship between psychological variables such as pain catastrophizing and the development of chronic pain (Chaves & Brown, 1987; Keefe et al., 1989; McCracken & Dhingra, 2002; Pavlin et al, 2005; Picavet et al., 2002; Spanos et al., 1979), our findings suggest that these variables do not predict the MSNA response to pain and likely do not play a role in the development of hypertension associated with chronic pain.

In almost all regions showing differences in signal intensity changes, signals increased in the increasing MSNA group and remained unchanged in the decreasing group. Moreover, most of these regions were positively correlated with maximum MSNA amplitude. One clear exception was the region of the dlPAG, in which the signal

intensity initially increased in both groups but then returned to baseline levels in the increasing MSNA group and continued to increase in the decreasing MSNA group. In addition, there was a negative correlation between signal intensity in the PAG and peak MSNA. It is well established in the cat that the PAG is activated by both superficial and deep pain and is critical for the expression of defensive behaviours (Bandler et al., 1991; Keay & Bandler, 2001). Keay and Bandler's review (2001) discusses studies in which cutaneous pain activates the lateral PAG, and direct stimulation of this PAG region evokes fight/flight behaviours that are associated with behaviourally-relevant increases in blood pressure, heart rate, and sympathetic activity (Bandler et al., 2000; Carrive, Bandler, & Dampney, 1988; Carrive, Dampney, & Bandler, 1987; Hilton & Redfern, 1986; Meller & Dennis, 1991; Van Bockstaele, Aston-Jones, Pieribone, Ennis, & Shipley, 1991; Yardley & Hilton, 1986). In direct contrast, deep pain, such as that arising from muscle, activates the ventrolateral PAG, and stimulation of this PAG region evokes quiescence with decreases in blood pressure, heart rate, and sympathetic activity (Carrive & Bandler, 1991; Lovick, 1992). Given these data, one would expect that tonic muscle pain in humans would evoke decreases in MSNA in all individuals, and that this would be associated with signal changes in the ventrolateral part of the PAG, which did not occur in the current study.

An alternate proposal of PAG organization is one based on whether the pain is interpreted as being escapable or inescapable. That is, when the situation is escapable, active coping strategies such as fight/flight and sympathoexcitation are evoked, whereas an inescapable stressor is met with passive coping mechanisms, such as a conservation-withdrawal response and sympathoinhibition (Keay & Bandler, 2001). In our experiment, the noxious stimulus is inescapable in the sense that the MRI

scanner is a confined environment and requires the subject to remain stationary. Alternatively, the stimulus could be deemed escapable - given that each individual is informed that they can stop both the pain and the scanning at any point. It might be that those individuals that deemed the stimulus inescapable displayed decreases in MSNA, whereas those that judged the stimulus as escapable displayed MSNA increases. If this were the case, one would then predict that decreased MSNA would be associated with increases in signal intensity in the ventrolateral PAG, and increased MSNA with lateral PAG signal increases, which again did not occur.

It is curious that the divergent MSNA responses seen in our study during long-lasting muscle pain are associated with different signal intensity changes in the region of the dlPAG, and not the lateral or ventrolateral PAG. Although stimulation of the dlPAG evokes active coping behaviours, unlike the lateral and ventrolateral PAG, it does not receive noxious inputs from the spinal cord or brainstem but, instead, receives inputs from primarily prefrontal and cingulate cortices (Floyd, Price, Ferry, Keay, & Bandler, 2000). The predominance of cortical inputs into the dlPAG has led to the suggestion that this region produces active coping strategies in response to psychological stressors (Keay & Henderson, 2010). This idea is supported by studies in the rat, which show active coping behaviours and dlPAG activation during threat signals such as the odour or sight of a cat (Canteras & Goto, 1999; Dielenberg, Leman, & Carrive, 2004). Furthermore, it has been shown in the rat that the dlPAG projects to, and receives input from the hypothalamus - which in turn receives input from the prefrontal and cingulate cortices (Beckstead, 1979; Domesick, 1969; Floyd, Price, Ferry, Keay, & Bandler, 2001; Gabbott et al., 2005). Microinjection of excitatory amino acids into the dlPAG in the rat evokes a significant increase in sympathetic nerve activity and respiratory

activity (Huang, Subramanian, Balnave, Turman, & Moi Chow, 2000; Iigaya, Horiuchi, McDowall, & Dampney, 2010). Imaging studies in humans have demonstrated the role of the dlPAG in the physiological responses to psychological stressors, such as induced panic attack (Reiman et al., 1989), or the approach of a virtual predator (Mobbs et al., 2007). Moreover, stimulation of the dlPAG in humans can evoke increases in blood pressure (Basnayake et al., 2011; Basnayake, Green, & Paterson, 2012) as well as increases in MSNA burst amplitude (Sverrisdóttir et al., 2014). Further investigations in rats have proposed that these increases are mediated via a pathway to the DMH (Horiuchi, McDowall, & Dampney, 2009), and since there is only a small direct projection from the dlPAG to the DMH (Thompson & Swanson, 1998), it has been suggested that the dlPAG produces sympathoexcitation through indirect projections to the DMH via the cuneiform and/or parabrachial nuclei (Dampney, Furlong, Horiuchi, & Iigaya, 2013; Fulwiler & Saper, 1984; Lam & Verberne, 1997). The DMH then projects directly to the RVLM and/or raphe pallidus, where it can excite sympathetic premotor neurons and increase sympathetic output, as shown in the rat (Fontes, Tagawa, Polson, Cavanagh, & Dampney, 2001).

Consistent with this proposed dlPAG output pathway and descending inputs, we found that in subjects in whom MSNA increased, signal intensity also increased in the dlPFC, sgACC, DMH, dorsolateral pons, and RVLM. The increased signal intensity in DMH and RVLM in the increasing MSNA group supports our hypothesis. Interestingly, within the dlPAG, signal intensity increased during the initial period of pain but then decreased towards baseline levels for the remainder of the scanning period whilst MSNA remained elevated. It has been suggested the dlPAG control of sympathetic activity may involve a facilitatory summation of inputs from the dlPAG and DMH

(Dampney et al., 2013; de Menezes, Zaretsky, Fontes, & DiMicco, 2009). This raises the possibility that, following the initial period of muscle pain, facilitatory effects of the dlPAG on the DMH, in combination with descending inputs from the prefrontal and cingulate cortices - which also project to the DMH (Beckstead, 1979; DiMicco, Samuels, Zaretskaia, & Zaretsky, 2002; Domesick, 1969; Floyd et al., 2001; Gabbott et al., 2005) - are adequate to maintain increased sympathetic drive without increased dlPAG activity.

Intriguingly, dlPAG signal increased and remained elevated during the entire pain period in individuals who displayed a fall in MSNA. In these subjects, signal intensity did not change in the prefrontal or cingulate cortices, DMH, dorsolateral pons or RVLM. Indeed, the only regions besides the dlPAG that showed signal changes were the precuneus and caudate nucleus, which displayed robust decreases in signal intensity. It is possible that in these subjects, inputs onto the dlPAG from the precuneus and caudate over-ride the pathway described above and, instead, elicit decreased dlPAG output and reduced muscle sympathetic activity. In fact, the dlPAG contains a high concentration of receptors for the inhibitory neurotransmitter gamma-aminobutyric acid (GABA; Barbaresi, 2005, 2010) and the blockade of these receptors elicits strong increases in sympathetic and respiratory activity (Iigaya et al., 2012). Given this, increased dlPAG signal intensity in the decreasing MSNA group could reflect increased excitation of tonic local GABAergic activity, resulting in a decrease in MSNA. Additionally, the dlPAG contains high concentrations of NADPH diaphorase and nitric oxide synthase (NOS; Bandler & Shipley, 1994; Herbert & Saper, 1992; Onstott, Mayer, & Beitz, 1993). Blocking NOS in the dlPAG evokes an increase in mean arterial pressure, and injection of nitric oxide donating compounds can evoke

hypotensive responses (Hall & Behbehani, 1997, 1998). Why differences in the effects of higher centres, such as the prefrontal and cingulate cortex, or precuneus and caudate nucleus, on the dlPAG occur in some individuals and not others remains unknown.

Finally, it is important to emphasize that, although we used a physical stressor (tonic muscle pain), we did not find differential signal changes in the region of the lateral or ventrolateral PAG, but instead found such changes in the dlPAG. Although this difference may reflect inter-species variability, we suggest instead that the natural behavioural-coupled cardiovascular responses to the physical stressor were not expressed due to the need for the individual to remain still. We speculate that the MSNA changes and associated changes in brain activity reflect differences in individuals' psychological stress responses to the unpleasant painful event. If this is true, it significantly alters the interpretation of brain imaging studies that explore PAG activity during physical stressors, since natural behavioural responses and their associated changes in cardiovascular control and even analgesic responses may not be properly expressed. Nevertheless, this experimental method of inducing pain provides insight as to how pain may modulate MSNA and its underlying central circuitry. However, in order to interpret these changes in chronic pain patients, one would need to know their baseline levels prior to the development of chronic pain.

4.7. Limitations

As with our previous studies on the sympathetic responses to muscle (or cutaneous) pain, it may be that there was a form of selection bias in our subjects. While we did not knowingly introduce a bias, one must recognize that those subjects who volunteered for the experiment did so knowing that they were going to have a microelectrode inserted into a nerve, another needle inserted under the skin, and a

cannula inserted into a muscle. Moreover, they knew they were going to experience strong muscle pain during the intramuscular infusion of hypertonic saline for up to an hour. As such, subjects with a needle phobia, or a morbid fear of pain, would not volunteer for this type of experiment. None of the subjects we did study reported having a fear of needles, and none fainted during the course of the experiment (although they were supine). Of course, it may well be that had we actively selected those with a needle phobia, or those with a tendency to faint, we may have uncovered further differences, but it is fair to say that all our subjects were essentially normal. Another potential limitation is that we always recorded MSNA from the right common peroneal nerve, ipsilateral to the intramuscular infusion of hypertonic saline. Accordingly, we must be circumspect in any consideration of the lateralization of the changes in BOLD signal intensity we uncovered, but it is fair to say that changes in MSNA are usually distributed symmetrically to muscles on either side of the body. The most significant limitation of this study is the spatial resolution of imaging brainstem structures such as the PAG. Whilst we argue that the differential signal intensity changes within the midbrain were located in the region of the dlPAG, given the relatively poor spatial resolution of human brain imaging at 3 T, it is possible that these changes were indeed located in other PAG columns. Recent developments in MRI at higher field strengths, such as 7 T, offer to provide superior spatial acuity and will provide greater confidence in the spatial accuracy of future imaging studies.

CHAPTER 5 Muscle sympathetic nerve activity-coupled changes in brain activity during sustained muscle pain

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5.1. Abstract

Long-lasting experimental muscle pain elicits divergent muscle sympathetic responses, with some individuals exhibiting a persistent increase in muscle sympathetic nerve activity (MSNA), and others a decrease. These divergent responses are thought to result from sustained functional changes in specific brain regions that modulate the cardiovascular responses to pain. The aim of this study was to investigate brain regions that are functionally coupled to the generation of an MSNA burst at rest and to determine their behaviour during tonic muscle pain. Functional magnetic resonance imaging of the brain was performed concurrently with microelectrode recording of MSNA from the common peroneal nerve during a 40min infusion of hypertonic saline into the ipsilateral tibialis anterior muscle of 37 healthy human subjects. At rest, blood oxygen level dependent signal intensity coupled to bursts of MSNA increased in the rostral ventrolateral medulla, insula, dorsolateral prefrontal cortex, posterior cingulate cortex, and precuneus, and decreased in the region of the midbrain periaqueductal grey. During pain, MSNA-coupled signal intensity was greater in the region of the nucleus tractus solitarius, midbrain periaqueductal grey, dorsolateral prefrontal, medial prefrontal, and anterior cingulate cortices, than at rest. Conversely, MSNA-coupled signal intensity decreased during pain in parts of the prefrontal cortex. These results suggest that multiple brain regions are recruited in a burst-to-burst manner and the magnitude of these signal changes is correlated to the overall change in MSNA amplitude during tonic muscle pain.

5.2. Introduction

Investigations in experimental animals have revealed that pain arising from the skin (cutaneous pain) evokes increases in blood pressure, heart rate, and sympathetic vasoconstrictor drive, whereas pain arising from muscle (deep pain) evokes decreases in these parameters (Keay & Bandler, 2001). These distinct autonomic responses are mediated by neurons in the midbrain periaqueductal grey matter (PAG), which are themselves under the influence of higher brain centres (Bandler et al., 2000). In humans, the sympathetic vasoconstrictor drive is revealed through either skin or muscle sympathetic nerve activity (SSNA or MSNA), which can be recorded via microelectrodes inserted directly into a peripheral nerve in awake human subjects, a technique called microneurography. MSNA, which is tightly coupled to cardiac rhythmicity, reflects the activity of postganglionic sympathetic neurons supplying the skeletal muscle vascular beds. An increase in MSNA causes vasoconstriction and thereby increases blood pressure (Macefield, 2013). Whilst distinctive cardiovascular responses to pain originating from different tissues have been observed in humans (Feinstein et al., 1954; Lewis, 1942), we recently reported that experimentally induced, transient (~6 minutes) cutaneous and muscle pain did not evoke such distinct cardiovascular responses. *Cutaneous* pain, elicited by subcutaneous injection of hypertonic saline solution, evoked increases in muscle sympathetic nerve activity (MSNA), blood pressure (BP) and heart rate (HR). On the other hand, *muscle pain*, elicited by intramuscular injection - on average - also evoked significant increases in these cardiovascular parameters (Burton et al., 2009). We speculated at the time that this unexpected pattern of change during acute muscle pain may be a characteristic of the short-lasting nature of the stimulus and the associated novelty.

We have since shown that prolonged (~45 minutes) muscle pain in humans evokes a mixed cardiovascular response, with some individuals showing a sustained MSNA increase, and others a sustained decrease (Fazalbhoy et al., 2012, 2014; Kobuch et al., 2015, 2016, 2017). These individual differences are reliable across multiple experimental sessions in individuals (Fazalbhoy et al., 2014), and are not influenced by sex, age, anxiety levels, attitudes to pain, or resting MSNA, blood pressure, or heart rate levels (Kobuch et al., 2015, 2016). Using concurrent functional magnetic resonance imaging (fMRI) and microneurography, we recently found that areas of the prefrontal, cingulate and precuneus cortices, hypothalamus, midbrain, and medulla displayed sustained increases in blood oxygen level dependent (BOLD) signal intensity in those individuals who displayed sustained increases in MSNA compared with those who displayed sustained decreases (Kobuch et al., 2017). That study explored continuous signal intensity changes throughout the whole scanning period, with subjects separated into two groups according to whether they exhibited an increase or a decrease in MSNA during pain (Kobuch et al., 2017). However, while investigating overall signal intensity changes associated with sustained increases or decreases in MSNA responses during tonic muscle pain, it did not explore MSNA-coupled changes in BOLD signal intensity, i.e. regions in which signal intensity increased or decreased during each MSNA burst. This is important, since sustained signal changes may be providing persistent modulatory inputs onto other brain regions that are actually driving each MSNA burst. This chapter is a separate burst-burst analysis of the same data presented in the previous chapter (Chapter 4).

For example, at rest, signal intensity within the prefrontal, insular and precuneus cortices, hypothalamus, and medulla are tightly coupled to each MSNA burst (see

Macefield & Henderson, 2016 for review). It is unknown whether, during sustained muscle pain, signal intensity within these areas remains coupled to MSNA, and/or whether other brain regions react in a similar manner. This is an important question since it is not known whether, in humans, there is an invariant set of brain regions in which activity is coupled to MSNA, or whether other brain regions are recruited during various autonomic challenges.

The aim of the current study was to identify brain regions that are functionally coupled to the generation of bursts of MSNA, both at rest and during tonic muscle pain. We hypothesized that, in addition to areas shown to be coupled to MSNA bursts at rest, additional regions, such as the cingulate cortex and midbrain, would also exhibit coupling to MSNA during pain, and that increases in BOLD signal intensity in these areas would parallel an increase in MSNA burst amplitude.

5.3. Methods

5.3.1. Participants

Experiments were performed on 37 healthy subjects (11 females; mean±SEM age: 21.9±0.5 years, range: 18-31 years). The subjects were recruited through an advertising flyer at the Western Sydney University School of Medicine. Exclusion criteria included a history of cardiovascular disease or chronic musculoskeletal pain. The subjects who volunteered for the experiment did so knowing that they were going to have a microelectrode inserted into a nerve, another needle inserted under the skin, and a cannula inserted into a muscle. Moreover, they knew they were going to

experience strong muscle pain during the intramuscular infusion of hypertonic saline for up to an hour. All experiments were conducted at 2pm, and the subjects were instructed to abstain from any strenuous exercise and from drinking caffeine. None of the subjects took any medication for cardiovascular disease or pain relief. Most participants were part of a larger study (Kobuch et al., 2016) that included the completion of the following questionnaires: Pain Catastrophizing Scale (PCS; Sullivan et al., 1995), Pain Vigilance and Awareness Questionnaire (PVAQ; McCracken, 1997), Pain Anxiety Symptoms Scale (PASS-20; McCracken & Dhingra, 2002), and the State and Trait Anxiety Inventory (STAI; Spielberger et al., 1983).

All procedures were approved by the Human Research Ethics Committees of Western Sydney University and the University of New South Wales. Written consent was obtained from all subjects in accordance with the Declaration of Helsinki.

5.3.2. Microneurography

The protocol for MSNA and cardiorespiratory parameters measurement were described in the general methods section.

5.3.3. MRI scanning and stimulus

Using a 3 Tesla MRI scanner (Philips Achieva, 32-channel SENSE head coil), two scans encompassing the whole brain were collected: a high-resolution 3D T1-weighted anatomical image (200 axial slices, echo time (TE)=2.5ms, repetition time (TR)=5600ms, raw voxel size=0.87mm^3), followed by a series of 250 gradient echo echo-planar blood oxygen level dependent functional magnetic resonance images

(fMRI: 46 axial slices, TE=40ms, TR = 8 s, raw voxel size=1.5x1.5x3.25mm^3). All 46 axial slices in each of the 250 fMRI brain volumes were collected during the first 4 seconds of the 8 second TR, which allowed for 4 seconds of clean MSNA recording per brain volume, i.e. without the potential for scanner artefact.

The noxious stimulation protocol was described in the general methods section. An infusion of hypertonic saline was commenced during the 50th volume of the fMRI scan at the rate of 0.25ml/min using an infusion pump (Harvard Instruments, USA), and this rate was constantly adjusted to maintain a pain level of 5 out of 10 on a Numerical Rating Scale (0 = no pain, 10 = most intense pain imaginable). Subjects knew they were going to experience pain, but they were not informed as to the start time of the infusion. Throughout the entire scanning period, subjects gave feedback about their pain level by pressing 4 colour-coded buttons; each colour was associated with either "pain onset", "pain at 5/10", "pain below 5/10", "pain above 5/10." At the completion of the scanning session, each subject was asked to complete a McGill Pain Questionnaire (Melzack, 1975) and draw the area of perceived pain on a standard diagram of the leg. This was then placed into ImageJ to determine the total area of spread (reported in number of pixels).

5.4. Analysis

5.4.1. MSNA

Individual bursts of MSNA were displayed as a mean-voltage neurogram, computed as the root-mean-square (RMS) processed signal with a moving time average window

of 200ms. The 4 seconds inter-scan OFF period (see below) allowed the measurement of MSNA bursts. This 4s period was divided into 1s increments; for each second, bursts were manually counted and the amplitude was measured from the RMS-processed nerve signal (*Figure 5.1B*, *Figure 5.2A*).

5.4.2. Brain image processing

Using SPM12 (Friston, 1994) and custom software, all fMRI images were realigned, and co-registered to each individual's T1-weighted image set. The T1 image was then spatially normalized to the Montreal Neurological Institute (MNI) template, and the normalization parameters applied to all fMRI images. We used the VBM8 toolbox DARTEL template. This template was derived from 550 healthy control subjects of the IXI database (http://brain-development.org/) and is in MNI152 space. In all subjects, no pain was reported to occur during the first 70 brain volumes (or 9.33 minutes) and thus we selected this period to be the *baseline period.* In contrast, we found that between volumes 181 and 250, subjects reported sustained pain, and there was a significant change in MSNA; we selected this 70-volume (9.33 minutes) period as the *tonic pain period.* These periods were selected and separated so that they could then be detrended separately, in order to remove the potential influence of sustained changes in overall intensity within each voxel. The 70 volumes in each baseline and tonic pain period were then linearly detrended, bias corrected, and spatially smoothed using a 6mm full-width at half-maximum (FWHM) Gaussian filter. In addition, we performed a brainstem-specific analysis in subjects for whom we acquired images that extended to the caudal medulla (n=30). In each subject, the brainstem was isolated from the T1-weighted anatomical image and the parameters applied to the coregistered, realigned, and detrended fMRI images. The T1 images set was normalized to a

brainstem template in MNI space using the SUIT toolbox and the parameters applied to the fMRI images sets (Diedrichsen et al., 2011). These fMRI brainstem image sets were then spatially smoothed using a 4mm FWHM Gaussian filter.

As mentioned above, during the fMRI scan, all axial slices were collected during the first 4 s of the 8 s TR. Signal intensity changes were measured during the subsequent 4 s (ON period), taking into account the (+) 5 s neurovascular coupling delay between a neuronal event and the peak BOLD signal (Logothetis et al., 2001), and the (-) 1 s required for the burst of MSNA to travel from the brain to the peripheral recording site (Fagius & Wallin, 1980). Because the images were collected in a caudal to rostral direction, the region of the brainstem from the caudal medulla to the rostral pons was scanned in the 1st second, the rostral pons to the diencephalon in the 2nd second, the diencephalon and surrounding cortex in the 3rd second, and the remainder of the cortex in the 4th second (*Figure 5.1A*).

In each subject, and for each of the 4 s periods, the brain volumes during periods in which there were no bursts were averaged to create a mean *no MSNA burst* image. Similarly, brain volumes during periods in which there were MSNA bursts were averaged to create a mean *MSNA burst* image. These two images were created for the baseline and tonic pain periods separately (*Figure 5.1B*). This resulted in a total of 4 images for all 4 seconds for each subject. For each of the 4 seconds: (1) burst image for baseline, (2) no burst image for baseline, (3) burst image for pain, (4) no burst image for pain.

To determine brain areas in which signal intensity was greater during MSNA bursts compared to periods of no MSNA bursts, we entered these two images for the baseline period into a second level, random effects paired t-test for each of the 4-second periods. An initial threshold of $p < .001$, uncorrected was used to display regional changes. We then performed cluster correction (family wise error $p < .05$) to limit the prospects of Type II errors (Woo, Krishnan, & Wager, 2014). Clusters were overlaid onto a mean whole-brain and brainstem T1-weighted anatomical image calculated from all subjects in the study.

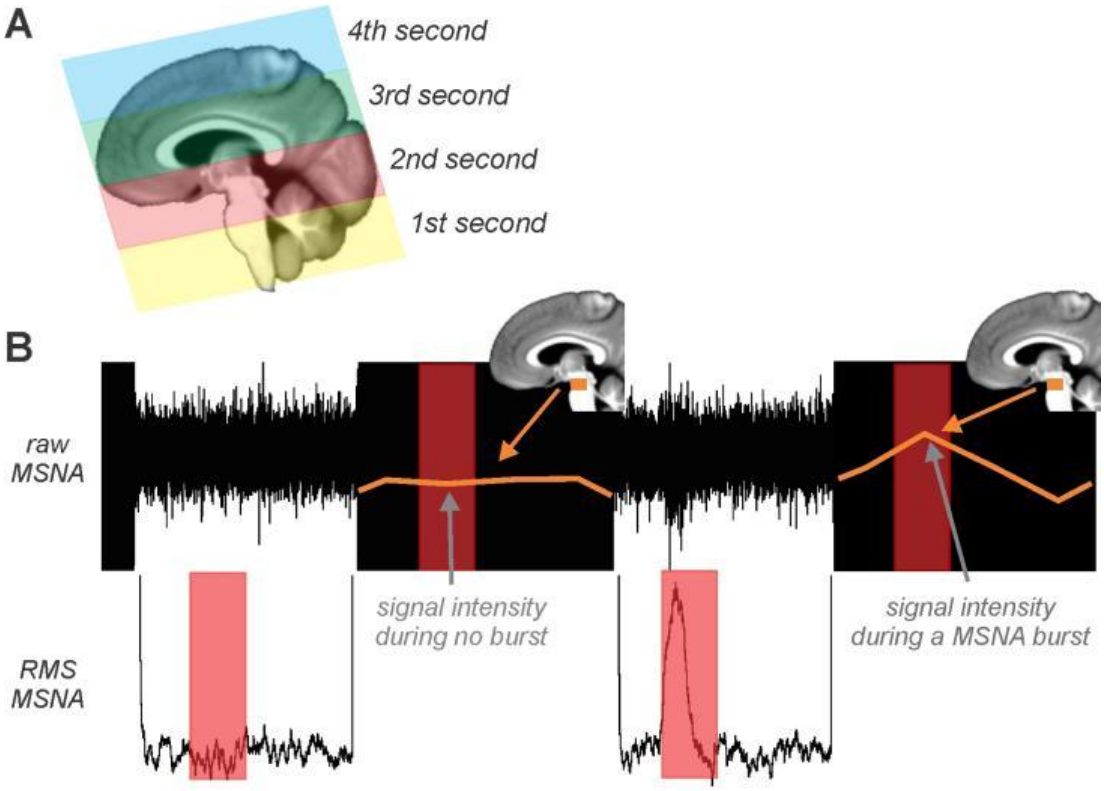

Figure 5.1: MSNA-coupled fMRI.

A: Sagittal section of the brain showing the location of functional magnetic resonances imaging (fMRI) scans collected during the 4 s period. Images were collected in a caudal to rostral direction, enabling the analysis to target specific brain regions by only using the input of muscle sympathetic nerve activity (MSNA) that occurred in the 1st (yellow), 2nd (red), 3rd (green), and 4th (blue) second of the 4 second interscan interval. B: Recording of MSNA in a

subject while performing fMRI of the brain. The filtered neurogram is shown in the top trace, the root-mean-square processed signal in the bottom trace. The black areas represent scanning artefacts during the 4 s "ON" periods when fMRI images were being acquired. The red shading corresponds to the 2nd second of the interscan interval. The signal intensity values for the highlighted brain region (orange rectangle) are schematically plotted as the orange line. The MSNA-coupled signal intensity change was calculated for images in each second by averaging signal during an MSNA burst and subtracting the signal during period of no bursts. MSNA: muscle sympathetic nerve activity; RMS: root-mean-square.

In addition, to determine changes in MSNA-coupled BOLD signal intensity evoked by tonic pain, we subtracted the mean *MSNA no burst* image from the mean *MSNA burst* image during the baseline and tonic pain periods. This resulted in a single baseline and a single tonic pain image for each subject, for each of the 4 seconds. Thereby, each voxel's signal intensity value reflected the difference between periods of no bursts compared to periods of MSNA bursts.

The percentage difference between these two images was then calculated for each voxel, resulting in a brain map in which each voxel's value was the percentage change in MSNA-coupled signal intensity during tonic pain compared with baseline. These brain maps were placed into a second level random effects analysis, where relationships between percentage change in MSNA-coupled signal intensity and percentage change in MSNA amplitude during tonic pain were determined ($p < .001$, uncorrected). Significant clusters were overlaid onto a mean whole-brain and brainstem T1-weighted anatomical image, calculated from all subjects in the study. Finally, for each significant cluster, the percentage differences in MSNA-coupled signal intensity changes during pain compared with baseline were extracted and plotted against the overall change in MSNA amplitude during pain.

5.5. Results

5.5.1. Participants

The resting mean blood pressure was 86.3±2.0 mmHg (range: 68.7-105.5mmHg), resting mean heart rate was 66.4±1.7 beats/min (range: 50-85 beats/min), resting MSNA frequency 12.0±1.5 bursts/min (range: 2-35 bursts/min), MSNA amplitude change for the last pain period was +42.7±16.6% (range: -91.7 to +388.1%), PCS: 12.0±1.2 (range: 0-27), PASS: 28.8±2.8 (range: 2-72), PVAQ: 34.7±1.6 (range: 16-49), state anxiety: 31.4±1.9 (range: 20-52), and trait anxiety: 35.1±1.4 (range: 24-50); see *Table 5.1* below.

Table 5.1: Subject information.

Age, sex, resting mean BP, resting HR, resting MSNA frequency, MSNA amplitude change during pain, PCS, PASS, PVAQ, and STAI averages of all participants.

	Mean	***SEM***
Sex	11F, 26M	
Age	22.0	0.5
Mean BP (mmHg)	86.3	2.0
HR (beats/min)	66.4	1.7
Resting MSNA (bursts/min)	14.3	1.7
MSNA amplitude during pain (% change)	42.7	16.6
PCS	12.0	1.2
PASS	28.8	2.8
PVAQ	34.7	1.6
State anxiety	31.4	1.9

	Mean	*SEM*
Trait anxiety	35.1	1.4

5.5.2. Psychophysics

In all subjects, intramuscular infusion of hypertonic saline elicited continuous muscle pain, which began, on average, 22 volumes (176 seconds) after the start of the infusion (i.e. at volume 72) and continued at a mean pain intensity of 5.8±0.1 (range: 5-7) for the remainder of the fMRI scan. The mean volume of infusion was 18.6±1.4 ml. The mean pain spread depicted on the McGill pain questionnaire involved an area of 1039±80 pixels (as measured from the area of pain subjects drew on an image of a leg). The most frequent descriptors chosen from the McGill pain questionnaire to describe the tonic pain were “dull”, “aching”, and “throbbing”.

5.5.3. MSNA rest vs pain:

The mean percentage change during the last 70 volumes of pain compared to the 70 volumes of baseline was +42.7±16.6%. The amplitude change during this period ranged between -91.7% and +388.1% across the 37 subjects (*Figure 5.2B*).

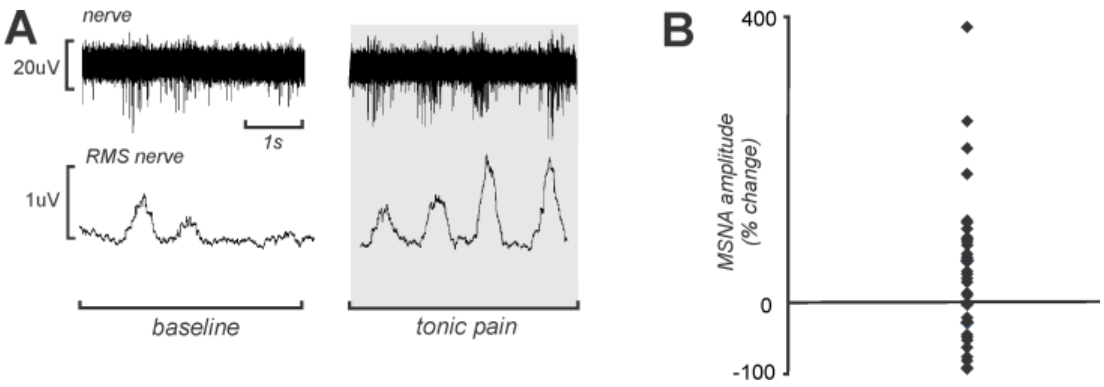

Figure 5.2: MSNA recording and percentage changes.

A: Extract of an MSNA recording from the common peroneal nerve in a subject in whom MSNA amplitude increased during intramuscular infusion of hypertonic saline into the tibialis anterior muscle (grey shading, right), compared to baseline (left). Grey shading indicates the tonic pain period. B: Plot of the mean percentage change in MSNA amplitude for the last 70 volumes of pain compared to baseline in 37 subjects. MSNA: muscle sympathetic nerve activity; RMS: root-mean-square; uV: microvolts.

5.5.4. MSNA-coupled signal intensity changes

5.5.4.1. Baseline

During the baseline period, increases in MSNA-coupled signal intensity occurred in the rostral medulla encompassing the area of the left rostroventrolateral medulla (RVLM; mean±SEM percent change burst versus no burst: 0.34±0.14), left mid-insula (0.17±0.03), right mid-insula (0.17±0.05), left posterior cingulate cortex (PCC; 0.14±0.04), left dorsolateral prefrontal cortex (dlPFC; 0.22±0.07) and left precuneus (0.27±0.07; *Figure 5.3*, *Table 5.2*). In contrast, decreases in MSNA-coupled signal intensity occurred in only one region, in the rostral midbrain encompassing the region of the left midbrain periaqueductal grey matter (PAG; -0.25±0.07).

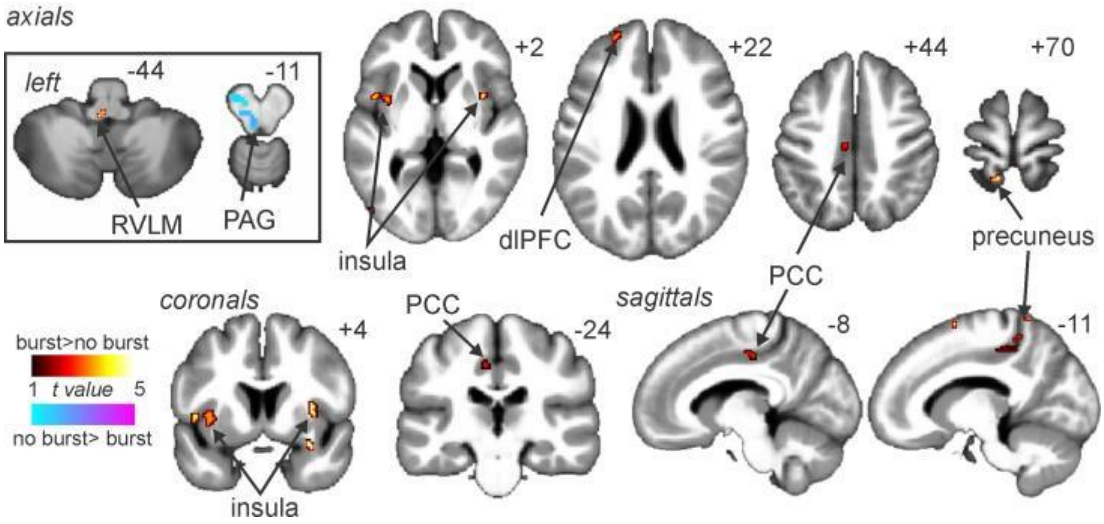

Figure 5.3: Signal intensity increases and decreases during MSNA bursts compared to periods of no bursts at rest.

Signal intensity increases (hot colour scale) and decreases (cool colour scale) during MSNA bursts compared to periods of no bursts, at rest in all 37 subjects. Significant clusters were overlaid onto a mean T1-weighted anatomical image set created from all 37 subjects. Slice locations in Montreal Neurological Institute space are indicated in the top right of each image. The left side is the side contralateral to the side of microneurography recording. The inset on the left represents the results from a brainstem-specific analysis. dlPFC: dorsolateral prefrontal cortex; PAG: periaqueductal grey; PCC: posterior cingulate cortex; RVLM: rostral ventrolateral medulla.

Table 5.2: Brain regions showing signal intensity increases or decreases during MSNA bursts compared to no bursts at rest.

Locations in Montreal Neurological Institute space, t values, and cluster sizes of regions in which signal intensity increased or decreased during muscle sympathetic nerve activity bursts compared with periods of no MSNA bursts while the subject was at rest.

Signal intensity increases	***x***	***y***	***z***	**t value**	**Cluster size**
Dorsolateral prefrontal cortex					
Left	-22	54	24	3.41	6
Insular cortex					

Signal intensity increases	***x***	***y***	***z***	**t value**	**Cluster size**
Left	-34	2	0	5.07	65
Right	38	6	2	3.62	15
Posterior cingulate cortex					
Left	-6	-22	44	3.84	13
Precuneus					
Left	-12	-52	70	3.55	7
Rostral ventrolateral medulla					
Left	-2	-40	-43	2.58	4
Signal intensity decreases	***x***	***y***	***z***	**t value**	**Cluster size**
Periaqueductal grey					
Left	-8	-26	-11	2.84	18

5.5.4.2. Tonic Pain

During tonic pain, a number of brain regions displayed significant correlations between percentage changes in MSNA-coupled signal intensity and MSNA-amplitude (*Figure 5.4*, *Figure 5.5*, *Figure 5.6*, *Table 5.3*). It can be seen in *Figure 5.5* and *Figure 5.6* that one subject had a very high MSNA value, accordingly we have performed statistical analyses with and without the outlier. Significant positive relationships occurred in the dorsal closed medulla in the region of the right nucleus tractus solitarius (NTS; r = 0.46, p = .01; r = 0.43, p = .02 without outlier), right midbrain in the region of the ventrolateral PAG (r = 0.41, p = .03; r = 0.57, p = .001 without outlier), left insula (r = 0.65, p < .001; r = 0.48, p = .004 without outlier), left medial prefrontal cortex (mPFC; r = 0.64, p < .001; r = 0.28, p = .10 without outlier), right dlPFC (r = 0.59, p < 0.001; r = 0.42 p = .01 without outlier) and the right anterior cingulate cortex (ACC; r = 0.57, p < .001; r = 0.28, p = .11 without outlier). Significant negative

relationships occurred in the left dlPFC (r = -0.55, $p < .001$; r = -0.55, $p < .001$ without outlier) and the left mPFC (r = -0.58, $p < .001$; r = -0.32, $p = .06$ without outlier). After excluding the outlier, significant positive relationships were still apparent for the NTS, PAG, left insula, and significant negative relationships for the right dlPFC and left mPFC.

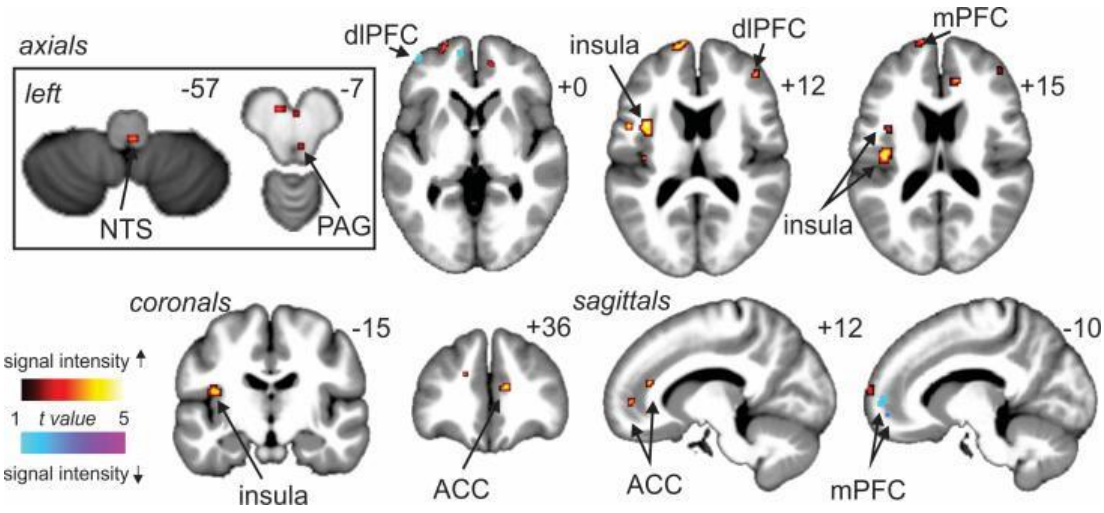

Figure 5.4: Brain regions in which MSNA-coupled signal intensity changes are significantly correlated with MSNA amplitude.

Brain regions in which muscle sympathetic nerve activity (MSNA) -coupled signal intensity changes were significantly positively (hot colour scale), or negatively (cool colour scale) correlated with MSNA amplitude change in 37 subjects. Significant clusters were overlaid onto a mean T1-weighted anatomical image set created from all 37 subjects. Slice locations in Montreal Neurological Institute space are indicated in the top right of each image. The inset on the left represents the results from a brainstem-specific analysis. ACC: anterior cingulate cortex; dlPFC: dorsolateral prefrontal cortex; mPFC: medial prefrontal cortex; NTS: nucleus tractus solitarius; PAG: periaqueductal grey.

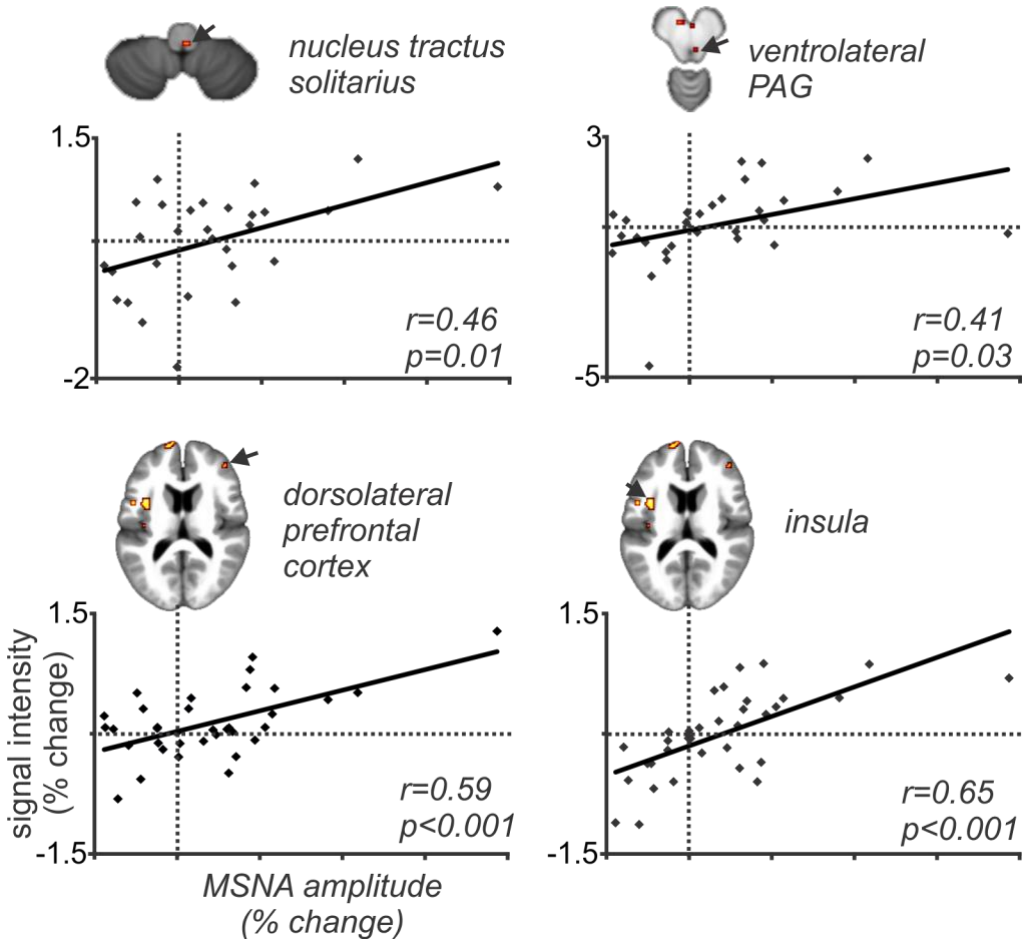

Figure 5.5: Relationship between MSNA-coupled signal intensity changes and MSNA amplitude change during pain.

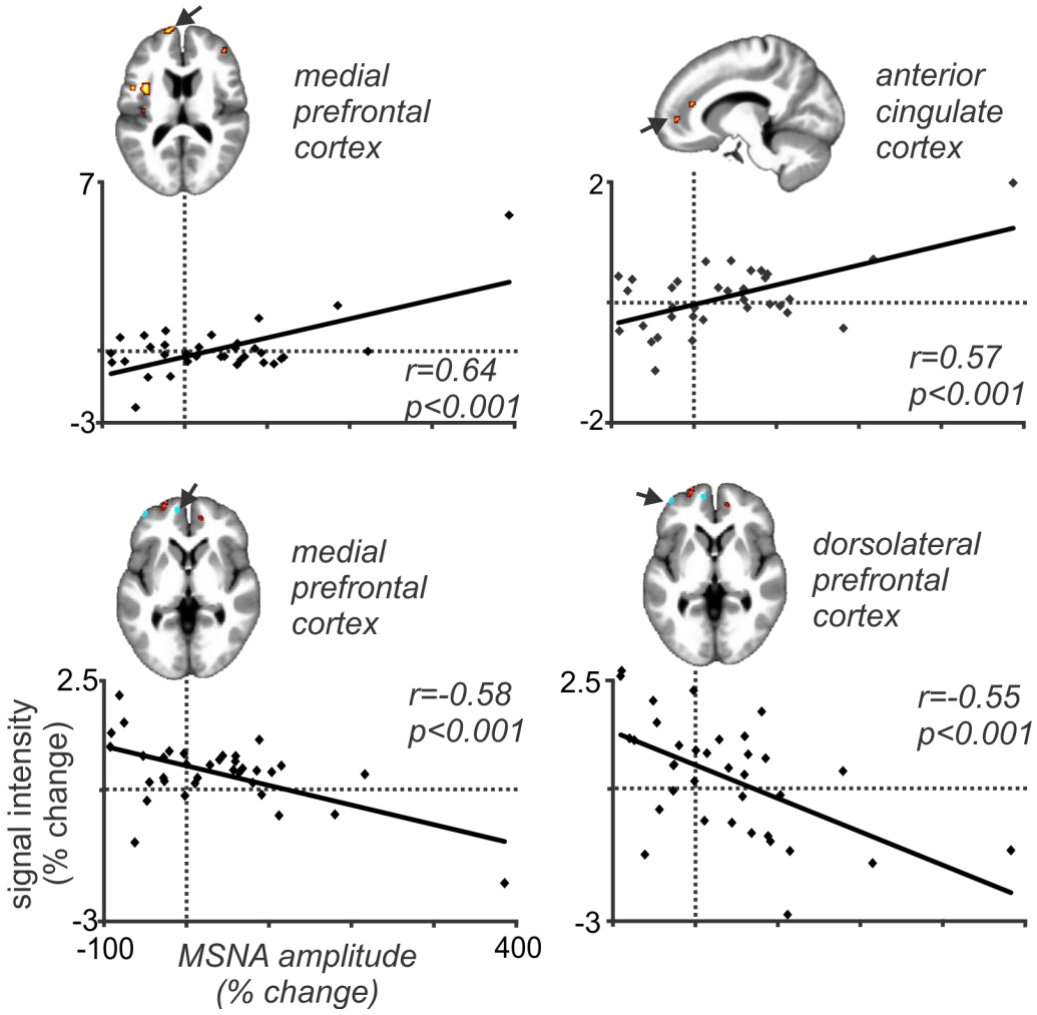

Figure 5.6: Relationship between MSNA-coupled signal intensity changes and MSNA amplitude change during pain (cont'd.)

Plots of the muscle sympathetic nerve activity (MSNA)-coupled signal intensity changes against MSNA amplitude change during tonic pain in all 37 subjects. In all regions, there is a significant linear relationship between MSNA burst-to-burst signal changes during tonic pain compared with baseline with the overall change in MSNA amplitude. PAG: periaqueductal grey.

Table 5.3: MSNA-coupled signal intensity changes during tonic pain compared to rest.

Locations in Montreal Neurological Institute space, t values, and cluster sizes of regions in which muscle sympathetic nerve activity (MSNA) coupled signal intensity changes during tonic muscle pain were significantly different to those at rest.

Signal intensity increases	***x***	***y***	***z***	**t value**	**Cluster size**
Anterior cingulate cortex					
Right	15	50	0	3.74	3
	10	34	16	3.81	4
Dorsolateral prefrontal cortex					
Right	38	36	14	3.64	4
Insular cortex					
Left	-40	-18	14	4.05	37
	-36	6	12	4.07	27
Medial prefrontal cortex					
Left	-6	64	8	5.04	45
Midbrain periaqueductal grey					
Right	4	-32	-7	2.33	1
Nucleus tractus solitarius					
Right	2	-44	-57	2.66	2
Signal intensity decreases	***x***	***y***	***z***	**t value**	**Cluster size**
Dorsolateral prefrontal cortex					
Left	-40	52	2	4.21	5
Medial prefrontal cortex					
Left	-8	56	4	4.07	27

5.6. Discussion

In this study, we reveal MSNA-coupled signal intensity changes produced by tonic muscle pain. Although many previous investigations have explored changes in signal intensity associated with changes in overall MSNA during various challenges, this is the first to compare signal intensity changes during MSNA bursts at baseline and during a challenge such as tonic muscle pain. We found that during tonic pain MSNA-coupled signal intensity changes were positively correlated to MSNA amplitude in areas of the brainstem known to regulate the cardiovascular responses to pain, such as the PAG. In addition, we found both positive and negative correlations between MSNA-coupled signal changes and MSNA amplitude in higher order association areas, such as the insular, cingulate, and prefrontal cortices. Some of these regions also showed MSNA-coupled signal changes at rest, as we had shown previously (James et al., 2013; Lundblad et al., 2014), indicating that they are modulated during tonic pain to evoke an overall change in MSNA.

Hypertonic saline infusion into the tibialis anterior muscle induced pain that was described as dull, aching and throbbing. Consistent with our previous investigations, we found that tonic muscle pain evoked increases in MSNA amplitude in some individuals and decreases in others (Fazalbhoy et al., 2012, 2014; Kobuch et al., 2015, 2016, 2017). These changes began approximately 20 volumes (160 seconds) after the start of the hypertonic infusion, at approximately the same time as subjects began to perceive pain and remained fairly stable for the duration of the pain period. Importantly, and unlike any previous investigation, we have determined brain regions that influence MSNA amplitude in a *burst-to-burst* (MSNA-coupled) manner rather than in a

sustained manner during a cardiovascular challenge. Previous studies investigating brain activation patterns during various cardiovascular challenges have explored gradual signal intensity changes associated with sustained changes in BP, HR, MSNA, or other autonomic variables (Beissner et al., 2013 for review; Harper et al., 2003 (cold pressor); Henderson et al., 2002 (Valsalva)). Although these studies are valuable, they are unable to explore signal intensity changes in brain regions that directly drive each MSNA burst. We find in this study a series of brain regions that are only revealed through such an analysis, which provides a basis for exploring regions that may be recruited only during various cardiovascular challenges and only in a burst-to-burst manner.

Consistent with our previous studies, we found that at rest, signal intensity changes coupled to MSNA bursts occurred in the region of the RVLM, insula, cingulate, prefrontal cortex, and precuneus (James et al., 2013; Lundblad et al., 2014; Macefield and Henderson, 2010). These results were consistent despite a subtle difference in the analysis methods, i.e. signal intensity changes coupled to MSNA burst *pattern*, versus mean signal during MSNA bursts compared with no bursts. One region that was different between this and our previous investigations was the PAG; we previously reported positive signal coupling with MSNA, whereas in this study we found decreased MSNA-coupled signal intensity in the PAG at rest (Lundblad et al., 2014). Why this disparity occurs is not clear, however it is possible that it arises from differences in methodological approaches. In addition to the PAG, tonic pain evoked MSNA-coupled signal changes associated with MSNA amplitude in other regions, which also display MSNA-coupled signal changes at rest, such as the NTS, and the insular, cingulate, and prefrontal cortices. This suggests that these regions are not

necessarily recruited during tonic pain itself, but instead are modulated so that their activities drive either increases or decreases in MSNA amplitude.

Consistent with this idea, we found that the PAG, the right dlPFC, and the right ACC each displayed both burst-to-burst *and* sustained signal intensity changes during tonic muscle pain (Kobuch et al., 2017). Indeed, we previously found that the dorsolateral PAG (dlPAG) displayed *sustained* increases in signal intensity during tonic pain that were negatively correlated to MSNA amplitude, i.e. the greater the signal change, the lower the MSNA amplitude change (Kobuch et al., 2017). In contrast, in the current study we found that positively correlated MSNA-coupled signal changes during muscle pain occurred in the region of the ventrolateral PAG (vlPAG).

The PAG is arranged in a columnar fashion, based on histological and functional differences (Keay & Bandler, 2001). In animals, it is well established that the PAG is activated by both superficial and deep pain. Indeed, superficial pain activates the lateral PAG column, and direct stimulation of this column of the PAG elicits active behavioural responses that are coupled with increases in blood pressure, heart rate, and sympathetic activity (Bandler et al., 2000; Carrive et al., 1987; Carrive et al., 1988; Hilton & Redfern, 1986; Meller & Dennis, 1991; Van Bockstaele et al., 1991; Yardley & Hilton, 1986). In contrast, deep pain activates the ventrolateral column of the PAG, and direct stimulation of this column evokes passive, quiescent coping behaviours, and decreases in BP, HR, and sympathetic activity (Carrive & Bandler, 1991; Lovick, 1992). Further, unlike the vlPAG, which receives noxious muscle and visceral inputs from the spinal cord and brainstem, the dlPAG receives inputs from primarily prefrontal and cingulate cortices (Floyd et al, 2000). This difference in input patterns

has led to the suggestion that the dlPAG produces active coping strategies in response to psychological stressors, whereas the vlPAG produces passive coping responses in response to deep physical stressors (Keay & Henderson, 2010). This idea is consistent with our own findings; although overall signal intensity within the vlPAG did not change during tonic muscle pain, MSNA-coupled signal intensity changed in a manner consistent with each individual's overall MSNA response. Increases in MSNA-coupled signal intensity in the vlPAG paralleled increases in MSNA amplitude, and MSNA-coupled signal intensity decreases in the vlPAG were accompanied by decreases in MSNA amplitude. It is possible that the vlPAG is involved in driving the change in MSNA burst amplitude on a burst-to-burst basis, whereas the dlPAG may provide more of a tonic modulatory role during tonic muscle pain.

Similar to the PAG, the ACC and dlPFC displayed MSNA-coupled signal intensity changes, which paralleled MSNA amplitude changes: increases in MSNA amplitude were accompanied by increases in MSNA-coupled signal intensity in these regions and decreases in MSNA amplitude were accompanied by decreases in MSNA-coupled signal intensity in these regions. Further, we previously showed that these two regions displayed sustained intensity increases during tonic muscle pain in a pattern that matched the increase in MSNA amplitude (Kobuch et al., 2017). A role for these regions in mediating changes in autonomic function has been recognized for some time. For example, the ACC and dlPFC are both activated during cardiovascular challenges such an inspiratory capacity apnoea (Kimmerly et al., 2013; Macefield et al., 2006). Furthermore, we have shown that these regions display MSNA-coupled signal changes even at rest.

In our previous study, we found that the insular cortex did not display sustained signal intensity changes coupled to sustained changes in MSNA during tonic muscle pain (Kobuch et al., 2017). This is surprising given the growing body of literature describing insular cortex changes during various cardiovascular challenges (Beissner et al., 2013; Butcher & Cechetto, 1995; Harper et al., 2003; Henderson et al., 2002; Macefield et al., 2006; Macey et al., 2003). However, we found in the current study that the left insula displayed robust changes in MSNA-coupled signal intensity that were indeed associated with overall changes in MSNA. Consistent with previous findings, the left and right insula exhibited MSNA-coupled signal intensity increases at rest (Fatouleh et al., 2014; James et al., 2013). During tonic pain, this MSNA-coupled signal change was located more dorsally in mid- and anterior insula and only on the left side, contralateral to the noxious stimulus. We have previously reported that intramuscular injection of hypertonic saline into the right tibialis anterior muscle causes sustained signal increases in the contralateral posterior insula and ipsilateral anterior insula and not in the contralateral anterior insula (Henderson et al., 2007). It has been proposed that these regions are involved in the link between sensory and emotional aspects of pain processing (Craig, 2011). The data presented here reveals that the contralateral mid- and anterior dorsal insula display MSNA-coupled signal changes during pain and are thus likely involved in mediating the cardiovascular aspect of pain. Interestingly, it has been revealed that the mid- and anterior insula are activated during a range of autonomic challenges such as the Valsalva manoeuvre, handgrip and cold pressor test, whereas the posterior insula is not (Macey et al., 2012). This raises the prospect that different insula regions may be preferentially involved in mediating the sensory, affective and cardiovascular response to sustained muscle pain.

In direct contrast to the above-mentioned regions, in which the magnitudes of MSNA-coupled signal changes alter during tonic muscle pain, a number of important brain regions did not display pain-related changes. For example, although we found that the RVLM and precuneus displayed MSNA-coupled signal changes at rest, consistent with our earlier studies (James et al., 2013; Macefield & Henderson, 2010), these signal intensity increases did not change in magnitude during each burst of MSNA during tonic muscle pain. However, both of these regions displayed *sustained* signal intensity increases in the increasing MSNA group, and decreases in the decreasing MSNA group (Kobuch et al., 2017). Therefore, the RVLM and precuneus are not recruited to fire either more or less in a burst-to-burst fashion during tonic muscle pain, but instead may provide a tonic modulatory role.

Finally, it is important to note that the areas identified in this study overlap with regions thought to be involved in descending pain modulation (Millan, 2002). Furthermore, it is interesting that there appears to be a relationship between the cardiovascular and descending pain modulation systems, since the greater the magnitude of cardiovascular response during the cold pressor test, the greater an individual's endogenous analgesic ability (Chalaye et al., 2013). Our findings that MSNA-related regions overlap with those involved in endogenous analgesia further support the idea that the two systems are intertwined.

5.7. Limitations

As with all studies there are limitations to the technique and methods of analysis. Whilst the brainstem is small relative to the spatial resolution obtained in most fMRI investigations, one of the major limitations of brainstem analysis is accurate spatial

normalization. To overcome this issue, we used a brainstem-specific toolbox, which isolates the brainstem; we then manually selected the brainstem in each individual subject. This was then used to spatially normalize the image sets, which creates greater consistency between subjects with respect to their brainstems' final location in MNI space. Given this we are confident that the changes we report in this study, particularly those in the brainstem, are accurate. Another limitation relates to the number of volumes chosen for the baseline and tonic pain periods. We collected 70 brain volumes prior to the onset of pain and, to be consistent, we chose 70 volumes during the tonic pain period, when the level of pain was stable. A greater number of volumes in both periods would have been desirable, to increase reliability, and in future investigations we aim to collect longer baseline periods. We did, however, find consistent MSNA-coupled signal changes at rest when compared to our previous studies in which we analysed 200 brain volumes, so we are confident that the results presented are indeed accurate.

Finally, we acknowledge that the regional hemodynamic response curve has a wide range, and therefore using a fixed 4-s ON and 4-s OFF protocol prevents us from taking into account individual variations. However, we have used this same protocol in many previous investigations where we concurrently recorded MSNA and fMRI, and have demonstrated that the BOLD signal intensity is temporally coupled to the bursts of MSNA recorded peripherally. Indeed, MSNA-coupled fMRI allows one to identify regions that are temporally coupled to the firing of an MSNA burst, which (with the exception of certain pathophysiological states) do not occur in every heartbeat. In our previous investigations, we had shown that both increases and *decreases* in BOLD signal occurred. Importantly, these were not *general* changes in BOLD intensity,

which one would expect from physiological noise related to ongoing cardiac (and respiratory) pulsations within the brain, but discrete. In this study we were not able to filter out potential physiological noise (Brooks, Faull, Pattinson, & Jenkinson, 2013) since we were exploring MSNA-coupled signal intensity changes, and MSNA bursts are tightly coupled to the cardiac rhythm. Removing frequencies (or their harmonics) that represent cardiac pulsatile signals would also remove MSNA-coupled signal changes. Since periods in which there were no MSNA bursts also contain cardiac beats, any potential effect of physiological noise would be equivalent during both periods. Furthermore, given that the discrete areas of increase or decrease in BOLD signal were localised to cortical and subcortical regions known to contribute to cardiovascular regulation in experimental animals, the most parsimonious explanation is that the observed changes in BOLD signal intensity reflect proxy markers of functional changes in neuronal activity, rather than physiological artefact.

5.8. Conclusions

For the first time, we have shown brain regions in which MSNA-coupled signal intensity changes are altered during a challenge that affects sympathetic outflow to the muscle vascular bed. Whilst it is important to understand brain regions that are associated with sustained changes in sympathetic drive, we show here that signal intensity changes in numerous brain regions occur only in a burst-to-burst manner. We provide evidence that some regions display both sustained and burst-to-burst changes whereas others display one or the other. These data are consistent with the idea that changes in MSNA result from a combination of sustained and burst-to-burst changes in activity in various brain regions. Whether these changes persist during chronic pain remains unknown. However, investigating these potential changes in chronic pain

would require knowing the baseline parameters prior to the development of chronic pain.

CHAPTER 6 The effects of audio-visual distraction on the muscle sympathetic responses to experimental muscle pain

Chapter 6 is published as:

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6.1. Abstract

Pain elicited by intramuscular infusion of hypertonic saline solution causes muscle sympathetic nerve activity (MSNA) to increase in some subjects, yet decrease in others. Although the direction of the response is not predictable based on baseline physiological and psychological parameters, we know that it results from sustained functional changes in specific brain regions that are responsible for the behavioural and cardiovascular responses to psychological stressors, as well as those involved in attention. The aim of this study was to investigate whether MSNA responses to experimental muscle pain in humans could be altered with an audiovisual stimulus that served to distract them from the pain. MSNA was recorded from the left common peroneal nerve of 20 young healthy individuals during a 45-minute intramuscular infusion of hypertonic saline solution into the ipsilateral tibialis anterior muscle. The distracting stimulus commenced 15 minutes after the start of the infusion and lasted for 15 minutes. Fifteen subjects showed an increase in mean burst amplitude of MSNA (to 176.4±7.9% of baseline), while 5 showed a decrease (to 73.1±5.2% of baseline); distraction had no effect on these profiles. These results indicate that even though the subjects were attending to the audiovisual stimulus, and were presumably distracted from the pain, it failed to alter the MSNA responses to muscle pain.

6.2. Introduction

The sympathetic nervous system contributes importantly in generating physiological responses to pain, with recent work in human subjects having characterized the changes in sympathetic outflow to muscle and skin during delivery of noxious stimuli to muscle or skin (for review see Burton et al., 2017). We recently demonstrated that

a prolonged (~45min) infusion of hypertonic saline into a leg muscle, which induces muscle pain for ~1 hour, causes a sustained increase in muscle sympathetic nerve activity (MSNA) amplitude in some subjects but a sustained decrease in others (Fazalbhoy et al., 2012, 2014; Kobuch et al., 2015, 2016, 2017). We cannot explain these inter-individual differences by sex, age, anxiety levels, attitudes to pain, or levels of resting MSNA, blood pressure, or heart rate (Kobuch et al., 2015, 2016). Moreover, they are reproducible over two experimental sessions, suggesting that there is something "hard-wired" in a given subject's pattern of response (Fazalbhoy et al., 2014). Using concurrent recordings of MSNA and functional magnetic resonance imaging (fMRI) of the brain, we recently found that certain cortical areas (precuneus, prefrontal, cingulate), as well as the hypothalamus, midbrain and medulla, displayed sustained differences in BOLD (blood oxygen level dependent) signal intensity in the two groups of subjects, despite the reported pain levels, and quality of pain, being statistically identical in the group of subjects in whom MSNA increased and the group in whom MSNA decreased (Kobuch et al., 2017, 2018). We suggested that the divergent sympathetic responses to an identical noxious input resulted from the activation of a neural pathway that included the dorsomedial hypothalamus and dorsolateral periaqueductal grey – which are thought to be responsible for the behavioral and cardiovascular responses to psychological stressors (Keay & Henderson, 2010). Furthermore, differences in BOLD signal intensity in the precuneus lead us to believe that attention to the noxious stimulus may influence the sympathetic response to muscle pain. Indeed, the precuneus is known to form part of the default mode network, which comprises specific areas of the brain that display high levels of activation when an individual is at rest with eyes closed but exhibit inactivation during an attention-demanding or goal-directed task (Fransson, 2006; Raichle et al., 2001).

The effects of distraction from a physical stimulus on the physiological responses to the stimulus has had mixed results. For example, some studies have shown that distraction reduces distress during pain (Eccleston, 1995; McCaul & Haugtvedt, 1982; Wack & Turk, 1984), while others have found no physiological or behavioral changes (Leventhal et al., 1979; McCaul et al., 1992). A study conducted by Terkelsen and colleagues showed that mental stress - but not attention to the stimulus - inhibited pain perception and resulted in changes in heart rate variability (Terkelsen et al., 2004). Furthermore, it has been reported that distraction can lead to dampening of the cardiovascular responses to anger recall and rumination (Gerin et al., 2006; Glynn et al., 2002; Neumann et al., 2004).

In this study, we set out to assess whether distraction – via engagement in an affectively neutral competing task (i.e. an audiovisual stimulus) – could influence the muscle sympathetic responses to muscle pain. Specifically, we tested the hypothesis that distraction would *reduce the increase in MSNA* in those subjects in whom MSNA increased during tonic muscle pain.

6.3. Methods

6.3.1. Participants

From the inception of the study we had decided to limit the number of experiments to twenty healthy individuals, reasoning that if distraction did have a significant effect on the pattern of sympathetic response it should be clear with 20 participants. We adopted

a gender-balanced design: 10 females and 10 males, with a mean age of 25.0±1.9 years. All participants provided written informed consent to the experimental procedures, which were conducted under the approval of the Human Research Ethics Committee of Western Sydney University and satisfied the requirements of the Declaration of Helsinki. No subject had any history of cardiovascular disease or chronic musculoskeletal pain.

6.3.2. Microneurography

The protocol for MSNA and cardiorespiratory parameters measurement were described in the general methods section.

6.3.3. Noxious stimulation

The protocol for the noxious stimulation has been described in detail in the general methods section.

6.3.4. Procedure

After 5 min of recording resting activity, the infusion was started, at a time unknown to the subject, at an initial rate of 0.25 ml/min; the infusion rate was adjusted throughout the duration of the experiment in order to maintain a pain level of 5-6/10 on a visual analog scale (VAS). Once a stable rating of 5-6/10 pain was reached, the physiological parameters were recorded continuously for 15 min (pain period 1). Then, while the infusion was continuing, an audiovisual stimulus – a documentary of the Australian landscape – was displayed via a video projector for 15 min (pain period 2). The documentary was chosen as it was deemed an emotionally neutral stimulus. During this time, the subjects were asked to focus on the documentary and to stop

rating the pain. The participants were told to notify the investigator if the pain reached a level that was higher than 5-6/10. This did not occur in any of the experiments. When the 15-minute audiovisual stimulus was finished, the infusion was maintained for another 15 min, and subjects were asked to resume rating the pain (pain period 3).

6.3.5. Analysis

Bursts of MSNA were displayed as a mean voltage neurogram, computed as the root-mean-square (RMS) processed signal with a moving time average window of 200ms. The signal was analysed using the Peak Parameters module of the LabChart 7 Pro software (ADInstruments, Sydney, NSW, Australia) to calculate the amplitude of each burst. The absolute values were averaged into 5min blocks, normalized to the baseline and reported as percentage change from baseline (100%). The average of all 5min blocks was taken in order to determine the direction of the MSNA amplitude response. Subjects in whom overall MSNA amplitude change was greater than 10% during pain were classified as 'increasing', and vice versa for the 'decreasing' group. Statistical analysis was performed using Prism version 6 for Mac OS X (GraphPad Software, San Diego, CA, USA). For normally distributed data we used a repeated-measures one-way analysis of variance (rmANOVA), coupled with Dunnett's multiple comparisons test, to determine changes over time; for non-normally distributed data we used Friedman's ANOVA, coupled with Dunn's multiple comparison test. Otherwise, the paired t-test or Wilcoxon signed-rank test was used. All data were expressed as means plus standard error of the mean (SEM). Probability levels of $p<0.05$ were deemed significant.

6.4. Results

6.4.1. Subjective experience of pain

The pain rating remained constant at 5-6 out of 10 throughout the duration of the infusion. Although the subjects were asked not to rate the pain during the distraction task, when asked afterwards no subject reported changes in their perception of pain intensity while watching the documentary.

Table 6.1: Subject information.

Subject characteristics, resting MSNA burst frequency and incidence, and cardiovascular parameters.

Variable	***Increasing MSNA Group (n=15)***	***Decreasing MSNA Group (n=5)***	***p value***
Sex	9F, 6M	1F, 4M	
Age	25.5±1.8	23.6±2.9	.6
Body mass index (kg/m^2)	27±2	21±1	.08
MSNA burst frequency (burst/min)	17±1	20±3	.5
MSNA burst incidence (bursts/100 heart beats)	24±2	31±5	.4
Systolic arterial pressure (mmHg)	137±6	141±3	.7
Diastolic arterial pressure (mmHg)	71±3	75±3	.5
Mean arterial pressure (mmHg)	91±3	95±4	.5
Heart rate (beats/min)	72±3	72±4	1.0

6.4.2. Effects of pain and distraction on cardiovascular parameters: population response

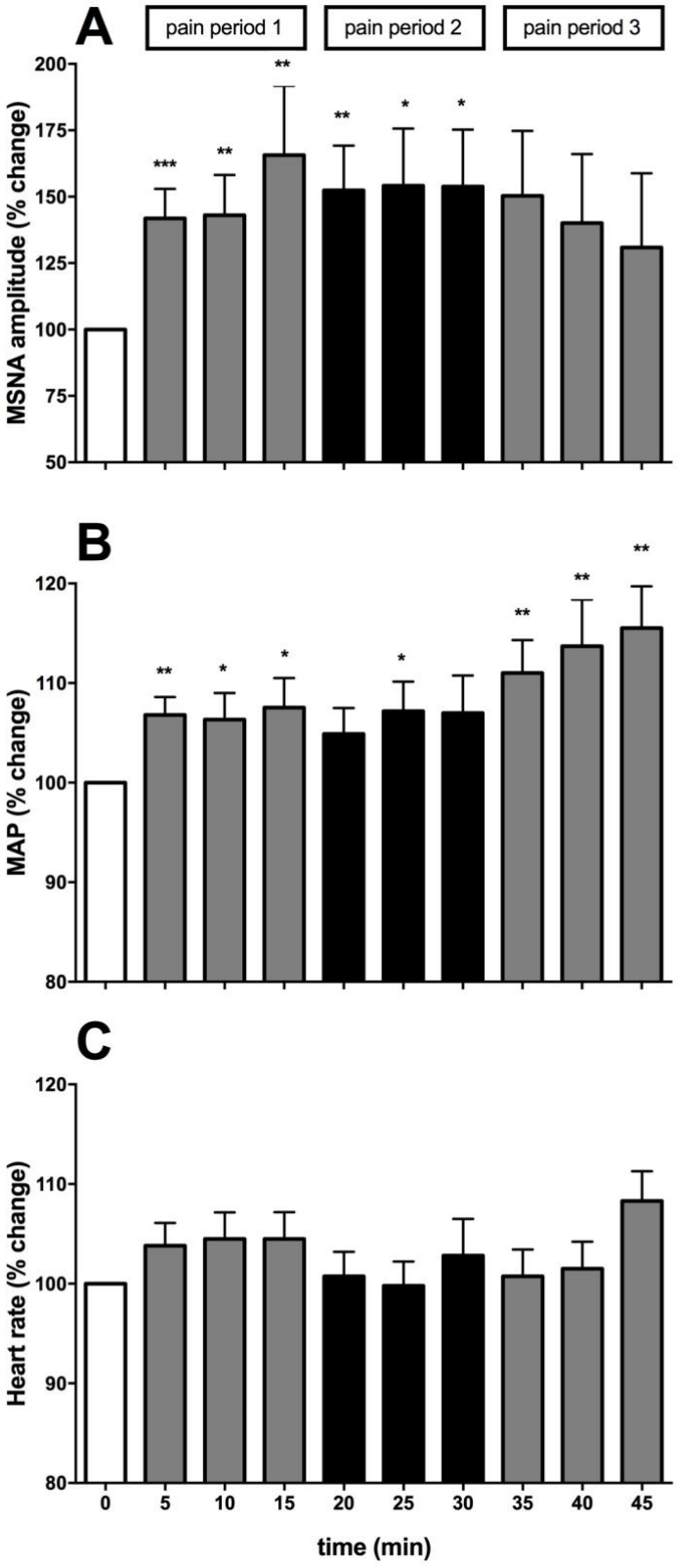

Figure 6.1 shows the pooled data, sampled every 5 minutes, for normalised MSNA, mean arterial pressure (MAP) and heart rate (HR). It can be seen that for the whole sample of 20 subjects, normalised MSNA increased during tonic muscle pain

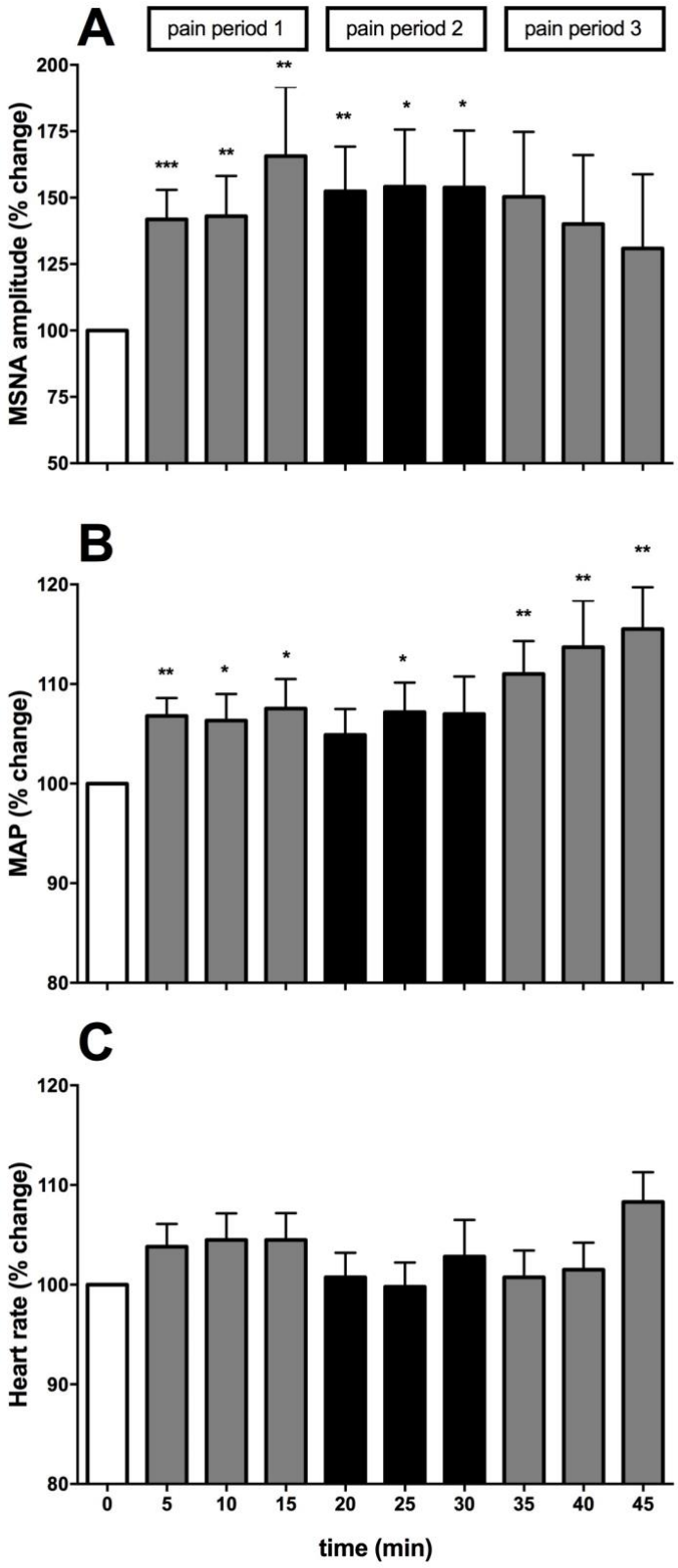

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Figure 6.1A). On average, MAP (*Figure 6.1B*) and HR

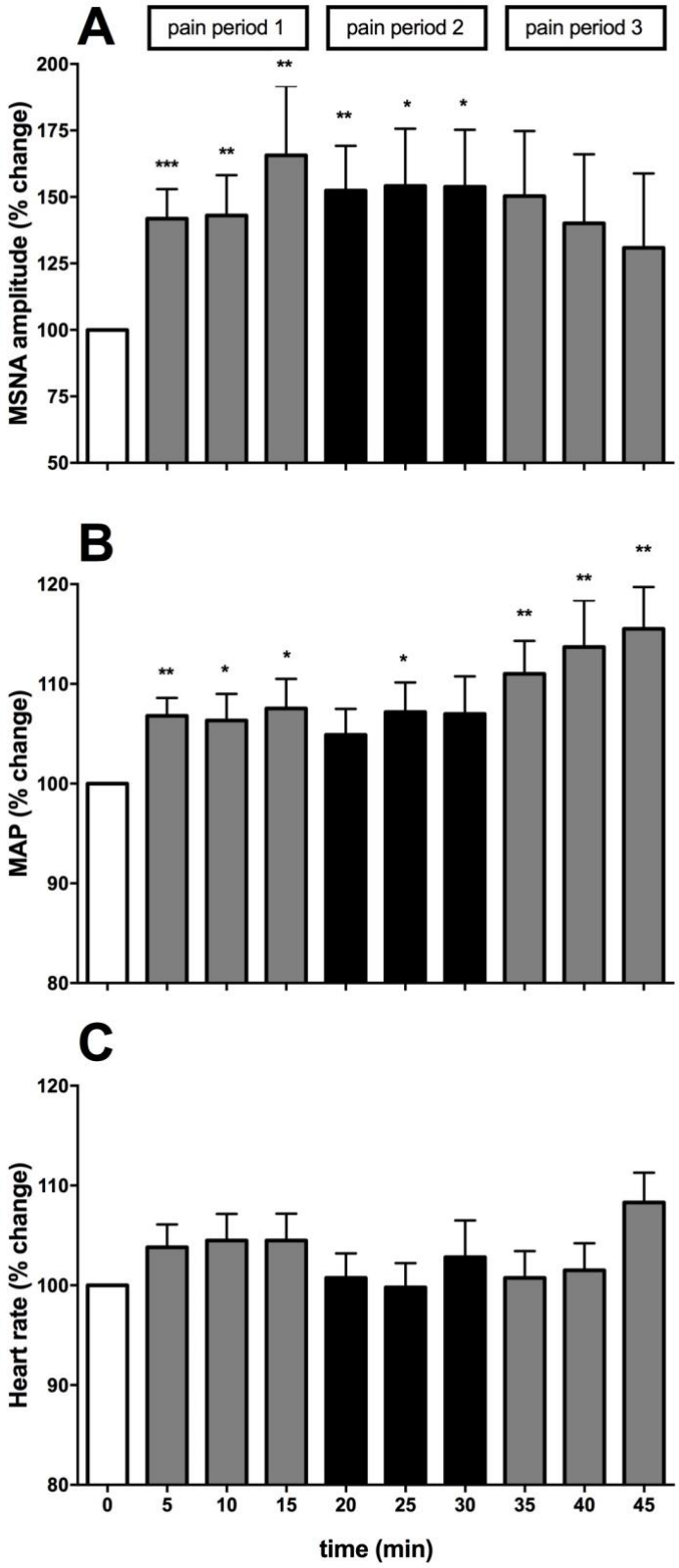

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Figure 6.1C) also increased significantly during the first 15 minutes of muscle pain (pain period 1). The 15-minute audiovisual stimulus, presented during pain period 2, had no significant effect on the trajectory of the MSNA response. Interestingly, there was a slight fall in MAP and HR while subjects were watching the documentary, which – while not statistically significant overall – would fit with them being engaged with the documentary. At the very least, the fact that neither MAP nor HR increased during the audiovisual stimulus, suggests that the documentary content was relaxing. By limiting the analysis to the first 5-min epoch of pain period 2 with the preceding 5-min epoch of pain period 1, heart rate was the only parameter to show a significant fall ($p = .048$; paired-test).

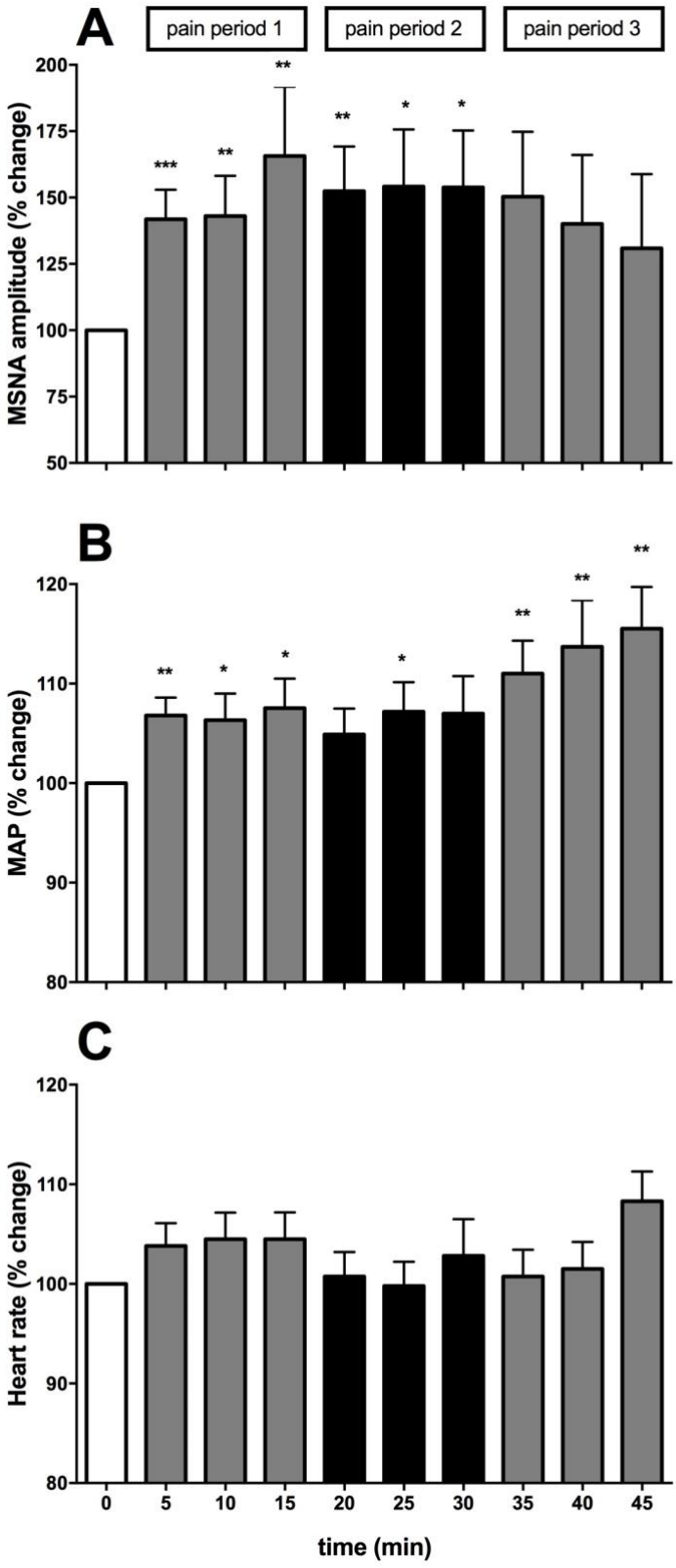

Figure 6.1: Mean MSNA amplitude changes during pain.

Mean (±SEM) changes in muscle sympathetic nerve activity (MSNA) burst amplitude, mean arterial pressure and heart rate at rest (white columns) and during tonic muscle pain – before

(pain period 1), during (pain period 2) and following (pain period 3) an audiovisual distraction task. Data from 20 subjects. *p < .05, **p < .01, ***p < .001.

6.4.3. Effects of pain and distraction on cardiovascular parameters: group responses

As we had shown previously, tonic muscle pain elicited a divergent muscle sympathetic response: the normalized mean burst amplitude increased in a sustained fashion in 15 subjects (to 176.4±7.9 % of baseline, p < .001, paired t-test), while five subjects showed a decrease (to 73.1±5.2% of baseline, p < .001, paired t-test). The fact that more subjects showed an increase in MSNA in this cohort of subjects explains

why the mean MSNA increased overall in

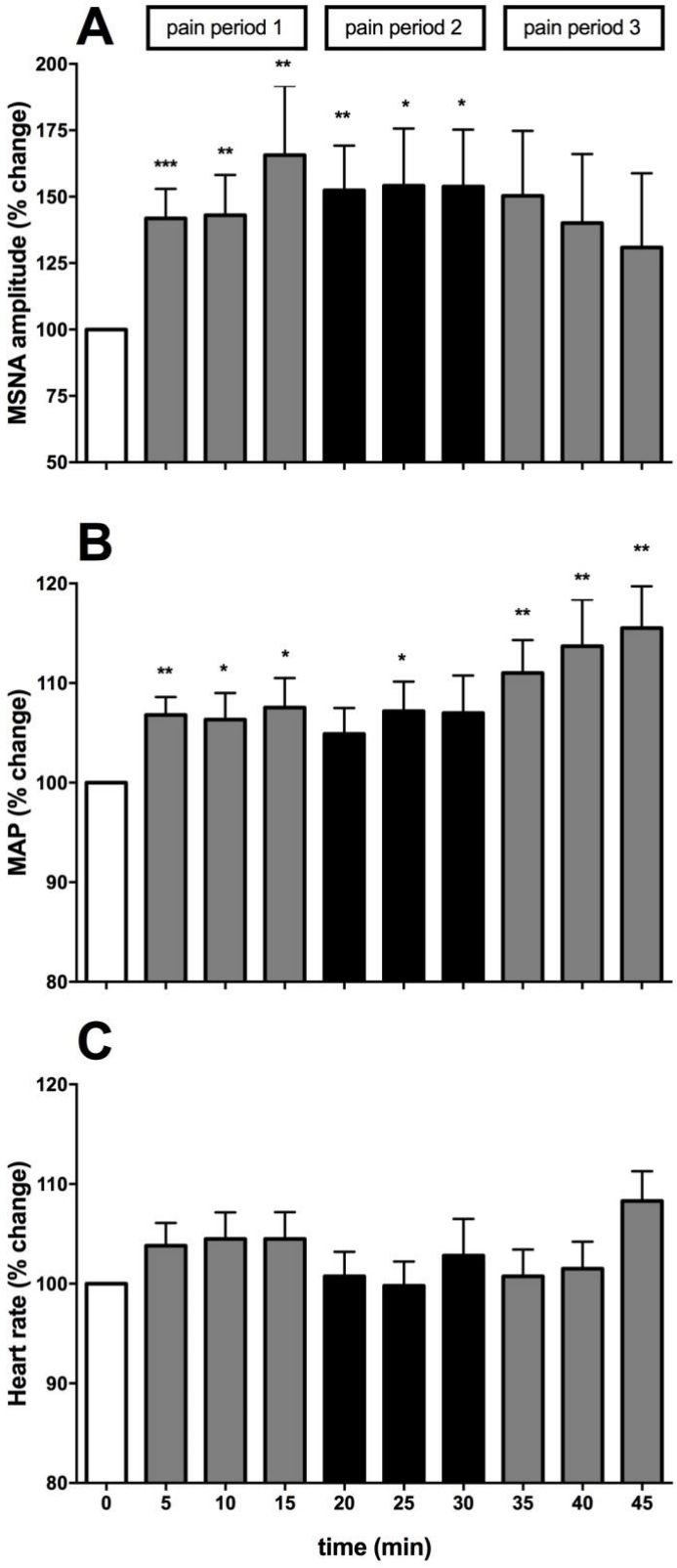

Figure 6.1

Table 6.1 provides baseline data for the two groups of subjects. It can be seen that, apart from differences in body mass index (although not statistically significant) between the increasing MSNA and decreasing MSNA groups (there were fewer females in the latter), there were no differences in MSNA burst frequency, MSNA burst incidence, or in systolic, diastolic or mean arterial pressures and heart rate. Moreover, there was no difference in the total volume of hypertonic saline infused in the two groups during the induction of tonic muscle pain (19 ± 1 vs 19 ± 1 ml).

Mean changes in MSNA normalized burst amplitude, MAP and HR are shown graphically in *Figure 6.2*. Overall, in either group, the distraction task had no effect on MSNA. The changes in mean normalised burst amplitude, and absolute changes in MSNA burst frequency, MSNA burst incidence, and systolic, diastolic and mean arterial pressure and heart rate during pain periods 1, 2 and 3 are shown for both groups of subjects in *Table 6.2*.

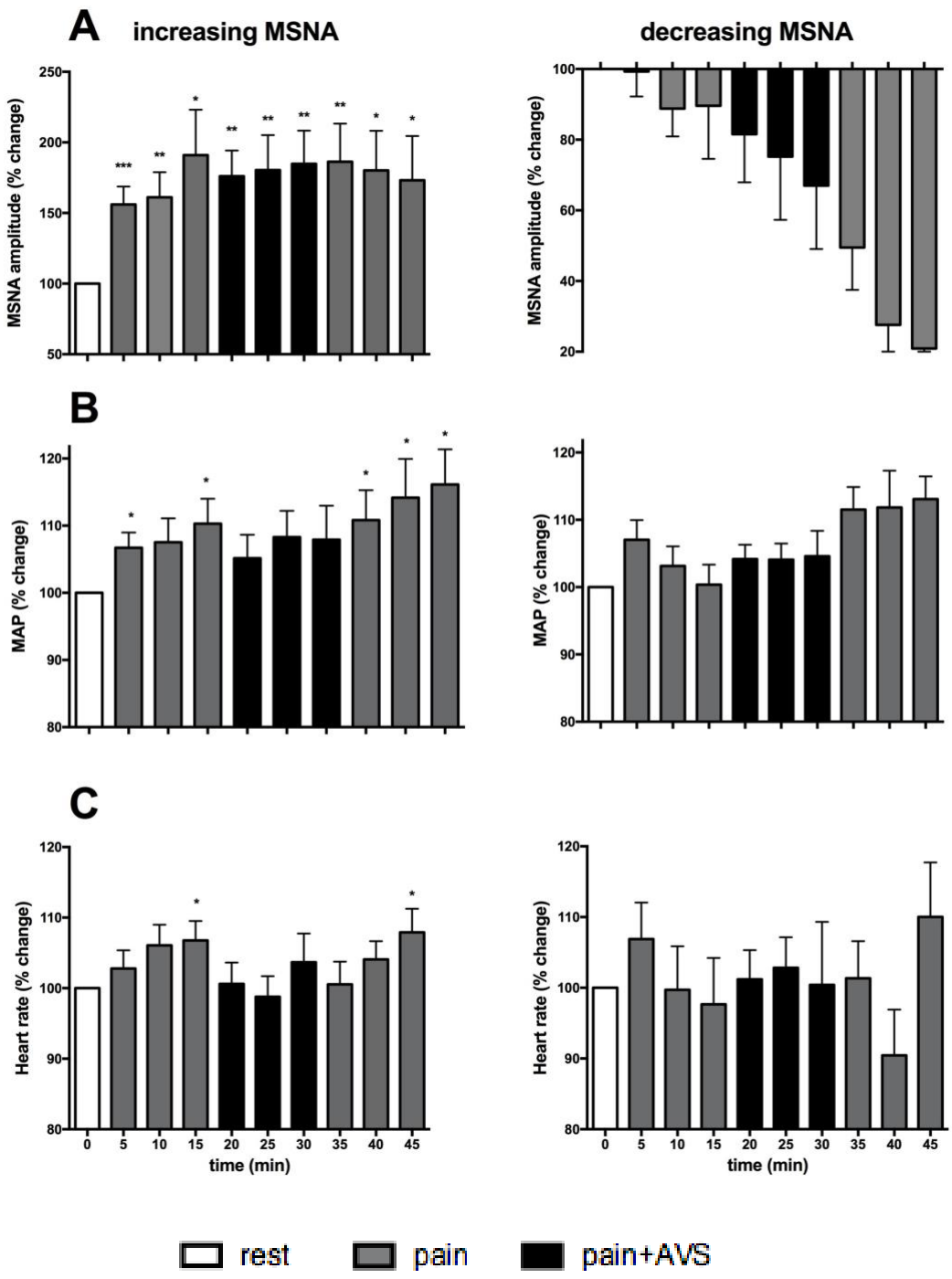

Figure 6.2: Changes in MSNA, MAP, and HR for both groups.

Mean (±SEM) changes in normalized muscle sympathetic nerve activity (MSNA) burst amplitude, mean arterial pressure and heart rate after separating the data of *Figure 6.1* into an increasing MSNA group (n=15) and a decreasing MSNA group (n=5). There was insufficient data in the decreasing group to perform statistical analysis. *p < .05, **p < .01, ***p < .001.

Table 6.2: Changes during tonic pain.

Mean changes in MSNA normalised burst amplitude and absolute changes in cardiovascular variables during tonic muscle pain. MSNA: muscle sympathetic nerve activity. *Significantly different from baseline. p values refer to differences between pain periods 1, 2, and 3.

Variable	***Increasing MSNA group (n=15)***				***Decreasing MSNA group (n=15)***			
	Pain 1	***Pain 2***	***Pain 3***	***p-value***	***Pain 1***	***Pain 2***	***Pain 3***	***p-value***
MSNA burst amplitude (%)	174±14*	180±13*	180±6*	.95	93±6	75±9	45±7*	.02
MSNA burst frequency (Δ bursts/min)	5±1*	5±1*	5±1*	.69	-1±1	-3±1	-9±1*	.06
MSNA burst incidence (Δ bursts/100 heart beats)	5±1*	7±1*	6±1*	.54	-5±2	-7±2	-14±3*	.16
Systolic arterial pressure (Δ mmHg)	9±2*	7±1	18±0*	.37	7±5	7±3*	13±0*	.42
Diastolic arterial pressure (Δ mmHg)	4±0*	3±0	6±0*	.69	2±1	3±0	7±1*	.12
Mean arterial pressure (Δ mmHg)	5±0*	4±0	8±0*	.69	3±2	4±1	8±1*	.18
Heart rate (Δ beats/min)	2±0	0±1	1±0	.38	1±0	1±0	1±1	.95

6.5. Discussion

We have shown that the divergent muscle sympathetic responses to tonic muscle pain are not affected through distraction using an audiovisual stimulus. However, that heart rate fell slightly during the audiovisual task suggests that the subjects were engaged, and presumably distracted from the painful stimulus. It is known that novel stimuli can lead to decreases in HR, coupled with an increase in sweat release, as part of the orienting response (Bradley, 2009), so it is possible that the slight fall in heart rate can be explained in similar terms. Although we have previously reported significant differences in blood pressure (mean, systolic, and diastolic) between subjects in whom MSNA amplitude increases compared with those in whom MSNA amplitude decreases (Kobuch et al., 2015), as well as differences in heart rate (Fazalbhoy et al., 2012), here we did not find significant differences between the two groups. It is possible that the increase in blood pressure in both the increasing and decreasing MSNA groups in this study is driven by nociceptor activation.

Recent research in our laboratory has shed light on the neurophysiological mechanisms through which experimental muscle pain elicits divergent sympathetic responses in healthy human subjects. By performing fMRI at the same time as recording MSNA we have reported signal intensity differences between the increasing and decreasing MSNA groups in distinct brain regions, such as the prefrontal and cingulate cortices, as well as the dorsomedial hypothalamus, dorsolateral periaqueductal grey (dlPAG), and the precuneus (Kobuch et al., 2017). It remains unknown as to why these patterns of activation in the brain are associated with either increases or decreases in MSNA during tonic muscle pain. The contribution of higher-order cortical regions, such as the prefrontal and cingulate cortices, which are involved in emotional cognitive functions, indicates the possibility of underlying emotional differences between the groups.

However, we have recently reported that state and trait anxiety levels, as well as pain catastrophizing, pain anxiety, and pain vigilance did not determine the direction of the response (Kobuch et al., 2016).

The contribution of the PAG and the precuneus hints towards the involvement of psychological stress and engagement. Indeed, research in cats has shown that direct stimulation of the lateral PAG evokes a fight-or-flight response associated with behaviourally-relevant increases in sympathetic activity, blood pressure, and heart rate (Bandler et al., 2000; Carrive et al., 1987; Keay & Bandler 2001). Furthermore, this column of the PAG receives input predominantly from cortical regions, which suggests that it produces active coping responses to psychological rather than physiological stressors (Keay & Henderson, 2010). Conversely, the precuneus is part of a network of brain regions which exhibits a uniform pattern of deactivation upon initiation of a goal-directed behaviour but is otherwise active when an individual is at rest (Raichle et al., 2001). Therefore, in this study, we had good reasons to investigate whether distraction from the noxious stimulus, and attention to an emotionally neutral stimulus, could modulate the sympathetic response.

Distraction techniques have been used in many experimental procedures as a means of pain reduction – using cognitive strategies ranging from emotion-centred tasks (Avia & Kanfer, 1980; Berntzen, 1987) to attentional tasks. Attentional tasks can require the subjects to either focus on the pain sensation (Leventhal, et al., 1979; McCaul & Haugtvedt, 1982; Ahles et al., 1983), or conversely, to engage in an affectively neutral competing task (Barber & Cooper, 1972; Beers & Karoly, 1979; Devine & Spanos, 1990). Most studies have found that distraction is effective in reducing the distress associated with the painful experience in healthy participants (Eccleston, 1995; McCaul & Haugtvedt, 1982; Wack & Turk, 1984). However, some studies have found

distraction not to reduce physiological or behavioral responses (Leventhal and Everhart, 1979; McCaul et al., 1992). Furthermore, it has been proposed that attention to physical sensations may enhance the perceived strength of those sensations, whereas distraction may minimize the perceived intensity of the sensation (Pennebaker & Lightner, 1980). We also know that mental stress increases MSNA in some subjects but not in others (Carter & Ray, 2009), and our own work has shown that this depends on the rate of rise in blood pressure (El Sayed, Macefield, Hissen, Joyner, & Taylor, 2016). Accordingly, one should not be surprised at inter-individual variability in responsiveness to a given stressor.

The fact that distraction from the ongoing noxious stimulation did not have an effect on the muscle sympathetic response to muscle pain is important, as it demonstrates that the response is independent of the subject's attention to the pain. This further demonstrates that the neurophysiological response to the noxious input is prioritized over the conscious attentional input. Nevertheless, given that we observed a slight slowing of heart rate during the audiovisual stimulus, this supports our contention that the subjects were adequately distracted from their pain by attending to the audiovisual stimulus. Of course, it may be that a greater deceleration would have occurred in the absence of pain, even though such cardiodeceleration is only in the order of 2-3 beats per minute (Bradley, 2009).

The main limitation of this chapter is the low number of subjects in whom MSNA decreased during pain.

6.6. Conclusions

We had posited that distraction would reduce the increase in MSNA during tonic muscle pain but found no such attenuation. Moreover, we saw no change in the group

of subjects in whom MSNA decreased during pain, although we had very few subjects in this group. Nevertheless, we conclude that the muscle sympathetic responses to experimental muscle pain does not depend on the attentional state of an individual.

CHAPTER 7 Conclusions

The aim of this thesis was to investigate the neurophysiological basis of the divergent sympathetic responses to long-lasting experimental muscle pain in humans. This chapter will summarize the main findings of each investigation, discuss the implications of these findings, and finally, provide recommendations for future research based on the questions that arose in this thesis.

7.1. Main findings

The first study exploring the basis of the divergent muscle sympathetic response to muscle pain, presented in Appendix A, revealed that the baseline physiological parameters measured do not predict whether an individual exhibits an increase or decrease in MSNA during long-lasting muscle pain. Indeed, both the direction of the response and the magnitude of change were independent of baseline MSNA, heart rate, blood pressure, heart rate variability, as well as age and body mass index. Furthermore, sex did not play a role in determining the direction of response to muscle pain. However, this study revealed a fairly even split in the propensity of subjects in whom MSNA and blood pressure increased, and those in whom MSNA decreased during the noxious stimulation. Importantly, an increase in MSNA was associated with significantly greater increases in blood pressure during muscle pain.

Having been unable to demonstrate the influence of baseline physiological parameters in Appendix A, Chapter 3 focused on psychological factors. This study tested the hypothesis that elevated anxiety levels and negative attitudes towards pain lead to a higher prevalence of increases than decreases in MSNA during tonic muscle pain. The psychological parameters were measured with the State and Trait Anxiety Inventory (Spielberger et al., 1983), the Pain Catastrophizing Scale (Sullivan et al., 1995), the

Pain Anxiety Symptoms Scale (McCracken & Dhingra, 2002), and the Pain Vigilance and Awareness Questionnaire (McCracken, 1997) in 66 subjects in whom MSNA was measured during a 45-minute infusion of hypertonic saline solution. None of these explained the divergent sympathetic responses, nor did they appropriately predict whether a given individual shows an increase or a decrease in muscle sympathetic nerve activity during long-lasting muscle pain.

In Chapter 4, the muscle sympathetic responses to muscle pain were recorded concurrently with functional brain imaging to investigate whether the responses were associated with differential changes in regional brain activity. The study tested the hypothesis that a sustained increase in MSNA during long-lasting pain is associated with sustained increases in blood oxygen level in important autonomic brain regions. This investigation revealed that the divergent MSNA responses were associated with different signal intensity changes in a number of brain regions, namely the prefrontal and cingulate cortices, precuneus, and dorsomedial hypothalamus, and brainstem regions such as the periaqueductal grey and rostroventrolateral medulla. These results suggest that, during tonic muscle pain, descending modulation of the brainstem circuits, which are thought to control the cardiovascular responses to pain, can evoke different MSNA responses in different individuals.

The findings of the fMRI investigation documented in Chapter 4 prompted questions regarding the influence of specific brain areas on the MSNA response, particularly which regions would show a change in signal intensity during each MSNA burst, rather than a sustained signal change as seen in Chapter 4. It is known that at rest, signal intensity within the prefrontal, insular and precuneus cortices, hypothalamus, and

medulla are tightly coupled to each MSNA burst (Macefield & Henderson, 2016). The aim of this study was to investigate whether during sustained muscle pain, signal intensity within these areas remains coupled to MSNA, and/or whether other brain regions react in a similar manner. The hypothesis to be tested was that in addition to areas shown to be coupled to MSNA bursts at rest, other regions, such as the cingulate cortex and midbrain, would also exhibit coupling to MSNA during pain, and that increases in blood oxygen level dependent signal intensity in these areas would parallel an increase in MSNA burst amplitude. Results of the investigation revealed that multiple brain regions, including the nucleus tractus solitarius, PAG, prefrontal and cingulate cortices are recruited in a burst-to-burst manner, and that the magnitude of these signal changes correlates with the overall change in MSNA amplitude during muscle pain.

The final study of this thesis (Chapter 6) described an exploration into whether distraction from the painful stimulus could influence the direction of the MSNA response. Since certain brain regions involved in attention (i.e. precuneus), as well as regions involved in the response to psychological stressors (i.e. PAG) had shown differences in activity in Chapter 4, the aim was to explore the effects of audio-visual distraction on the MSNA response to tonic muscle pain. The hypothesis was that distraction via an engagement in an affectively neutral competing task could bring about changes in the MSNA responses to tonic muscle pain. The hypothesis was not supported, and the findings indicate that audio-visual distraction does not appear to influence the muscle sympathetic response to muscle pain.

7.2. Implications

The findings of this thesis indicate that, despite the work outlined here, relatively little is known about the physiological and psychological basis of the divergent muscle sympathetic response to long-lasting experimental muscle pain, but that differential central pathways are implicated in this response. Questions remain as to *why* these divergent responses occur, and whether there are predicting factors that dictate whether an individual will have a sustained increase in MSNA and blood pressure during pain, or a sustained decrease. Furthermore, whether these changes in MSNA amplitude persist once chronic pain is established remains unknown.

Animal and human studies had extensively explored the effects of acute noxious stimuli on the cardiovascular system (Burton et al., 2008, 2009a,b,2016; Feinstein et al., 1954; Horeyseck & Jänig, 1974; Lewis, 1942; Nordin & Fagius, 1995; Sato et al., 1984), suggesting that the cardiovascular responses to a noxious stimulus were dependent on the origin of the pain (Keay & Bandler 2001; Feinstein et al., 1954; Lewis, 1942). Few however, had focused on a longer lasting stimulus, until Fazalbhoy and colleagues discovered the divergent MSNA response to long-lasting experimental muscle pain (Fazalbhoy et al., 2012, 2014). Extensive work was then necessary in order to understand the basis of this phenomenon. Appendix A provides the first in-depth understanding that this response cannot be attributed to the age, sex, or BMI of an individual, or his/her baseline heart rate, blood pressure, and MSNA levels (Kobuch et al., 2015). As previously reported (Fazalbhoy et al., 2012), in the group of subjects in whom MSNA increased there was also a significant increase in blood pressure. This is an important finding as it raises the question as to whether the rise in MSNA and the

associated increase in blood pressure in certain people is responsible for the development of hypertension that is seen in many chronic pain patients (Bruehl et al., 2005).

Since the physiological parameters presented in Appendix A were not implicated in determining the direction of the MSNA response to long-lasting experimental muscle pain, the next step was to determine whether high anxiety levels and negative attitudes towards pain could be associated with an increase in MSNA. There has been growing evidence that patients with anxiety are at greater risk for cardiovascular disease (Musselman et al., 1998; Rosengren et al., 2004), and disturbed sympathetic firing patterns in these patients have been reported (Lambert et al., 2006,2008,2010). Furthermore, in 2002, Keay and Bandler showed that the autonomic responses to a noxious stimulus were also associated with a specific behavioural reaction, depending on whether the stimulus was deemed escapable or inescapable. Indeed, an escapable situation is associated with sympathoexcitation and a fight/flight behaviour – whereas one that is inescapable is met with sympathoinhibition and a conservation-withdrawal response (Keay & Bandler, 2002). Since there were no differences in the anxiety levels and attitudes to towards pain in our subjects, it may well be that certain individuals identified the stressor as escapable and thereby showed an increased sympathetic response, while others found it inescapable, and therefore was met with passive coping mechanisms, which are associated with sympathetic depression (Keay & Bandler, 2002). However, this is difficult to assess in this study, as the noxious stimulus is inescapable in the sense that the microneurography with or without the MRI scanner depicts a restricted environment and requires the subjects to remain immobile, but could be deemed escapable in the sense that the subjects knew they had the option to

opt out. Furthermore, the fact that distraction from the noxious stimulus did not elicit changes in MSNA amplitude during pain implies that the reactions may be representative of defensive reactions rather than conscious attentional effort. Research in humans has shown that defensive reactions are associated with a variety of existing personality constructs (Perkins et al., 2010). For example, increased fear is correlated with the tendency to orient oneself away from the threat (Perkins & Corr, 2006). A questionnaire investigating perceived threat intensity may provide some answers to the psychological stress that may be associated with the MSNA response to pain.

Keay and Bandler (2002) showed the importance of different columns of the midbrain periaqueductal grey as being the sites in the CNS responsible for either of the autonomic and behavioural responses to pain. In humans, the PAG has been shown to be involved in the physiological responses to psychological stressors (Reiman et al., 1989; Mobbs et al., 2007), and stimulation of particular columns can evoke changes in blood pressure and MSNA (Basnayake et al., 2011, 2012; Sverrisdottir et al., 2014).

The results presented in Chapters 4 and 5 revealed that multiple brain regions are responsible for the sustained changes in MSNA amplitude during pain. Specifically, the results indicate that the PAG is playing a crucial role in this response. It would be interesting to investigate the activity of the specific columns of the PAG during other stressors which are known to elicit sympathoexcitation or inhibition in humans using magnetic resonance imaging. Furthermore, the concurrent microneurography and imaging studies presented in Chapters 4 and 5 provide evidence that several brain regions (including the PAG), known to be associated with changes in sympathetic drive, are recruited both in a sustained and burst-to-burst manner during a painful

stimulus. Whether other stressors elicit similar patterns of activity in autonomic brain regions is an interesting question and worthy of exploration.

7.3. Future research

If the effects of long-lasting pain on the sympathetic nervous system are to be fully understood, the first priority is to conduct experiments with a longer noxious stimulus. Indeed, one hour of muscle pain produced by infusion of hypertonic saline solution does not truly reflect the chronic nature of pain, and such a model is not applicable to draw conclusions on the physiological responses to persistent pain. Therefore, it would be interesting to see whether the changes in MSNA are sustained with ongoing pain, such as delayed onset muscle soreness. Furthermore, since the sustained activation of the sympathetic nervous system is thought to be associated with the development and maintenance of chronic pain (Ali et al., 2000; Benarroch, 2006), it would be interesting to see whether the subjects who showed an increase in MSNA are more prone to develop chronic pain than the subjects in whom MSNA decreased. This could involve a simple questionnaire exploring the pain history of the participants involved in the studies that contributed to this thesis. Furthermore, it would be useful to measure the blood pressure of individuals who have developed chronic pain, and compare it to those who have not, as it is known that patients with post-surgical chronic pain have nearly twice the prevalence of clinical hypertension than medical patients without pain (Bruehl et al., 2005). Accordingly, we could postulate that a person who consistently exhibited increases in MSNA and blood pressure during experimental muscle pain may - if he or she developed chronic pain - go on to develop hypertension.

Few studies have measured MSNA in chronic pain conditions, and the results are conflicting. In a study involving a single patient with chronic regional pain syndrome (CRPS), no difference in the sympathetic outflow was found between the painful and non-painful limb (Casale & Elam, 1992). In contrast, pain intensity in 25 fibromyalgia patients was positively correlated to MSNA frequency levels (Zamunér et al., 2015). It would be interesting however, to measure the MSNA levels of chronic pain patients, and repeat a measurement after chronic pain has ceased. Although, it may be difficult to draw conclusions on the MSNA amplitude levels, as these are dependent on the distance of the tip of the electrode and active fibres, and therefore vary between multiple measurements of MSNA at rest. However, burst frequency and burst incidence are reproducible; arterial pressure may also be an important indirect measure to investigate.

Finally, the studies presented in Chapters 4 and 5 provide a precedent for future concurrent functional brain imaging and microneurography studies during autonomic challenges. It is important to note that brain regions can show sustained or MSNA-burst-coupled activation during an autonomic challenge, or both. Therefore, it is essential to including both a model that detects sustained signal intensity changes *and* MSNA burst-coupled signal intensity changes when eliciting a continuous autonomic response.

CHAPTER 8 References

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Appendices

APPENDIX A. Inter-individual responses to experimental muscle pain: baseline physiological parameters do not determine whether muscle sympathetic nerve activity increases or decreases during pain

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A.1. Abstract

We have previously reported that there are inter-individual differences in the cardiovascular responses to experimental muscle pain, which are consistent over time: intramuscular infusion of hypertonic saline, causing pain lasting ~60min, increases muscle sympathetic nerve activity (MSNA)—as well as blood pressure and heart rate—in certain subjects, but decrease it in others. Here, we tested the hypothesis that baseline physiological parameters (resting MSNA, heart rate, blood pressure, heart rate variability) determine the cardiovascular responses to long-lasting muscle pain. MSNA was recorded from the common peroneal nerve, together with heart rate and blood pressure, during a 45-min intramuscular infusion of hypertonic saline solution into the tibialis anterior of 50 awake human subjects (25 females and 25 males). Twenty-four subjects showed a sustained increase in mean amplitude of MSNA (160.9 ± 7.3%), while 26 showed a sustained decrease (55.1 ± 3.5%). Between the increasing and decreasing groups there were no differences in baseline MSNA (19.0 ± 1.5 vs. 18.9 ± 1.2 bursts/min), mean BP (88.1 ± 5.2 vs. 88.0 ± 3.8 mmHg), HR (74.7 ± 2.0 vs. 72.8 ± 1.8 beats/min) or heart rate variability (LF/HF 1.8 ± 0.2 vs. 2.2 ± 0.3). Furthermore, neither sex nor body mass index had any effect on whether MSNA increased or decreased during tonic muscle pain. We conclude that the measured baseline physiological parameters cannot account for the divergent sympathetic responses during tonic muscle pain.

A.2. Introduction

Pain is important for survival by helping to avoid tissue damage, mobilizing all relevant homeostatic systems for a fight-and-flight response or, alternatively,

promoting conservation of energy, and thus promoting healing (Craig, 2002). It is well known that pain originating in deep structures may evoke very different behavioural and cardiovascular responses than pain originating in superficial structures. Indeed, Lewis (1942) observed that pain originating in skin evokes "a rise of pulse rate" and a "sense of invigoration" whereas pain originating in deep structures evokes quiescence, a "slowing of the pulse" and "falling of the blood pressure" (Lewis, 1942). Subsequent studies confirmed Lewis' findings that muscle pain was associated with a fall in blood pressure and bradycardia in awake human subjects (Feinstein et al., 1954). However, since these early observations by Lewis and Feinstein, very few studies have examined the effects of pain on the cardiovascular system in awake human subjects.

We have been using subcutaneous or intramuscular injection of hypertonic saline—a specific stimulus for nociceptors (Graven-Nielsen & Mense, 2001)—to study the effects of acute pain on the cardiovascular system in awake human subjects. We showed that a bolus (0.5 ml) injection of hypertonic saline into the tibialis anterior muscle caused a *sustained* increase in muscle sympathetic nerve activity (MSNA), and a modest increase in blood pressure and heart rate (Burton et al., 2009a), while there was only a *transient* increase in skin sympathetic nerve activity (SSNA)—the latter being consistent with an arousal rather than reflex response (Burton et al., 2009b). More recently, we used intramuscular infusion to produce a sustained, steady-state, level of pain lasting for approximately 1h (Fazalbhoy et al., 2012, 2014). We showed that about half of the subjects showed a sustained increase in MSNA, blood pressure, and heart rate during tonic muscle pain, while the other half showed sustained decreases (Fazalbhoy et al., 2012, 2014).

These data call into question the idea that noxious stimuli produce invariant responses and raise the prospect that these differential responses may be related to an individual's particular traits, which may be reproducible over time. That is, in some individuals muscle pain *always* evokes increases in MSNA, blood pressure and heart rate, whereas in others it consistently evokes decreases. Indeed, we recently showed that subjects who generate increases in MSNA, blood pressure and heart rate during one session also show increases in a second session; the same is true for those who show parallel decreases in MSNA, blood pressure and heart rate (Fazalbhoy et al., 2014). Moreover, we showed that there were no differences in resting MSNA, blood pressure or heart rate between the two recording sessions (Fazalbhoy et al., 2014), but we do not know whether differences in these baseline physiological parameters *across* individuals determine whether MSNA increases or decreases during tonic muscle pain. Indeed, in our first study we showed that resting levels of MSNA were higher in the group that showed an increase in MSNA than in the group that showed a decrease, but these differences failed to reach statistical significance—presumably because of the low subject numbers (n = 12). Against this background, the aim of the current study was to determine whether baseline physiological parameters—including resting MSNA, blood pressure and heart rate—could account for the divergent MSNA responses to tonic muscle pain. Our earlier studies (Fazalbhoy et al., 2012, 2014) were based on small subject numbers and were not sex-balanced. Here, we have studied a larger sample (n = 50), comprising 25 males and 25 females.

A.3. Methods

Experiments were performed on 25 female and 25 male healthy subjects, aged 18–39 years. Data from 35 new participants were pooled with those from 15 participants

reported previously (Fazalbhoy et al., 2014). All subjects provided informed written consent to the experimental procedures, which were conducted under the approval of the Human Research Ethics Committee of the University of Western Sydney and satisfied the requirements of the Helsinki Declaration. No subject had a history of cardiovascular disease or former chronic musculoskeletal pain. Prior to commencement height, weight, body mass index (BMI), and total muscle mass were measured for each subject using a body-composition analyser (SA165A, Tanita, Japan).

A.3.1. Experimental procedures

The subjects were seated in a comfortable reclined position with the legs supported in an extended position. The room was kept quiet and at a constant temperature of 22◦C. The course of the common peroneal nerve was identified via external stimulation (2–10 mA) using a 1 mm surface probe which delivered 0.2 ms pulses at 1Hz from an isolated stimulator (Stimulus Isolator; ADInstruments, Sydney, Australia). Spontaneous bursts of muscle sympathetic nerve activity (MSNA) were recorded from muscle fascicles of the common peroneal nerve supplying the ankle or toe extensor or foot everter muscles via tungsten microelectrodes (FHC, Bowdoin, ME, USA) inserted percutaneously at the level of the fibular head. Multi-unit neural activity was amplified (gain 20 000, bandpass 0.3–5.0 kHz) using an isolated amplifier (NeuroAmp EX, ADInstruments, Sydney, Australia) and stored on computer (10-kHz sampling) using a computer-based data acquisition and analysis system (PowerLab 16SP hardware and LabChart 7 software; ADInstruments, Sydney, Australia). ECG (0.3–1.0kHz) was recorded with Ag–AgCl surface electrodes on the chest and sampled at 2 kHz. Blood pressure was recorded continuously using finger pulse plethysmography (Finometer

Pro, Finapres Medical Systems, The Netherlands) and sampled at 400 Hz. Respiration (DC-100Hz) was recorded using a strain-gauge transducer (Pneumotrace, UFI, Morro Bay CA, USA) wrapped around the chest.

A.3.2. Noxious stimulation

A 7% hypertonic saline solution was prepared by diluting sterile, 20% hypertonic saline with sterile water. Two syringes of 10 ml each were filled with the 7% hypertonic saline, placed in an infusion pump (Harvard Instruments, USA), and connected to a three-way tap via a 75cm extension tubing primed with hypertonic saline. A 23 gauge butterfly needle was then attached to the three-way tap via a cannula, primed, and inserted 1.5 cm deep into the belly of the ipsilateral tibialis anterior muscle, about 5 cm lateral and 10 cm inferior to the tibial tuberosity. The cannula was inserted as soon as a stable recording of spontaneous MSNA was achieved. Prior to infusion of the saline solution, a 5 min baseline recording of MSNA, blood pressure, respiration, and heart rate was obtained. Infusion of the 7% hypertonic saline solution was started at a time unknown to the subject, and was maintained for 45 min; as described previously (Fazalbhoy et al., 2012, 2014), the pain lasted for ~60min. The initial rate of infusion was set at 0.25ml/min and was constantly adjusted to maintain a pain level of 5–6/10 on a Numerical Rating Scale (NRS). Subjects were asked to rate their pain continuously by sliding a linear potentiometer (Response Meter, ADInstruments, Sydney, Australia) that was calibrated to the NRS, with a rating of "0" meaning "no pain/discomfort" at all, and a rating of "10" being equivalent to the "worst pain the subject ever had experienced." When the pain level dropped below 4/10 or rose above 6/10, the infusion rate was changed by 0.02 ml/min accordingly. After the infusion was completed, the recording was continued until the pain stopped. At the conclusion

of the experiment, each subject completed a McGill Pain Questionnaire, in which subjects described the quality of the pain using a standard set of descriptors.

A.3.3. Analysis

LabChart 7 Pro software (ADInstruments, Sydney, Australia) was used to record the following parameters: muscle sympathetic nerve activity (burst amplitude and frequency), heart rate, blood pressure, respiration, pulse pressure, heart rate variability (HRV), and pain ratings. Individual bursts of MSNA were displayed as a mean-voltage neurogram, computed as the root-mean-square (RMS) processed signal with a moving time average window of 200 ms. This signal was then analysed using the "Peak Analysis" module of the LabChart 7 Pro software to calculate the amplitude of each burst. The absolute values were averaged into 5-min blocks and reported as percentages from the "baseline" values. An average of all blocks was taken to determine the direction of the response. Subjects with overall average MSNA amplitude 10% lower than baseline were arbitrarily assigned to the decreasing group; averages 10% higher than baseline were considered as increasing. Baseline MSNA amplitude was compared to the 5-min block with the mean value calculated over the entire infusion period, and to the highest average for the increasing group and to the lowest average value for the decreasing group. Changes in mean heart rate and mean blood pressure were also measured in 5 min epochs, normalized to the baseline value prior to the infusion of hypertonic saline. HRV was assessed over a 5-min steady state period before the infusion, and then again over 5 min when the subject experienced a steady-state level of pain during the infusion. The parameters of HRV that were analysed included the low frequency (LF) and high frequency (HF) power, as well as the Root Mean Square Successive Difference of cardiac intervals (RMSSD). Statistical

analysis—non-paired two-tailed *t*-tests for normally distributed data and Mann-Whitney tests for non-normally distributed data—was performed using Prism version 6 for Mac OS X (GraphPad software, San Diego, California, USA). All values are expressed as means and standard error. Probability levels of $p < .05$ were deemed significant.

A.4. Results

A.4.1. Subjective experience of muscle pain

In all subjects, intramuscular infusion of hypertonic saline induced a steady state level of muscle pain in the tibialis anterior muscle. The level of pain was kept constant, typically around 5 out of 10—throughout the period of infusion by adjusting the rate of infusion according to the subject's tracking of the pain level. The mean pain rating was 4.9 ± 0.1. Using the McGill Pain Questionnaire, 36 of the 50 subjects (72%) described the pain as "aching," 48% described it as "heavy" and 48% as "dull." After these, "throbbing," "cramping," "hurting," "discomforting," and "continuous" were the most frequent descriptions used.

A.4.2. Muscle sympathetic nerve activity during tonic muscle pain

Experimental records from two subjects are shown in *Figure A-1* and *Figure A-2*. Muscle sympathetic nerve activity (MSNA) increased during tonic pain in the subject depicted in *Figure A-1*; it is apparent that blood pressure also increased. Conversely, the subject illustrated in *Figure A-2* exhibited a sustained decrease in MSNA and blood pressure during the infusion.

As expected, when all subjects were analysed according to their pattern of MSNA response to muscle pain two distinct groups of responses emerged: 24 subjects (48%) showed a significant increase in burst amplitude over the entire infusion period (132.6 ± 6.1% $p < .0001$, *t*-test), while 26 subjects (52%) showed a significant decrease (72.6 ± 3.0%, $p < .0001$, *t*-test), relative to baseline. The peak changes in the increasing and decreasing groups, measured over 5 min, were 160.9 ± 7.3% and 55.1 ± 3.5% respectively; these were significantly different from baseline ($p < .0001$, *t*-test). The time at which the peak increase in MSNA occurred (29 ± 3 min) in the increasing group, and the time at which the peak fall occurred (32 ± 3 min) in the decreasing group, were not significantly different ($p = .5077$, Mann-Whitney test). There was no significant difference in the mean pain rating in the increasing and decreasing groups (4.7 ± 0.2 vs. 5.1 ± 0.2, respectively; $p = .18$, *t*-test).

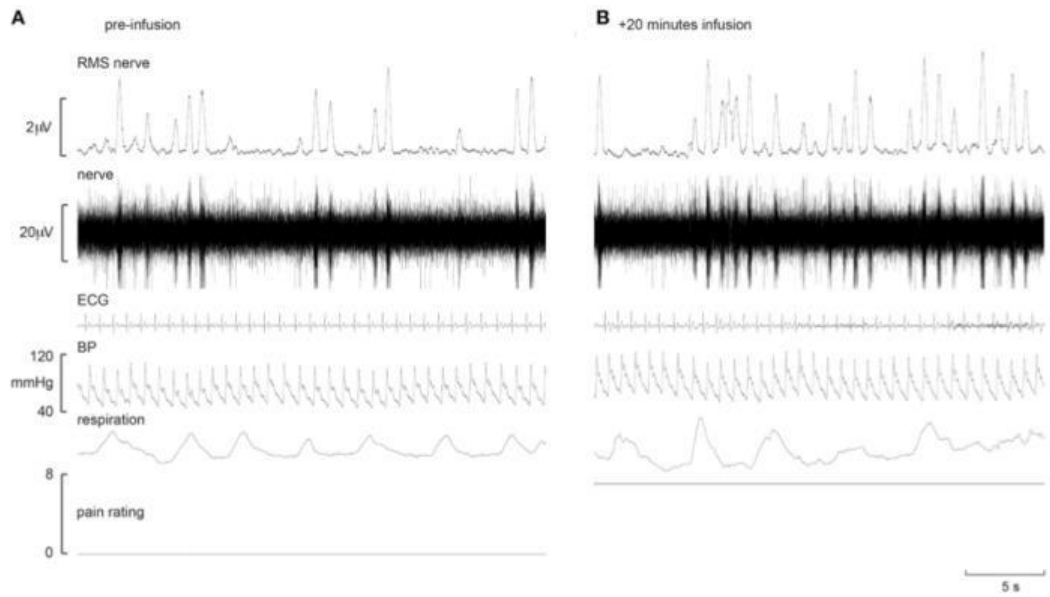

Figure A-1: Subject in whom MSNA increased during intramuscular infusion of hypertonic saline.

Baseline is shown in the left panel (A) while the right panel (B) shows a sample at which MSNA was at its maximum.

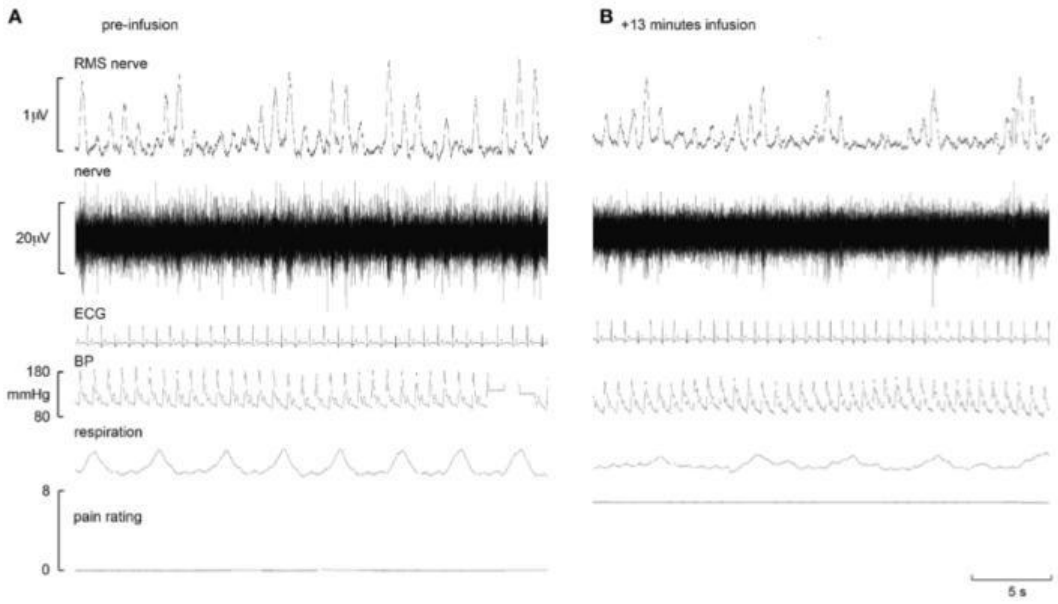

Figure A-2: Subject in whom MSNA decreased during intramuscular infusion of hypertonic saline.

Baseline is shown in the left panel (A), while the right panel (B) shows a sample at which MSNA was at its minimum.

A.5. Blood pressure and heart rate during tonic muscle pain

Interestingly, those subjects who showed an increase in MSNA showed a significantly larger increase in blood pressure than those in whom MSNA decreased. Systolic pressure increased from 132.0 ± 5.5 (baseline) to 159.9 ± 5.8 mmHg (steady level of pain) in the increasing group but from only 133.0 ± 4.7 to 142.7 ± 5.3 in the decreasing group. Diastolic pressure increased from 70.2 ± 5.2 (baseline) to 86.6 ± 4.5 mmHg (steady level of pain) and from 75.1 ± 4.2 to 76.7 ± 4.3 in the increasing and decreasing groups, respectively. Relative changes in blood pressure, heart rate and MSNA in the two groups are presented in *Figure A-3*. In the increasing group, data from two subjects were excluded from the calculated mean of all parameters as they showed much larger increases in amplitude of MSNA (396 and 520%), as defined by running an Outliers Test (Prism, GraphPad software), which would have skewed the results.

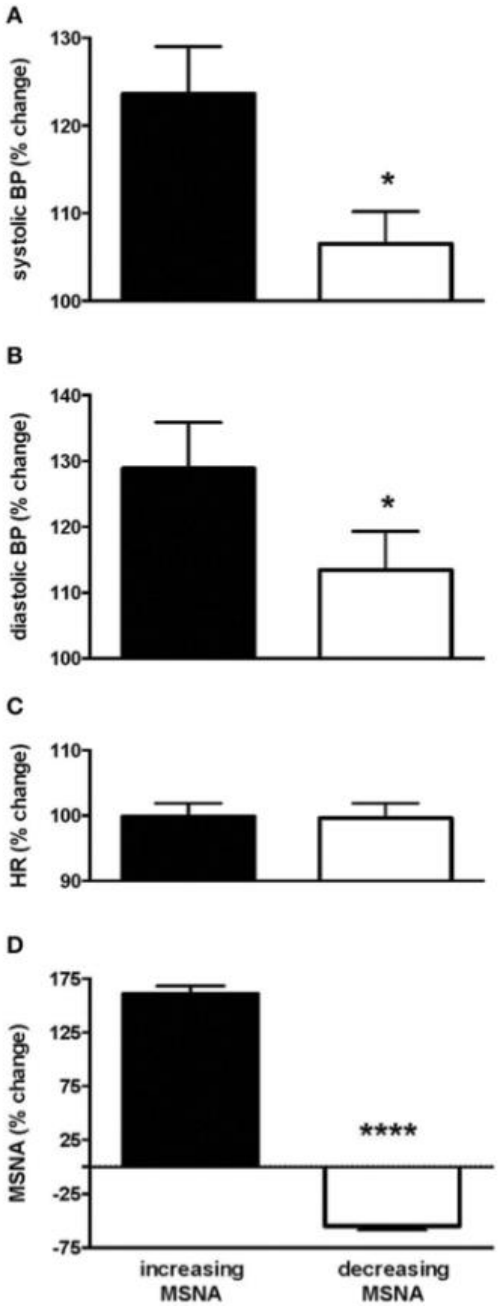

Figure A-3: Peak changes in blood pressure, heart rate, and MSNA for the increasing and decreasing MSNA groups.

Systolic and diastolic blood pressures were significantly higher in the increasing group, as depicted by the asterisk. Results are compared to baseline levels (i.e. 100%).

A.6. Resting levels of MSNA and BP

When comparing the increasing and decreasing groups, there were no differences in baseline MSNA (19.0 ± 1.5 vs 18.9 ± 1.2 bursts/min; p = .99, t-test) that could account for these divergent responses. Moreover, as shown in *Table A-1* there were no differences in resting blood pressure parameters, heart rate or heart rate variability, and no effect of body mass index or total muscle mass.

Table A-1: Baseline data for both MSNA groups.

Baseline data for the group showing an increase in MSNA (n=24) during tonic muscle pain and the group showing a decrease (n=26).

	Increasing MSNA	**Decreasing MSNA**	**P-value**
Number of subjects	11 female, 13 male	14 female, 12 male	.78
Age (years)	22.1±1.3	22.4±0.9	.34
Height (cm)	168.7±1.6	170.1±2.1	.60
Weight (kg)	65.7±2.6	68.0±2.9	.56
BMI (kg/m^2)	23.1±1.0	23.4±0.8	.65
Muscle mass (kg)	49.5±2.2	48.7±2.3	.80
Pain rating (/10)	4.7±0.2	5.1±0.2	.18
MSNA (bursts/min)	19.0±1.5	18.9±1.2	.99
SAP (mmHg)	132.0±5.5	133.0±4.7	.52
DAP (mmHg)	70.2±5.2	75.1±4.2	.32
MAP (mmHg)	88.1±5.2	88.0±3.8	.78
HR (beats/min)	74.7±2.0	72.8±1.8	.44
LF HRV (nu)	56.9±3.8	59.4±4.0	.80
HF HRV (nu)	38.4±3.4	35.6±3.7	.58
LF/HF HRV	1.8±0.2	2.2±0.3	.38
RMSSD HRV (ms)	40.5±4.1	40.8±4.0	.99

A.7. Sex differences

Of the 24 subjects in whom MSNA increased, 11 were female and 13 were male, while there were 14 females and 12 males in whom MSNA decreased. These data indicate that there was no difference in the *propensity* of males or females to exhibit an increase or decrease in MSNA during long-lasting muscle pain (p = .78, Fisher's Exact test). Moreover, the data illustrated in *Figure A-4* show that there were no differences in the peak *magnitude* of change in MSNA between females and males in either the increasing group (158.0 ± 11.3% vs. 163.2 ± 10.0%; p = .40, Mann-Whitney) or the decreasing group (44.1 ± 4.7% vs. 46.4 ± 5.4%; p = .77, Mann-Whitney). There was no significant difference in the mean pain rating between females and males (4.9 ± 0.2 vs. 4.9 ± 0.2, respectively; p = .87, *t*-test).

There were no statistically significant differences in resting MSNA between the female and male subjects (18.8 ± 1.5 vs. 20.2 ± 1.5 bursts/min; p = .19, unpaired Mann-Whitney test), and no significant differences in any of the other baseline cardiovascular parameters (*Table A-2*). The only statistically significant differences between males and females were in BMI and muscle mass, both of which were significantly higher in the males (p = 0.05 and p < .0001, respectively), and age (p < .01)—on average, the females were one year older, though this is of no consequence because ages were not significantly different in the increasing and decreasing groups (cf *Table A-1*).

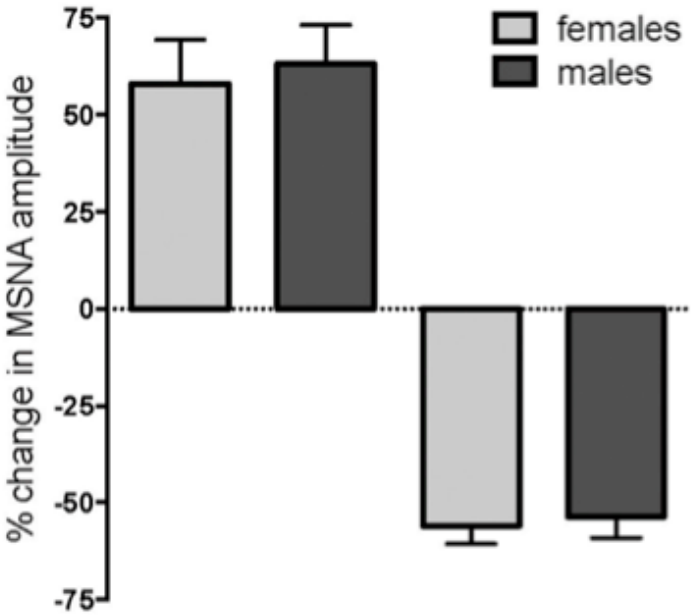

Figure A-4: Changes in MSNA in females and males during tonic muscle pain.

Table A-2: Baseline data for males and females

Age, BMI, total muscle mass, MSNA frequency (normalized to baseline), systolic arterial pressure (SAP), diastolic arterial pressure (DAP), mean arterial pressure (MAP), heart rate (HR) and specific components of heart rate variability (HRV) at baseline, divided by sex. Nu (normalized units).

	Females	**Males**	**P-value**
Number of subjects	25	25	
Age (years)	22.8±0.8	21.8±1.5	<.01
Height (cm)	164.0±1.1	175.0±1.7	<.0001
Weight (kg)	61.5±2.7	72.6±2.2	<.02
BMI (kg/m^2)	22.9±1.0	23.4±0.5	.05
Muscle mass (kg)	43.3±0.8	57.8±2.1	<.0001
Pain rating (/10)	4.9±0.2	4.9±0.2	.87
MSNA (bursts/min)	18.8±1.5	20.2±1.5	.19
SAP (mmHg)	131.7±6.3	133.3±3.3	.96
DAP (mmHg)	72.7±5.4	72.7±3.9	.69
MAP (mmHg)	89.5±5.5	86.6±3.3	.96
HR (beats/min)	75.0±1.9	72.3±1.9	.32
LF HRV (nu)	61.7±3.5	57.4±3.2	.21
HF HRV (nu)	33.8±3.2	37.2±3.0	.16
LF/HF HRV	2.2±0.3	1.8±0.3	.15
RMSSD HRV (ms)	39.0±3.9	41.9±4.5	.64

A.8. Discussion

This study extends the recent work conducted in our laboratory on the effects of experimental muscle pain on the sympathetic nervous system (Burton et al., 2008, 2009a,b; Fazalbhoy et al., 2012, 2014; Hall et al., 2012). In our first study of 12 subjects we found that tonic muscle pain, produced by intramuscular infusion of hypertonic saline for 45min created divergent changes in muscle sympathetic outflow: one group (n = 7) showing an increase in MSNA and another (n = 5) showing a

decrease (Fazalbhoy et al., 2012). In a second study of 15 subjects, we had reported that 11 subjects showed consistent increases (n = 5) or decreases (n = 6) in MSNA when assessed on two occasions at least 2 weeks apart (Fazalbhoy et al., 2014). Here we have confirmed the findings of divergent sympathetic responses to long-lasting muscle pain, but with a much larger sample size (n = 50): one group of people (n = 24) showed an increase in MSNA and another group (n = 26) showed a decrease.

A.8.1. Baseline physiological parameters

The findings of the current study suggest that the cardiovascular responses to long-lasting muscle pain are not determined by our measured baseline physiological levels; both the direction of the response and the magnitude of change were independent of baseline MSNA, heart rate, blood pressure, heart rate variability, as well as age, sex, and BMI. This is consistent with studies showing comparable control and sensitivity of the sympathetic baroreflex in young men and young women (Tank et al., 2005; Studinger et al., 2009; Hart et al., 2011). Whether, these findings remain with increasing age is beyond the scope of this study. However, it would be interesting to know whether the pattern of response remains unchanged with age.

In this larger sample of subjects, we found no correlation between MSNA and heart rate, unlike the parallel changes observed in the smaller data sets reported previously (Fazalbhoy et al., 2012, 2014). Because of the dual innervation of the heart, it may well be that the increase in sympathetic outflow to the vascular bed in muscle is matched by a parallel increase in cardiac sympathetic drive, which would increase heart rate, but that a competing parasympathetic influence via the vagus nerve counteracts this.

A.8.2. MSNA and blood pressure

Although, there was no difference in the changes in heart rate and the change in MSNA between the two groups, in the group of subjects in whom MSNA increased during tonic muscle pain blood pressure was significantly higher than in the group in whom MSNA decreased. This suggests that the increase in MSNA was driving the increase in blood pressure, as an increase in blood pressure should, via the baroreflex, lead to a fall in MSNA. Indeed, the latter mechanism may explain why in some subjects MSNA fell despite an increase in blood pressure: in these cases, it would appear that the increase in blood pressure was causing a baroreflex-mediated reduction in MSNA, while in other instances a reduction in both blood pressure and MSNA could be the result of a nociceptor-driven withdrawal of MSNA. However, for those subjects in whom both MSNA and blood pressure increased during tonic muscle pain, we would like to suggest that nociceptor-driven increases in blood pressure could potentially be a risk factor for the development of clinically significant high blood pressure in the future, given that some individuals with chronic pain go on to develop hypertension. Indeed, patients with post-surgical chronic pain have nearly twice the prevalence of clinical hypertension than medical patients without pain (Bruehl et al., 2005). Accordingly, we could postulate that a person who consistently exhibited increases in MSNA, blood pressure, and heart rate during experimental muscle pain may—if he or she developed chronic pain from an injury in the future—go on to develop hypertension.

A.8.3. Heart rate variability

Heart rate variability is widely reported to reflect the degree of sympathetic and parasympathetic control over the heart. The LF band is proposed to represent

(primarily) sympathetic cardiac activation (Malliani et al., 1991), while the HF band is proposed to reflect vagal cardiac control (Bernston et al., 1997). Subsequently, the LF/HF ratio has been suggested as an index of the sympathovagal balance (Cohen et al., 2000; Martinez-Lavin, 2004; Staud, 2008; Reyes del Paso et al., 2011). The value of HRV in distinguishing between cardiac sympathetic and parasympathetic outflow is debatable (Goldstein et al., 2011). However, that there was no difference between any HRV parameters at either baseline or during tonic pain indicates that HRV is not related to whatever is responsible for the divergent sympathetic responses to muscle pain seen in this study.

A.9. Limitations

The intramuscular infusion of hypertonic saline occurred at a time unknown to the subject, who was asked to continuously report the development of pain, as a rating out of 10, via the linear potentiometer provided. Infusion rates were titrated—by increasing or decreasing the rate of infusion in increments of 0.02ml/min—to maintain a constant level of pain. Although we did not routinely record either the rate of infusion, or the total volume infused, in each subject, we never exceeded 20 ml (as noted in Methods we used two syringes of 10ml each). Nevertheless, there were no differences in total muscle mass in the group in whom MSNA increased and the group in whom MSNA decreased and, given that the infusion caused a notable distension of the muscle belly in both groups, it is reasonable to assume that any changes in plasma osmolality were limited to the muscle compartment and that comparable depolarization of small-diameter axons by the hypertonic saline occurred in the two groups. In other words, the noxious sensory input was the same in the two groups, as reflected in the fact that there were no significant differences in mean pain ratings

between the two groups. The same was true when we separated the cohort into males and females: the only significant differences here being the higher BMI and lower total muscle mass in the females, both of which are expected. Of course, one could argue that the intramuscular infusion of hypertonic saline would have a greater effect in a smaller muscle (in the females), but in our experience, we see no differences in mean pain ratings in small muscles (e.g., intrinsic muscles of the hand) and large muscles (e.g., flexor carpi radialis, deltoid, tibialis anterior), and pain ratings were the same in males and females.

A.10. Implications

We have shown, in a large sample of subjects (n = 50), that the baseline physiological parameters measured here do not predict whether an individual exhibits an increase or decrease in MSNA during long-lasting muscle pain. Furthermore, sex appears to play no role in determining the direction of response to muscle pain. Unlike the short-lasting pain we had previously induced by bolus injections (Burton et al., 2009a,b), we believe the physiological responses to tonic pain will more closely replicate episodes during which chronic pain patients are suffering and coping with their pain. Persistent deep pain in experimental animals has been shown to provoke a passive coping response—i.e., conservation/withdrawal (Keay & Bandler, 2002). Of course, while tonic muscle pain lasting only 20 min has been used as a model for chronic musculoskeletal pain (Capra & Ro, 2004), we should stress that this only reflects continuous nociceptive pain and not the neuropathic pain typically associated with chronic pain. Nevertheless, this method of inducing pain offers the advantage of allowing a controlled investigation into how pain may modulate MSNA, blood pressure, and heart rate. Conversely—assuming everything else is equal—one would

need to know the level of MSNA in a person prior to the development of chronic pain in order to interpret any changes in muscle sympathetic outflow. Microelectrode recordings of sympathetic nerve traffic in human subjects have found no differences in sympathetic outflow to a painful limb compared to the contralateral non-painful limb in patients with complex regional pain syndrome, suspected to be sympathetically maintained because of the marked cutaneous vasoconstriction (Casale & Elam, 1992). In order to understand the neurophysiological basis of the divergent sympathetic responses to experimental muscle pain, further investigations are needed, as the current results fail to demonstrate that baseline physiological parameters, BMI or sex, play a role in the cardiovascular responses to long-lasting muscle pain in humans.

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APPENDIX B. Pain Catastrophizing Scale

Sullivan, M.J.L., Bishop, S., Pivik, J. (1995). The pain catastrophizing scale: development and validation. *Psychol. Assess.* 7:432–524.

PCS

Client No.: _____ Age: _____ Sex: M(__) F(__) Date: _____

Everyone experiences painful situations at some point in their lives. Such experiences may include headaches, tooth pain, joint or muscle pain. People are often exposed to situations that may cause pain such as illness, injury, dental procedures or surgery.

We are interested in the types of thoughts and feelings that you have when you are in pain. Listed below are thirteen statements describing different thoughts and feelings that may be associated with pain. Using the following scale, please indicate the degree to which you have these thoughts and feelings when you are experiencing pain.

0 – not at all **1** – to a slight degree **2** – to a moderate degree **3** – to a great degree **4** – all the time

When I'm in pain …

1. ☐ I worry all the time about whether the pain will end.
2. ☐ I feel I can't go on.
3. ☐ It's terrible and I think it's never going to get any better.
4. ☐ It's awful and I feel that it overwhelms me.
5. ☐ I feel I can't stand it anymore.
6. ☐ I become afraid that the pain will get worse.
7. ☐ I keep thinking of other painful events.
8. ☐ I anxiously want the pain to go away.
9. ☐ I can't seem to keep it out of my mind.
10. ☐ I keep thinking about how much it hurts.
11. ☐ I keep thinking about how badly I want the pain to stop.
12. ☐ There's nothing I can do to reduce the intensity of the pain.
13. ☐ I wonder whether something serious may happen.

… Total

APPENDIX C. Pain Vigilance and awareness questionnaire

McCracken, L.M. (1997). "Attention" to pain in persons with chronic pain: A behavioural approach. *Behaviour Therapy*, 28:271–284.

PVAQ

Below is a list of statements about how much (or how little) you notice pain. Please rate how accurately these statements describe you. Record your rating on the line next to each item.

Never					Always
0	1	2	3	4	5

_____1. I am very sensitive to pain.

_____2. I am aware of sudden or temporary changes in pain.

_____3. I am quick to notice changes in pain intensity.

_____4. I am quick to notice effects of medication on pain.

_____5. I am quick to notice changes in location or extent of pain.

_____6. I focus on sensations of pain.

_____7. I notice pain even if I am busy with another activity.

_____8. I find it easy to ignore pain.

_____9. I know immediately when pain starts or increases.

_____10. When I do something that increases pain, the first thing I do is check to see how much pain was increased.

_____11. I know immediately when pain decreases.

_____12. I seem to be more conscious of pain than others.

_____13. I pay close attention to pain.

_____14. I keep track of my pain level.

_____15. I become preoccupied with pain.

_____16. I do not dwell on pain.

APPENDIX D. Pain anxiety symptoms scale (PASS-20)

McCracken, L.M., Dhingra, L. (2002). A short version of the pain anxiety symptoms scale (PASS-20): preliminary development and validity. *Pain Res. Manag.* 7:45–50.

Pain Anxiety Symptom Scale Short Form 20

Please rate each item in terms of frequency, from 0 (Never) to 5 (Always).

Item Numbers		Never					Always
1.	I can't think straight when in pain	0	1	2	3	4	5
2.	During painful episodes it is difficult for me to think of anything besides the pain	0	1	2	3	4	5
3.	When I hurt I think about pain constantly	0	1	2	3	4	5
4.	I find it hard to concentrate when I hurt	0	1	2	3	4	5
5.	I worry when I am in pain	0	1	2	3	4	5
6.	I go immediately to bed when I feel severe pain	0	1	2	3	4	5
7.	I will stop any activity as soon as I sense pain coming on	0	1	2	3	4	5
8.	As soon as pain comes on I take medication to reduce it	0	1	2	3	4	5
9.	I avoid important activities when I hurt	0	1	2	3	4	5
10.	I try to avoid activities that cause pain	0	1	2	3	4	5
11.	I think that if my pain gets too severe it will never decrease	0	1	2	3	4	5
12.	When I feel pain I am afraid that something terrible will happen	0	1	2	3	4	5
13.	When I feel pain I think I might be seriously ill	0	1	2	3	4	5
14.	Pain sensations are terrifying	0	1	2	3	4	5
15.	When pain comes on strong I think that I might become paralyzed or more disabled	0	1	2	3	4	5
16.	I begin trembling when engaged in activity that increases pain	0	1	2	3	4	5
17.	Pain seems to cause my heart to pound or race	0	1	2	3	4	5
18.	When I sense pain I feel dizzy or faint	0	1	2	3	4	5
19.	Pain makes me nauseous	0	1	2	3	4	5
20.	I find it difficult to calm my body down after periods of pain	0	1	2	3	4	5
	Total Score	_____					

Cont'd ▶

APPENDIX E. State and trait anxiety inventory (STAI)

Spielberger, C.D., Gorsuch, R.L., Lushene, R., Vagg, P.R., Jacobs, G.A., (1983).

Manual for the State-Trait Anxiety Inventory. Consulting Psychologists Press, Palo Alto, CA.

SPIELBERGER'S STATE AND TRAIT ANXIETY INVENTORY

SELF-EVALUATION QUESTIONNAIRE
DEVELOPED BY C.D. SPIELBERGER, R.L. GORSUCH AND R. LUSHENE

NAME_____DATE_____

DIRECTIONS: A number of statements which people have used to describe themselves are given below. Read each statement and then blacken in the appropriate circle to the right of the statement to indicate how you *feel* right now, that is, *at this moment*. There are no right or wrong answers. Do not spend too much time on any one statement but give the answer which seems to describe your present feelings best.

	Almost never	**Sometimes**	**Often**	**Almost always**
1. I feel calm……..	☐	☐	☐	☐
2. I feel secure……..	☐	☐	☐	☐
3. I feel tense……	☐	☐	☐	☐
4. I am regretful……	☐	☐	☐	☐
5. I feel at ease……	☐	☐	☐	☐
6. I feel upset……	☐	☐	☐	☐
7. I am presently worrying over possible misfortunes……	☐	☐	☐	☐
8. I feel rested……	☐	☐	☐	☐
9. I feel anxious……	☐	☐	☐	☐
10. I feel comfortable……	☐	☐	☐	☐
11. I feel self-confident……..	☐	☐	☐	☐
12. I feel nervous……	☐	☐	☐	☐
13. I am jittery……	☐	☐	☐	☐
14. I feel "high strung"……..	☐	☐	☐	☐
15. I am relaxed…….	☐	☐	☐	☐
16. I feel content……	☐	☐	☐	☐
17. I am worried……	☐	☐	☐	☐
18. I feel over-excited and "rattled"……..	☐	☐	☐	☐
19. I feel joyful……..	☐	☐	☐	☐
20. I feel pleasant……	☐	☐	☐	☐

NAME_____DATE_____

DIRECTIONS: A number of statements which people have used to describe themselves are given below. Read each statement and then blacken in the appropriate circle to the right of the statement to indicate how you *generally* feel. There are no right or wrong answers. Do not spend too much time on any one statement but give the answer which seems to describe how you generally feel.

	Almost never	**Sometimes**	**Often**	**Almost always**
21. I feel pleasant……..……	☐	☐	☐	☐
22. I tire quickly……..…	☐	☐	☐	☐
23. I feel like crying……..	☐	☐	☐	☐
24. I wish I could be as happy as others seem to be……	☐	☐	☐	☐
25. I am losing out on things because I can't make up my mind soon enough……..	☐	☐	☐	☐
26. I feel rested……	☐	☐	☐	☐
27. I am "calm, cool, and collected"……	☐	☐	☐	☐
28. I feel that difficulties are piling up so that I cannot overcome them……	☐	☐	☐	☐
29. I worry too much over something that really doesn't matter……..	☐	☐	☐	☐
30. I am happy……...	☐	☐	☐	☐
31. I am inclined to take things hard…….	☐	☐	☐	☐
32. I lack self-confidence……..	☐	☐	☐	☐
33. I feel secure……	☐	☐	☐	☐
34. I try to avoid facing a crisis or difficulty……	☐	☐	☐	☐
35. I feel blue……	☐	☐	☐	☐
36. I am content……	☐	☐	☐	☐
37. Some unimportant thought runs through my mind and bothers me ……	☐	☐	☐	☐
38. I take disappointments so keenly that I can't put them out of my mind…….	☐	☐	☐	☐
39. I am a steady person……	☐	☐	☐	☐
40. I get in a state of tension or turmoil as I think over my recent concerns and interests……	☐	☐	☐	☐

APPENDIX F. McGill Pain questionnaire

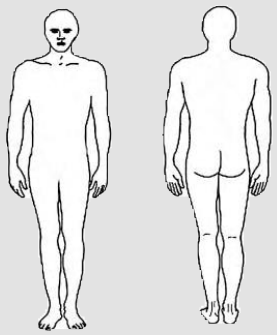
Melzack, R. (1975). The McGill pain questionnaire: Major properties and scoring methods. *Pain* 1:277–299.

McGill Pain Questionnaire

PATIENT'S NAME _____ DATE _____ TIME _____ AM/PM

PRI: S (1-10) A (11-15) E (16) M (17-20) PRI(T) (1-20) PPI

1	FLICKERING		11	TIRING		BRIEF	RHYTHMIC	CONTINUOUS
	QUIVERING			EXHAUSTING		MOMENTARY	PERIODIC	STEADY
	PULSING		12	SICKENING		TRANSIENT	INTERMITTENT	CONSTANT
	THROBBING			SUFFOCATING				
	BEATING		13	FEARFUL				
	POUNDING			FRIGHTFUL				
2	JUMPING			TERRIFYING				
	FLASHING		14	PUNISHING				
	SHOOTING			GRUELLING				
3	PRICKING			CRUEL				
	BORING			VICIOUS				
	DRILLING			KILLING				
	STABBING		15	WRETCHED				
	LANCINATING			BLINDING				
4	SHARP		16	ANNOYING				
	CUTTING			TROUBLESOME				
	LACERATING			MISERABLE				
5	PINCHING			INTENSE				
	PRESSING			UNBEARABLE				
	GNAWING		17	SPREADING				
	CRAMPING			RADIATING				
	CRUSHING			PENETRATING				
6	TUGGING			PIERCING				
	PULLING		18	TIGHT				
	WRENCHING			NUMB				
7	HOT			DRAWING				
	BURNING			SQUEEZING				
	SCALDING			TEARING				
	SEARING		19	COOL				
8	TINGLING			COLD				
	ITCHY			FREEZING				
	SMARTING		20	NAGGING				
	STINGING			NAUSEATING				
9	DULL			AGONIZING				
	SORE			DREADFUL				
	HURTING			TORTURING				
	ACHING			PPI				
	HEAVY		0	NO PAIN				
10	TENDER		1	MILD				
	TAUT		2	DISCOMFORTING				
	RASPING		3	DISTRESSING				
	SPLITTING		4	HORRIBLE				
			5	EXCRUCIATING				



E = EXTERNAL
I = INTERNAL

COMMENTS:

The descriptors fall into four major groups: sensory, 1 to 10; affective, 11 to 15; evaluative, 16; and miscellaneous, 17 to 20. The rank value for each descriptor is based on its position in the word set. The sum of the rank values is the pain rating index (PRI). The present pain intensity (PPI) is based on a scale of 0 to 5.

Other output during the candidature

Kobuch, S., Macefield, V.G., Henderson, L.A.: Resting regional brain activity and connectivity varies with resting blood pressure but not sympathetic nerve activity in normotensive humans: an exploratory study. *Journal of Cerebral Blood Flow and Metabolism*, 0271678X18798442.

Kobuch, S., Fatouleh, R.H., Macefield, J., Henderson, L.A., Macefield, V.G.: Regional grey matter volume differences with varying resting blood pressure and muscle sympathetic nerve activity in healthy humans. *Under review in Human Brain Mapping*