The Effect of Early Post-Natal Castration on Subsequent Electroencephalogram Response to Tail Docking in Lambs

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Abstract

The objective of this study was to investigate the effect of early age painful stimulation on the cortical response to subsequent painful stimulation in lambs. Using the electroencephalogram (EEG), the current study measured the effect of early age castration at one day of age on the cortical pain response to tail docking at 23 days of age in lambs.

Lambs were randomly assigned to rubber ring castration (n=12) or handling (n=12) at one day of age. At 23 days of age lambs were tail docked under a minimal plane of anaesthesia maintained using halothane in oxygen (P_EHal = 1%). EEG data was recorded for two minutes pre-docking, and for eight minutes following tail docking. EEG median frequency, spectral edge frequency and total power were derived using fast Fourier transform. Data were analysed for group (castrated versus handled), time and group by time effects using mixed model analysis, as well as for the effect of group on pre-docking EEG.

Castrated lambs showed an increased cortical response to pain, demonstrated by a greater increase in EEG median frequency (Mixed model analysis; F = 5.45, P = 0.03) and greater reduction in total power (F = 5.15, P = 0.03) in response to subsequent tail docking.

These findings indicate that early age noxious stimulation results in an increased cortical response to subsequent noxious stimulation at approximately three weeks of age in lambs. The greater cortical response in the castrated lambs would likely correspond to an increased perception of pain, and therefore the potential for a greater degree of suffering and welfare compromise in response to subsequent painful injuries, for example lambing, injury and footrot.

There was also a tendency toward a higher pre-docking total power of the EEG in the castrated lambs when compared with handled lambs (Satterthwaite's t-test; T = 1.86, P =

0.08). The higher pre-docking total power may indicate a greater background activity in the nociceptive centres of the castrated lambs. However, the significance of this finding is not clear at this stage, and further work is necessary to better define the basis and clinical importance of this observation.

Key Words: Pain, electroencephalogram, sheep, lamb, castration, hyperalgesia

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List of Abbreviations

CNS	Central nervous system
CT	Computed tomography
EEG	Electroencephalogram
F50	Median (50^{th} percentile) frequency of the electroencephalogram
F95	Spectral edge (95^{th} percentile) frequency of the electroencephalogram
FFT	Fast Fourier transform
fMRI	Functional magnetic resonance imaging
Hz	Hertz
IASP	International Association for the Study of Pain
KHz	Kilohertz
LTP	Long term potentiation
PHP	Painful husbandry procedure
Ptot	Total power of the electroencephalogram
SE	Standard error

1 Introduction

Studies in lambs have shown that neonatal pain can modify their behavioural response to later painful stimulation (Pifeleti, 2011, McCracken et al., 2010). McCracken et al. (2010) showed that lambs castrated at one day of age demonstrate a greater pain-related behavioural response to tail docking at 30 days of age than do lambs castrated at ten days of age. Indicating that castration in first few days of life can affect the behavioural response to subsequent painful stimulation. Pifeleti (2011) showed that lambs castrated at one day of age demonstrate a greater active behavioural response to tail docking at 21 days of age than do lambs handled at one day of age. Again, this finding indicates that early age castration in lambs results in a change in the behavioural response to subsequent painful stimulation that may be reflective of a greater perception of pain in these animals.

Castration and tail docking are routinely performed in New Zealand, with an estimated 24.8 Million lambs being tailed in 2010-2011 year (Beef and Lamb New Zealand, 2011), and roughly half that number also being castrated (personal observation, as these data are not routinely collected). Both castration and tail docking are procedures that, without appropriate analgesia or anaesthesia, cause considerable acute pain and distress in lambs (Mellor and Stafford, 1999, Molony et al., 1993). While the acute behavioural and physiological effects of castration and tail docking are relatively well understood, the potential long term effects of these early painful stimuli on later pain sensitivity and perception are not.

The current New Zealand PHP code (2005) recommends that lambs are castrated and tail-docked as young as is possible, and advises that these procedures may be undertaken without the provision of pain relief before six months of age. Generally, in New Zealand

lambs are castrated and tail docked within the first few weeks of life, and the use of analgesia at the time of castration and tail docking is not common practice due to the extra time, labour and costs required.

The behavioural hyperalgesia described by Pifeleti (2011) and McCracken (2010), is only seen in response to castration of neonatal lambs and is not expressed in response to castration of more developmentally mature lambs. As the nervous system is still in developing at this early stage, exposing neonatal lambs to nociceptive stimuli during a critical developmental window may affect the normal development of the nociceptive and pain pathways, with the potential to have long lasting effects on the pathways involved in the sensation and perception of pain that is not expressed in more developmentally mature lambs (Sandkühler, 2009). Sensitization of these pathways in response to early age castration in lambs may result in a greater cortical perception of pain to subsequent nociceptive stimuli, and consequently an alteration in their behavioural response to subsequent painful events.

Pain is an unpleasant sensory and emotional experience (Loeser and Treede, 2008), with the behavioural responses being mediated by the cortex (Carrasquillo and Gereau, 2008). The electroencephalogram (EEG) is a method of directly assessing cortical activity by way of measuring the electrical activity over a defined area of the cortex (Neidermeyer and Da Silva, 2005). Studies in lambs have shown that predictable changes in the frequency components of the EEG correspond to the cortical nociceptive response to tail docking under anaesthesia (Johnson et al., 2009, Johnson et al., 2005a). It has been shown that the EEG response to potentially painful stimuli is similar across lightly anaesthetised and conscious animals (Morris et al., 1997, Murrell et al., 2007, Murrell et al., 2003).

Furthermore, assessment of changes in the frequency components of the EEG provide direct insight into the magnitude of cortical response to nociceptive stimulation (Murrell and Johnson, 2006), thereby providing a direct method to assess the cortical response to painful procedures such as castration and tail docking in lambs.

This study assessed whether lambs castrated at one day of age showed a difference in their cortical response to subsequent tail docking at 23 days of age, when compared with control lambs that were handled at one day of age. Using EEG techniques, the current study evaluated whether previously described hyperalgesic behaviours in lambs in response to early nociceptive stimulation (McCracken et al., 2010, Pifeleti, 2011) correspond with an increased cortical response. A greater cortical response to subsequent tail docking in the castrated lambs would suggest a greater perception of pain, and therefore the potential for a greater degree of suffering and welfare compromise in these animals.

Structure of the Thesis

There are five main sections to this thesis: The introduction above provided a basic introduction to the study, including the most pertinent information on the basis for the study, the method employed, and objectives of the study.

The literature review provides the necessary background for understanding the methodology and findings of the study. The first section reviews pain as a study, with specific focus on castration and tail docking in lambs, including a definition of pain in animals, discussion of the pain pathways, and the main approaches to evaluating pain in scientific studies. The second section discusses the significance of early pain in precocial

mammals and the potential effects on later pain sensitivity, principally hyperalgesia. It includes a definition for hyperalgesia, consideration of the general mechanisms and experimental evidence for developmental hyperalgesia. The third section reviews the key methods for evaluating pain in animals, leading to an introduction for the electroencephalogram, its uses and application to evaluating pain in animals, as well as the key considerations and potential limitations for the electroencephalogram in the current study. The final section provides a summary of the study and its aims.

The methods section clearly lays out the technical details of carrying out the study, data manipulation and analysis. The results section details the findings of the study.

The discussion places the findings of the study in context of the current literature, considering both the principal and minor findings. It also includes a consideration of the potential limitations of the current study, and recommendations for future work. Lastly, I close with a brief section on the application of the findings and a conclusion summarising the findings and implications of the study.

Please note that there are a number of definitions for key terms provided in text. A number of these definitions are based on the taxonomy presented by the International Association for the Study of Pain (IASP), which was most recently updated May 22, 2012. The updated IASP list of definitions can be accessed on the IASP website: www.IASP-pain.org/taxonomy.

2 Literature Review

2.1 The Study of Pain

2.1.1 Definition of Pain and Nociception

Pain and nociception are two distinct physiological phenomena. The International Association for the Study of Pain (IASP) defines pain as: “An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”. The IASP definition goes further to state that: “the inability to communicate verbally does not negate the possibility that an individual is experiencing pain”. In recognition of the fact that pain in animals and humans is similar, the veterinary profession has adopted the IASP definition of pain (Paul-Murphy et al., 2004). The problem with the above definition for pain in animals is that it is based on subjective self-reporting using verbal expression (Viñuela-Fernández et al., 2007), which limits its usefulness for the current study.

A definition specific for pain in animals was proposed by Molony and Kent (1997) as:

> An aversive sensory and emotional experience representing an awareness by the animal of damage or threat to the integrity of its tissues. It changes the animal’s physiology and behaviour to reduce or avoid the damage, to reduce the likelihood of recurrence and to promote recovery.

This definition is more valuable for evaluating animal pain as it encompasses the nature of the animal’s response to pain, and therefore is the definition of pain that is used in the current study.

It is important to be aware that pain is both a sensory and emotional experience, and the animal must be both sentient and conscious to experience suffering. In the case of animals, sentience requires sufficiently sophisticated neural mechanisms to receive and interpret afferent sensory information, and consciousness is the state of awareness which allows the animal to perceive and be cognitively aware of the sensory information (Mellor and Diesch, 2006). These prerequisites are satisfied in the neonatal lambs within the first moments of birth, such that they are able to perceive pain and potentially suffer as a consequence (Mellor and Diesch, 2006).

By contrast, the International Association for the Study of Pain (IASP) defines nociception as: "The neural process of encoding noxious stimuli". The IASP goes further to state that: "[The] consequences of encoding may be autonomic (e. g. elevated blood pressure) or behavioural (motor withdrawal reflex or more complex nocifensive behaviour)… [And that] Pain sensation is not necessarily implied". Nociception is the process by which potentially harmful stimuli are transformed into physiological signals by neuronal receptors at, or near to, the site of insult. These neuronal signals are then relayed to the spinal cord and higher centres of the central nervous system (CNS) for sensory and emotional association that constitutes pain. Therefore, where nociception is a necessary step for noxious stimuli to result in the sensation of pain it is not synonymous with pain, and does not necessarily result in the perception of pain.

Throughout this thesis the term "nociceptive stimulation" is used to describe the input to the nervous system provided by tail docking. This term is preferred over "painful stimulation", as lambs were tail docked under general anaesthesia, and as such were not capable of consciously experiencing the emotional component of the pain response (Murrell

and Johnson, 2006). The rationale behind inferring cortical pain response from a nociceptive stimulus response recorded under anaesthesia will be discussed later under section 2.3.5, The Electroencephalogram and Anaesthesia.

2.1.2 Castration and Tail Docking as Painful Husbandry Procedures

Castration and tail docking are two painful husbandry procedures that are routinely carried out on farms in New Zealand, with an estimated 24.8 Million lambs being tailed in the 2010-2011 farming year (Beef and Lamb New Zealand, 2011), and close to half that number also being castrated (personal observation, as statistics on castration are not routinely collected). Castration and tail docking are both acutely painful procedures, involving both physical and emotional components that cause the animal distress. Castration and tail docking in lambs results in raised plasma cortisol levels, indicative of a stress response, as well as causing an increase in behavioural measures of pain and distress (restlessness, rolling, kicking and stamping) (Molony et al., 1993, Mellor and Murray, 1989).

Due to the pain involved they are termed painful husbandry procedures, the recommendations for which are given under the New Zealand Animal Welfare Painful Husbandry Procedures Code (2005). In this code a painful husbandry procedure is defined as:

> Any procedure carried out with or without instruments which involves physical interference with the sensitive soft tissue or bone structure of an animal and is carried out for non-therapeutic reasons. It does not apply to those procedures used to treat animals with existing injuries or disease (National Animal Welfare Advisory Committee, 2005).

Castration is performed for the purpose of flock management and effects on meat quality. It negates the risk of indiscriminate breeding, and reduces fighting and injury through inter-ram aggression (National Animal Welfare Advisory Committee, 2005). Tail docking is performed to make dagging (removing faecal dags from the tail), crutching and shearing easier, and to prevent the accumulation of faecal dags on the wool around the anus, which reduces the risk of animals getting fly-strike (National Animal Welfare Advisory Committee, 2005). Fly-strike is a disease in which flies lay their eggs in the faecal clumps. Once hatched the parasitic maggots burrow into the flesh of the host sheep and secrete poisonous ammonia. It can cause death within 3-6 days from onset, causing the animal severe pain and discomfort during this time and representing a significant welfare concern (Morris, 2000).

Methods of Castration and Tail Docking

The method of castration or docking can affect the pain experienced by the lamb. There are three different techniques commonly used to castrate lambs: rubber ring castration, emasculation (also called castration clamp and burdizzo) and surgical castration (Mellor and Stafford, 1999). Similarly, tail docking can be performed by rubber ring, hot iron (cautery) or surgically (Mellor and Stafford, 1999).

Rubber ring castration and tail docking involve the application of a tight rubber band that causes occlusion of the vessels and atrophy of the distal tissues over four to six weeks. Surgical tail docking is typically performed with a knife that is used to sever the tail. And hot iron cautery uses a specially designed heated, chiselled device to cauterise and cut through the tissues of the tail (Mellor and Stafford, 1999).

Pain and distress caused by the different methods of castration have been measured to assess what method affords the least welfare compromise. Surgical castration with a knife induces greater elevations in serum cortisol and behavioural changes indicative of pain and distress than either rubber ring or emasculation castration techniques (Mellor and Stafford, 1999). Similar results have been shown in cattle, with surgical castration causing greater distress than rubber ring castration (Fell et al., 1986), or castration with an emasculator (King et al., 1991). It must be noted that without appropriate analgesia or anaesthesia all methods of castration and tail docking cause considerable pain and distress in lambs (Mellor and Stafford, 1999, Molony et al., 1993).

In addition to the pain and distress, there are a number of further considerations for the use of different castration techniques, including ease of procedure, haemostasis and post-operative complications including swelling, haematomas and infection. Rubber ring and emasculation castration are typically bloodless and have a reduced risk of infection when compared with surgical castration, as there is no perforation of the skin (Melches et al., 2007).

Data for the method of castration in lambs is not routinely collected on farms in New Zealand. Generally, rubber ring castration seems to be the favoured method of castration on farms in New Zealand, as it is quick, cheap, easy to perform, effective, and can be performed by a single operator (Personal observation). While data for castration techniques on lambs are not available, a survey of castration methods in cattle in New Zealand showed the popularity of rubber ring castration, reporting that 74% of 3,788 respondents castrated calves on farm, and of these 85% used a rubber ring, 18% carried out surgical castration and only 0.009% used an emasculator (Stafford et al., 2000).

Rubber ring castration is performed by placing a rubber ring around the neck of the scrotum, proximal to the testes and distal to the nipples, using an elastrator. The ring interrupts blood supply, resulting in hypoxia and eventually anoxia, and the tissue then atrophies and drops off over a period of four to six weeks (Mellor and Stafford, 1999). Pain is a result of the mechanical crushing of the tissue, the ischaemic visceral pain, and inflammatory pain due to tissue damage (Molony et al., 1993). In lambs, cortisol levels remain elevated for approximately three to four hours following castration and tail docking with a rubber ring, indicating that the acute pain response may last for several hours (Mellor and Stafford, 1999).

Castration and tail docking are generally performed at the same time (personal observation), and many of the considerations outlined above for castration apply to tail docking. However, the pain of castration includes visceral nerve supply to the testes, in addition to the nerves supplying the skin and other tissues, and is therefore a qualitatively and quantitatively different pain experience to tail docking. Behavioural responses suggest that rubber ring castration may cause severe acute pain in very young lambs, including a failure to suckle and disruption of maternal bonding (Paul Kenyon, personal communication). For this reason it is not advised to tail dock lambs less than 12 hours old, and this recommendation has been adhered to in the current study.

Due to the likely greater use of rubber ring application for castration and tail docking on lambs in New Zealand, and the fact that the acute pain response is relatively well understood (Mellor and Stafford, 1999), this method has been chosen for use in the current study.

2.1.3 Types of Pain

Pain can broadly be divided into three types: somatic, visceral and neuropathic (Landa, 2012). Somatic pain comprises pain from the skin (cutaneous), muscles and connective tissues, including bone. The pain from tail docking is due to afferent somatic nociceptors. Visceral pain comprises pain from the viscera, or internal organs. Castration with a rubber ring involves visceral pain which is transmitted through afferent nerves in the spermatic cord (Molony and Kent, 1997), in addition to the somatic pain from the skin, muscle and connective tissues. Neuropathic pain is due to neurological damage or changes, including neuropathies and central pain states, as such the phenomena hyperalgesia and allodynia can be considered a form of neuropathic pain (Cervero, 2008). The phenomena of hyperalgesia and allodynia will be covered in detail in the next section of the literature review.

2.1.4 Pain Pathways

The pain pathways are the neurological circuits by which nociceptive signals are transduced and coded at the periphery, relayed to the central nervous system, and then projected to different regions of the brain for modulation, sensory association and perception. In rats, the placing of experimentally induced lesions has demonstrated that the two main ascending nociceptive pathways in the spinal cord, the spinothalamic and dorsal column pathways, both contain somatic and visceral nerve fibres (Palecek et al., 2002). Furthermore, both somatic and visceral afferents can exhibit sensitization of the spinal neurons of the dorsal horn, representing a potential pathway for central sensitization and consequent hyperalgesia (Sandkühler, 2009, Gebhart, 2000). While such studies have not

been performed in lambs, the work in rats suggests that the nociceptive signals of castration and tail docking are carried in these ascending nociceptive pathways, and that the spinal cord is a possible site of sensitization following these procedures.

The pain pathway can be broken down into four key stages: transduction, transmission, modulation and perception (Hathway and Fitzgerald, 2008). While the whole pathway together is termed “pain pathway”, until the point of perception it is more accurate to refer to the pathway as a “nociceptive pathway”, and this distinction has been applied throughout the thesis.

Transduction

Transduction is the process whereby peripheral nociceptors convert noxious stimuli (thermal, mechanical or chemical) into a physiological signal which is coded electrical activity (action potentials) in the neuron, this coded electrical activity is then relayed up the afferent nociceptive pathways (Hathway and Fitzgerald, 2008). Nociceptors are the peripheral nerve endings of primary sensory neurons, with their cell bodies located in the dorsal root ganglia of the spinal cord and trigeminal ganglia of the brainstem (Hathway and Fitzgerald, 2008, Carrasquillo and Gereau, 2008). There are two principal types of peripheral nociceptors: A-delta neurons which respond to thermal and mechanical nociceptive stimuli and C-type neurons that are polymodal nociceptors responding to high intensity mechanical, chemical and thermal stimuli (Hathway and Fitzgerald, 2008, Carrasquillo and Gereau, 2008). In castration and tail docking both fibre types are involved, with A-delta fibres being involved in the initial short sharp mechanical pain, and C-type fibres being responsible for the longer duration of ischaemic and inflammatory pain following castration.

Transmission

Transmission is the process whereby peripheral nociceptors relay the encoded electrical signals up the ascending spinothalamic and dorsal column pathways (Hathway and Fitzgerald, 2008). Both classes of peripheral primary neurons project to lamina I, II and V in the dorsal horn of the spinal cord.

Modulation

Two major systems of modulation have been identified in the nociceptive pathways: local spinal cord modulation and descending modulation. Local spinal cord modulation occurs at the level of the dorsal horn of the spinal cord, involving excitatory and inhibitory interneurons that act to influence the synaptic conductions between the primary (peripheral nociceptors) and secondary neurons (projection neurons of the central nervous system) of the nociceptive pathways (Carrasquillo and Gereau, 2008). Descending modulation is mediated by higher centres of the CNS, primarily neuronal centres that originate in the periaqueductal grey matter of the medulla, that project to act on the primary afferent neurons and dorsal horn of the spinal cord. These projections can act to both inhibit (dampen) and facilitate (enhance) the activity of afferent peripheral nociceptors and projection neurons at the level of the dorsal horn (Carrasquillo and Gereau, 2008).

Following local and descending modulation, projection neurons carry the signal up the spinal cord to the supraspinal centres, including the brainstem, midbrain, hypothalamus, thalamus and amygdala (Hathway and Fitzgerald, 2008, Carrasquillo and Gereau, 2008). These sites of modulation are central to current theories on the development of

hyperalgesia and allodynia, which will be discussed in detail shortly in section 2.2.2, General Mechanisms of Hyperalgesia.

Perception

Pain perception is the conscious appraisal of ascending nociceptive signals that, through the subjective cognitive and emotional association, can result in an animal suffering as a consequence of pain (Carrasquillo and Gereau, 2008) (Figure 2.1).

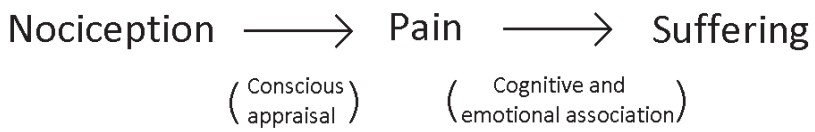


Figure 2.1: Flow diagram showing the relationship between nociception, pain and suffering in animals.

It is known that the ascending signals from peripheral nociceptors diverge to many different brain centres, with the main centres being the hypothalamus, limbic system, reticular formation, basal ganglia, insula cortex and the primary somatosensory cortex (Carrasquillo and Gereau, 2008). These centres communicate extensively to result in the perception of pain, accounting for the conscious, emotional, and motivational aspects of pain, which combine to effect a change in behaviour. The necessary role and extent of cortical involvement in the processing of pain has been established by medical imaging techniques which demonstrate the functional activity of the brain in response to painful stimulation,

namely magnetic resonance imaging, computed tomography and positron emission tomography (Treede et al., 1999, Jones et al., 1992, Talbot et al., 1991).

In clinical terms the stages of pain sensation and behavioural response can be summarised into five stages: application of the nociceptive stimulus, ascending nociceptive signal, local spinal reflexes, pain perception, and lastly the behavioural response (Figure 2.2).

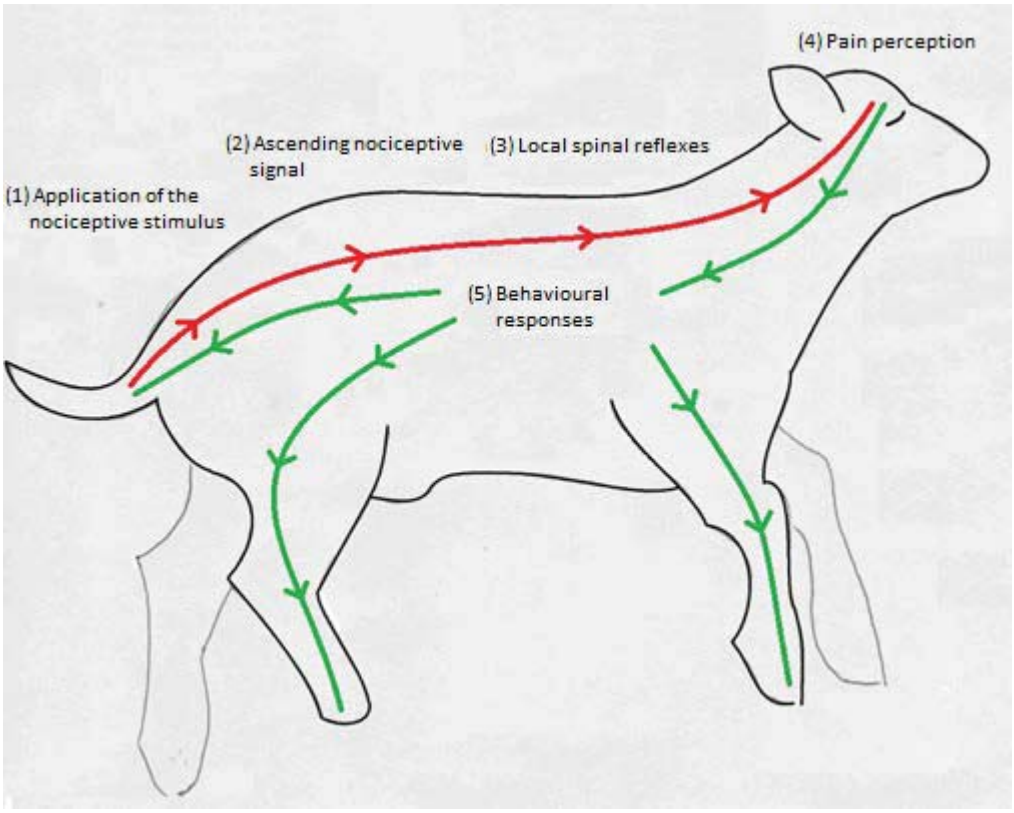


Figure 2.2: Schematic diagram of pain pathway from stimulus to perception, and then to behavioural response in the lamb.

Pain can be inferred from responses to stimulation of peripheral nociceptors that occur upstream of cortical pain perception, including local spinal withdrawal reflexes and physiological changes mediated by the autonomic nervous system, for example elevated

heart rate (Sneddon et al., 2014). However, these upstream responses are not specific as responses to pain as they do not require cortical appraisal of the noxious insult.

It is only at the level of the cortex that nociceptive signals take on the sensory and emotional characteristics of pain. Consequently, pain perception can only be directly assessed by measuring the activity of the appropriate populations of cortical neurons.

2.2 Hyperalgesia and the Potential Longer Term Effect of Early Pain Exposure

2.2.1 Defining Hyperalgesia

Hyperalgesia is defined by the IASP as: “Increased pain sensitivity”. More specifically, hyperalgesia is an increased response to stimuli that would normally activate afferent nociceptive fibres, and would therefore be painful stimuli even in the absence of increased pain sensitivity (Cervero, 2008, Sandkühler, 2009) (Figure 2.3). Allodynia, by contrast, is defined by the IASP as: “pain in response to non-nociceptive stimulus”, for example soft touch being sensed as painful at a site of injury (Cervero, 2008). It must be stressed that allodynia can only be used appropriately where it is known that stimulus in question is not normally able to activate nociceptors (Sandkühler, 2009) (Figure 2.3).

“Hyperalgesia” is sometimes used as an umbrella term for increased pain sensitivity, including both a reduced threshold for nociception (allodynia) and an increased response to supra-threshold stimuli (hyperalgesia). This generalised use of the term hyperalgesia should be avoided, as it confuses the two distinct phenomena of hyperalgesia with allodynia.

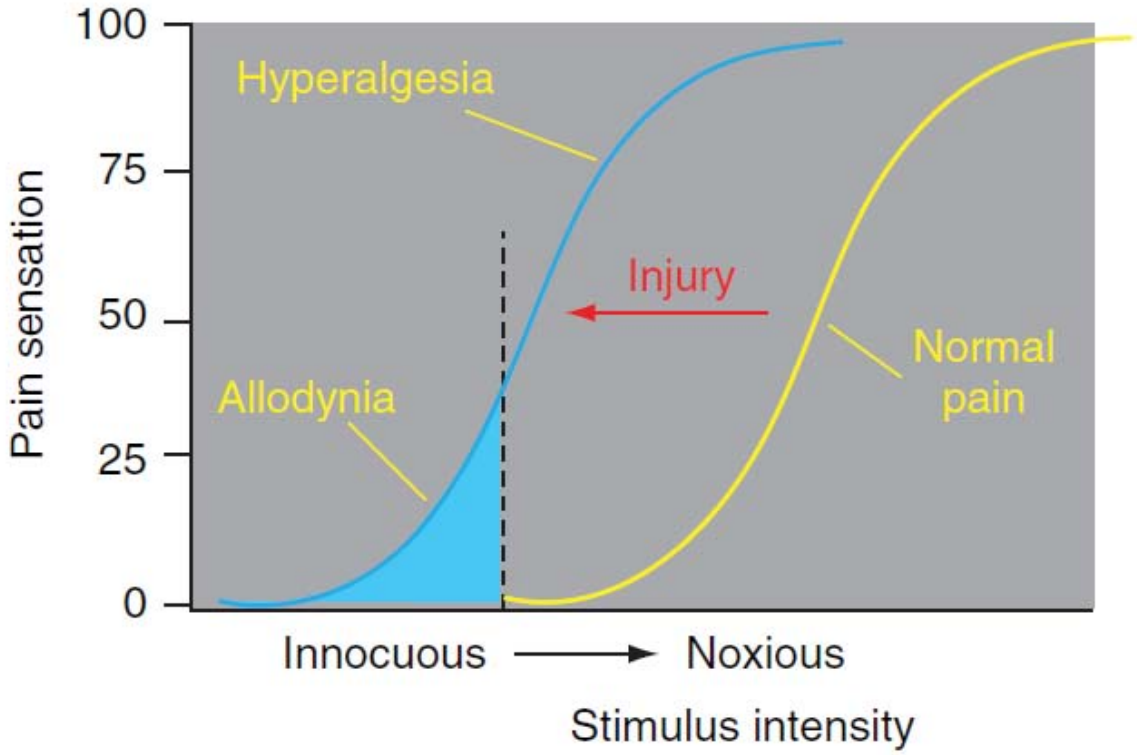


Figure 2.3: Diagram representing the distinct phenomena of hyperalgesia and allodynia; where hyperalgesia represents an increase pain sensation of painful stimuli, and allodynia represents a pain sensation in response to normally innocuous stimuli. Figure source: Cervero (2008).

Hyperalgesia can be primary, which is an increased sensitivity at the site of original injury or noxious insult; and/or secondary, which is an increased sensitivity at areas adjacent or removed from the original site of injury (Sandkühler, 2009). Primary hyperalgesia is thought to be due to both sensitization of the peripheral nerve endings and central sensitization (Sandkühler, 2009). By contrast secondary hyperalgesia, due to it being at a site removed from the initial insult, is thought to be due to central sensitization exclusively (Sandkühler, 2009); where central sensitization is defined by the IASP as: “increased responsiveness of

the nociceptive neurons in the central nervous system to their normal or subthreshold afferent inputs".

2.2.2 General Mechanisms of Hyperalgesia

In general terms the key physiological mechanisms underpinning the development and maintenance of hyperalgesia are sensitization of peripheral nerve endings, and central sensitization. Sensitization of peripheral nerve endings rarely outlasts the primary cause for pain and is anatomically restricted to the site of injury (Sandkühler, 2009). Therefore, while sensitization of peripheral nerve endings may potentially be important in primary hyperalgesia, it is not considered necessary for the phenomenon of secondary hyperalgesia investigated in the current study.

Central sensitization as a mechanisms for hyperalgesia is thought to be mainly due to sensitization of the synapses and neuronal pathways in the dorsal horn of the spinal cord, where afferent nociceptive signals are relayed to the cortex through ascending projection neurons (Sandkühler, 2009). It is proposed that sensitization of adjacent synapses, and of synapses that are common to more than one anatomical region of nociceptive sensation may account for the development of secondary hyperalgesia (Sandkühler, 2009, Carrasquillo and Gereau, 2008).

There are three types of central neuronal sensitization described at the level of the dorsal horn: wind-up, long term potentiation and classical central sensitization (Carrasquillo and Gereau, 2008). Wind-up occurs in response to a train of repeated stimuli in rapid succession. It lasts only as long as the duration of the stimuli, and as such is not directly relevant when considering prolonged hyperalgesia. Long term potentiation occurs in response to excessive

stimulation (either a high frequency or high intensity of stimulation), and it has been shown that long term potentiation can result in an increase in pain sensitivity (Carrasquillo and Gereau, 2008). However, the significance of LTP in hyperalgesia remains debated (Carrasquillo and Gereau, 2008). LTP occurs only at the synapse that is being stimulated, and does not appear to affect adjacent synapses (Carrasquillo and Gereau, 2008). This would mean that LTP is an unlikely explanation for secondary hyperalgesia in the developing nervous system. Lastly, classical central sensitization is seen in response to nociceptive insult and injury, and results from sensitization of the projection neurons of the dorsal horn of the spinal cord, partly due to a reduction in the inhibitory tone of the local interneuron network (Carrasquillo and Gereau, 2008). Unlike wind-up and LTP, classical sensitization occurs in adjacent neurons as well as the activated neuron(s) (Carrasquillo and Gereau, 2008), an observation which may help explain the phenomenon of secondary hyperalgesia.

Evidence for post-natal development of the pain pathways

Fine tuning and refinement of the sensory and nociceptive pathways is an important developmental process that demonstrates a greater developmental plasticity early in post-partum life (Mellor and Lentle, 2014). During this early post-partum refinement of the nervous system may be particularly susceptible to the effects of noxious stimulation, such as early age castration and tail docking in lambs.

The current New Zealand Animal Welfare (Painful Husbandry Procedures) Code of Welfare (2005) recommends that lambs are castrated and tail-docked as young as is possible, and advises that these procedures may be undertaken without the provision of pain relief before six months of age. Typical farming practice in New Zealand is to perform castration

and tail docking at the same time once there are sufficient lambs to justify the labour of herding, yarding, and the procedure of castration, tail-docking and applying ear markers (Personal observation). Currently, there are no statistics available on the average age of lambs at tailing and castration on New Zealand farms, as these data are not routinely collected. It is my experience that the majority lambs would be castrated and tail docked within the few weeks of life, and that the use of analgesia at the time of castration and tail docking is not common practice due to the extra time, labour and costs required.

Lambs of different ages demonstrate quantitatively and qualitatively different behavioural and physiological (electroencephalogram) responses to castration over the first weeks of life (Johnson et al., 2009, Johnson et al., 2005a, Molony et al., 2002, Thornton and Waterman-Pearson, 1999, McCracken et al., 2010). For example, lambs of different ages demonstrated qualitatively different behavioural responses to castration, with lambs castrated at 10 days of age showing a greater incidence of abnormal behaviours indicative of pain and distress than did lambs castrated at one day of age (McCracken et al., 2010). The reduced pain-related behavioural response of the one day old lambs may reflect a true difference in severity of pain perceived when compared with the ten day old lambs, or it may reflect a developmental difference in the ability of the one day old lambs to express these behaviours.

Using EEG techniques, it has been shown that two week old lambs undergo a qualitatively different cortical pain response to tail docking when compared with four week old lambs (Johnson et al., 2005a). More recent work showed that one day old lambs did not show a significant cortical response to castration, whereas ten day old lambs showed an “adult-like” response, suggesting that there were significant changes in pain perception over the first

ten days of life in lambs (Johnson et al., 2009). It must be noted that the response of the ten day old lambs was described as “adult-like” by the authors of Johnson et al. (2009), although this response was still not the same as the EEG response of the four week old lambs, suggesting that there may be further development of the EEG between ten days and four weeks of life.

The finding that older lambs have a reduced behavioural response to thermal stimulation contrasts with the findings of Johnson et al. (2009), who showed that younger lambs showed a reduced cortical response to castration. It is possible that this difference owes to the different modalities of nociceptive stimulation, or that the older lambs are showing a reduced behavioural response and yet are subject to a similar or greater degree of cortical nociceptive response.

Development of the cortical response to painful stimuli in early post-natal life has also been shown in rat pups and wallabies. Rat pups showed a progression from isoelectric EEG activity (no activity) at 5-7 days old with no response to tail clamping, developing in to intermittent EEG activity at greater than 12 days old, with a greater baseline power and greater response to tail clamping in the older rats (Diesch et al., 2009). This same progression has also been shown in wallabies, developing from isoelectric EEG activity up to 127 days old, with an increase in EEG power with increasing age up to 261 days old (Diesch et al., 2010), and similarly a greater EEG response to tail clamping in the older animals. These studies suggest that there is continued development of the nociceptive pathways throughout the neonatal period, though the extent and rate of this development appears to vary across species, for example rats and wallabies discussed above (Diesch et al., 2010, Diesch et al., 2009).

The role of differing developmental timeframes across species

Different mammalian species are born at very different stages of developmental maturity, and this generalisation is true for the development of the nociceptive pathways and cortical pain centres. Broadly speaking, at birth mammals can be placed on a continuum from developmentally immature at birth (altricial) to developmentally mature at birth (precocial) (Mellor and Lentle, 2014). Lambs and other ruminants are considered to be highly developmentally mature at birth, and are able to see hear, stand and walk within minutes to hours of birth. Rodents and human infants are, by comparison, considered moderately developmentally mature at birth and show minimal activity for the first few weeks of life (Mellor and Lentle, 2014). For example, in these moderately developmentally mature species hearing and sight are not present at birth and typically do not become active until two to three weeks of age depending on the species (Mellor and Lentle, 2014). Marsupials, for example wallabies, are by contrasts extremely developmentally immature at birth and do not develop the capacity for hearing and sight until approximately 130-140 days of age (Mellor and Lentle, 2014).

These differing developmental timeframes are linked to the individual parenting approach and survival demands of each species in early life, and the behavioural maturity at birth also correlates to the stage of neurological development. Electroencephalogram studies in Tammar wallabies (Diesch et al., 2010), rats (Diesch et al., 2009) and lambs (Johnson et al., 2009) allow for direct comparison of the EEG across these three species that represent three stages of developmental maturity at birth (Diesch et al., 2008). For example, Tammar Wallabies show no EEG activity recordable at birth, but develop toward mature patterns of EEG activity by 2-5 months of age. In addition to the EEG evidence, the cerebral cortex is

composed of a relatively undifferentiated structure two cell layers thick at birth, which resembles the cortex of 26 day old sheep embryo (Mellor and Lentle, 2014). In comparison, rats show an intermittent and immature EEG pattern at birth that becomes continuous with mature patterns of activity evident by 1-3 weeks of age, and lambs show mature continuous EEG patterns for several weeks pre-partum that remains continuous with mature patterns of EEG activity following birth (Mellor and Lentle, 2014).

One key measure of neurological development is the establishment of thalamo-cortical connections that allow for sufficient neural integration to permit perceptual awareness, where the advent of perceptual awareness is central to the cognitive and emotional association of ascending nociceptive signals and thus to pain perception (Diesch et al., 2008). Functional thalamo-cortical connections are established between two to five months of age in marsupials, by 2-3 weeks in rodents, and are operational several weeks pre-partum in lambs (Diesch et al., 2008). This variation in developmental timeframes highlights the potential importance of developmental maturity across species, and underscores why it is necessary to be careful when applying studies done in rodents to the development of pain perception and hyperalgesia in lambs. It also highlights the relative sense of the term “early pain”, and that what is considered developmentally ‘early’ pain may differ between different mammalian species. This is not to say the findings in rodents cannot be applied to lambs, and in many cases this is necessary as there are often no primary studies done in lambs. Furthermore, while lambs are born at a developmentally more mature stage than humans, rats and wallabies, it has been shown that there is still significant post-natal development of the central nervous system and more specifically the nociceptive pathways and central processing of ascending pain signals in lambs.

The effect of age on the sensitization of the developing nociceptive pathways

As a physiological explanation for the behavioural hyperalgesia observed in lambs in response to early age castration (Pifeleti, 2011, McCracken et al., 2010), I propose that there is a critical developmental period in which the nociceptive pathways are uniquely susceptible to nociceptive inputs; and that this same plasticity and susceptibility of the nociceptive pathways is not expressed in more developmentally mature lambs. Preliminary work by Pifeleti (2011) and McCracken et al. (2010) suggests that this critical developmental window exists at less than ten days of age. These preliminary observations are the basis for castrating lambs at one day of age in the current study, as I one day of age would most likely fall within this potential critical developmental period.

Generally speaking the dorsal horn of the spinal cord is more excitable in neonates than in physiologically mature animals , and this increased excitability has been proposed to be, at least in part, due to the reduced influence of descending inhibition on the dorsal horn of neonates (Sandkühler, 2009, Fitzgerald and Jennings, 1999, Gonzalez et al., 1993, Fitzgerald, 1985). The greater excitability of the spinal cord in neonatal animals is thought to be linked to the exaggerated behavioural responses to pain that can be seen in response to noxious stimulation in neonates, for example the increased and more generalised withdrawal reflex in response to nociceptive stimulation of the paw outlined above (Sandkühler, 2009). Though it must be noted that comparison of behavioural pain response between neonatal and more developed juvenile or even adult animals is limited due to the under-developed physical abilities in neonates when compared with older animals (Mellor and Lentle, 2014).

Studies have demonstrated that the neonatal nociceptive system in neonates exhibits a critical developmental period of activity dependent physiological and functional plasticity,

which is not observed in developmentally mature, adult nociceptive system. (Sandkühler, 2009, Gonzalez et al., 1993, Beggs et al., 2002, Grunau et al., 2006).

In the majority of mammalian species (including sheep, rodents and humans, and not including those that are exceptionally immature at birth) the nociceptive pathways are fundamentally formed in utero, and are subjected to post-natal refinement through activity dependent extinction and reinforcement (Sandkühler, 2009, Castro-Lopes et al., 1993, Fitzgerald and Jennings, 1999, Gonzalez et al., 1993).

In human neonates peripheral cutaneous nociceptors (which signal to the dorsal horn of the spinal cord) have relatively large receptive fields that are developed through activity dependent fine-tuning of both peripheral nociceptors and their afferent tracts over the first few weeks of life (Sandkühler, 2009, Fitzgerald and Jennings, 1999). Human neonates show nociceptive fine tuning in the local spinal cord reflex loops, with a general reduction in the magnitude of behavioural withdrawal reflex to mechanical stimulation over the first 35 weeks of age (Andrews and Fitzgerald, 1994). Human infants under 30 weeks of age showed a greater response to subsequent cutaneous stimulation, following non-painful mechanical stimulation of the skin, when compared with the response of infant over 30 weeks of age (Andrews and Fitzgerald, 1994). This observation indicates that there is an early developmental period of cutaneous sensory plasticity in human infants at less than 30 weeks of age.

In rodents this spatial fine-tuning of the cutaneous nociceptor receptive fields occurs predominantly over the first 14 days of life, with pinching or brushing of the hindlimb in rats resulting in long lasting discharges in the dorsal horn of the spinal cord that were of 30-90 seconds duration up to three days post-natal, and reduced in amplitude and duration with

increasing age over the first 15 days post-natal (Fitzgerald, 1985). Furthermore, neonatal rats show an exaggerated and more generalised behavioural withdrawal reflex in response to a painful prick of the foot, a response that becomes reduced in magnitude and more specific in space with increasing post-natal maturity (Sandkühler, 2009, Fitzgerald and Jennings, 1999).

These studies suggest that there is a consistency in the process of post-natal refinement in cutaneous sensory processing in humans and rats, and furthermore that these processes occur over markedly different developmental timeframes between these two species. To the best of the authors knowledge, the exact dates of this cutaneous fine tuning and spinal cord excitability has not been studies in lambs. However, it seems likely that lambs undergo a similar period of postnatal refinement of cutaneous nociceptor receptive fields, as this is not a process of development that can be meaningfully achieved and fine-tuned in utero.

The potential role of descending inhibition in early post-natal development

One proposed explanation for the greater response to noxious stimulation seen in the neonate, is that there is a reduced, or possibly absent, descending inhibition of the ascending and dorsal horn nociceptive pathways in neonates (Sandkühler, 2009). Afferent nociceptive stimulation of the developing neonatal dorsal horn may result in exaggerated development of long term potentiation and classical sensitization resulting in centrally mediated hyperalgesia, due to the fact that these neurons are generally more excitable and are under a reduced influence of descending inhibition (Sandkühler, 2009).

In rats the lumbar dorsal horn cells demonstrate an age related response to stimulation of the dorsolateral funiculus (the brain centre responsible for projecting descending inhibition

signals in the rat) that affects the processing of nociceptive response to mechanical stimulation of the skin of the hind paw (Fitzgerald and Koltzenburg, 1986). The study showed that normal descending inhibition was present in 22-24 day old rats but reduced with decreasing age, with only half of the cells demonstrating an influence of descending inhibition at 12 days of age, and no cells responding at nine days of age or younger (Fitzgerald and Koltzenburg, 1986). Again, to the best of the author's knowledge the age related development of descending inhibition of the dorsal horn has not been studied in lambs and it is quite possible that, due to the very different neuro-developmental timeframes of lambs and rats (Mellor and Lentle, 2014), the neural centres and pathways of the spinal cord may show a very different stage and rate of post-natal development.

Having outlined the physiological basis for the development of hyperalgesia in the neonate, I will now outline the key studies which have demonstrated the development of behavioural hyperalgesia and its link to early age noxious stimulation in mammals.

2.2.3 The Evidence for Hyperalgesia in Response to Early Age Noxious Stimulation

It has been shown that noxious stimulation during an early developmental window can alter later pain responsiveness in humans (Grunau et al., 2006, Taddio et al., 1997, Taddio et al., 1995), rats (Anand et al., 1999, Wang et al., 2004) and lambs (Pifeleti, 2011, McCracken et al., 2010).

Work in human neonates has shown that noxious stimulation during the neonatal period can alter later pain sensitivity, however the direction of this change appears to vary and may depending on the timing, type and extent of the noxious insult (Grunau et al., 2006, Lidow, 2002). There are also varied findings as to whether the alterations in pain responsiveness

are transient or permanent (Lidow, 2002). Human infants circumcised at 5 days or younger showed an increased behavioural response to vaccination at 4-6 months when compared with non-circumcised controls, or neonates treated with EMLA (Eutectic Mixture of Local Anaesthetics; comprising lignocaine and prilocaine at the time of circumcision (Taddio et al., 1997, Taddio et al., 1995). Further work has shown that prematurely born human neonates exposed to repeated routine painful procedures presented with a lower threshold to tactile stimulation (greater sensitivity) and greater responses to supra-threshold stimuli (hyperalgesia) as adolescents aged 12-18, when compared with the response of adolescents born at term who were not subjected to repeated routine painful procedures (Buskila et al., 2003). These data suggest that the effects of early painful stimuli in humans may be significant many years after the initial insult. However it is also possible that the differences in later pain responses may, in part, be a consequence of developmental differences between neonatal and full term infants. However, the longer duration of the increased pain responsiveness reported by these studies, may be a composite effect of repeat painful procedures and the social and behavioural stressors in the NICU environment (Lidow, 2002, Grunau et al., 2006).

Studies in rats have shown that repeated pin prick stimulation of the paw from one to seven days of life causes a reduced pain threshold to later thermal stimulation at 16, 22 and 65 days of age (Anand et al., 1999). Further studies in rats have shown that inflammatory injury to the hind paw in neonates can produce increased pain response to subsequent noxious inflammatory event as an adult (Lidow et al., 2001). Further observation showed that the increased pain response was observed in the limb contralateral to the initial site of noxious insult, as well as in both fore paws (Wang et al., 2004, Ren et al., 2004). Subsequent work showed that somatic injury in three day old (neonatal) rats induced alterations in visceral

and somatic pain processing, which was not observed in control rats, or rats that experienced somatic hind paw injury at 14 days of age (Wang et al., 2004). These studies show that early neonatal pain in a critical period of development, less than 14 days in rats, can modify both visceral and somatic pain responses in adult life, both at the site of initial injury and at other sites in the body. Lastly, two week old rats show a faster return to baseline limb withdrawal latencies following hind paw incision, than did four and 16 week old rats (Ririe et al., 2003). It must be noted that the rats in the study were all at least two weeks old, and as such were not neonates. Nonetheless, the more rapid decrease in mechanical allodynia in the younger rats, when compared with older rats, indicates an age dependent difference in development of the pain pathways following noxious stimulation that is expressed beyond the neonatal period.

In lambs, early age castration has been shown to alter behavioural response to later tail docking. For example, McCracken et al. (2010) showed that lambs castrated at one day of age showed a greater behavioural response to rubber ring tail docking at 30 days of age than did lambs castrated at ten days of age, with the lambs castrated at one day of age showing a greater incidence of rolling, standing unsteadily and standing with abnormal posture. The observation that lambs castrated at one day of age showed a greater behavioural pain response to subsequent tail docking than did lambs castrated at ten days of age suggests that the earlier castrated lambs showed a greater behavioural hyperalgesia. This observation highlights the importance, in terms of development and sensitivity, of the timing of noxious stimulation in causing behavioural hyperalgesia. As there were no controls, it is possible that the lambs castrated at ten days of age also showed behavioural hyperalgesia in response to subsequent tail docking, and that it was simply to a lesser degree than the lambs castrated at one day of age. Furthermore, as both groups were tail docked at approximately 30 days of

age, those castrated at 1 day of age had a much greater interval to tail docking. It seems improbable that a greater duration between castration and tail docking is responsible for the increased response to tail docking. Therefore, a greater behavioural response in the animals castrated at one day of age likely indicates that it is the early age castration, and not duration between castration and tail docking, that is responsible for the greater behavioural response.

Pifeleti (2011) further assessed the effects of age at castration on later behavioural response to tail docking, as well as the effect of duration between castration and tail docking. The study compared the behavioural response to tail docking across six groups: (1) castrated or (2) handled at one day of age and tail docked at 21 days of age, (3) castrated or (4) handled at 21 days of age and tail docked at 42 days of age, and (5) castrated or (6) handled at one day of age and tail docked at 42 days of age. Pifeleti's study found that prior castration altered the behavioural response to later tail docking, with castrated animals showing a greater active behavioural response to tail docking than did handled lambs. The effect on subsequent behavioural response was greatest when lambs were castrated at one day of age and tail docked at 21 days of age, when compared with castration at 21 days of age and tail docked at 42 days of age. These findings indicate that the modified behavioural response were a consequence of early age castration and not the duration between stimuli. Furthermore, there was no significant difference in the behavioural response between lambs castrated at one day of age and tail docked at 42 days of age, and with lambs handled at one day of age and tail docked at 42 days of age. This finding indicates that the altered behavioural response observed at three weeks of age, was transient and resolved by six weeks of age. The findings reported in Pifeleti (2011) informed the selection of treatment ages for the current study.

While the behaviours in lambs described above are not singularly indicative of pain, they are reliable indicators of discomfort and distress (see review by Murrell and Johnson 2006), and the aforementioned studies have evidenced an increase in behaviours that are indicative of distress and discomfort in response to early nociceptive stimuli exposure.

In summary, both McCracken et al. (2010) and Pifeleti (2011) showed that early age castration affects later behavioural response to tail docking in lambs. These studies suggest that there is a period of greater sensitivity in early neonatal life in precocial mammals such as lambs, which may lead to transient hyperalgesia of several weeks duration in response to early age painful events. As there is development of the lamb's cortical pain response and pain processing pathways during the first weeks of life (Johnson et al., 2009, Johnson et al., 2005a), it is possible that exposing a very young animal to pain during critical periods of neurological development may affect subsequent pain processing, resulting in prolonged hyperalgesia and consequently an increased pain sensitivity to subsequent noxious stimuli, for example lambing, injury and footrot in sheep.

2.3 Evaluating Pain and the Electroencephalogram

2.3.1 Key Considerations for Evaluating Pain in Animals

The foremost barrier to the measuring of pain is that pain is an inherently subjective experience (Carrasquillo and Gereau, 2008), and for this reason self-reporting is considered to be the gold standard for assessment in humans (Chapman et al., 1985, Chen et al., 1989, Chen and Rappelsberger, 1994). Animals, however, are incapable of verbal expression. Therefore, it is not possible to prove that an animal is suffering an adverse state that is analogous to pain in humans (Sneddon et al., 2014).

Bateson (1991) provided a series of criteria which, where satisfied, provided a framework for assessing whether animals can be considered capable of experiencing pain. His criteria were as follows: possession of nociceptors; pathways from nociceptors to the brain; brain structures analogous to the human cerebral cortex that process pain; opioid receptors and endogenous opioid substances in a nociceptive neural system; a reduction in adverse behavioural and physiological effects after administration of analgesics or painkillers; learning to avoid potentially painful stimuli and that this learning is rapid and inelastic. All of these criteria have been demonstrated across numerous mammalian species (Sneddon et al., 2014), including lambs (McCracken et al., 2010, Johnson et al., 2009, Mellema et al., 2006, Grant, 2004, Graham et al., 1997, Cottrell and Molony, 1995, Mellor and Stafford, 1999, Johnson et al., 2005a), suggesting that beyond reasonable doubt lambs are capable of experiencing pain in response to noxious stimulation, including castration and tail docking.

2.3.2 Evaluating Pain through Behavioural and Physiological Measures

Pain in animals has typically been studied through measures of behaviour or physiological changes (Murrell and Johnson, 2006, Sneddon et al., 2014). However, both behavioural and physiological parameters are indirect measures that lack specificity to pain, and are poorly correlated to self-reported pain in humans (Bateson, 1991, Chapman et al., 1985).

Candidate behaviours for study are selected based on their repeatable association with noxious stimulation, analogy to human pain behaviours, repeatability and amelioration by the appropriate use of analgesia (Sneddon et al., 2014). Examples of pain related behaviours in sheep in response to castration and tail docking include: increased incidence of active behaviours, such as rolling, standing up and sitting down, walking backwards, and the

incidence and duration of abnormal behaviours, such as standing with abnormal posture, standing unsteadily, kicking, and watching the site of noxious stimulation (McCracken et al., 2010, Mellor and Stafford, 1999). However, none of these behaviours are specific indicators of the magnitude of pain perception in lambs.

There are a number of key limitations to behavioural studies to consider, with the main problems being that pain behaviours differ among species, individuals of the same species, and at different ages during development, as well as being context specific (Paul-Murphy et al., 2004). Pain response can also vary depending on factors such as location and type of injury, meaning that comparison of different husbandry techniques can be difficult. For example lambs show qualitatively different behavioural responses to knife versus ring castration methods (Mellor and Stafford, 1999). Furthermore, Behavioural responses can be elicited by stimuli less intense than what would be considered to be the pain threshold (for example limbs withdrawal in response to soft touch), and often the magnitude of these responses does not correspond to the intensity of the nociceptive stimuli (Anil et al., 2002, Chapman et al., 1985).

Physiological variables measured to evaluate pain typically include: autonomic changes such as heart rate and heart rate variability, blood pressure, respiratory rate, body temperature, plasma glucocorticoid levels (cortisol in lambs), and adrenaline (Sneddon et al., 2014, Mellor and Stafford, 1999). These parameters are all measures of the generalised stress response, and are a consequence of negative states in the animal (Sneddon et al., 2014). They are not specific to pain, and can also be activated by non-painful stressors in a manner that is comparable to that elicited by pain, for example transportation, handling, restraint and social isolation in lambs (Molony and Kent, 1997, Grandin, 1997, Thornton and Waterman-

Pearson, 1999, Mellor and Stafford, 1999).

In many cases behavioural and physiological measures of pain are not considered to be an accurate reflection of an animal's cortical perception of pain (Woodbury et al., 2002, Mellor and Stafford, 1999). Therefore, a more direct means to assess the perception of pain in an individual must be considered. The difficulty here lies in finding an objective means to assess a subjective cognitive state in animals. Recent developments in brain imaging techniques have provided a means to get a quantitative insight into the activity of the brain. One method that has shown promise in measuring the general cortical responses to pain is the electroencephalogram (Murrell and Johnson, 2006).

2.3.3 Introduction to the Electroencephalogram

The electroencephalogram is a method of directly measuring the nett electrical activity of cortical neuronal populations. An electroencephalogram is a recording of the electrical activity of the brain between two or more points of recording on the scalp, with the raw EEG waveform being a summation of all electrical activity across the area of cortex between the measuring electrodes. The electrodes measure the far-field potentials, which are thought to be dominated by the summation of the individual electrical potentials formed by electrical impulses across neuronal synapses, and are therefore a summation of the activity of excitatory post-synaptic potentials and inhibitory post-synaptic potentials expressed between connecting neurons (Neidermeyer and Da Silva, 2005). As the electrodes record the net activity of an area of cortex, and individual synapse activity only produces minute electrical fields, the EEG recording is predominantly sensitive to neuronal populations that fire synchronously in a co-ordinated pattern (Neidermeyer and Da Silva, 2005) – here

synchronously refers to firing at the same time as a population, and is not to be confused with the phenomena of EEG synchronisation and desynchronisation that will be discussed later in this section.

The principal contributors to the EEG waveform are believed to be the pyramidal cells of layers IV and V of the cortex, as they are regularly arranged in a palisade formation perpendicular to the scalp, meaning that any changes in post-synaptic potentials result in a maximal change in the electrical potential between two different sites on the scalp (Neidermeyer and Da Silva, 2005). Furthermore, the pyramidal cell populations typically fire at the same time as a group, resulting in a summation of activity that is detectible as the sum of far-field potentials by recording electrodes on the scalp (Murrell and Johnson, 2006, Neidermeyer and Da Silva, 2005).

The pyramidal cells of the cortex are the primary excitation units of the mammalian cerebral cortex, and involved in signal projection throughout the cortex (Elston, 2003). The cortex can be divided into general functional layers, from I to VI. For the layers noted above, layer IV is primarily involved in receiving thalamocortical connections that are central to primary sensory cortex involved in localisation of peripheral sensation (including pain sensation), and layer V is primarily involved in giving off efferent projections to the basal ganglia, brain stem and spinal cord (Swenson, 2006). The role of the pyramidal cells in the EEG recording appears to be due to their role as pattern generators in the deeper brain regions, providing synchronous firing that is involved in generating the main frequencies of the EEG that can be related to different cortical functions (Neidermeyer and Da Silva, 2005). The cortical and physiological implications of different frequency patterns of EEG activity will be discussed shortly under section 2.3.7, Fast Fourier Transform and the Electroencephalogram Power

Spectrum.

2.3.4 Uses of the Electroencephalogram

The EEG waveform is presented as a trace of the electrical potential difference (volts) against time and has been used to evaluate cortical pain responses in both humans (Chen and Rappelsberger, 1994, Chen et al., 1989) and animals (Murrell and Johnson, 2006). Interpretation of the EEG has a wide range of research and clinical applications, including: studies in pain across a range of mammalian species (Kongara et al., 2013, Murrell et al., 2010, Kongara et al., 2010, Gibson et al., 2007, Murrell et al., 2005, McGregor, 2005, Johnson et al., 2005b, Johnson et al., 2005a, Murrell et al., 2003, Diesch et al., 2010, Diesch et al., 2009, Johnson et al., 2009); assessing anaesthetic depth in dogs (Kongara et al., 2013, Kongara et al., 2010) and horses (Johnson et al., 2003, Murrell et al., 2003); loss of consciousness in cattle at slaughter (Gibson et al., 2009d, Gibson et al., 2009c, Gibson et al., 2009b, Gibson et al., 2009a, Gibson et al., 2007); and studying brain development of highly altricial wallaby joeys (Diesch et al., 2010) and its comparison with the developmental timeframes of other species (Diesch et al., 2008).

Alongside its use in animal studies, the EEG has been used for a host of human clinical and research applications for many decades, including: monitoring anaesthesia and sleep wake cycles, coma diagnosis, assessing neurocognitive function, diagnosing brain pathologies and developmental abnormalities, neuropharmacological studies and more recently for seizure prediction (Neidermeyer and Da Silva, 2005, Traast and Kalkman, 1995, Jessop and Jones, 1992).

2.3.5 The Electroencephalogram and Anaesthesia

Recording of the EEG can be done in animals that are awake or under general anaesthesia. Recording under general anaesthesia is preferable as the unconsciousness and muscle relaxation removes the presence of movement artefacts, which can 'drown' sections of the EEG recording in extraneous electrical noise, meaning that the data are irretrievably lost (Van Drongelen, 2007). General anaesthesia acts on the central nervous system, and drugs can be to an appropriate dose or concentration that will result not only in loss of consciousness and muscle relaxation, but also insensitivity to pain (Australian and New Zealand College of Anaesthetists, 2015).

In lambs restlessness and increased movement are common behavioural responses to nociceptive stimulation (Dinniss et al., 1999, McCracken et al., 2010). For this reason general anaesthesia is particularly useful when studying the EEG response to nociceptive stimuli in lambs, as movement would likely severely impair EEG recording were the animals conscious. Previous studies have shown that EEG recording can be safely and effectively recorded under general anaesthetic in lambs using volatile inhalational agents delivered in oxygen, and that it is relatively simple to establish and maintain an appropriate level of anaesthesia in these animals (Johnson et al., 2009, Johnson et al., 2005a).

The main inhalational anaesthetics used in veterinary medicine, halothane, isoflurane and sevoflurane, are all volatile anaesthetic agents which means that they are liquid at room temperature but can be readily evaporated and delivered to the lungs. The disadvantage of using volatile anaesthetic agents in studies assessing pain responses is that they are known to depress cortical activity, and thus blunt the EEG response to what noxious stimuli (Murrell et al., 2008, Murrell and Johnson, 2006, Molony et al., 2002). Halothane is an older

anaesthetic agent that is less popular in clinical practice than many newer anaesthetics (personal observation). However, it has properties that prove favourable for its use in EEG studies. Halothane causes less cortical depression than other volatile anaesthetic agents such as servoflurane, desflurane and isoflurane, and consequently it results in less depression of the EEG waveform (Murrell et al., 2008). Furthermore, in contrast to isoflurane and sevoflurane, halothane is not considered to have any direct anti-nociceptive properties (Murrell et al., 2008, Murrell and Johnson, 2006).

Despite some cortical depression under anaesthesia, the EEG response to nociceptive insult is similar in lightly anaesthetised and conscious lambs. Studies in conscious lambs demonstrated an increase in the midrange frequencies of the EEG in response to noxious stimulation, including castration and tail docking (Jongman et al., 2000) and electrical stimulation (Ong et al., 1997), that is consistent with an increase in the EEG median frequency (F50) previously reported in anaesthetised lambs (Johnson et al., 2009, Johnson et al., 2005a). To the best of the author's knowledge there are no studies directly comparing the EEG response of conscious and anaesthetised animals to noxious stimulation.

The current study employed the minimal anaesthesia model developed by Johnson and colleagues, which involves placing the animal under a light plane of halothane anaesthesia that is sufficient to stop the animal being consciously aware of the nociceptive insult, yet allows the animal's brain to demonstrate a minimally depressed EEG response that closely resembles the changes that would be seen in an awake, conscious animal (Murrell and Johnson, 2006, Murrell et al., 2003).

2.3.6 Placement of Electrodes

The placement of the recording electrodes is a principal determinant of the EEG recording, as the location of the electrodes defines the boundaries over which the potential electrical difference (voltage) is measured. A different placement of the electrodes therefore results in a different axis being recording, in the same way as measuring the electrocardiogram of the heart along different leads, with recording electrodes in different locations, allows for a very different appearance of the waveform from the same overall electrical activity of the heart (Figure 2.4).

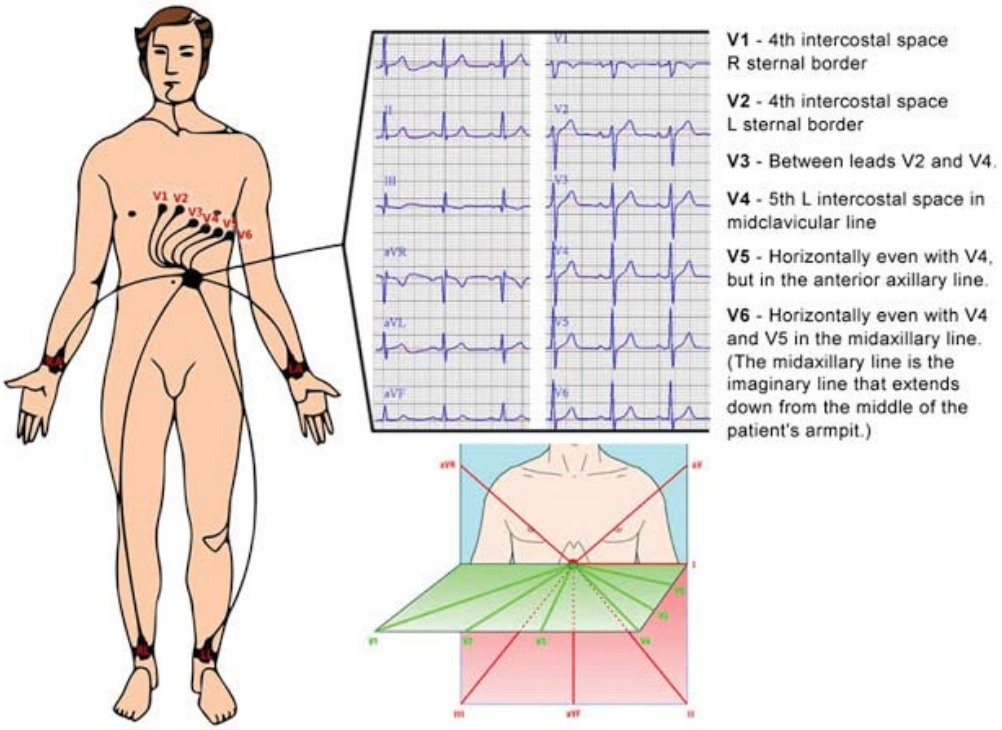


Figure 2.4: Diagram showing the relationship between electrode placement and electrocardiogram presentation of a normal ECG recording from the heart. Note that the waveforms show varied appearance depending on the axis of recording despite being a recording of the same nett electrical activity of the heart. Figure source: http://www.ivline.org/2010/05/quick-guide-to-ecg.html, accessed 14 July 2015.

Electrode placement in human subjects is internationally standardised based on the 10-20 system (American Electroencephalographic society), where the “10” and “20” refer to the distance between electrodes being 10% and 20% of the total length and width of the skull from the nasion to inion (Figure 2.5) (Malmivuo and Plonsey, 1995, Sharbrough et al., 1991), which allows for direct comparison across studies.

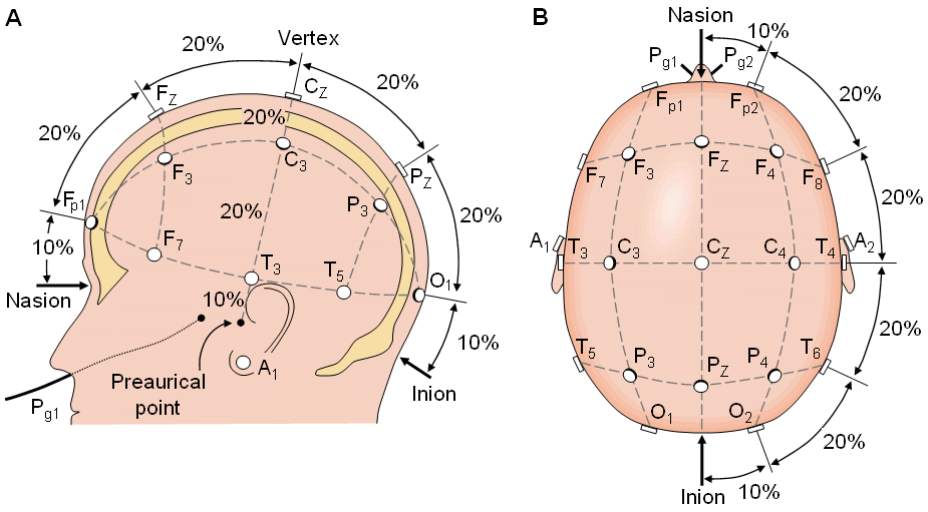


Figure 2.5: The international 10-20 electrode placement system for EEG recording, as standardised by the American Electroencephalographic Society, a total of 21 electrodes are used in the 10-20 EEG montage. Figure source: Sharbrough et al. (1991).

Currently EEG electrode placement in animals is not standardised, and typically animal studies use far fewer electrodes than the human 10-20 system (Kongara et al., 2013, Murrell et al., 2010, Kongara et al., 2010, Gibson et al., 2007, Murrell et al., 2005, McGregor, 2005, Johnson et al., 2005b, Johnson et al., 2005a, Murrell et al., 2003, Diesch et al., 2010, Diesch et al., 2009, Johnson et al., 2009, Murrell et al., 2007). A standardised EEG recording method has been developed for use in animals (reported in Johnson and Taylor, 1997). The method

uses a four-electrode montage for recording activity in both hemispheres, with individual left and right recording electrodes placed superficially over the left and right mastoid processes, a common recording electrode placed on the frontal midline between the medial canthi of the eyes, and a common ground electrode placed over the nuchal process of the skull (shown in Figure 3.1, Materials and Methods). This electrode montage has been successfully applied to a number of mammalian species to study the cortical response to noxious stimuli (Johnson and Taylor, 1997, Johnson et al., 2005b, Kongara et al., 2013, Kongara et al., 2010, Diesch et al., 2010, Johnson et al., 2009, Gibson et al., 2007, Murrell et al., 2005, Murrell et al., 2003, Johnson et al., 2003).

2.3.7 Fast Fourier Transform and the Electroencephalogram Power Spectrum

Changes in the frequency components of the EEG waveform can provide inferences about the cognitive state of the individual (Neidermeyer and Da Silva, 2005), including pain perception in both human (Chen et al., 1989, Chen and Rappelsberger, 1994) and non-human mammalian species (Murrell and Johnson, 2006). Neuronal cell activity is determined by the rate and temporal coding of action potentials, which are an all-or-nothing response, with neurons oscillating between on (firing action potentials) and off states as the basis for information coding (Neidermeyer and Da Silva, 2005). Therefore, analysing neuronal activity based on the frequencies of electrical activity gives insight into the pattern of neuronal oscillation between these on and off states, and therefore provides insight into the predominant patterns of activity for neuronal populations.

The raw EEG waveform is a biological signal of time versus amplitude (volts), and therefore requires transformation of the data to allow for analysis of frequency patterns. The French

mathematician Jean Joseph Fourier (1768—1830) demonstrated that "[an] arbitrary function, continuous or with discontinuities, defined in a finite interval by an arbitrarily capricious graph can always be expressed as a sum of sinusoids" (Gao and Yan, 2011). Put more simply, a waveform can be expressed (or approximated) by a weighted sum of a series of representative sinusoidal wave functions (Gao and Yan, 2011).

This principle can be applied to EEG analysis in transforming the raw EEG waveform from the time domain into the frequency (spectral) domain. Using an algorithm based on the fast-Fourier transform the raw EEG can be transformed from the time domain into a power spectrum representing the relative contribution of the different frequency components of the EEG waveform (Cooley and Turkey, 1965) (Figure 2.6), allowing us to analyse the frequency patterns and relative contributions to the EEG waveform.

The frequency spectrum of the EEG can be expressed graphically as amplitude (μV) over frequency (Hz), and can be expressed numerically by key summary statistics of this power spectrum; for example median frequency (50^{th} percentile), spectral edge frequency (95^{th} percentile) and total power (figure 2.6) (Murrell and Johnson, 2006). The importance of these three summary statistics for the mammalian EEG is discussed below.

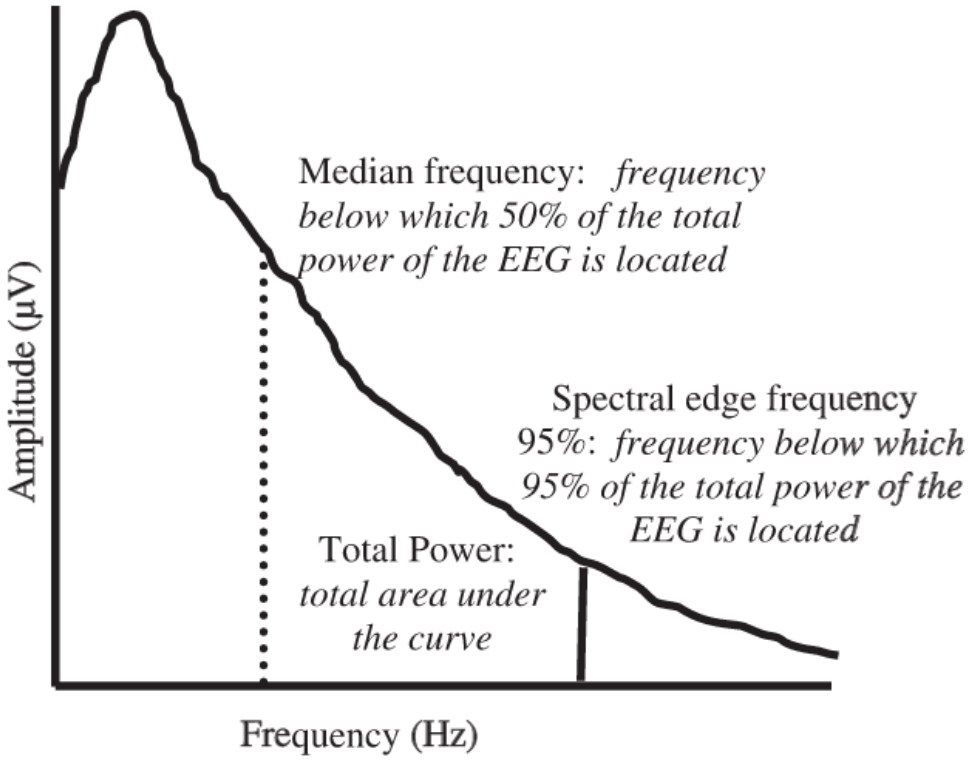


Figure 2.6: Schematic representation of an EEG power spectrum. The dashed line represents median (50%) frequency, the solid line represents spectral edge (95%) frequency. Figure source: Murrell and Johnson (2006).

There are other methods available for analysing the frequency component of a signal recorded in the time domain (such as EEG), and the other key method considered would be wavelet analysis. Where FFT describes the frequency components of a signal, wavelet analysis tell us both the frequency (Bandwidth) components and at what time (temporality) these frequencies occur (Gao and Yan, 2011). Wavelet analysis is therefore useful for analysing the signal changes in transient, intermittent scenarios. In the current study it is known when the stimulus is applied, and the variable of interest is the EEG frequency changes in pre-tail docking versus post-tail docking. Therefore, FFT is an appropriate method

of data transformation, and the extra information of temporality of the frequencies is not necessary for the analysis. For this reason, FFT was used to convert the raw EEG data from the time domain into frequency domain for analysis of the frequency distribution of the EEG pre and post tail docking.

2.3.8 Human and Animal Power Spectra

For analysis the frequency components of the EEG are broken down and represented as either frequency bands (Neidermeyer and Da Silva, 2005), or as summary statistics that provide information on the frequency distribution of the power spectrum (Murrell and Johnson, 2006).

The human EEG is typically analysed based on the relative contribution of five frequency bands to the total power of the signal (Neidermeyer and Da Silva, 2005). The standard five frequency bands are: Delta, 0-4 Hz; Theta, 4-8 Hz; Alpha, 8-13 Hz; Beta 13-30 Hz and Gamma, 30+ Hz. However, this system is not universal, for example some systems calculate the power contribution of Gamma based on frequencies above 36 Hz, and In addition these five bands are often further divided e.g. Alpha 1, Alpha 2 (Neidermeyer and Da Silva, 2005). The neurological states corresponding to different patterns of EEG activity are well studied in humans, and these frequency bands are assigned based on the different patterns and of human neurological activity (Neidermeyer and Da Silva, 2005). For example, activity in the alpha frequency band is associated with relaxation in humans and has been used in diagnosing and monitoring brain activity that is concordant with certain coma states (Blum and Rutkove, 2007), as well as providing insight into patient prognosis (Iragui and McCutchen, 1983, Scollo-Lavizzari and Bassetti, 1987).

The representative frequency bands of the power spectrum described in humans have been applied to EEG studies in sheep (Ong et al., 1997) and lambs (Jongman et al., 2000). However, a system based on our understanding of human EEG frequency patterns does not necessarily correspond to the neurological state of sheep and lambs, as we do not understand the neuronal activity of sheep and lambs to the same depth as we do the human species. Importantly, it has not been demonstrated that these frequency bands correspond to the same neurological states as in sheep as they do in humans (Jongman et al., 2000, Ong et al., 1997). For these reasons the use the established human system in an EEG study of lambs is not currently justified.

A simplified system of EEG interpretation has been developed for use in non-human mammalian species, whereby the EEG is assessed on three summary statistics which represent the frequency distribution of the power spectrum: Median frequency, spectral edge frequency, and total power (Murrell and Johnson, 2006).

The median frequency, or F50, is the mid-point of the power spectrum, with 50% of the total power below and 50% above this frequency (Murrell and Johnson, 2006). Median frequency is a robust measure of the spread of the data, and has been shown to increase in response to noxious stimulation under anaesthesia in a number of mammalian species including sheep (Johnson et al., 2009, Johnson et al., 2005a), rats (McGregor, 2005), deer (Johnson et al., 2005b), cattle (Gibson et al., 2007), horses (Murrell et al., 2003) and dogs (Kongara et al., 2010, Kongara et al., 2013).

The total power, or Ptot, is the sum of the power contained in the EEG (Murrell and Johnson, 2006). It is preferentially sensitive to lower frequency EEG activity, as the lower frequencies contain more power (i.e. have higher amplitude) than the higher frequencies and therefore

make a greater contribution to the total power (Neidermeyer and Da Silva, 2005). The total power has been shown to reduce in response to noxious stimulation in a number of mammalian species under anaesthesia including rats (McGregor, 2005), deer (Johnson et al., 2005b), cattle (Gibson et al., 2007) and dogs (Kongara et al., 2010). However, a reduction in total power in response to noxious stimulation is not a consistent finding in the literature, with other studies reporting no significant reduction in the total power following noxious stimulation (Johnson et al., 2005a, Murrell et al., 2003).

The Spectral edge frequency, or F95, is the frequency below which 95% of the total power of the EEG is contained. It is a sensitive indicator of the high frequency components of the EEG recording, and has shown variable response to noxious stimulation. While some studies have shown an increase in the spectral edge frequency in response to noxious stimulation (Gibson et al., 2007, Johnson et al., 2005b, McGregor, 2005), this is not a consistent finding following noxious stimulation (Kongara et al., 2013, Kongara et al., 2010, Johnson et al., 2005a, Murrell et al., 2003). It has been proposed that spectral edge frequency corresponds to the general level of arousal, and is therefore a better measure of the depth of anaesthesia in these animals than of the magnitude of cortical response to noxious stimulation (Kongara et al., 2013, Kongara et al., 2010, Murrell et al., 2003).

2.3.9 Potential Limitations of the Electroencephalogram

There are a few key limitations of the EEG as a method to directly assess cortical response to noxious stimulation in animals. The key general limitations of the EEG are: the fact that EEG measures electrical activity from superficial recording electrode sites and therefore is much less sensitive to neuronal activity in deeper neuronal populations; the relatively poor spatial

resolution of the EEG when compared with other neurophysiological recording techniques; the risk of aliasing occurring due to an inappropriate sampling frequency; and that the FFT analyses the frequency components of the raw EEG waveform but does not preserve the time component of the signal. Each of these limitations will be briefly outlined and explained in the context of the current study.

EEG is a recording from electrodes in the scalp, and as such the recording is dominated by activity of more superficial structures of the brain and is less sensitive to the activity of deeper neuronal populations (Neidermeyer and Da Silva, 2005). However, as the deep and superficial structures communicate extensively in the processing of pain signals, the EEG recording will be influenced by activity of the deeper neuronal populations, though to what extent remains speculation at this stage (May, 2007). While this may potentially limit the usefulness of the EEG for recording the activity of deeper brain regions, this is not directly a limitation for this study. It has been demonstrated that EEG recordings of the superficial brain structures are a reliable indicator of the conscious perception of pain in mammals (Murrell and Johnson, 2006, Chen et al., 1989, Chen and Rappelsberger, 1994), despite the fact that the underlying mechanisms remain poorly understood.

The EEG has relatively poor spatial resolution when compared with other neurological imaging techniques, for example functional magnetic resonance imaging (fMRI) and computed tomography (CT) (Liu et al., 2006). This is especially true in the current study, where the EEG of each cerebral hemisphere is measured between just two recording electrodes. This is because the electrical signal is recorded as a far-field potential with a relatively large distance between electrodes, and the signal has to pass through a number of tissue layers (including the soft tissues of the meninges, cerebrospinal fluid, muscle and skin,

and hard bone tissue) before reaching the electrodes. The key advantage of the EEG over other neurological imaging techniques is that it is a direct measure of nett electrical activity of neuronal populations, and has a very high temporal resolution – being able to measure events that happen on a millisecond timescale (Liu et al., 2006, Neidermeyer and Da Silva, 2005). In contrast to EEG, both fMRI and CT are indirect measures of the metabolic activity of tissues through showing variation in blood flow and blood oxygenation levels, they have a much slower temporal resolution, and are indirect measures of cortical neuronal activity (Volkow et al., 1997, Liu et al., 2006).

The poorer spatial resolution in not limiting in the current study, as I am interested in comparing the cortical response to nociception in two groups of lambs, and not aiming to localise any differences anatomically. Therefore, despite its relatively lower spatial resolution the fact that the EEG provides a direct measure of neuronal activity of the cortex makes it more appropriate as a measure of cortical response to noxious stimulation than other common neuro-imaging techniques of fMRI and CT.

Avoiding the phenomenon of aliasing is a chief concern for electrical signal recording. Aliasing can occur when converting an analogue signal into a digital signal, for example when recording the biological (analogue) activity of the brain as a digitised EEG signal on a computer (Van Drongelen, 2007). Aliasing occurs as a consequence of an inappropriate sampling frequency, and is especially important in the current study due the fact that discrete points on an amplitude versus time recording, such as an EEG, can be represented by multiple sine waves where one of them is at a frequency more than half of the sampling frequency (Van Drongelen, 2007) (figure 2.7). The consequence of this is that the digitally recorded signal may no longer accurately represent the original signal, as the approximation

of the signal becomes contaminated by lower frequency approximations than those that were originally represented in the raw signal (Murrell and Johnson, 2006).

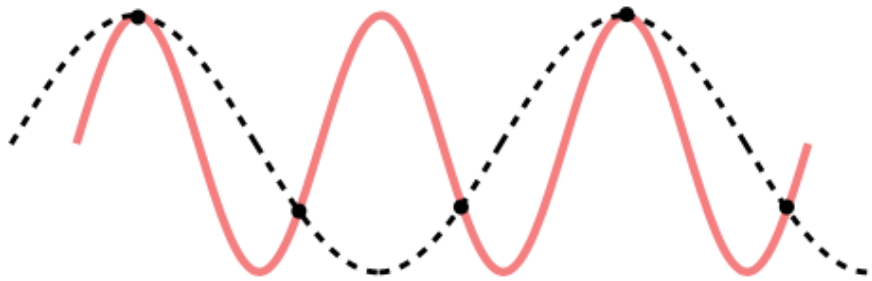


Figure 2.7: Sample points may be represented by more than one frequency of sine wave, where one of them is at a frequency more than half of the sampling frequency. Image source: Wikipedia, created by user Pluke for the article: https://en.wikipedia.org/wiki/Nyquist%E2%80%93Shannon_sampling_theorem, accessed 15 July 2015.

To avoid aliasing, the minimum sample frequency must be more than twice the maximum frequency of interest represented in the signal, termed the Nyquist Frequency. This means that the sine wave frequencies that are fitted to the recorded (digitised) signal are an accurate representation of the signal frequencies present in the raw signal, and not misleading lower frequency approximations (Van Drongelen, 2007). All data above the Nyquist Frequency should be removed with a low pass filter to avoid contamination through aliasing of these higher frequencies (Murrell and Johnson, 2006, Van Drongelen, 2007). For EEG studies the frequencies of interest are typically between 1-50 Hz (Neidermeyer and Da Silva, 2005), and the sampling frequency for recording mammalian EEG using the method employed in the current study is set at 1 KHz (Johnson et al., 2009, Johnson et al., 2005b,

Johnson et al., 2005a), a sampling frequency that is well above the Nyquist frequency for EEG data, and therefore is appropriate to avoid aliasing of the signal.

One further potential limitation of using the EEG in the current study is that when the raw EEG signal is converted from the time domain to the frequency domain, the temporal (time) component of the signal is lost (Gao and Yan, 2011). This means that changes in the signal over time cannot be compared. One approach to this problem is to analyse discrete windows of the data, for example one second epochs used in the current study, and compare changes in the signal across subsequent epochs and against a baseline segment of the data. This approach does not however, directly address the issue, and is a smoothing technique for the data rather than restoring a time component to the data. Comparison of post treatment epochs against a baseline epoch allows for analysis of pre and post treatment changes, and can therefore provide an indication of the effect of treatment on different frequency measures of the power spectrum, and this approach is more thoroughly explained in the methods section 3.6, Analysis of Electroencephalogram.

In addition to the above considerations, accurate recording of the EEG is best performed under general anaesthesia (2.3.5, Electroencephalogram and Anaesthesia), which adds an additional requirement for anaesthetic equipment, veterinary expertise, monitoring and potential anaesthetic risk to the animal. However, other neurological imaging techniques, such as fMRI and CT also require the animal to be anaesthetised as they are very sensitive to movement due to their acute spatial resolution and relatively slow temporal resolution (Volkow et al., 1997, Liu et al., 2006), meaning that this is not a limitation that is unique to the EEG.

In summary, the electroencephalogram offers a direct means to measure the far field potential of cortical neuronal activity between recording electrodes. EEG signal analysis is based on frequency distribution of the power of the EEG waveform following transformation with a modified algorithm of the fast Fourier transform. The current study used a simplified power spectrum analysis, based on the summary statistics of median frequency (F50), total power (Ptot) and spectral edge frequency (F95); with previous studies in animals showing an increase in median frequency and reduction in the total power following noxious stimulation under anaesthesia. While there are a number of potential limitations of the EEG for use in the current study, the EEG is a direct measure of cortical neuronal activity, and EEG recording in anaesthetised and awake individuals show similar reproducible response to noxious stimulation that correlates to the magnitude of the offending stimulus. These characteristics make EEG the most appropriate method for the current study comparing the magnitude of cortical response to subsequent tail docking in lambs castrated or handled at one day of age.

2.4 Summary and Aims of the Study

Castration and tail docking are painful husbandry procedures that are routinely performed on lambs in New Zealand. In addition to the acute pain caused, these procedures may also negatively affect later pain sensation and perception. Preliminary work has shown that early age castration results in a greater behavioural response to tail docking in lambs (Pifeleti, 2011, McCracken et al., 2010).

There are two probable explanations for the change in behavioural response to pain as a consequence of early nociceptive stimulation:

(1) An increase in the behavioural response to nociceptive stimuli may be due to a greater perception of pain.

(2) Or, it may represent a different behavioural strategy to deal with the afferent nociceptive signals. In this case it is simply the organisation of the behavioural response to the painful stimulus that is altered, a response that may not necessarily correspond to the degree of cortical response and pain perceived.

The objective of the current study was to assess the effects of early painful stimulation on later pain perception in a precocial mammalian animal model. More specifically, the current study used EEG to measure cortical response to nociceptive stimulation, to assess whether lambs castrated at one day of age demonstrate a greater cortical response to tail docking at 23 days of age than do lambs handled at one day of age. This study will aid in determining whether the changes in pain related behaviours in response to early nociceptive stimulation previously reported in lambs (Pifeleti, 2011, McCracken et al., 2010) reflect an increase in the pain perceived.

I hypothesised that both the castrated and handled lambs will show a cortical response to tail docking at 23 days of age indicative of pain, and that the lambs castrated at one day of age will show a greater cortical response than the handled lambs. More specifically, I hypothesised that both groups would show a trend toward EEG desynchronisation, with an increase in the median frequency and reduction in the total power of the power spectrum following tail docking. The magnitude of EEG desynchronisation is predicted to be greater in the lambs castrated at one day of age. The greater magnitude of cortical response in the

castrated lambs would indicate a greater magnitude of perceived pain in these animals, and therefore, as a consequence of early age castration, the potential for a greater degree of suffering following subsequent painful stimulation, for example lambing, injury, and footrot.

3 Materials and Methods

3.1 Animals and General Care

All procedures were approved by the Massey University Animal Ethics Committee (MUAEC Protocol 11/46). Twenty-four male, Romney-cross lambs were included in this study. After birth, lambs were left undisturbed with their dams for at least 12 hours to allow time for proper maternal bonding (Paul Kenyon, personal communication). All lambs were kept on pasture with their dams at Massey University's Large Animal Teaching Unit under normal husbandry conditions, except during the period of anaesthesia and data collection which took place in a custom built laboratory. The study was conducted between the dates of 28th August to 5th October, 2011.

3.2 Study Design and First Treatment

Twelve lambs were assigned to each of two treatments groups, castration or handling (control), alternately based on order of birth. In the case of male twins one lamb was assigned to each treatment group. Paddocks were checked every 12 hours, in the morning and early evening. New lambs were noted on each visit, and then treated at the next visit to ensure that all lambs were older than 12 hours. Consequently, the initial treatment was applied between 12 and 24 hours of age.

Each lamb was caught in the paddock where the treatment was applied. Castration was carried out using an elastrator to apply a rubber ring, placed proximal to the testes and distal to the nipples. Handled lambs were subject to the same treatment of being caught and the elastrator held in place, the only difference being that no ring was applied.

Following this first treatment lambs were left undisturbed on pasture with their dam for approximately 23 days.

3.3 Anaesthesia and Tail-Docking

At 23 days of age (23.06 ± 0.18 SE) lambs were caught and transported approximately ten minutes by vehicle to the laboratory, in groups of two to five based on the number of lambs of appropriate age on that day. Lambs were caught and transported in the same order as for the initial treatment. There was some variation in the age at tail docking due to limitations on how many lambs could be anaesthetised and recorded in a single day. Therefore, lambs were treated between 22 and 24 days of age. Once at the laboratory lambs were placed in a 2m by 2m holding pen. Lambs were allowed 15 minutes undisturbed time to acclimatise to the laboratory setting before any procedures began.

Lambs were individually anaesthetised for recording of the electroencephalogram (EEG). Anaesthesia was induced with 4% halothane (Pfizer Laboratories Ltd, Manakau, New Zealand) in oxygen using a precision gas vaporiser delivered by face mask (Flurotec 3, Cyprane Ltd, Keighly, UK). Anaesthesia was maintained with a facemask at a light place of anaesthesia, in accordance with the Minimal Anaesthesia Model for EEG studies described in Murrell and Johnson (2006). End tidal halothane tension was maintained as close as possible to 1.0% (±0.1%). The plane of anaesthesia was monitored using eye position, jaw tone and palpebral response. The total duration of anaesthesia for each individual animal was 30-40 minutes, depending on how smooth the induction.

Following induction of anaesthesia animals were instrumented for anaesthetic monitoring and to establish electrocardiogram (ECG) and EEG recordings. Anaesthetised animals were

placed in left lateral recumbency, on a towel laid over a circulating hot water blanket at 40^0C (Gaymar, New York, NY, USA). Expired end tidal oxygen, carbon dioxide and halothane were monitored using an anaesthetic gas monitor (Hewlet Packard M1025B, Hewlet Packard, Hamburg, Germany). Pulse rate and peripheral arterial blood oxygen saturation were measured by pulse-oximeter placed on the tongue (M1025A; Hewlett Packard, Hamburg, Germany). Heart rate and electrical activity were monitored by a simple lead ECG with stainless steel electrodes (Medlec™, Radiometer, Auckland, New Zealand), with the inverting electrode placed over the right axilla, the non-inverting electrode over the right withers, and the ground electrode over the occipital process (common with EEG leads). All physiological measures were used to monitor the state of anaesthesia and the general condition of the animal, they were not analysed as a part of this study.

3.4 Electroencephalogram Set-Up

For measurement of EEG stainless steel electrodes (Medlec™, Radiometer, Auckland, New Zealand) were placed transdermally, based on the system reported by Johnson and Taylor (1997). Four electrodes were placed to establish a three electrode montage for both the left and right side, with individual left and right non-inverting electrodes placed over the mastoid process, the common inverting electrode placed on the frontal midline between the medial canthi, and the common ground electrode placed over the occipital process (Figure 3.1). The left channel electrodes were intermittently in contact with the table the lambs was placed on, and thus subject to a greater degree of movement artefact. As a consequence of this movement artefact the left EEG channel was discarded and only the right EEG channel was analysed.

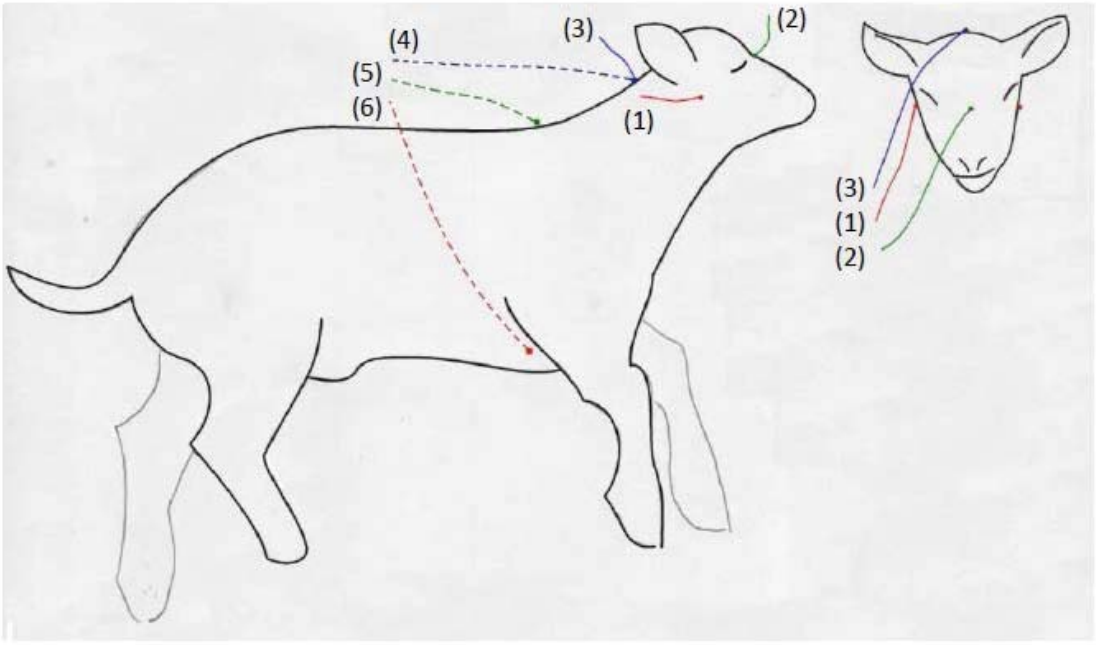


Figure 3.1: Placement of EEG electrodes (solid lines) for the bilateral three electrode montage, with non-inverting (1), common inverting (2), and ground (3) leads; and the ECG electrodes (dashed lines), with inverting (4), non-inverting (5) and shared ground (6) leads.

Input was filtered through an isolated biological amplifier (World Precision Instruments Inc., Saratosa, FL., U.S.A.). The EEG electrode cables fed into two identical break-out boxes, one for each channel, with each break-out box plugged into a physiological signal amplifier (Iso-Dam isolated physiological signal amplifier, World Precision Instruments, Sarasota FL, USA). Signal was recorded at a sample rate of 1 KHz, with the amplifiers providing a signal gain of 1000 and a pass band between 1 Hz (high pass) and 0.5 kHz (low pass).

Each amplifier fed into an analogue-to-digital converter (Powerlab, ADInstruments Ltd, Australia). The EEG signal was digitised at 1000 points per second, and was displayed and recorded continuously using Chart™ 5.2.2 software (ADInstruments Ltd, Sydney Australia) installed on an Apple™ Macintosh® desktop computer (Imac G3®, Apple Inc., California, U.S.A.) connected to PowerLab™ 4/20 data recording system (PowerLab™ data acquisition systems®, AD Instruments Ltd., Sydney, Australia).

3.5 Data Collection

Once the EEG leads were attached the lamb was monitored for a minimum of five minutes to allow for a stable plane of anaesthesia to be established. Once stable, two minutes of pre-docking EEG was recorded. The lamb was then tail docked by application of a rubber ring using an elastrator. The ring was placed between two tail vertebrae leaving enough tail to cover the anus as is recommended in the New Zealand Animal Welfare (Painful Husbandry Procedures) Code of Welfare (2005).

EEG output was recorded for a further 8 minutes following tail-docking. At the conclusion of the data collection period lambs were given 3mg/kg of ketoprofen (Pfizer Laboratories Ltd, Manakau, New Zealand) subcutaneously for pain relief and to reduce inflammation, and monitored for recovery. Once recovered, lambs were returned to their dam on pasture. In total lambs were away from their dams for a maximum of four hours.

3.6 Analysis of the Electroencephalogram

Raw EEG data were manually inspected using Chart™ 5.2.2 software (ADInstruments Ltd, Sydney Australia), and any segments of data reflecting artefact due to over scale or under scale (<1 Hz and >0.5 kHz, respectively) were excluded from further analysis, as they were outside the limits of the recorded frequencies. Due to their amplitude these excluded segments were deemed unlikely to be due to cortical activity and most likely reflected movement artefact, or other forms of high frequency interference (personal communication, Craig Johnson). Where a data-point was removed the value was replaced with the value for the data point immediately prior. The five seconds of data on either side

of the time of tail docking were also removed from analysis, due to the contamination of these data by movement artefact as a consequence of rubber ring application.

Data were submitted to fast Fourier transformation using specialised software (Spectral Analyser, C.B. Johnson, 2002) to calculate median frequency (F50), spectral edge (F95) and total power (Ptot) variables for each one second epoch. For a description of how each variable is derived from the power spectrum please review section 2.3.7, Fast Fourier Transform and the Electroencephalogram Power Spectrum. For each variable, data were smoothed using a nine-point centred moving mean. Using a nine point centred moving mean meant that a value could not be calculated for the first four seconds and the last four seconds of the EEG recording (due to insufficient data points on either side). Data from ten consecutive one second epochs were then averaged to provide a single data point for every 10 second period.

Data for analysis comprised a single representative 10 second baseline value taken 60 seconds before ring application, along with 47 values after tail docking. A ten second baseline data point was selected for consistency with the post tail docking data points, as opposed to using an average of the whole 110 second pre-docking period. Two minutes (120 seconds) pre-docking data were recorded, however only 110 seconds was available for analysis. Smoothing by a nine point moving mean excluded the first four seconds as a nine point centred moving mean could only be produced for the fifth value onwards, the five seconds immediately prior to ring application were removed due to movement artefact, and the 120th second was counted as the point of tail-docking and thus was not included as a pre-docking data point. The representative baseline point was taken from 60 seconds prior to tail docking, as I did not want a point too close to tail docking where there was more

likely to be an effect of disturbance when the lamb or equipment were being prepared for tail docking.

All data points for analysis (including the single representative 'baseline' value) were converted to a percentage of the average pre-docking value calculated over the full 110 second pre-docking period. This was done to maintain the natural variation in the baseline data among lambs, which would otherwise artificially become zero, increasing the likelihood of finding statistically significant differences from baseline.

For the interested readers there is a flow diagram summarising the data manipulation steps included as an appendix (6.1).

3.7 Statistical Analysis

All data were tested to check whether they met the assumptions for parametric analysis (normal distribution and homogenous variance). None of the percentage data were normally distributed, so were normalised using Blom's transformation (Blom, 1958). The effects of treatment, time and their interaction on F50, F95 and Ptot were tested using a mixed model analysis with repeated measures for time and lamb as the random effect and treatment as the group effect (SAS version 9.3, SAS Institute Inc, Cary, NC, USA). Where significant effects of time or the interaction between group and time were found, post-hoc tests were performed with p-values manually corrected for multiple comparisons.

As well as evaluating the effects of treatment on percentage change from baseline after docking, I wanted to see whether treatment had any effect on baseline F50, F95 or Ptot. In preliminary analyses no effects of time on the baseline data measured over 110 seconds

were observed for any variable. Therefore, I looked at the treatment effect on baseline averaged over the whole 110 second period. The baseline data were normally distributed for all variables, but Ptot had unequal variance for the two treatment groups. The effect of treatment on the average baseline value was then evaluated using Student's t-test for F50 and F95, and Satterthwaite's t-test (unequal variance t-test) for Ptot (Ruxton, 2006).

Means and standard errors of untransformed data are presented and differences are considered significant at $p < 0.05$, with tendencies reported at $p < 0.09$.

4 Results

4.1 Mixed Model Analysis of Group, Time, and Group by Time Effects

Median frequency (F50)

There was a significant effect of treatment group on F50 (as a percentage of baseline) following tail docking, with the castrated lambs showing a significantly greater increase in F50 (mean ± SE, 105.75% ± 2.59%) than did the handled lambs (98.53% ± 0.92%) (Table 4.1, Figure 4.1). There was also a significant overall effect of time on F50 that was independent of group (Table 4.1). However, after correction for multiple comparisons, no individual post-docking time points were significantly different from baseline (Figure 4.2). The only significant differences were between individual post-docking time points. There was no significant group by time interaction effect for F50 (Table 4.1).

Table 4.1: Results of the mixed model analysis for F50, F95 and Ptot following tail docking; values were calculated as a percentage of the baseline.

Variable	**Treatment group**		**Time**		**Group x Time**	
	F (1, 22)	**P**	**F (47, 1034)**	**P**	**F (47, 1034)**	**P**
F50	5.45	**0.03**	1.67	**0.004**	0.85	0.75
F95	1.58	0.22	1.36	0.06	0.99	0.50
Ptot	5.15	**0.03**	1.57	**0.01**	0.96	0.56

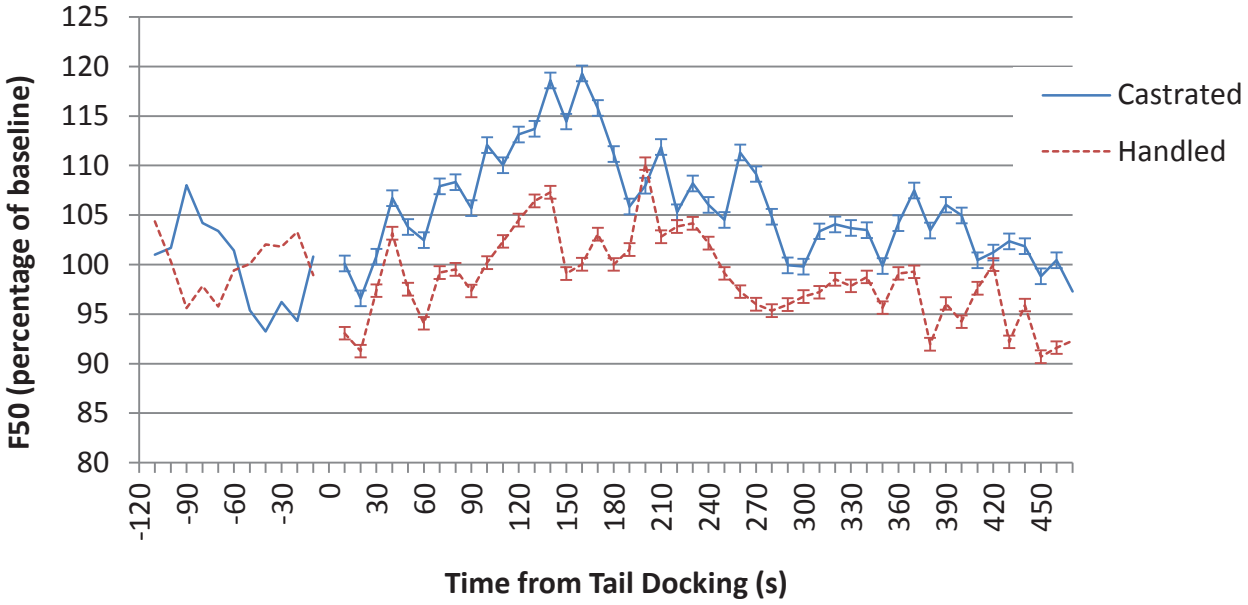


Figure 4.1: Raw (non-transformed) average median frequency for castrated (solid line) and handled (broken line) lambs as a percentage of the average 110 second pre-docking period. Time 0 marks tail docking. Vertical bars indicate standard error. Pre-docking data is included for comparison, but was not statistically analysed. Standard error was calculated for post tail docking data, and therefore it has not been applied to the pre-docking data.

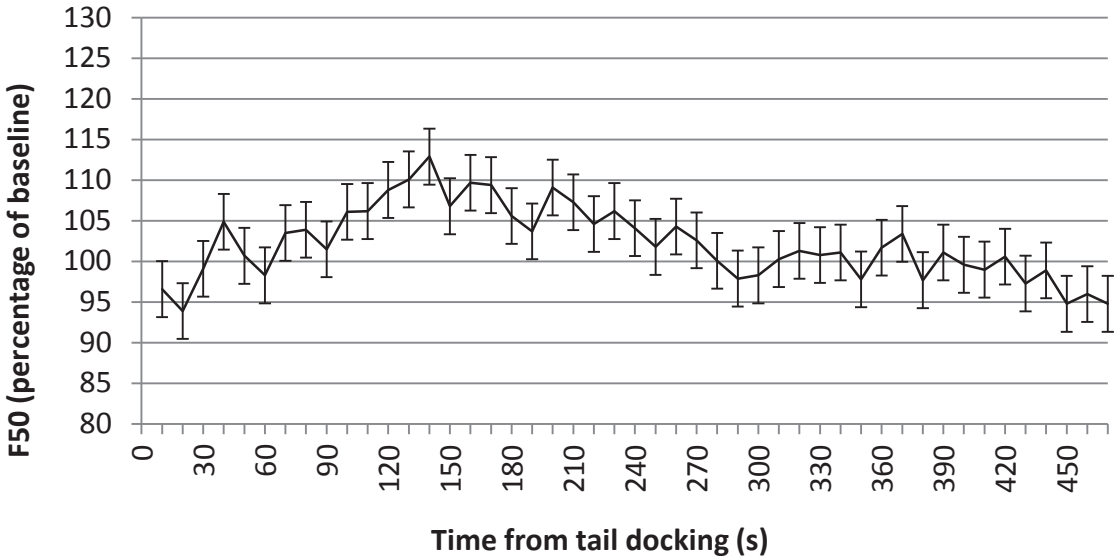


Figure 4.2: Raw (non-transformed) average median frequency (pooled castrated and handled data) response following tail docking, shown as a percentage of baseline. Time 0 marks tail docking. Vertical bars indicate standard error.

Spectral edge frequency (F95)

There were no significant treatment group, time, or group by time interaction effects on F95 (Table 4.1, Figure 4.3). Overall, there was a tendency for a time effect on F95 (Table 4.1). Nine Individual time points tended to be different from baseline, occurring 20 - 130 seconds after tail docking (Figure 4.4).

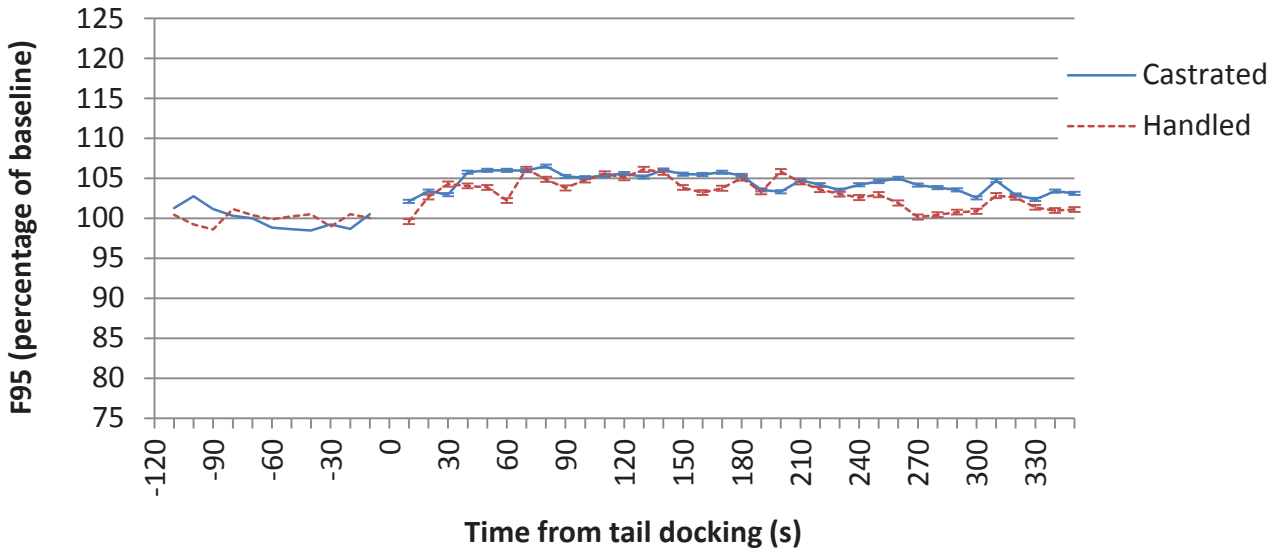


Figure 4.3: Raw (non-transformed) average spectral edge frequency for castrated (solid line) and handled (broken line) lambs as a percentage of the average 110 second pre-docking period. Time 0 marks tail docking. Vertical bars indicate standard error. Pre-docking data is included for comparison, but was not statistically analysed. Standard error was calculated for post tail docking data, and therefore it has not been applied to the pre-docking data.

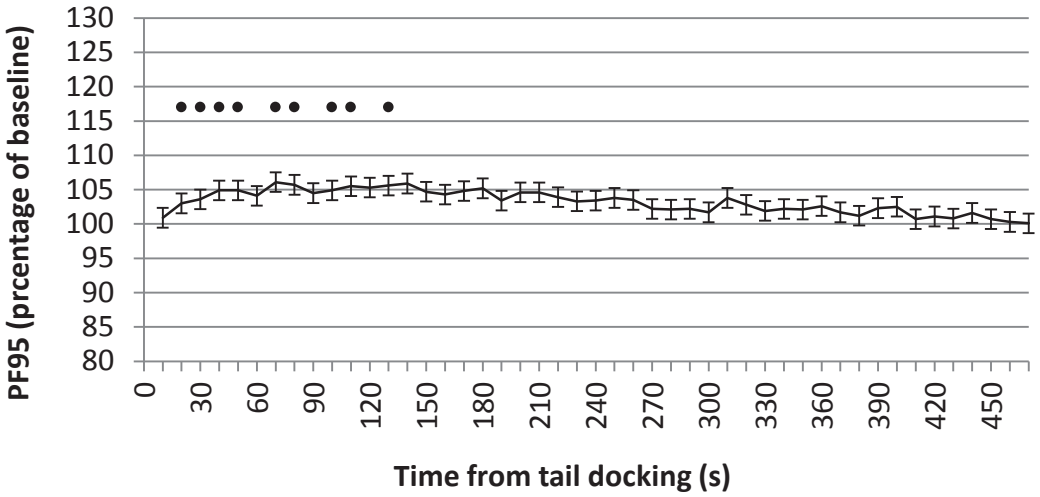


Figure 4.4: Raw (non-transformed) average spectral edge frequency (pooled castrated and handled data) response following tail docking, shown as a percentage of baseline. Time 0 marks tail docking. Vertical bars indicate standard error. Filled circles (•) indicate time points that tended to be different from baseline.

Total power (Ptot)

There was a significant effect of treatment group on Ptot following tail docking, with the castrated lambs showing a significantly greater reduction in Ptot (88.07% ± 1.68%) than did the handled lambs (92.40% ± 1.30%) (Table 4.1, Figure 4.5). Overall, there was also a significant effect of time on Ptot (Table 1). Thirty eight individual time points were significantly different from baseline, occurring 10 - 440 seconds after tail docking (Figure 4.6). There was no significant group by time interaction effect on Ptot (Table 4.1).

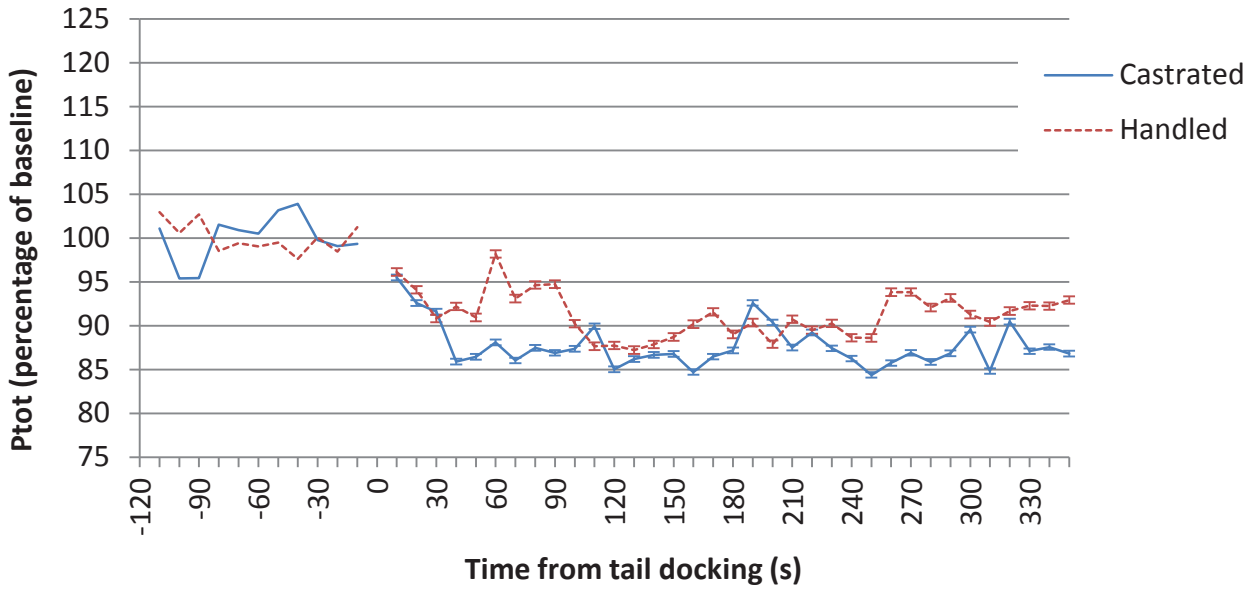


Figure 4.5: Raw (non-transformed) average total power for castrated (solid line) and handled (broken line) lambs as a percentage of the average 110 second pre-docking period. Time 0 marks tail docking. Vertical bars indicate standard error. Pre-docking data is included for comparison, but was not statistically analysed. Standard error was calculated for post tail docking data, and therefore it has not been applied to the pre-docking data.

Note for figure 4.5 above, for consistency the pre-docking data is also shown as a percentage of the average 110 second pre-docking period, masking the absolute difference seen in the raw pre-docking data presented below in section 4.2, Analysis of Baseline.

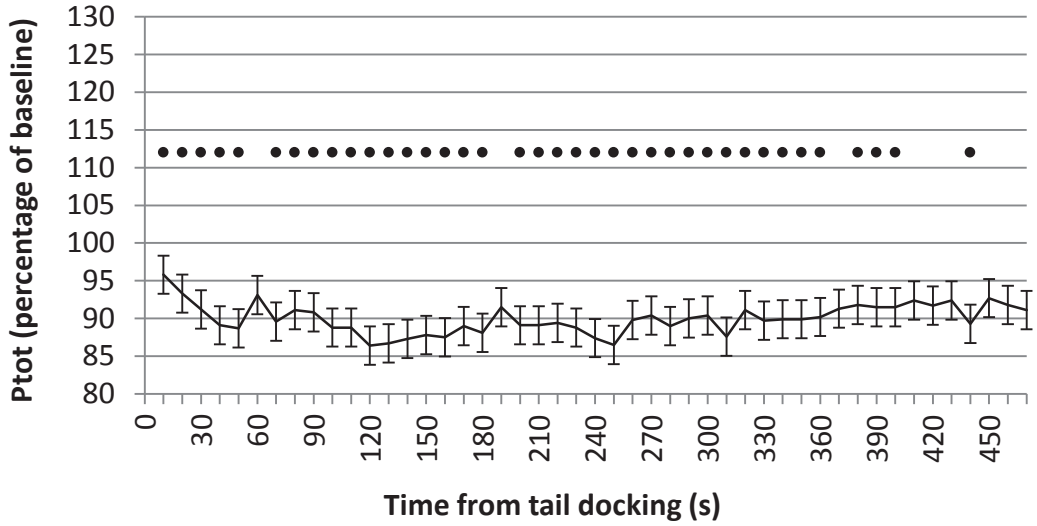


Figure 4.6: Raw (non-transformed) average total power (pooled castrated and handled data) response following tail docking, shown as a percentage of baseline. Time 0 marks tail docking. Vertical bars indicate standard error. Filled circles (•) indicate time points that are significantly different from baseline.

4.2 Analysis of Baseline

In preliminary analyses no significant effects of time on the raw baseline (values calculated for 10 second blocks recorded over 110 seconds) for F50, F95 or Ptot data were observed. Therefore, I looked at the effect of treatment group on the average values calculated over the entire 110 second baseline period. There was no significant effect of treatment group on the baseline for F50 or F95 (Table 4.2). However, there was a tendency toward an effect of treatment group on the Ptot baseline, with the castrated lambs tending to have a higher Ptot baseline than the handled lambs (Table 4.2, Figure 4.7).

Table 4.2: Treatment group mean and standard error of baseline for F50, F95 and Ptot averaged over 110 second pre-docking period, and T-test results for group comparison.

Variable	Castrated	Handled	T*	P
F50 (Hz)	3.26 ± 0.14	3.36 ± 0.35	-0.57	0.58
F95 (Hz)	19.88 ± 0.33	20.10 ± 0.21	-0.56	0.58
Ptot (Watt)	30.62 ± 2.39	25.82 ± 0.95	1.86	**0.08**

*F50 and F95 evaluated using Student's t-test, df = 22. Ptot evaluated using Satterthwaite's t-test, df = 14.42.

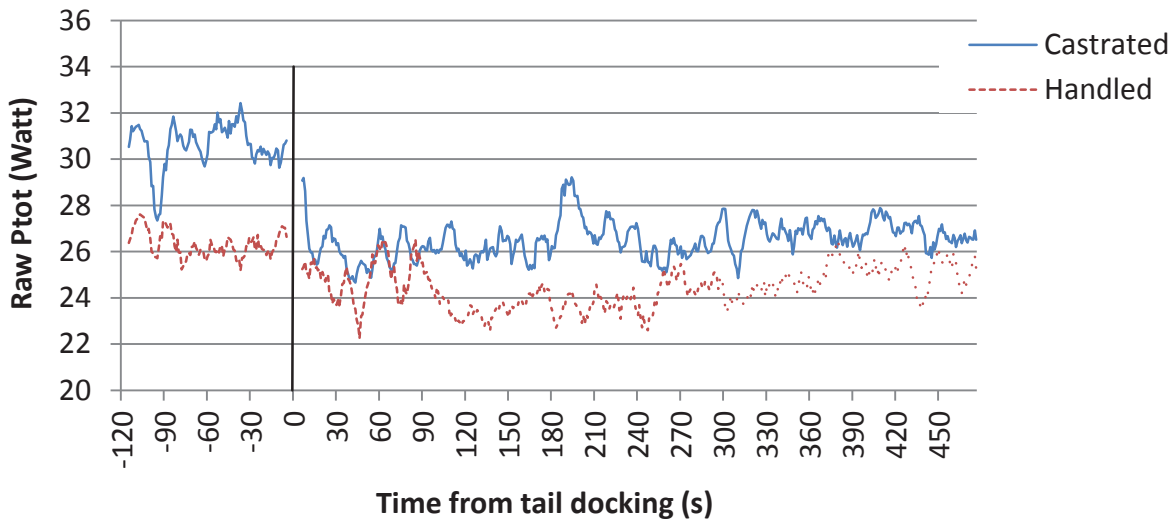


Figure 4.7: Raw total power of castrated and handled lambs, recorded at 1 Hz. Time of tail docking is marked by the vertical black line (time = 0). Pre-docking standard error is 0.09 for castrated lambs, and 0.05 for handled lambs. EEG recording began 115 seconds before tail docking, and 5 seconds of data either side of tail docking was excluded from analysis, giving 110 seconds pre-docking recording. Post tail docking raw data are included on this graph for visual reference, but were not included in the statistical analyses.

5 Discussion

5.1 Review of the Aims of the Study

This study aimed to assess the effects of early painful stimulation on later pain perception in a precocial mammal. More specifically, the current study aimed to assess whether the previously reported change in pain related behaviours after tail docking in lambs subjected to earlier castration (Pifeleti, 2011, McCracken et al., 2010) were concordant with an increase in cortical response to nociceptive stimulation.

I hypothesised that both lambs castrated and lambs handled at one day of age would show a cortical response to tail docking at three weeks of age, and that this response would be of greater magnitude in the castrated lambs. Using the EEG model, I hypothesised that the castrated lambs would show a greater increase in median frequency and a greater reduction in total power, which is indicative of an increase in high frequency, low power EEG activity termed desynchronisation.

A greater cortical pain response to tail docking in the castrated animals would likely reflect a greater degree of pain experienced, and therefore the potential for greater suffering and welfare compromise in these animals. Were the castrated and handled lambs to show a similar response to subsequent tail docking, this would suggest that the castrated lambs were simply demonstrating a different behavioural strategy to cope with a similar degree of perceived pain.

5.2 Principle Findings

5.2.1 Summary of Principle Findings

In accordance with the hypothesis, both the lambs castrated and those handled at one day of age showed an increase in the median frequency and decrease in the total power of the EEG spectrum in response to tail docking under anaesthesia at 23 days of age. Furthermore, the lambs castrated at one day of age showed a greater increase in median frequency and greater reduction in total power than did lambs handled at one day of age.

The EEG desynchronisation in response to tail docking in both the castrated and handled lambs indicates that tail docking resulted in a cortical response to nociceptive stimulation in anaesthetised lambs that would have been perceived as painful in conscious lambs. The greater cortical response in the castrated lambs indicates that there would have been a greater degree of perceived pain in these animals, were they awake. Importantly, these findings suggest that early painful stimulation resulted in a change in the cortical response of lambs to subsequent painful stimuli at three weeks of age. The greater magnitude of cortical response to castration in the castrated lambs likely reflects a greater degree of pain perception in response to tail docking at three weeks of age, and therefore the potential for a greater degree of suffering and welfare compromise in these animals.

None of the three EEG variables, median frequency, spectral edge frequency and total power, showed a significant interaction between group and time. These results indicate that, while there may have been a difference in the overall magnitude of response between castrated and handled lambs, the two groups did not significantly differ in the timing, onset, or the pattern of their response over the eight minute period following tail docking.

5.2.2 Physiological Basis for Hyperalgesia, and the Potential for Allodynia

The main findings of the current study present evidence for a hyperalgesic effect of early age castration, which represents an increased pain response to a supra threshold stimulus (normally painful). The current study has not evaluated potential allodynic effects, whereby the animal's response to non-painful stimuli is altered such that non-painful stimuli elicit a pain response.

Secondary hyperalgesia (hyperalgesia at a different site to the initial insult) as a consequence of early age noxious stimulation is thought to be primarily due to central sensitization in the dorsal horn of the spinal cord (review section 2.2.2, General Mechanisms of Hyperalgesia). This sensitization may result in a greater strength of ascending nociceptive signals relayed through the dorsal horn and up to the cortex, and consequently a greater activation of the pain centres of the cortex and greater cortical pain perception.

Furthermore, dorsal horn sensitization may result in both and increased sensitivity to painful stimuli (hyperalgesia), and a reduced threshold for sensation of stimuli as painful (allodynia) (Anderson and Muir, 2005, Sandkühler, 2009), and it is possible that sensitization of the dorsal horn during a critical window of development may also contribute to a prolonged allodynia following castration in lambs. As allodynic responses typically only elicit a low threshold pain response (Woolf, 2011), for example touch eliciting a painful sensation, testing for allodynia using EEG would require the EEG to be sensitive to low grade non-nociceptive responses, and to the best of the author's knowledge the sensitivity of EEG recording for low grade pain has not been tested in lambs. Noting that all of the EEG pain studies in precocial mammals cited throughout this thesis have been in response to

supramaximal stimuli (Johnson et al., 2009, Johnson et al., 2005a, Jongman et al., 2000, Johnson et al., 2005b, Murrell et al., 2003).

With regards to allodynia, while there is the potential for allodynia following early age castration in lambs, the usefulness of the EEG model for the study of allodynic responses to early age painful stimulation in precocial mammals has yet to be assessed. Future studies should therefore assess the validity of EEG recording for detecting low grade pain in precocial mammals, and whether the EEG technique would be a useful technique to assess the development of allodynia following early age painful stimulation in lambs and other precocial mammals.

5.2.3 Use of EEG variables to Evaluate Pain in Animals

In support of the above interpretation of the results, previous EEG studies in animals using the three parameters of the EEG (Median frequency, spectral edge frequency and total power), have shown that nociceptive stimulation results in an increase in median frequency (Murrell et al., 2003, Gibson et al., 2007, Johnson et al., 2005b, Johnson et al., 2005a, Kongara et al., 2010), and reduction in total power (Kongara et al., 2010, Gibson et al., 2007, Johnson et al., 2005b, Murrell et al., 2003). The transition toward high frequency, low power EEG activity, termed desynchronisation, represents an increased state of arousal that is thought to be indicative of a cortical pain response (review section 2.3.7, Fast Fourier Transform and the Electroencephalogram Power Spectrum). In support of the assertion that tail docking elicited a cortical nociceptive response in lambs, it has been shown that both castration and tail docking elicit behavioural responses that are considered indicative of pain in lambs (Molony et al., 2002, Mellor and Stafford, 1999).

The EEG response to painful stimuli has been shown to be reduced or abolished by the use analgesics in both deer and horse (Johnson et al., 2005b, Murrell et al., 2003), and has been correlated to self-reporting of pain in humans (Neidermeyer and Da Silva, 2005, Chen et al., 1989), indicating that the EEG response described is specific as a measure of cortical pain response. For example, in horses and ponies castration under anaesthesia resulted in an increase in the median frequency and decrease in the total power of the EEG spectrum (Murrell et al., 2003), this response which was abolished by the use of intravenous lignocaine analgesia at the time of castration (Murrell et al., 2005). Additionally, antler removal in red deer caused an increase in the median frequency and reduction in the total power of the EEG power spectrum (Johnson et al., 2005b). Local lignocaine analgesia of the antler pedicle prior to antler removal abolished the increase in median frequency, and significantly reduced the decrease in total power when compared with animals not given local anaesthesia. The finding that appropriate pain relief can abolish or reduce the cortical nociceptive response to castration and antler removal indicates that the increase in high frequency low power activity in the EEG is due to nociceptive stimulation, which would be perceived as painful in conscious animals. These findings indicate that the EEG response seen in this study is indicative of a cortical response to the nociceptive component of tail docking, and not due to other features of the study.

Predictable patterned changes in the EEG activity closely corresponds with human pain reporting in man, with the magnitude of the power of the EEG response being correlated with self-reporting of the degree of pain (Chen et al., 1989). Pain sensitive human volunteers reported a greater degree of pain sensation in response to cold-pressor test (cold pain) that was also expressed as a greater magnitude of EEG response to painful stimulation, reflected in a greater power in the beta and delta frequencies, when compared

with pain tolerant individuals (Chen et al., 1989). This observation indicates that the EEG demonstrates a graded response to pain sensation, with a greater magnitude of perceived pain being reflected in a greater magnitude of EEG response to painful stimulation.

With regard to spectral edge frequency, the current study showed that spectral edge frequency tended to increase following tail docking regardless of treatment group. Two previous EEG studies have also reported an increase in F95 in response to nociceptive stimulation, following antler removal in deer (Johnson et al., 2005b), and scoop dehorning in cattle (Gibson et al., 2007). However, it has been proposed that F95 better represents the level of general CNS depression, and that it is therefore an indicator of anaesthetic depth rather than being specific to nociceptive stimulation (Kongara et al., 2013, Kongara et al., 2010, Murrell et al., 2003).

These findings suggest that spectral edge frequency is not an accurate indicator of the degree of cortical nociceptive response in lambs, and therefore the change in spectral edge frequency following tail docking likely reflects other aspects of the experimental set-up such as anaesthetic depth. In the current study end tidal halothane levels were maintained within a narrow range, while maintaining a satisfactory plane of anaesthesia. It is possible that the tendency for an increase in F95 following tail docking was due to changes in anaesthetic depth as a consequence of stimulation through noise and movement during the placing of the rubber ring around the tail. Therefore, the tendency toward an increase in F95 may reflect a cortical response via an increase in activity of higher frequencies of the EEG in response to tail docking.

EEG desynchronisation in response to noxious stimulation is not a unanimous observation across mammalian species. Two studies in anaesthetized neonatal rats and Tammar

wallabies have reported a transition toward lower frequency EEG activity, termed synchronisation or paradoxical arousal, in response to painful stimulation. There are a number of potential explanations for the observed variations in EEG response reported, including differences in developmental timeframes, differences in EEG recording technique, and potential species differences, each of which will be briefly outlined.

The first study by Diesch et al. (2009) in rats, showed a reduction in median frequency in response to 10 seconds of tail clamping in 12-14 day old rat pups, and a reduction in spectral edge frequency in 12-14 and 21-22 day old rat pups. And the second study by Diesch et al. (2010), in young Tammar Wallabies (187-261 days of age), showed a decrease in median frequency in response to 30 seconds of toe clamping.

The differences between studies showing synchronisation in rat pups and wallabies, and those showing desynchronisation may owe to developmental stage at birth in the different species (Diesch et al., 2009). On the continuum from precocial to altricial, both rats and wallabies are highly altricial species being born with very little mobility or ability to survive independently. Rat pups are born hairless, blind and with minimal ambulatory skills. Rats have a gestation period of 21-24 days, they begin to crawl at about 10 days of age, and open their eyes at about 14 days of age. Wallaby pups are born exceptionally immature after a 28-34 day gestation, and are considered to resemble a 26 day sheep embryo at birth (the gestation length in sheep is 142 days) (Mellor and Lentle, 2014). Wallaby pups do not open their eyes and explore outside of the pouch until approximately 100-114 days of age (Diesch et al., 2010), whereas in lambs the traits of opening their eyes and exploring the environment are expressed within minutes to hours of birth (Mellor and Lentle, 2014).

In opposition to the hypothesis that the observed difference owes to developmental maturity at birth, EEG desynchronisation in response to nociceptive stimulation has been shown in adult dogs (Kongara et al., 2010) and humans (Chen et al., 1989), which are both moderately altricial species. However, both dogs and humans are not as markedly altricial as wallaby or rat pups, and the studies in dogs and humans were conducted on adult animals and not juveniles as the rat studies were. Adult rats show no change in the median frequency or total power of the EEG spectrum in response to mechanical stimulation (tail clamping with artery forceps), with only some animals showing an increase in median frequency in response to thermal stimulation (tail in 55^0C water bath for 5 seconds) (Murrell et al., 2007). A more recent study in adult rats demonstrated that most animals showed a reduction in median frequency throughout ovariohysterectomy (OHE) surgery, with a reduction in total power in response to ligation of the ovaries and cervix (Murrell et al., 2010). Furthermore, both wallaby joeys and rat pups showed a more marked response to tail clamping with increasing age (Diesch et al., 2009, Diesch et al., 2010), suggesting that synchronisation as a response to tail clamping may become more developed with age in these species, and is not an early developmental response that then later transitions to desynchronisation in more mature animals.

The findings of Deisch et al. (2009, 2010), suggest that there is not a developmental transition from synchronisation to desynchronisation in response to nociceptive stimulation. However, as there are currently no studies that have repeatedly tested the EEG response to nociceptive stimulation in animals of different ages, it is not certain how the EEG response changes through the early developmental period in rats and wallabies, and how these changes may relate to the current study in lambs.

In both of the above studies in adult rats (Murrell et al., 2010, Murrell et al., 2007) cortical electrical activity was recoded from dural electrodes placed over the somatosensory cortex, rather than the electrode montage used in the current study. The absence of a significant change in F50 in response to mechanical or thermal stimuli in these studies may reflect a minimal activation of the somatosensory cortex in response to nociceptive inputs, with the increase in median frequency seen in only some of the rats owing to a more generalised activation of electrical neuronal pathways that is not specific to nociceptive pathways (Murrell et al., 2007). This suggests that the differences in recording electrode depth and location may have contributed to the disparities seen between the EEG responses in these studies and the current study. Furthermore, the duration of OHE surgery in rats (Murrell et al., 2010) compared with castration and tail docking may have contributed to the disparity with previous studies that used EEG responses to infer cortical response to nociceptive stimuli (Kongara et al., 2010, Gibson et al., 2007, Johnson et al., 2005b, Johnson et al., 2005a, Murrell et al., 2003).

Both Deisch et al. (2009) and Murrell et al. (2010) acknowledged the consistent association between nociceptive stimulation and EEG desynchronisation in other mammalian species, stating that the reasons for a reduction in median frequency during nociceptive stimulation in rats and wallaby pups were “unclear”. EEG synchronisation in response to nociceptive stimulation has not been reported in lambs, or sheep. Therefore, it seems likely that there is a species difference in the EEG response to nociceptive stimulation in lambs when compared with rats and wallabies, though the reasons for this difference are not yet clear.

In summary, I propose that the greater magnitude of EEG desynchronisation observed in castrated lambs following tail docking suggests a greater degree of cortical response to

nociceptive stimulation in these animals, which would represent a greater magnitude of perceived pain in awake animals. This is the first study to show changes in EEG in response to tail docking as a consequence of early age castration, and suggests that an early age painful event in neonatal animals can increase the magnitude of later pain perception in response to subsequent painful stimuli.

5.2.4 Behavioural Evidence for the Effects of Early Painful Events on Later Pain Responses

In support of the findings that castration at one day of age results in an increased cortical response to tail docking at 23 days of age, early age castration has been shown to alter behavioural response to later tail docking in lambs. McCracken et al. (2010) showed that lambs castrated at one day of age showed a greater pain-related behavioural response to rubber ring tail docking at 30 days of age than did lambs castrated at ten days of age. As there were no controls in McCracken et al. (2010), I cannot say whether the lamb castrated at ten days of age were demonstrating behavioural hyperalgesia, just to a lesser degree than the lambs castrated at one day of age, or whether they were exhibiting a 'normal' behavioural response to castration under those circumstances.

Expanding on these findings, Pifeleti (2011) explored both the effect of early age castration on later behavioural pain response and the effect of duration between castration and tail docking. In agreement with the findings of the current study, she reported that lambs castrated at one day of age showed a greater behavioural pain response to tail docking at 21 days of age than did lambs handled at one day of age and castrated at 21 days of age. Furthermore, they reported that lambs castrated at one day of age showed a greater

behavioural pain response to tail docking at 21 days of age than lambs castrated at 21 days of age and tail docked at 42 days of age. This finding indicates that it was the developmental timing of castration, and not the duration between castration and tail docking which correlated with the behavioural hyperalgesia.

The final key finding of Pifeleti (2011) for the current study was that the increased behavioural response to tail docking seen at 21 days of age was resolved by 42 days of age, meaning that the behavioural response to tail docking at six weeks of age did not differ between lambs castrated and those handled at one day of age. The current study demonstrated that there was a significant difference in the cortical response to tail docking at three weeks of age. It did not examine that duration of the increase in cortical response beyond three weeks of age. Based on the findings of Pifeleti (2011), I hypothesise that the increased cortical response to noxious stimulation as a consequence of early age noxious stimulation would be resolved before six weeks of age in lambs, and this investigation may be the target of future studies.

In contrast, work in human infants has suggested a much longer duration of hyperalgesia that the three to six weeks reported by Pifeleti (2011). For example, human infants circumcised at five days or younger showed an increased behavioural response to vaccination at four to six months when compared with non-circumcised controls, or neonates that were circumcised with appropriate analgesia (Eutectic Mixture of Local Anaesthetics; comprising lignocaine and prilocaine) (Taddio et al., 1995, Taddio et al., 1997). In addition to this preterm human infants exposed to repeated, routine painful procedures as neonates demonstrated a greater sensitivity to mechanical stimulation of the skin as adolescents between 12-18 years old (Buskila et al., 2003). These data suggest that the

increased sensitivity owing to early painful stimuli may be significant many years, even decades, from initial insult in human neonates, a duration much greater than the six weeks reported in lambs (Pifeleti, 2011).

It is possible that the greater duration of hyperalgesia in response to early age noxious stimulation in humans may in part be due to their greater developmental immaturity at birth when compared with the precocial lamb. The relative immaturity of the human nociceptive system at birth may mean that noxious stimulation has a greater, more lasting, influence on the synaptic reinforcement of the developing nociceptive pathways (discussed in section 2.2.2, General Mechanisms of Hyperalgesia). There are two key considerations for the findings of Buskila et al. (2003), firstly they compared the responses of preterm (very low birthweight) infants with infants born at full term; and secondly the preterm infants were subjected to repeated painful stimuli. It is possible that the relative immaturity of the preterm nociceptive pathways may help explain the greater duration of hyperalgesia, as these pathways may be more sensitive to noxious stimulation. In addition to this the preterm infants were subjected to a large number of repeated painful stimuli when compared with the lambs in the current study, who were all born at term and subjected only to the single noxious stimulus of castration. Typically repeated stimulation across a synapse results in a greater sensitization of that pathway than does a single stimulus (Sandkühler, 2009), and consequently the greater duration of sensitization reported in human infants may owe to the repeated nature of painful stimulation, in addition to the greater developmental immaturity of preterm infants.

Hyperalgesia in response to early nociceptive stimulation is not a unanimous observation, with a number of studies in rodents showing a reduced responsiveness to pain following

early age nociceptive stimulation. Mouse pups subjected to exploratory laparotomy surgery at one day of age showed reduced nociceptive sensitivity to thermal tail withdrawal tests as adults, when compared with mice not subjected to the surgery (Sternberg et al., 2005). However, the same study also found that mouse pups that underwent exploratory laparotomy or sham surgery (no incision) both showed a reduced behavioural response to thermal withdrawal testing as adults, when compared with control mice not subjected to the stresses of handling and separation from their dam. Furthermore, mice that were handled and placed in isolation for 12 minutes once daily from two to 19 days of age showed reduced behavioural response to thermal nociceptive stimulation at 5 weeks of age, when compared with mice that were not handled (D'Amore et al., 1995). Similarly, mice stressed by once daily handling and 15 minutes isolation, for the first 14 days postnatally, demonstrated reduced behavioural stress response as adults (10-12 weeks of age) (Sternberg and Ridgway, 2003).These findings suggest that the reduced behavioural response to nociceptive testing reported in some rodent studies may be a consequence of the neonatal stress, rather than a specific response to earlier pain in mice.

The possibility of handling and stress exposure as a confounder to interpreting the effects of pain highlights the importance of appropriate controls. In the current study the handled lambs acted as a control population, and were exposed to the same stressors (handling, transport, removal from dam) as the castrated lambs, meaning that the potential for confounding effects of stress and other influences were minimised. As the application of the rubber ring for castration was the only difference in handling and manipulation of the two groups, the potential confounding effects of stress on the cortical nociceptive response were minimised and were unlikely to have significantly affected the results.

The key limitation of behavioural studies in pain is that while behaviours can be a reliable indicator of discomfort and distress, they are not specific as measures of pain (Anil et al., 2002, Chapman et al., 1985). The value of the current study is that the EEG allows us to measure the cortical response to nociceptive stimuli that is specific to pain, which when taken in concert with the findings of the behavioural studies discussed gives a more accurate and complete picture of the effects of early painful stimulation on later nociceptive response. Furthermore, the main limitation in applying work done on humans and rodents to interpretation of the current findings is that sheep and human and rodent neonates are born at very different stages of developmental maturity. Therefore, behavioural effects owing to painful stimulation in altricial mammals may not correlate with the observations in neonatal lambs, or other precocial mammals.

In summary, where previous studies in lambs have shown that early nociceptive stimulation causes changes in subsequent pain related behaviours (Pifeleti, 2011, McCracken et al., 2010), the current study is the first to demonstrate that in lambs this altered behaviour corresponds to a greater cortical response to nociceptive stimulation. Together these data suggest that early painful stimulation results in a change in both the cortical and behavioural response to subsequent painful stimuli, up to at least three weeks of age in lambs, and that the altered behavioural response to subsequent painful stimuli likely represents a hyperalgesic state in these individuals.

5.3 Minor Findings

5.3.1 Higher Pre-docking EEG Total Power in previously castrated lambs

The current study found that lambs castrated at one day of age tended to have a higher pre-docking total power than did lambs handled at one day of age. It is important to note that the pre-docking EEG was recorded without any nociceptive input, and therefore, is not a reflection of the responses specific to the nociceptive pathways, but rather represents an alteration in the EEG signal of the cortex that is independent of the subsequent nociceptive stimulation.

With regard to the primary findings of the study, the observation that the castrated lambs tended to show a higher pre-docking total power may have been a factor in the finding that castrated lambs showed a greater reduction in the total power of the EEG following tail docking. The post-docking EEG data was expressed as a percentage of the average pre-docking EEG recording, to allow each animal to acts as its own control. The key interest for this study was in the relative response recorded in the EEG power spectrum for castrated versus handled lambs following tail docking. Therefore, the post-docking EEG data are a reflection of the relative response to tail docking shown by each individual. On this basis, the greater reduction in total power following tail docking in the castrated group is reflective of a different response to tail docking in this castrated compared with handled lambs. However, without a better understanding of the physiological and clinical significance of the higher pre-docking power in the castrated lambs, it is difficult to comment on the influence of the higher pre-docking total power on the greater reduction of the total power following tail docking seen in the castrated lambs.

With regard to the clinical importance of an increase in the pre-docking total power in castrated lambs, a greater power component of the EEG may be a consequence of a greater number of action potentials over the one second epoch, or due to a greater power signal from the individual action potentials. However, as action potentials are typically an all-or-nothing response (Neidermeyer and Da Silva, 2005), it seems likely that the greater EEG power is a consequence of a greater number of action potentials being fired. What the increased EEG power may therefore indicate is that overall there is a greater neuronal activity in the castrated lambs over the area of cortex measured.

It may be that the increase in power is reflective of an increase in the background activity of the nociceptive centres of the brain, or it may reflect an increase in the power of non-nociceptive regions that are also captured in the area subject to the EEG recording - remembering that the EEG is a recording of the sum of electrical activity from all of the neurons between the two recording electrodes. The baseline EEG recording used in the current study is therefore not specific to the activity of nociceptive centres, rather it allows for the recording of changes in the EEG spectrum that are specific to the magnitude of cortical response to nociceptive stimulation. It is possible that a greater activity in the nociceptive pathways in early castrated lambs results in an increase in the background activity of the nociceptive pathways that relay to the cortex. This increased activity is then reflected in the baseline recording of the EEG centred over the nociceptive centres of the pyramidal cortex. Due to the limited spatial accuracy of the EEG, it is not possible to be more specific about the likely source of the increase in baseline power in the castrated lambs.

In addition to the electro-physiological evidence presented in the current study, Pifeleti (2011) observed that prior to tail docking (pre-docking recording) lambs castrated at one day of age showed different behaviour patterns at 21 days of age than did lambs handled at one day of age, with lambs castrated at one day of age showing a reduced incidence of walking backwards, standing unsteadily and time in a normal position in the 30 minutes of observation. During the observation period lambs were exposed to a number of stressors, including separation from their dam, being placed in stock yards, mixing with unfamiliar lambs, and the presence of people and equipment in the yards. It is possible that the different behaviour in the castrated lambs was due to the effects of, and associations with, the stressors listed above and may not have been due solely to the nociceptive components of early age castration (Pifeleti, 2011). Nonetheless, were the difference due to other stressors this would still indicate a difference in the later stress responsiveness as a consequence of early age castration.

The current study is the first to report a change in the pre-docking EEG as a consequence of early age nociceptive stimulation. The reasons for a tendency towards a higher baseline EEG total power in lambs castrated at one day of age are not clear, but are consistent with finding that early age castration alters behaviours prior to tail docking.

In the current study it is possible that lambs castrated at one day of age had a greater stress response to handling, transport and induction of anaesthesia than handled lambs. This could be due to the negative association of handling with the pain and discomfort of rubber ring castration at the time of initial handling, or simply due to an increase in stress reactivity (e.g. CNS change due to castration). The heightened stress in these animals may have manifested as an increase in the total power of the EEG. However, the EEG is a gross measure of the

sum of neuronal activity between two points, and has a low spatial accuracy which limits my ability to comment more specifically on the likely source of the increased baseline power in the castrated lambs. Further work is needed to better define this response and to study the basis and clinical importance of this response.

5.3.2 Duration of the EEG Response to Tail Docking

The current study demonstrated a significant effect of time on the median frequency and total power of the EEG, with an increase in median frequency and decrease in total power following rubber ring tail docking in lambs - discussed under the principle findings above. Post-hoc analysis of the individual time points relative to baseline EEG values showed that the reduction in total power following tail docking was not completely resolved (returned to pre tail-docking values), by the end of the eight minute recording period. However, as only one of the last seven data points (70 seconds worth of recording) showed a significant difference from baseline data, it would appear that the response had resolved, at least in any meaningful sense.

McGregor (2005) proposed that total power is the most sensitive measure of nociceptive somatic stimulation in rats, when compared with median and spectral edge frequencies. The finding that changes in both median frequency and spectral edge frequency appeared clearly resolved within the eight minute window, whereas the response seen in the total power showed variation from baseline up to 440 seconds following tail docking, appears to support this hypothesis. This finding is of only minor consideration, as the results were nonetheless able to address the main objectives of the study. Future studies may consider a

longer post-stimulus recording window to be assured that the EEG response is fully resolved.

5.4 Potential Limits of Study Design and Recommendations for Future Studies

5.4.1 Potential Limitations of Summarising the EEG spectrum as F50, F95 and Ptot

Summarising the EEG power spectrum as three variables (F50, F95 and Ptot; Figure 4.4) can potentially limit the interpretation, given the existing limited knowledge of the distribution of the power spectrum based on these three variables.

General understanding of the human EEG power spectrum is much greater than that of the lamb (Neidermeyer and Da Silva, 2005), and this is reflected in the human power spectrum typically being broken down into six or more frequency bands (Delta, 0-4 Hz; Theta, 4-8 Hz; Alpha, 8-13 Hz; Beta 13-30 Hz and Gamma, 30+ Hz), which correlate with different states of neurological activity (Neidermeyer and Da Silva, 2005). These same frequency bands have been successfully applied to EEG studies of electrical stimulation in sheep (Ong et al., 1997), and castration in lambs (Jongman et al., 2000). Ong et al. (1997) commented on the difference in absolute power in each frequency band based on the magnitude of electrical stimulus intensity. They summarised stating that similar changes have been reported in human EEG studies, but did not provide a discussion on the meaning and importance of the individual bands in sheep. Jongman et al. (2000) similarly provided a summary of the mean power values across all bandwidths, but did not provide an interpretation for each individual bandwidth. The fact that neither of these papers provided comment on the significance of the individual bandwidths suggests that summary variables would have provided similar information for meaningful analysis.

The frequency bands used in human EEG studies are based on an understanding of human EEG patterns, and the same frequency intervals do not necessarily correspond to the same neurological state in other mammals. Furthermore, there are changes in the distribution of the power spectra of the EEG throughout development in rats and lambs (Diesch et al., 2010, Johnson et al., 2005a) and humans (Somsen et al., 1997, Gasser et al., 1988) that typically show a movement toward a greater representation by higher frequency spectra with increasing developmental age. Therefore, to justify the application of specific frequency bands to the interpretation of the EEG you would also need to match the developmental stage between species, humans and lambs for example, for which the data is not currently available.

The value of using the three variable system for interpreting the power spectrum, is that it reduces the complexity of the data while appearing to accurately represent the magnitude of cortical nociceptive response of mammals to noxious inputs (Murrell et al., 2003, Gibson et al., 2007, Johnson et al., 2005b, Johnson et al., 2005a, Kongara et al., 2010, Murrell and Johnson, 2006). The use of the three summary variables proved sufficient to display a meaningful difference between castrated and handled lambs in the current study, and proved successful in achieving the aims of the study.

In summary, while there are possible limitations in using only three summary variables to represent the EEG, the use of three variables proved sufficient for the aims of the current study and provided a data-set appropriate for analysis. The frequency bands used in human EEG studies have been applied to sheep and lambs (Jongman et al., 2000, Ong et al., 1997), but both of these studies summarised their findings based on overall power trends, suggesting that the use of summary statistics in these studies would have yielded similar

results with a simpler data set for analysis and interpretation. Future work may assist in developing better understanding of the development and significance of narrow frequency bands in the EEG of lambs, until such time it seems most appropriate to use the three summary variables for analysis of the EEG repose to nociception in lambs as has been done in the current study.

5.4.2 Potential Effects of Anaesthetic Depth on the EEG

The EEG power spectrum reflects the state of CNS arousal (Neidermeyer and Da Silva, 2005, Murrell and Johnson, 2006), and can be affected by the depth of anaesthesia, with a reduction in the high frequency low power activity and a reduced responsiveness to afferent stimuli at a deeper plane of anaesthesia (Kongara et al., 2013, Kongara et al., 2010, Murrell et al., 2003).

A number of lambs in the current study transitioned to a very light plane of anaesthesia following tail docking (personal observation, data not recorded), suggesting that the stimulation of tail docking caused a reduction in the plane of anaesthesia in some lambs. It is possible that the reduction in the plane of anaesthesia may have been partly responsible for the overall desynchronisation seen following tail docking. However, as both groups were treated similarly with regard to anaesthesia and tail docking, it is unlikely that there was any systematic difference in the plane of anaesthesia following tail docking that may have artificially generated a group effect on the EEG response to tail docking.

A number of lambs showed periods of apnoea during general anaesthesia. These periods of apnoea were not formally recorded, and therefore cannot be analysed. The periods of apnoea were typically of less than 30 seconds duration (personal observation). During this

apnoeic period there would have been little delivery of isoflurane to the brain, and it is probable that the anaesthetic plane would have become lighter as a consequence. As the apnoeic periods were not recorded I am unable to comment on the potential for a group effect on anaesthetic depth and EEG output. To the best of my knowledge, there are no studies in lambs demonstrating an effect of early age castration on the response to anaesthetic. Future studies could record and analyse the anaesthetic depth, and periods of apnoea, to evaluate any confounding effect of castration on the response to general anaesthesia in lambs.

The current study aimed to provide a smooth anaesthetic induction, with minimal stress and excitation. For these reasons it was decided that induction by face mask was preferable to intubation. End tidal halothane was monitored, but not recorded, and the plane of anaesthesia was judged to be acceptably stable. There are no studies that have directly assessed the role of intubation and IPPV on periods of apnoea and anaesthetic depth in lambs. Two previous studies have used tracheal intubation in lambs to allow for accurate measurement of end tidal halothane levels, and intermittent positive pressure ventilation (IPPV) during periods of apnoea (Johnson et al., 2009, Johnson et al., 2005a). In these previous studies, intubation of lambs proved a difficult and disruptive process. Previous work in the horse has shown that similar EEG changes occur in response to variations in end tidal halothane with (Johnson and Taylor, 1998) and without (Johnson et al., 1994) IPPV. However, horses are not as prone to period of apnoea under anaesthesia as are lambs (Craig Johnson, personal communication).

In summary, variations in anaesthetic depth as a consequence of arousal following tail docking may have contributed to the effect of time on the EEG response observed in the

current study. It is however unlikely that variation in anaesthetic depth affected castrated and handled lambs differently, and is therefore unlikely to have contributed to the group effects reported. Future studies should consider recording end tidal halothane as a measure of anaesthetic depth, to allow for evaluation and control of the effects of anaesthetic depth on EEG responses.

5.4.3 Potential Limitations of Sample Size

The current study found a non-significant tendency toward an effect of group on total power baseline ($p = 0.08$), and a non-significant tendency for a time effect on the spectral edge frequency variable ($p = 0.056$), the implications for each have been covered earlier in the discussion. While it is possible that a larger sample size may have given a more conclusive statistical answer for the observations above, there are a number of considerations to justify the appropriateness of sample size used in the current study.

Most importantly, the sample size of 24 lambs was sufficient to address the primary aims of the study. A post hoc power calculation of median frequency data suggested that the sample size was appropriate, with 80% power and observed differences between groups would have been statistically significant with only 5 animals per group (Performed by Ngaio Beausoleil). The effect of group on total power baseline and the time effect on spectral edge frequency were unexpected results; they were not the primary aims of the study and therefore were not the basis for selecting the sample size in the current study. In the case of future work, the current study provides a statistical basis for power analyses, and gives a more definite indication of the number of animals that would be appropriate.

The failure to find statistically significant results in all of the variables is not in itself indicative of an insufficient sample size. A larger sample size may have yielded a greater number of statistically significant observations in the data, particularly for the two observations above, but the question is whether these statistical observations would be clinically significant. It must also be noted that while generally accepted, $p = 0.05$ is an arbitrary value for significance, and should not be treated as an absolute, hence the reporting of a non-significant tendency for P-values between 0.05 and 0.09.

In summary, I have reported two findings where a larger sample size may have provided a clearer statistical result. However, the sample size proved to be sufficient to address the primary aims of the study, which suggests that it was appropriate for the study. Future studies may require a larger sample size if they wish to assess the effect of castration on baseline total power, or the effect of time from tail docking on the EEG response.

5.4.4 Limitation of Evaluating EEG Recording from Only the Right Cerebral Hemisphere

For recording of the EEG lambs were placed in left lateral recumbency, which meant that the left non-inverting EEG lead over the mastoid process (figure 3.1, Materials and Methods) was in contact with the table, and subject to substantial movement artefact due to only slight movement of the lamb's head. I was not aware of the degree of movement artefact on the left side channel until all of the data had been collected, as traces were only briefly skimmed immediately following recording and were not fully assessed until after completion of all the recordings. The extent of movement artefact meant that the left EEG channel was discarded, and only the right channel analysed.

It would have been preferable to have the head lifted and supported on the dependent side in a way that would protect the electrodes from movement, even by something as simple as a few well cut foam pads, which would allow for meaningful recording and analysis of both left and right hemispheres.

While it is expected that the recording from left and right hemispheres would be correlated, it is possible that the left and right hemispheres may have reacted differently, and having data from both channels may have provided more information on the EEG response to tail-docking in lambs. For example, human subjects showed consistently stronger EEG activity in the left-hemisphere in response to nociceptive heat stimulation of both the left and right hand, based on analysis of EEG recordings from the parasylvian cortex (Schlereth et al., 2003).

Observation of a more marked response to nociceptive stimulation in one hemisphere relative to the other in lambs would inform us on which channel to focus recording on in future studies, as it would be preferable to have the most important, or responsive, hemisphere's recording subject to the least interference from potential contact with the table.

5.4.5 Future Studies and the Inclusion of an Analgesia Control Group

In future studies there is the potential for inclusion of a group exposed to early age castration castrated with appropriate analgesia, which would inform us on the potential for appropriate analgesia to reduce or eliminate the hyperalgesic effect of early age painful stimulation in lambs.

Appropriate analgesia at the time of painful procedures can minimise the sensation of pain, and can obtund the EEG response to nociceptive stimuli in precocial mammals (Murrell et al., 2005, Murrell et al., 2003, Johnson et al., 2005b). Behavioural work in human infants has shown that appropriate analgesia can obtund the development of later hyperalgesic behavioural responses as a consequence of early painful procedures (Peters et al., 2003, Taddio et al., 1997, Taddio et al., 1995). However, there are no studies to date that have shown whether appropriate analgesia at the time of nociceptive stimulation can obtund or prevent the development of increased pain perception, as demonstrated in the current study, to later painful stimuli in precocial mammals.

The two main classes of analgesia used in mammalian species and non-steroidal anti-inflammatory drugs and opioids, both of which have centrally acting mechanisms of analgesia (Bovill, 1997, Dickenson, 1995). The primary physiological mechanism underlying developmental hyperalgesia is currently thought to be central sensitization of the dorsal horn of the spinal cord, as a consequence of increased activity in afferent pain fibres and reduced descending inhibition (review section 2.2.2, General Mechanisms of Hyperalgesia). Based on this hypothesis for developmental hyperalgesia, it seems likely that effective analgesia with either NSAIDs or opioids at the time of castration would reduce the development of centrally mediated hyperalgesia in these individuals.

Therefore, where the current study has provided evidence suggesting that early age castration in lambs increases the cortical response to later painful stimuli, it is now the task of future studies to assess whether the increased cortical response seen in the current study can be ameliorated by routine analgesia at the time of castration.

5.4.6 Testosterone as a Potential Confounder

In addition to providing an initial painful stimulus, castration also removed the lamb's source of endogenous testosterone, which may have affected the EEG response to tail docking. In primates an increase in serum testosterone correlates with relatively more high frequency EEG activity (Poblano et al., 2004, Poblano et al., 2003). It is possible that entire (handled) lambs would have had higher circulating testosterone levels than the castrated lambs, and that this may have resulted in a greater representation of high frequency, low power activity in the handled group. However, as circulating levels of testosterone in lambs are very low during early developmental stages, prior to 12 weeks of age (Khalifa et al., 2013), it is unlikely that variations in circulating testosterone had a meaningful or significant effect on the EEG response to nociceptive stimulation at four weeks of age.

In the current study there was a tendency toward a higher baseline (prior to tail docking) total power in the castrated lambs, and there was no difference noted in the baseline median frequency or spectral edge frequency variables between castrated and handled lambs. As median frequency and spectral edge frequency are better measures of high frequency activity, this result suggests that castration alone had little effect on pre-docking high frequency EEG activity in this study.

5.5 Application of the Findings

5.5.1 Application of the Findings to the Current Animal Welfare Recommendations in New Zealand

The current study shows that the current Animal Welfare (Painful Husbandry Procedures) Code of Welfare (2005) recommendation to castrate and dock lambs as young as is possible, may have negative impacts on longer-term pain sensitivity, and thus longer term animal welfare. While the recommendation to perform PHPs as early as possible may be valid in terms of the amount of acute pain experienced by lambs at the time of the procedure, this consideration must be weighed up against the potential for longer term welfare compromise. In this respect, the findings of this study do not support the current Animal Welfare (Painful Husbandry Procedures) Code of Welfare (2005) recommendation that castration and tail docking should be performed as young as possible in lambs.

From this study I am not able to suggest a best age to perform PHPs in lambs, as I do not know how long after birth the potential for developmental hyperalgesia lasts. Based on the behavioural observation that lambs castrated at ten days of age show a reduced behavioural hyperalgesia when compared with lambs castrated at one day of age (McCracken et al., 2010), caution would suggest that lambs should not be castrated or tail docked at younger than ten days of age. However, it is unclear whether the lambs castrated at ten days of age also expressed hyperalgesia, just to a lesser degree than the lambs castrated at one day of age, thereby highlighting the limits of our current understanding of the development of hyperalgesia in lambs.

The current Animal Welfare (Painful Husbandry Procedures) Code of Welfare (2005) recommends pain relief be provided at all ages, where reasonable to do so, but is only

required in animals over six months of age. The findings of this study emphasize the value of providing pain relief when exposing animals to PHPs, as the animals are subjected not only to the acute pain of the procedure but may also develop long-lasting increased pain response (hyperalgesia) to subsequent painful stimuli as a consequence.

In summary, the findings of the current suggest that performing PHPs in neonatal lambs may increase the pain sensation of future painful stimuli, and the findings do not support the current PHP recommendations in New Zealand to castrate and tail dock labs as young as is possible. There is not a clear age that can be recommended as a minimum age for performing PHPs, and for this reason these findings underscore the importance of providing appropriate pain relief in neonatal animals undergoing PHPs, to prevent the development of hyperalgesia in these individuals, as well as to address the acute pain sensation.

5.5.2 Variation in Developmental Timeframes between Species

The variation in developmental timeframes that is observed across different mammalian species is one of the major limitations in the interpretation and broader application of the findings of this study, and was reviewed in section 2.2.2, General Mechanisms of Hyperalgesia. A large portion of the research done in the field of developmental neurophysiology cited in this thesis has been done on humans and rats, both of which are altricial animals, and therefore have a very different developmental timeframe to lambs and other precocial mammals. One pertinent example of varying developmental time frames is the establishment of afferent fibres in the spinothalamic tract extending to the thalamus – which relay nociceptive information to the cortex. The establishment of this tract occurs at 19 days postnatally in rats (Higashi et al., 2002), with the equivalent synapses developing

much earlier in sheep, at about 100 days gestation (Rees et al., 1994), thereby exemplifying the potential timing of developmental differences between species.

A great deal of further work needed to tease out the role of spinal and potential supra-spinal sensitisation in the development of pain signalling pathways, and to understand how these mechanisms and their development differ between species. Currently, while work in human infants and rat pups may suggest potential mechanisms underlying the development of hyperalgesia in lambs our understanding is too limited to apply more than the basic principles to the interpretation of the current study, a fact that must be taken in to account when considering the human clinical relevance of the findings.

5.5.3 The Human Clinical Relevance of the Findings

The findings of this research may have important implications for human as well as veterinary medicine, as the general principle of hyperalgesia as a consequence of early painful procedures discussed through this thesis may also apply to human neonates.

Due to current limitations in our understanding of pain and analgesia in infants, the majority of painful procedures performed on human neonates in neonatal intensive care units are done without the provision of effective analgesia (Walker, 2008, Carbajal et al., 2008). There is the potential for the development of hyperalgesic states in these individuals as a consequence of these early painful procedures without analgesia, which may result in human infants that have a greater sensitivity to later pain and experience greater suffering as consequence of later painful events (Taddio et al., 1997). Furthermore, over the past 30 years human infants have been surviving from earlier gestational ages, and the number of premature births is continuing to increase (Seaton et al., 2013, Marlow and Bryan Gill,

2007). As prematurely born infants are more likely to be subjected to invasive and painful medical interventions than full term infants (Barker and Rutter, 1995), understanding the long term consequences of nociceptive insult during the early developmental period is becoming increasingly important in human neonatal medicine.

In principle, the potential for developmental hyperalgesia as a consequence of early painful procedures merits further investigation in human medical studies. However, studies of this nature are limited in humans, due to the ethics of inflicting pain on neonates that cannot provide informed consent to partake in pain studies. As would be advised for lambs and other non-human mammals, I would propose that the priority should be determining effective pain management in human neonates. Not only to address the acute noxious insult of these painful procedures, but also to minimise the potential for longer-term increased pain sensitivity.

In summary, there is the potential for development of hyperalgesic states in human infants as a consequence of early age painful procedures performed in neonatal intensive care units. While the current study does not offer direct evidence for the potential development of hyperalgesia in human neonates, the general principles outlined in this discussion may be applicable to human neonates.

5.6 Conclusions

In conclusion, this study provides experimental evidence that early painful stimulation can result in an increased cortical response to later painful stimuli in lambs. The findings of this study demonstrate that neonatal castration in lambs results in changes in the cortical response to tail docking at three weeks of age. McCracken et al. (2010) and Pifeleti (2011) Showed that early age castration increases the incidence of pain related behaviours in response to subsequent painful stimulation in lambs. The current study shows that this behavioural hyperalgesia is concordant with a greater magnitude of cortical pain response to subsequent painful events. The greater magnitude of cortical pain response in the castrated animals indicates that these animals are likely exposed to a greater degree of perceived pain, and therefore may be subject to a greater degree of suffering and a greater welfare compromise.

There is much work to be done to elucidate the mechanisms underlying this developmental hyperalgesia, understand what early nociceptive events may lead to its development, and evaluate its duration, scope, and clinical significance in both precocial and altricial animal species, as well as in human neonates. The described developmental hyperalgesia has implications for painful husbandry procedures in production animals and possibly early painful procedures in companion animals, as well as potentially having important application to human infants undergoing painful procedures that are undertaken during the early neonatal period.

Specifically, the findings do not support the current Animal Welfare (Painful Husbandry Procedures) Code of Welfare (2005) recommendation that lambs be castrated and tail docked as young as is possible. While I cannot provide a specific age recommendation for

PHPs in lambs, preliminary behavioural work by McCracken et al. (2010) indicates that the developmental hyperalgesia shown in neonatal lambs is not seen in ten day old lambs, suggesting that the critical window for developmental hyperalgesia is at less than ten days of age. The findings of the current study highlight the potential long term hyperalgesic effects of early age castration and tail docking, which must be considered and weighed up alongside the acute painful effects of these procedures. These findings thereby underscore the importance of appropriate analgesia when undertaking PHPs in neonatal lambs as well as other precocial mammals, as appropriate analgesia can best manage both the acute and longer term effects of these procedures. Based on these considerations and until more complete information is available, I would advise castration and tail docking should be avoided in lambs younger than ten days of age, and effective analgesia should be provided for lambs exposed to painful husbandry procedures at an early age.

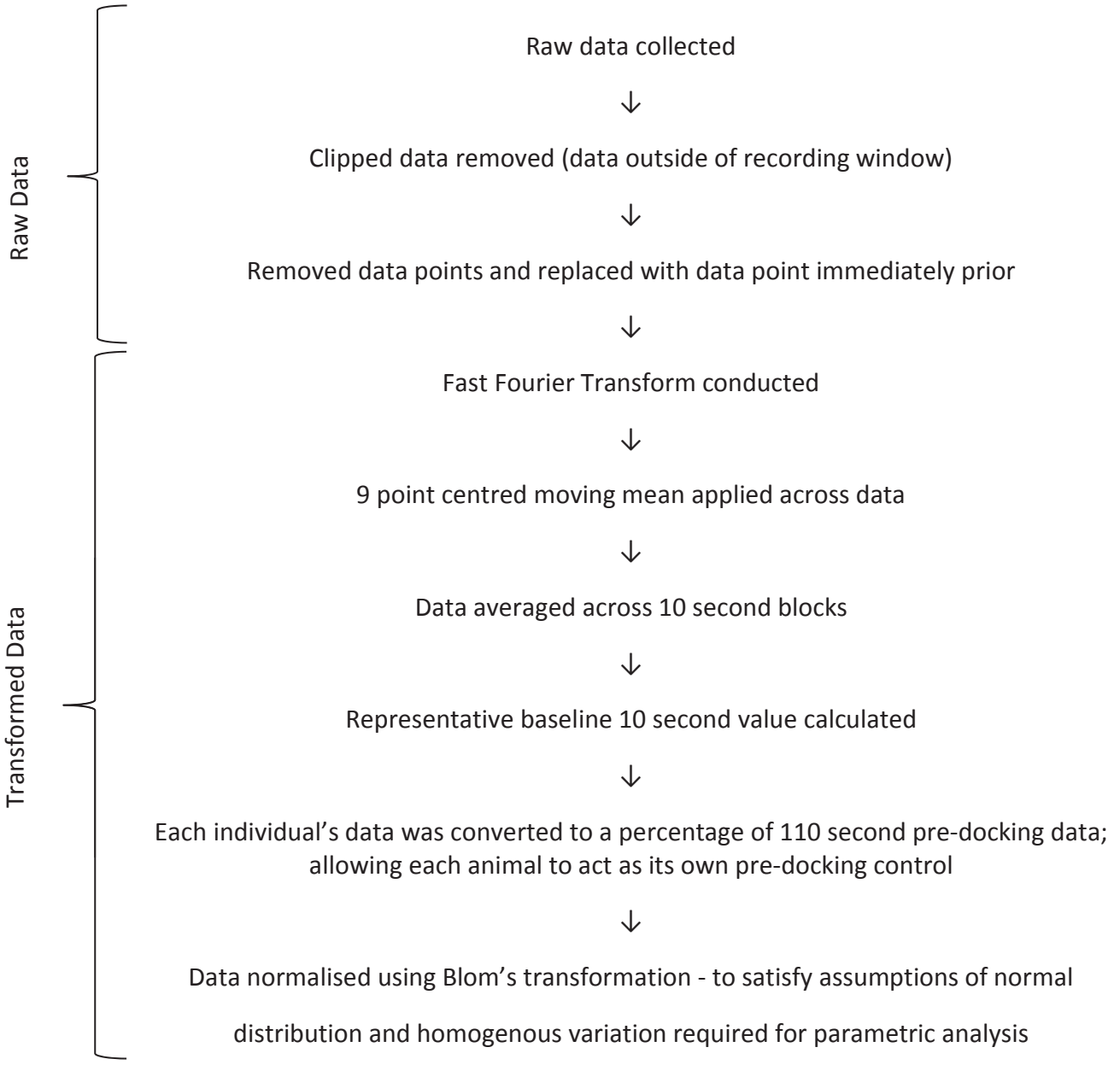
In addition to the primary findings, the current study also showed that lambs castrated at one day of age tended to have a higher total power during baseline EEG recordings than did control animals, suggesting that there may be differences in the baseline EEG spectra between these two groups. This tendency towards a higher predocking baseline power suggests that painful stimulation in neonatal lambs may produce a fundamental change in the cortical activity at 23 days of age. The potential clinical significance and permanence of this is not clear at this stage, and further work is necessary to better define and understand this observation.

It is hoped that this research will help to encourage and guide future work in pain development, help inform future welfare legislation for painful procedures in animals, and find practical application in veterinary medicine.

6 Appendices

6.1 Flow Chart for Steps in Data Manipulation

The following is a summary diagram of data manipulation steps for linear mixed model analysis. It was developed to aid my own understanding of the data processing, and has been included as a useful reference for the reader.



6.2 Paper Abstract, World Congress of Veterinary Anaesthesiology

The following is the written abstract that accompanied the presentation of this research at the World Congress of Veterinary Anaesthesiology in 2012. While I am first author on the paper, the presentation was given by then Associate Professor Craig Johnson.

Effect of early post-natal castration on subsequent EEG response to tail docking in lambs

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Objective To investigate the effects of noxious stimulation in the early post-natal period on electroencephalographic responses to subsequent noxious stimulation.

Study design Prospective randomized experimental study.

Animals Twenty four Romney-cross lambs.

Methods Lambs were randomly assigned to two groups and either castrated by application of a rubber ring (n=12) or handled (n=12) within 12 – 24 hours of birth. At 23.1 (0.1) days of age, lambs were anaesthetised using halothane in oxygen and maintained at a minimal plane of anaesthesia (P_EHal = 1%). Electroencephalogram (EEG) was recorded and following a two-minute baseline period, both groups underwent tail docking by application of a rubber ring and recording continued for a further 8 minutes. The EEG data were processed using Fast Fourier Transformation and the median frequency (F50), 95% spectral edge frequency (F95) and total EEG power (ptot) derived off line. Differences in baseline recordings and responses to tail docking were analysed using a generalised linear mixed model analysis in SAS 9.3 (SAS Institute Inc., Cary, NC, USA).

Results The castrated lambs demonstrated a significantly greater increase in F50 (p=0.035) and decrease in ptot (p=0.042) following tail docking than did the handled lambs. Baseline ptot was also significantly lower (p=0.037) in the castrated animals.

Conclusions Castration at one day of age results in a greater EEG response to subsequent tail docking. These results confirm previous studies indicating increased pain-related behaviour following early noxious stimulation and demonstrate the suitability of EEG recording to identify long-term hyperalgesia.

Clinical relevance Noxious stimulation in the early post-natal period may result in long-term hyperalgesia.

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