ABSTRACT

Title of Document: ELECTROENCEPHALOGRAPHY (EEG) AND MEASURES OF NOCICEPTION IN DOMESTIC CATTLE (Bos taurus)

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The first known bovine laser evoked potential (LEP), an EEG response to noxious laser heat stimuli, was measured in 2-3 year old Holstein cows (n=5). The amplitude of the bovine LEP correlated significantly (P<. 05) with behavior scores, the surrogate for self-reporting in human studies. Importantly, and comparable to human studies, the LEP occurs at a latency within which it is considered that cortical potentials reflect increasingly complex cognitive processes, rather than those that are reflexive and non-conscious. Differences between the bovine and human LEP were also determined, that cannot be fully explained at this time. The lack of standardization for large animal EEG-investigations is problematic regarding data

sharing across labs. A proposed standard method, for collecting and processing EEG in cattle was developed and is presented. Compared to human studies, signal processing of bovine data required significantly more stringent rejection criteria for data analysis. For example, while wavelet denoising is often used in human EEG; it was found essential for extracting a bovine LEP. In addition, explicitly addressing whether of not cortical potentials were being recorded was necessary to provide foundational background knowledge of bovine EEG. To this end, EEG was recorded under conditions designed to simulate the suppression and excitation of the primary visual cortex, as is measured in humans using eyes-open and eyes-closed. The simulation contrasted a dark and light environment. I propose this protocol to be used in the future large animal studies to verify that cortical potentials are being measured before EEG data recording. My results demonstrate that bovine EEG is a useful bovine cognitive science method, but more sophisticated signal processing techniques are needed to ameliorate issues of artifact. Lowered signal to noise ratios is considerably problematic for evoked response studies in large animals. Importantly, this research determined that a bovine LEP is measurable, and by analogy to human perceptual studies, I contend this demonstrates the cow experiences both the sensation and perception of noxious stimulus as painful.

ELECTROENCEPHALOGRAPHY (EEG) AND MEASURES OF NOCICEPTION IN DOMESTIC CATTLE

(*Bos taurus*)

By

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Aβ	A BETA, MECHANICAL NERVE FIBER
AEP	AUDITORY EVOKED POTENTIAL
ACC	ANTERIOR CINGULATE CORTEX
Aδ	A DELTA NOCICEPTIVE FIBER
BSE	BOVINE SPONGIFORM ENCEPHALOPATHY; COMMONLY KNOWN AS MAD COW DISEASE
BSS	BLIND SOURCE SEPARATION
CNS	CENTRAL NERVOUS SYSTEM
CS	CONDITIONED STIMULUS
CWT	CONTINUOUS WAVELET TRANSFORMATION
Cz	ELECTRODE ON VERTEX OF SCALP/CROWN
ECG	ELECTROCARDIOGRAM
EEG	ELECTROENCEPHALOGRAPHY
EOG	ELECTRO-OCCULOGRAM
ERD	EVOKED RESPONSE DESYNCHRONIZATION
ERP	EVOKED RESPONSE POTENTIAL
ERS	EVOKED RESPONSE SYNCHRONIZATION
FIR	FINITE IMPULSE RESPONSE
fMRI	FUNCTIONAL MAGNETIC RESONANCE IMAGING
HBS	HIGH BEHAVIOR SCORE
HPC	HEAT, PINCH AND COLD
Hz	HERTZ
IC	INTER-CANTHAL LINE
ICA	INDEPENDENT COMPONENT ANALYSIS
ISI	INTER-STIMULUS INTERVAL
LBS	LOW BEHAVIOR SCORE
LEP	LASER EVOKED POTENTIAL
MEG	MAGNETOENCEPHALOGRAPHY
Nd:YAG	NEODYMIUM-DOPED YTTRIUM ALUMINUM GARNET LASER
NS	NOCICEPTIVE SPECIFIC
N140	SOMATOSENSORY VERTEX POTENTIAL ELICITED BY ELECTRIC STIMULATION
N2	FIRST POTENTIAL IN THE LASER EVOKED POTENTIAL, N2P2
N2P2	LASER EVOKED POTENTIAL N FOR NEGATIVE, 2 FOR 200 MS, P FOR POSITIVE
NNMD	NON -NEGATIVE MATRIX DECOMPOSITION
OP	OCCIPITAL PROTUBERANCE
P2, P3, P3a	POSITIVE POTENTIALS ELICITED BY STIMULI SUCH AS A NEW SOUND
PCA	PRINCIPAL COMPONENT ANALYSIS
PLF	PHASE LOCKING FACTOR
SI	PRIMARY SOMATOSENSORY CORTEX
SII	SECONDARY SOMATOSENSORY CORTEX
PET	POSITRON EMISSION TOMOGRAPHY
SEF	SPECTRAL EDGE FREQUENCY
SEP	SOMATOSENSORY EVOKED POTENTIAL (SUCH AS ELECTRICALLY EVOKED POTENTIALS)
SF50	THE FREQUENCY IN A SPECTRUM AT WHICH 50% OF THE POWER LIES BELOW
SF80	THE FREQUENCY IN A SPECTRUM AT WHICH 80% OF THE POWER LIES BELOW
sLORETA	LOW-RESOLUTION BRAIN ELECTROMAGNETIC TOMOGRAPHY
STT	SPINOTHALAMIC TRACT

US	UNCONDITIONAL STIMULUS
VEP	VISUAL EVOKED POTENTIAL
VMN	VENTRAL MEDIAL NUCLEUS
VMPO	POSTERIOR COMPONENT OF THE VENTRAL MEDIAL NUCLEUS
WDR	WIDE DYNAMIC RANG

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Chapter 1 Introduction

Attaining a full understanding of pain has been a long-standing challenge for scientists. Pain is ultimately a private subjective state that is unique to the individual experiencing the phenomenon. Science is an objective process. A dedication to objectivity among scientists has often resulted in a reluctance to venture into subjectively experienced phenomena, such as pain. A somewhat foundational contention of this dissertation is that a general neglect in addressing the topic of pain has been especially true relative to investigations of pain in animals, particularly those commonly used in the production of food for humans, i.e., cows, pigs, chickens, etc. This is not to say that aspects of pain, and even the very topic of pain, have not been, and should not be addressed by scientists – and philosophers.

Scientists have provided a sound explanation for the anatomical and physiological sensory mechanisms that underlie the perception of pain. Chemical transmissions cause electrical currents to rapidly propagate from one neuron to another facilitating communication between somatic and cortical regions. Pain and suffering can result when the chemical-electrical-communication processes associated with somatic or visceral trauma reach cortex. In somewhat lay terms, the integration of neural communication across regions of the brain results in the individual "feeling pain."

Humans can conceptualize and even verbally communicate among ourselves what it means to experience pain. In the lexicon of some neuro-scientists, philosophers of mind, investigators of artificial intelligence, and others, we humans are said have the ability to form a representation of pain,

i.e., a mental representation of how it feels to have the experience of pain. Through representation and experience, we have some ability to predict the potential painfulness of an anticipated stimulus, and can recall episodic memories of pain previously experienced. Humans also empathize readily with each other, to the extent that the sympathetic observer's emotional cortices in the limbic system are activated in ways that would occur if the experience were personal. This cortical empathy does not require verbal communication, but only watching another in pain can initiate feelings of empathy, so specific is the behavior associated with pain. We generally interpret certain facial expressions, loud and atypical verbalizations, a limping ambulatory gait, etc. as possible indications of pain in others. To understand pain in animals, human-type verbal communication is obviously not possible. However, as Stamp-Dawkins (1998) and others have argued, behavioral cues from animals can also be useful in understanding what an animal is experiencing, and some sections of this dissertation will be based in part on this viewpoint.

Evolutionary biology and cognitive ethology contend that pain is adaptive. Some 2 to 3 billion years ago, individuals that developed a DNA-based ability to avoid noxious or harmful stimuli held an advantage over those of their kind that lacked this ability. The individuals that avoided harm produced more offspring, i.e., they had greater genetic fitness. Interestingly, neurons are not necessary for an organism to avoid a noxious stimulus. Single-celled organisms such as paramecia and amebae avoid or move away from conditions that are harmful. Multi-cellular organisms, such as jelly-fish, developed the specialized cells that today are identified as neurons. Ultimately,

some species evolved in much multi-cellular complexity and organization such that their neuronal system included a spinal cord and central brain.

Of particular relevance to this dissertation is the class of animals known as Mammalia. Notably, humans are members of this class along with a number of other species including cattle. This scientific classification is of course based on the large degree of commonality shared by these species. To paraphrase Darwin, "We humans differ from the other animals only in degree and not in kind." Darwin also acknowledged that the differences in degree are considerable and significant. The contentions of this dissertation are not intended to disagree. However, this dissertation does emphasize that the anatomical and physiological neuronal structures and function across the mammalian species share more in common than they have as differences. Specifically, this dissertation does hold that there will be instances that it is possible to extrapolate and draw inferences based on cross-species comparisons of data, behavior, physiology, and in this instance electroencephalogram (EEG) data.

The origin of the idea that led to the data collected and presented herein came from an awareness of human pain research based on EEG data collected in association with laser heat stimuli (Chen et al., 1979, Bromm et al., 1983, Arendt-Nielsen and Bjerring, 1988). The EEG feature elicited by the laser stimuli is the laser evoked potential (LEP), and correlates with the magnitude of subjective pain reported. Because humans can self-report the relative level of pain experienced, the LEP could be evaluated accordingly. The LEP was determined to be proportional to the intensity of the laser stimulus administered.

Therefore, it was proposed that the LEP technique had possibilities as a method of quantifying pain in animals. However, because animals cannot self-report, the data interpretation would need to be based, in part, on behavioral responses. The species selected for investigation were domestic cattle (*Bos taurus*).

Pain in cattle is important from both ethical and pragmatic viewpoints. A number of practices associated the production and treatment of cattle cause pain to the animal – sometimes unrelieved by analgesic or anesthetic. These practices can include castration, dehorning, branding, tail-docking, etc. From a practical viewpoint, pain and suffering in cattle can cause loss of weight, less milk produced, carcass condemnation, etc. Thus, pain in cattle in association with beef and milk production can have an economic impact. From an ethical viewpoint, these practices continue to be used in part because of tradition, but also because there remains among some animal producers and even some veterinarians a degree of non-acceptance of the concept that animals such as cattle experience pain (Anil et al., 2005). Thus, a research-based greater understanding of pain in cattle could have significant consequences from both ethical and economic viewpoints. Accordingly, the investigations reported herein are based on EEG recordings taken from cows. The cow was not used because she is considered to be a good model for pain research in general. Rather, the studies that are presented were designed specifically to gain a greater understanding of nociception, thus pain, in cattle.

The terminology used by pain researchers is not always consistent. Thus, the reader occasionally encounters the terms pain and nociception used

interchangeably in the literature. In this document, the two words will be defined as follows. Nociception will be used primarily in reference to the individual's objective perception of an unpleasant and potentially noxious stimulus, which might be unpleasant, and when continued could result in pain and potentially tissue damage. Pain will be used when referring to a more holistic experience involving a more subjective, emotional perception of an intense stimulus, encompassing an affective component that makes the experience extremely unpleasant, and involves a negative cognitive state. That said, a key concept critical to understanding how the terms and concepts are presented herein is that an intact nervous system cannot experience pain without nociception. Specifically, both nociception and pain share the same-labeled line pathways up until the projection from the thalamus terminated in the limbic system. Hence, neurophysiologically it is difficult to explain the difference, or why it exists.

Also, in the literature the two phrases "nociceptive processing" and "pain processing" are used interchangeably. The reason for this confounding is as a consequence of both involving the same supporting neuroanatomy. However, nociceptive processing will generally be used when referring to the role of the somatosensory cortices. The nociceptive system is part of the general somatosensory system but in this proposal, a distinction must be made between the system that processes the painful stimuli, and the system that processes benign stimuli such as touch. Therefore, the words tactile, haptic, non-nociceptive or touch in reference to the somatosensory system will be used in reference to any other aspect of the somatosensory system using peripheral receptors not involved in pain processing.

Because pain as defined in this proposal is the product of affective and sensory discriminative processes, and its experience is ultimately subjective, it is at best difficult, if not impossible, to study objectively using only neurophysiological methods such as EEG. A single evoked response potential or frequency response cannot represent such a multidimensional entity such as pain, but I will contend (and present supporting evidence) that EEG measures when properly recorded, analyzed and presented can be considered as neurophysiological markers of nociception stimulation that is necessary in the pathways to pain perception. Additionally, an argument can be made that it would be unethical to cause a living subject actual pain. For these reasons, my contention is that it is nociception that ultimately should be investigated, even though other authors often describe their research as pain-related. My studies presented herein were, therefore, investigations of nociceptive processing in cattle. As in the research of human pain, the laser-evoked potential that I have measured in cattle I will argue can be used as a neurocorrelate of pain perception.

Chapter II: Literature Review

Historically, pain research using electroencephalography (EEG) dates back at least to 1979 with the discovery that there was a potential that was interpreted as being a direct surrogate for subjective pain perception (Chen et al., 1979). More recently, pain research has focused specifically on gaining a greater understanding of nociceptive evoked potentials. The following overview of the published research includes a discussion of pain and nociception and the use of evoked potentials to study pain.

While much of the literature focuses on the study of pain in humans, and there are obvious cross-species consistencies, the very nature of pain as a subjective experience makes such comparisons difficult. This dissertation does not attempt to satisfy all lay and scientific definitions of pain.

Pain is a multidimensional experience, involving global cortical processing, and encompassing both sensory-discriminative and affective components. These dimensions are sub-served by diverging central pathways, the lateral and medial thalamic pathways, coding for sensory discriminative and affective components, respectively (Treede et al., 1999, Treede et al., 2000, Luo and Wang, 2008). The discriminative pathway projects from the lateral thalamic nucleus to the primary somatosensory (SI) and secondary somatosensory cortex (SII) (Treede et al., 1999, Timmermann et al., 2001), and insula, and codes for detection, intensity, location and quality (cold or hot) of stimulus.

SI and SII are known to be involved in nociceptive processing, but their role is still debated. Even though SI and SII generally code only for

somatosensory, rather than affective, aspects of pain, there is evidence that they have an unspecified role in the latter, counter-intuitively to our current understanding of the role of somatosensory cortices. The medial pathway, codes for the hedonic, or 'suffering,'(Sewards and Sewards, 2002) and projects from the medial thalamic nuclei to the insula and the anterior cingulate cortex (ACC) (Willis, 1985, Bushnell et al., 1999, Treede et al., 1999).

Even though nociception is a somatosensory function, the conscious appreciation of non-nociceptive and nociceptive stimuli is very different. Pain is coded for through both labeled lines and convergence on spinal relay cells, similarly to other sensory coding schemes (Sewards and Sewards, 2002, Craig, 2003). However, the pain pathway is largely anatomically distinct from the other somatosensory pathways beginning at the peripheral receptors. There are only two types of peripheral nociceptors, whereas there are seven types of mechanoreceptors that encode tactile stimuli. Therefore, tactile perception is a result of a convergence of afferent information from these receptors, whereas nociception is accomplished via excitation of any one of the nociceptors. Nociceptors are only excited by nociceptive or painful stimuli. The afferent information is sent via labeled lines through the spinothalamic tract (STT) to the thalamus and then to the cortex, where anatomical separation between nociception and somatosensory perception is preserved by the projections that result from either medial or lateral thalamus. Projections from medial thalamus tend to go to the cortices that code for emotion, whereas projections from lateral thalamus go to somatosensory cortices, historically thought only to code for 'what' and 'where.' Pain is also

distinct from other somatosensory systems in higher mammals because it is processed in parallel, rather than serially in the somatosensory cortices (Craig, 2003, Liang et al., 2011). Parallel processing is thought to be more evolutionarily primitive than serial processing and this has caused speculation that the pain pathway is more primitive than other somatosensory systems (Garcia-Larrea et al., 2003).

What is pain and what are its main pathways?

Pain is a multidimensional experience comprised of a somatosensory discriminative component and an affective component (Bromm and Lorenz, 1998, Rios et al., 1999, Treede et al., 1999, Peyron et al., 2000, Treede et al., 2000). These two components, while sharing some common pathways serve two different functions. The somatosensory discriminative component codes for the modality, intensity and location of nociceptive or tissue damaging stimuli (Rios et al., 1999, Treede et al., 1999), while the affective component is responsible for the unpleasantness, or suffering aspect of pain (Rios et al., 1999, Treede et al., 1999, Derbyshire, 2000, Sewards and Sewards, 2002). The affective component is also responsible for emotion and memory resulting from a painful stimulus (Treede et al., 2000). The activation of both of these pathways is significantly affected by attention or distraction (Derbyshire, 2000, Peyron et al., 2000, Treede et al., 2000, Yamasaki et al., 2000). Although these pathways are often described as distinct, actual pain results from a complex interaction between the peripheral and central component pathways (Luo and Wang, 2008).

Most recently, the 'saliency' of the stimulus has been shown to be the critical factor in determining the variance of the potential's magnitude (Mouraux and Iannetti, 2009a). However, this is most likely to be due to top down processing, and whether or not saliency and pain perception amount to different cognitive aspects, the end result is still a subjective negative state.

The original 'gate' theory of pain contended that pain was the result of a convergence of signals on primary spinal relay cells in the spinothalamic tract (STT) and their thalamic projections, disregarding the possibility of cortical processing of pain (Melzack, 1982). It was soon shown, however, that there can be no pain without cortical processing (Treede et al., 1999), but exactly how pain is processed and represented in the cortex is not fully understood. In a review, Craig (2003) described the pain pathway as comprising two separate sub-systems differentiating as early as the dorsal horn of the spinal cord. One sub-system uses almost exclusively labeled lines (STT lamina I), and the other uses convergent coding within a labeled line architecture (in which information within a series of cells transmitting information to the brain remains within separate channels or lines). This picture of the pain system suggests that pain is a separate system, discrete from other somatosensory systems, which is consistent with anatomical evidence. In a review of the pain system, Sewards and Sewards (2002) presented anatomical evidence suggesting that there is a dedicated pain pathway that uses labeled lines, but that, the somatosensory discrimination aspect was largely shared with other non-pain related somatosensory systems. This is explanatory to the finding of Iannetti et al. (2009) that laser potentials can be represented by principal components that also represent benign mechanical, somatosensory stimuli.

That painful stimulus perception does is not the direct result of activation of peripheral and spinal nociceptive pathways as Melzack (1982) suggested, it cannot happen with an intact nociceptive pathway. That the patency of the peripheral and central nervous system are imperative to pain perception is not disputed, but since Melzack (1982) the significant role of central processing in its experience has been well described. Craig's review (2003) focuses on the labeled lines structure of the laminae in STT, and Sewards and Sewards (2002) focuses on the fact that nociceptors also converge on wide dynamic neurons (WDR), dorsal horn cells in the medial lemniscal tract, which transmit tactile and proprioceptive information to SI.

Although there must be a somatosensory, or discriminative component to pain, it is unclear if there are dedicated areas in SI that represent pain as there are other stimulus modalities. Nociceptor density in SI has been determined to be sparse. Ogino et al. (2005) demonstrated a separate and somatotopically arranged pain area in SI of man, but Chen et al. (2008) found that tactile and pain areas overlapped, explaining Iannetti et al (2009) findings. Pain is processed by the somatosensory cortices in parallel, such that information is sent to both SI and SII simultaneously rather than from SI to SII (Garcia-Larrea et al., 2003, Kakigi et al., 2004, Shibasaki, 2004), as occurs in primate and higher mammalian non-nociceptive somatosensory processing. The fact that non-nociceptive somatosensory processing in some lower mammals is in parallel, rather than the serial arrangement of higher mammals, suggests that parallel processing is evolutionarily older. That the pain pathway is organized in parallel suggests that it, too, is a more primitive system (Garcia-Larrea et al., 2003) than other somatosensory systems.

Peripheral, spinal and supraspinal: A and C fibers, spinal and thalamic pathways

The anatomy of the peripheral and spinal pain pathway has been extensively researched. [For reviews see Craig (2003), Millan (1999), Bloedel & McCreery (1975), and Willis (1985)]. The experience of pain begins with activation of two types of peripheral free nerve endings in the skin, the Aδ and C fibers, which have distinctly different properties and code for different sensations. The result of nociceptive laser heat stimulus is a perception of a sharp first pain, and a burning second pain at least 700 ms later mediated by Aδ and C fibers, respectively (Bromm et al., 1983, Bromm and Treede, 1984, Bromm and Lorenz, 1998, Millan, 1999, Mouraux et al., 2003, Opsommer et al., 2003, Forss et al., 2005). The conductance speed of these fibers corresponds to the timings of the two pain sensations as Aδ fibers are small myelinated fibers with a conductance speed of about 4-30 m/s as opposed to the unmyelinated C fibers with a conductance speed of 0.4-1.8 m/s (Bromm and Lorenz, 1998, Millan, 1999, Kakigi et al., 2004).

C fibers have the highest density of all the cutaneous afferent fibers comprising 70% of the cutaneous somatosensory receptors (Millan, 1999, Stuckey et al., 2001). Aδ accounts for just 10% of cutaneous receptors, and Aβ, the mechanoreceptors, excited only by tactile stimuli under normal situations, accounts for the remaining 20% (Millan, 1999). A∂ fibers respond uniquely to either intense noxious stimuli, or are excited by heat, pinch and cold (HPC). They have a high excitation threshold, and continue to fire after excitation with a graded intensity as stimulus intensity increases. This is in

stark contrast to the multimodal C fibers, 60% of which are excited by heat and chemical stimuli (Bromm and Lorenz, 1998), and adapt quickly to increasing intensity of stimulus (Millan, 1999). One commonality between the fibers is the complex pattern of receptive fields that vary with the stimulus modality, the type of skin, and with species (Millan, 1999).

The nociceptive fibers enter the STT and synapse with two functionally different neurons in the laminae of the STT, lamina V and I, which project to the thalamus. A∂ and C fibers synapse directly with nociceptive specific neurons (NS) in lamina I, which code specifically for noxious stimuli (Bromm and Lorenz, 1998, Millan, 1999), and therefore the nature of the stimulus, and location (Willis, 1980), but not for intensity. They also synapse in lamina I with spinal polymodal HPC (not to be confused with the HPC A∂ fiber) neurons that respond incrementally to increasing stimulus intensity. HPC cells show a graded response activity to increasingly cold temperatures above skin temperature meaning that the HPC synapses can also code for changes in environment rather than just coding for nociceptive/non-nociceptive stimuli, and can play a role in homeostasis (Craig, 2003). Lamina I, therefore, codes for sudden and intense noxious stimuli, as well as subserving a homeostatic function by coding information about gradual and potential noxious environmental temperature changes. This is different from lamina V's function.

Lamina V contains predominantly modality non-specific (Willis, 1985) wide dynamic range neurons (WDR) with large receptive fields receiving all primary cutaneous afferent fibers (Craig, 2003), including proprioceptors. They are therefore suitable to code for intensity of the

stimulus (Willis, 1980, Bromm and Lorenz, 1998, Treede et al., 1999, Craig, 2003). Although the fact that they respond to all somatosensory afferents seems to refute the fact that the pain system is distinct from other somatosensory systems, it is the labeled line aspect of the system, i.e., signals from these lamina V neurons are considered nociceptive, that preserves the separation. Lamina V neurons have multiple spinal relays, project to the ventral horn neurons and the cerebellum as well as terminating in the thalamus, which means their activation can have a large effect on behavioral and postural response to nociceptive stimuli. The projection of these pathways to the cerebellum at first feels strange, as this is the cortex most associated with motor activity and balance. However, C fibers have been shown to constitute the majority of the nociceptive projections to the cerebellum, and they have been hypothesized to motivate nocifensive behavior requiring speedy balance changes (Fan et al., 2009).

The thalamus is the endpoint for the laminal projections from the STT and it is the point at which the sensory discriminative and the affective component of pain appear to diverge. Lamina I strongly projects to the medial thalamus which in turn projects to the insula and the anterior ACC, and more weakly to SI and SII (Bloedel and McCreery, 1975, Willis, 1985, Millan, 1999, Treede et al., 1999, Craig, 2003, Baumgartner et al., 2006, Kobayashi et al., 2009). Lamina V weakly projects to the medial thalamus, but has stronger projections to the lateral thalamus, which in turn projects mainly to SI, SII and, more weakly, the insula. Because of their projection targets, medial and the lateral thalamic pathways are commonly considered to represent the affective pain component and the somatosensory component, respectively.

The projections of and to the thalamic nuclei, and thus the STT relays, of primates and non-primates have been identified by Craig et al. (2003) and suggest that the sensation of pain could be different in non-primate mammals, although this is difficult to prove. In the primate, the posterior part of the ventral medial thalamus (VMpo) is large and is conjoined with the ventral medial nucleus (VMN) (Craig, 2003). In non-primates, these two parts of the medial nuclei are distinct, and the VMpo is under-developed. The VMN receives projections from visceral afferents in all species. The VMpo only receives input from lamina I of the STT. Because these two thalamic areas are essentially one in the primate, it could be considered that there is a single representation of the homeostatic state of the entire body found in the thalamic nuclei, i.e., VMpo and VMN (Craig, 2003, Critchley, 2005). Because these areas are discrete in non-primates, the subsequent projections to the insula from the medial thalamic nuclei are, by definition integrated rather than one single projection. This unique primate feature is described as the primate bypass (Willis, 1985, Craig, 2003, Critchley, 2005), which becomes significant in the light of hypothesized functions of the cortices to which this pathway projects. Regardless of species, however, the lateral thalamic nuclei projects to SI, SII and insula, whereas the medial thalamic nuclei mainly projects to the ACC, insula, S1 and SII. One potential result of the bypass might be that non-primates experience pain in an unpleasant hedonic manner, but could be less able to discern abdominal from peripheral pain.

Cortical processing of pain, the affective pathway and the limbic system

The ACC receives projections from the medial thalamic pathway, it is consistently bilaterally activated in pain related studies (Peyron et al., 2000), and there are nociceptive-specific neurons found in the ACC of rats (Johansen et al., 2001), humans, monkeys (Rios et al., 1999, Treede et al., 1999, Craig, 2003), rabbits, and mice, thereby demonstrating that it is an integral part of pain processing. It is unlikely that the ACC codes for sensory discrimination because its nociceptive-specific neurons have very large receptive fields (Treede et al., 1999), which are not suitable for localization and it has been shown that it probably does not code for intensity (Peyron et al., 2000). There is a consensus in the literature that the ACC is part of the affective component of the pain pathway, and it is suited to this as it resides in the limbic system and projects and receives information from the amygdala, insula, and other limbic areas. The ACC's role in the affective component of pain is further supported by evidence that cingulotomy reduces the affective component of pain while preserving the sensory discriminative aspect (Lenz et al., 1998, Rios et al., 1999, Treede et al., 1999, Peyron et al., 2000).

The insula is considered part of the affective component of the pain pathway for similar reasons as the ACC. In primates, the insula may have one of the most important roles in pain perception and conscious experience. It has been conclusively demonstrated that it is part of the pain pathway because it receives medial and lateral nociceptive thalamic neurons, and imaging studies show consistent insular activity in pain related studies (Peyron et al.,

2000). In addition, stimulation of the insular cortex causes memories of pain, or induces the feeling of pain in specific body areas (Treede et al., 1999, Craig, 2003). The insular cortex, however, receives projections from the VMpo/VMN. The VMpo/VMN receives all sympathetic and parasympathetic afferent flow (Friedman and Murray, 1986, Craig, 2003), as well as visceral and gustatory information. The VMpo/VMN substrate also has a representation of lamina I neurons which are modality specific, as well as carrying homeostatic information. The VMpo/VMN projects directly to the insula, which also receives information from the somatosensory cortices. These projections along with the insula's connectedness to the limbic system enables it, in primates at least, to have an entire representation of the body, or to act as an interceptive cortex (Craig, 2003).

Craig (2003) suggests that though non-primates posses an insular cortex, the primate bypass discussed above definitively implies that non-primates would have a greatly degraded affective component of pain, if they possess one at all. What he might mean, is that though they feel pain, they may not have the 'woe is me' aspect. The evidence he presents is that the VMpo is small to non-existent in non-primates, and as stated above, it is distinct from the VMN. Therefore there may be a degraded representation of lamina I information, which is highly pain specific. Furthermore, because this information is not directly transmitted with the other afferent somatic information there is not a strong, clear projection of the state of the body to the insula. He thus concludes that a lack of a substantial VMpo and the absence of the combined VMN/VMpo substrate precludes a representation of the internal homeostatic state of the body in the insula of non-primates, thus

preventing them from being capable of interoception (sense of the physiologic condition of the body). Craig believes this is the key to the human emotion of pain, and possibly self-awareness. They are therefore unlikely to experience pain as primates do.

Whether or not this is true, and how degraded the affective component may be can be debated. Although there is a primate bypass, there is no evidence that the information conveyed via the medial thalamic relays in non-humans does not reach the insula in its entirety, albeit integrated rather than direct. It might be dangerous, therefore, to unequivocally agree with Craig's conclusion given the limitation of self-report in animals.

Cortical processing of pain: the somatosensory cortices and the sensory discriminative pathway

The somatosensory cortices receive projections from nociceptive neurons in the lateral thalamus. Some studies have shown they are activated by nociceptive stimuli, providing evidence that they have some function in pain perception. This function is likely to be sensory discriminative given the function of somatosensory cortices in tactile perception. Their role in pain perception is still debated, particularly for SI. This debate is fueled by inconsistent findings in pain-related studies, and results that seem to contradict logical assumptions based on knowledge of the neuroanatomy and nociceptive neuronal response properties in SI. Only half the imaging studies aimed at elucidating SI's role in pain perception have even shown SI activation in response to painful stimuli, either heat or electrical (Peyron et al., 2000, Nir et

al., 2008). There are also discrepancies about the location of nociceptive processing such that some find pain and tactile activated areas in SI are the same (Coghill et al., 1994), while some have shown they are distinct (Ohara et al., 2004b).

The nociceptive neurons in SI have small receptive fields (Treede et al., 1999) and have been found by at least one author to be somatotopically arranged (Ogino et al., 2005), suggesting that SI codes for localization of the stimulus. This is further supported by lesion studies in monkeys demonstrating that ablation of SI causes a loss in localization ability (T.L., 1944), but these neurons are very sparsely populated, casting doubt on the significance of their function (Craig, 2003, Garcia-Larrea et al., 2003).

Furthermore, attention and cognitive state can significantly reduce SI's activation to painful stimuli (Bushnell et al., 1999). Allowing distraction to reduce the ability to localize tissue injury quickly seems like a large evolutionary cost, however. Finally, if localization was the function of SI, it ought to be activated in response to all painful stimuli, but imaging studies do not demonstrate this. Therefore, although SI has not been shown conclusively to code for localization, it is surely likely that either it shares this function with other cortices, or it has other significant functions in pain perception.

Interestingly, although the SI nociceptive neurons are not ideally suited to representing intensity because of their response properties discussed above, many studies indicate that they do. Ohara et al. (2004b) demonstrated that SI field potentials show graded response amplitudes to increasing stimulus intensity. Iannetti et al. (2005) and Nir et al. (2008) also found that SI may

code for stimulus intensity with EEG and standardized low-resolution brain electromagnetic tomography (sLORETA), respectively. Using magnetoencephalography (MEG), Timmerman et al. (2001) found that SI codes for intensity, and the amplitude of the SI response is linear with stimulus intensity and reported pain perception. Although this may imply that SI is also involved in representing the affective aspect of pain, it is probably because as stimulus intensity increases, pain generally does too.

There is also a lack of conclusive evidence about SII's role in nociception and pain perception, although anatomical studies provide evidence that SII plays a role in the somatosensory discriminative pathway. For example, WDR neurons of the lateral thalamic tract project to SII and are responsive to nociceptive stimuli (Frot et al., 1999), and NS neurons have been identified in SII of cats, rats, and monkeys (Nakahama, 1975, Frot et al., 2001), even though they appear to be rare (Sewards and Sewards, 2002). The rarity of these neurons was suggested by Treede et al. (2002), as a function of looking in the wrong place. Traditionally NS neurons were identified by locating tactile somatosensory neurons first, and then looking for nociceptive responsive neurons (Sewards and Sewards, 2002). Physiological and imaging studies also provide evidence of its role in nociceptive somatosensory discrimination. For example, stimulating SII can cause an unpleasant tingling and lesions may cause nociceptive detection deficits and reduction of pain (Bloedel and McCreery, 1975, Nakahama, 1975, Sewards and Sewards, 2002). Furthermore, unlike SI, SII is consistently activated in response to electric and heat stimuli in studies using MEG, EEG, PET and fMRI (Peyron et al., 2000, Apkarian et al., 2005). However, like SI its exact role,

i.e., if it codes for intensity, location or modality, is not clear.

The strongest evidence so far points to a role in coding for intensity (Coghill, 1999; Nakahama, 1975; Treede et al., 2005). Timmerman et al. (2001) found that SI coded for intensity of all somatosensory stimuli, whereas SII only coded for intensity above the pain threshold. This would make the role of the nociceptors in SII the recognition that a stimulus is nociceptive, and the coding for its intensity. Frot et al. (2007) also concluded that SII codes for intensity of noxious stimuli from sub-threshold to above threshold, but unlike Timmerman he found there was a ceiling effect once pain was experienced, maintaining the debated role of SII.

Several authors have suggested explanations as to why the role of the somatosensory cortices in nociception has been difficult to identify. Bushnell et al. (1999) and Bromm and Lorenz (1998) reviewed some of the reasons that separating SI's roles in tactile and nociceptive processing has been difficult. These include a difference in pyramidal cell orientation between the tactile and nociceptive SI homunculi, different cognitive states of subjects not accounted for in study designs, varying stimuli modalities, differences in statistical analyses, and not always achieving the significant spatial and temporal summation needed to activate SI neurons (Forss et al., 2005).

The same types of methodological problems also apply to the difficulty in identifying SII's role in nociception. Another possibility is the result of the SII and insular anatomy. In the primate, SII and the insula are contiguous and the volumes of the areas are highly variable, making it difficult for imaging tools to distinguish which region is being activated (Frot et al., 1999, Craig, 2003, Frot et al., 2007). The insula and SII nociceptive neurons

have slightly different properties. Although they both have increasing activation with increasing intensity, SII is more somatotopically arranged than is the insula (which may not be at all), and the insula is part of the limbic system, whereas SII is not. This suggests that their functions are slightly different. Therefore, the wrong inferences about function could be drawn if one area was mistaken for the other.

Though it is possible that methodological problems account for some of the problems in pinning down the somatosensory cortices' roles in nociceptive sensory discrimination, there are other possibilities more fundamentally related to brain function. Recently discovered is that, while there are separate homunculi for nociceptive and tactile processing in SI, they may overlap (Chen et al., 2008), and the nociceptive area may modify the response of the tactile area (Chen et al., 2009). In addition, it is possible that thermally responsive SI nociceptors occupy yet a separate area then do mechanical nociceptive neurons.

Perhaps one of the main reasons that roles in nociceptive somatosensory discrimination are hard to pin down is because nociception and pain perception are not functionally modular, but distributed (Coghill et al., 1999). That this is the case is supported by coactivation patterns of SI and SII that show that SI activation is not causal to SII in pain processing. In fact, contralateral SII and insula, part of the limbic system, appear to be the earliest areas activated following painful stimuli (Frot and Mauguiere, 2003, Garcia-Larrea et al., 2003). SI's activation is also modulated by a top down system (Coghill et al., 1999) which means its activation, and therefore functional significance, may be able to be changed according to past experience,

environment and stimulus. It is likely, then, that because individual studies ask specific questions aiming to assign one sensory discriminative role to a particular cortex, or area of a cortex, and that each study entails different circumstances, the results differ because the functional networks being activated for particular nociceptive discriminative tasks also differ.

Studying pain

Pain processing has been studied extensively using different modalities of stimuli such as capsasin, cold water, electrical stimulation, and laser stimuli. However, laser stimuli delivered by medical lasers have evolved as the stimulus of choice in pain-related EEG studies in humans. This is because a laser only excites nociceptors leaving other peripheral somatosensory receptors inactivated (Iannetti et al., 2004). The lasers have been high powered CO2 or neodymium-doped yttrium aluminum garnet; (Nd: YAG) or y*ttrium aluminum perovskite* (Nd: YAP) lasers that have the property of heating the skin rapidly causing excitation of the nociceptors in as little as 2ms, while not causing tissue damage. The stimuli are reported to feel like a pin prick pain, followed by a slow burning pain, mediated by A∂ and C fibers, respectively. Humans tolerate these stimuli very well, although they are reported to be unpleasant. Electrical stimuli have also been used but these are probably less useful for pain related studies for reasons given below.

Laser evoked potential (LEP)

The evoked response potential (ERP) resulting from a laser stimulus is known as the laser evoked potential (LEP) and has been named the N2P2. This robust component is maximal over the vertex, and consists of an initial negative component, the N2, followed by a positive component, or P2. The peak latencies of these components are about 180-230 ms for N2, and about 290-400 ms for the P2 (Arendt-Nielsen et al., 1999). Curiously, the LEP only reflects A∂ activation, but never C fiber activation, unless A∂ fibers have been blocked. In this case, a very late potential, with topology and morphology similar to the N2P2 appears at about 800 ms. Thus far, there is no satisfactory explanation for this. Electrical stimulation elicits the N140 ms, so called because its peak latency is 140 ms, and it too is maximal over the vertex. Because electrical stimuli also excite Aβ as well as the nociceptors, the morphology and temporal characteristics of the subsequent component are very different.

Although the N2P2 is a vertex component, sharing similar response characteristics to other somatosensory vertex components, it has some features that suggest that it is more specifically nociceptive than are ERPs like the N140 ms. Its magnitude is highly correlated with intensity of pain perception, rather than just stimulus intensity, and its magnitude continues to increase after pain threshold has been reached, unlike the N140 ms (Arendt-Nielsen, 1994). The N2P2 peak amplitude is also reduced by even mild analgesics (Bromm, 1985). This, combined with the fact that it results from unique excitation of nociceptors, makes the N2P2 useful for studying pain.

LEPs have been used in animals only to investigate the suitability of a

species as a biomedical model for human pain processing. LEPs have been found in rats and monkeys using intracranial electrodes. Monkeys have been found to be very suitable for more invasive pain-related studies because of the cortical homology and the similar morphology and topology of the LEP. However, conscious monkeys will not tolerate the laser stimuli (Baumgartner et al., 2006), so these studies have been done on anesthetized monkeys, limiting the usefulness of the monkey as a model for human pain perception. Rats do show an LEP but it is very dissimilar in behavior and morphology to the human LEP, even comprising a visible component corresponding to C fiber activation (Stienen et al., 2006).

Because most of the extant literature using animals as models has been intended to understand pain in humans, EEG research in non-human animals with the goal of studying the processing of pain in any particular species, has been very limited. No studies have used lasers in conjunction with EEG. Most have been done while the animal was anesthetized, and anesthetic significantly affects the power spectra of the EEG.

The susceptibility of horses to die under anesthesia has spurred one of the largest sets of studies in animals that have also included pain-related studies (Otto and Short, 1991, Ekstrom et al., 1993, Johnson et al., 1994, Johnson and Taylor, 1998, Johnson et al., 2003). Initial research was aimed at identifying anesthetic states of horses in order to prevent excessive anesthetic depth and subsequent death (Johnson et al., 1994). These studies evolved to investigate EEG response to painful surgical stimuli, such as castration. There have been consistent results suggesting that it might be possible to identify both anesthetic depth and pain associated with surgical stimulation using EEG frequency

measures (Otto et al., 1996, Murrell et al., 2003, Haga and Dolvik, 2005). However, because these studies were done in unconscious animals, their applicability to objectively identifying pain in conscious animals is limited.

A small number of studies aimed at identifying a neurocorrelate of pain in farm animals have been done. Some of these studies have also aimed to investigate the usefulness of somatosensory evoked potentials (SEP) to diagnose, ante-mortem, spongiform encephalopothies, such as bovine spongiform encephalopathy (BSE) (Strain et al., 1986a). One pain-related SEP study in sheep and one in cattle, aimed at studying nociception, demonstrated reproducible SEP using electrical stimulation (Strain et al., 1992, Ong et al., 1997a). SEP were not used, in the end, for BSE diagnosis, but visual evoked potentials were used in a cow to diagnose central blindness (Strain et al., 1897). Auditory evoked potentials were also done in cattle with the possibility that they could be used for diagnosing central nervous system (CNS) lesions or disease (Strain et al., 1989b). An ante-mortem diagnostic test using ERP was never developed.

Laser evoked potentials

The research leading to the understanding of how acute pain is processed, both peripherally and centrally, has largely used experimental manipulation with a small range of validated nociceptive stimuli. A review of the stimuli modalities, including capsasin, cold water, and hypertonic saline, and the activity patterns elicited by them, can be found in Garcia-

Larrea (2003) and, Arendt-Nielson and Chen (2003). The most commonly used stimuli by far, however, are electrical cutaneous shock, and laser heat stimuli. Both of these stimuli produce a robust vertex potential (Cz; at the crown of the head). However, they share similar characteristics to other sensory elicited vertex potentials (Arendt-Nielsen, 1994), suggesting that these potentials may not be specific to nociception. For example, they are a similar shape to potentials evoked by sudden somatosensory stimuli, they are maximal over the vertex, they are modulated by attention, and their amplitude decreases with repetitive stimulations (Arendt-Nielsen, 1994).

That said, there is reason to believe that laser evoked potentials (LEP) are more nociceptive-specific than electrically evoked potentials (SEP). Therefore, although LEPs may belong to the general class of vertex potentials, they may also be an appropriate neurocorrelate of nociception. The two potentials result from different receptor activation patterns, their vertex potentials have different morphologies, and psychophysical experiments using them have produced different results. Laser heat stimuli selectively activate the cutaneous nociceptors, A$\partial$and C, whereas electrical stimuli cause an unnaturally large, synchronous, volley resulting from activation of not only A∂, C, but also of Aβ mechanoreceptors, activated first because of their lower activation thresholds. In short, LEPs are the result of specific nociceptor activation. The result of a different pattern of receptor activation is a morphological and temporal difference between the two potentials.

The LEP, also known as the N2P2 shown in Figure II-1, has a negative component with mean peak latency 180-290 ms (N2), followed by

a larger positive component with a mean peak latency of 300-450 ms (P2) when stimulating the dorsum of the hand (Bromm et al., 1983, Bromm and Treede, 1984, 1987). These latencies may vary with the laboratory according to Arendt-Nielsen (1994), and if MEG is used, the latencies are shorter (Frot et al., 1999). The SEP, known as the N140, has a large negative component with peak latency of between 140 -190 ms (Treede et al., 1988). The peak amplitude of the N140 can also be as high as 21μV, whereas an LEP has peak amplitude in the range of 5-10μV. The short latency and large amplitude of the N140 is explained by the fast conductance speed of the Aβ fibers, and their synchronous activation (because of the nature of the electric stimulus), respectively. Laser heat stimuli do not synchronously activate fibers like electric stimuli do, which means afferent signals are more temporally distributed, reducing the amplitude of the resulting potential.

Response differences between the N140 and N2P2 across experimental conditions, and the correlation of their responses to psychophysical tests provides strong reason to believe that the N2P2 has at least some nociceptive specificity. The N2P2's amplitude increases with stimulus intensity, and, importantly, with the perceived pain level (Bromm et al., 1983, Arendt-Nielsen and Bjerring, 1988, Timmermann et al., 2001, Ohara et al.,2004c, Iannetti et al., 2005). This is in contrast to the behavior of other vertex potentials, including the N140, which increase linearly with stimulus intensity, but then plateau shortly after the pain threshold has been achieved (Arendt-Nielsen, 1994). The N2P2 amplitude is also reduced even in the presence of a mild analgesic such as aspirin (Bromm and Lorenz, 1998). Because the ACC has been shown to be one of the sources of the N2P2 by

Ohara et al. (2004b), it is possible that the N2P2 may also represent, at least in part, the perception of pain as well as the somatosensory discrimination. Of course, one component cannot completely represent a complex endogenous process such as pain perception, (Chen et al., 1979), and Iannetti et al. (2008) demonstrated that it can only be interpreted as an 'indirect' representation of nociception. However, the LEP remains a useful tool for indirectly studying pain perception, and has therefore become one of the most trusted ways to elicit pain-related vertex potentials and thus to study pain pathways (Cruccu et al., 2004, Iannetti et al., 2004, Garcia-Larrea, 2006, Cruccu et al., 2008, Perchet et al., 2008).

Changes in peak latency of the N2P2 have been less consistent. It has generally been found that increasing the intensity of the stimulus will decrease the peak latency of the complex (Arendt-Nielsen et al., 1999), but this is not correlated with perception.

By studying what cognitive processes affect pain-related evoked potential amplitudes and peak latencies, researchers are indirectly studying what cognitive states affect pain perception. Studies have shown that if subjects are distracted they report less pain (Nakajima and Imamura, 2000, Yamasaki et al., 2000), and the resulting SEP or LEP amplitudes are greatly reduced, regardless of intensity of stimulus (Ohara et al., 2004a). Anxiety and affective state also affect pain perception and amplitudes of the SEP and LEP, whereby both anxiety and negative affective state increase both subjective pain perception and amplitudes (Kenntner-Mabiala and Pauli, 2005, Warbrick et al., 2006). The inference from the above work suggests that attention, anxiety, and affective state are all important in the processing

and perception of pain.

The N2 and P2 are affected differentially by different types of attention (Lorenz and Garcia-Larrea, 2003). However, it is difficult to directly compare the literature because different experimental designs have been used by different researchers to answer questions specific to their research. Siedenberg and Treede (1996) used an oddball paradigm in two sets of experiments. The subjects had to push a button (experiment 1) when recognizing the target stimulus, or make a mental count of stimuli (experiment 2). Each experiment repeated the stimuli while reading distracted the subject. They found that in both experiments the amplitude of P2 (mean peak latency of 400 ms) was significantly larger ($P< 0.0001$) for targets than frequent stimuli and larger again for the frequent stimuli compared to the distracted condition. They also found the latencies of the mean peak amplitudes to significantly increase as the degree of distraction was reduced, suggesting that the active cognitive act of reading was interfering with the pain processing. Contrary to these findings, Kanda et al. (1996) found that the amplitude and latency of N2P2 remained the same for both target and non-target stimuli, although Kanda did not include a distraction condition. One important difference between the studies was the inter-stimulus interval (ISI). Kanda et al. (1996) used a random ISI of 3-7 seconds. Siedenberg and Treede (1996) used 10 seconds in experiment 1 and 6 seconds in experiment 2. Constant ISIs can increase expectation of the stimulus and therefore change the subject's attentional focus.

Later, Legrain et al. (2002) elegantly disentangled the affect of inter-modality attention and distraction. They found that only P2 (400ms) was

affected by stimulus probability and that N2 (230ms) was affected by spatial attention, meaning that it was affected by what part of the body was being attended to. Mouraux and Plaghki (2007) asked subjects to attend to the same stimulus modality, but to the two different pain sensations [sharp pin prick pain (A∂) followed by the slow burning C]. They found that attending the second pain increased the amplitude and peak latency of both N2 and P2. Thus, as Lorenz and Garcia-Larrea (2003) summarized in their review, the components of the LEP appear to be modulated differentially by different types of attention and cognitive states, e.g., non-specific vigilance, inter-modal attention, and intra-modal attention. A constant ISI can increase anticipation and attention to the stimulus, which would increase component amplitude.

Because of the latency of P2, and because it is affected by attention, the question arises if P2 and P3 [see Polich (2007) for a recent review of P3] might be functionally the same (Lorenz and Garcia-Larrea, 2003). P3, consisting of two components, P3a and P3b, are associated with updating information and memory, respectively, which are cognitive processes. P3 is also largely unaffected by stimulus intensity indicating that it is a result of purely internal cognitive processing. If P2 were functionally equivalent to P3, it would imply that P2 might represent a cognitive aspect of pain processing. Two of the main reasons given for the hypothesis that P2 and P3 may be functionally the same were that P2 can temporally overlap the P3 complex and it is sensitive to rare stimuli in oddball studies, as is P3. However, researchers who attempted to tease out the P2 from P3 components consistently found a separate P3-type wave associated with the LEP (Siedenberg and Treede,

1996, Kanda et al., 2002, Legrain et al., 2002, Perchet et al., 2008), meaning P2 is not a functional equivalent to P3. The LEP-related P3 was also distinguished from the N2P2 on the basis of scalp topography, which is one of the criteria for identifying ERP components (Handy, 2005).

Although there is clearly a separate P3 associated with the N2P2, it is possible that P2 does represent cognitive processing. Legrain et al. (2002) showed that late portions of the P2 responded to stimuli similarly to P3a. P3a is a subcomponent of P3 associated with orienting responses to new salient stimulus suggesting that P2 may represent an orienting response to the painful stimulus. Iannetti et al. (2008) found that P2 magnitude is greatly reduced when there is a temporal expectancy, meaning the saliency of the stimulus is an important modulating factor in P2. Given the results of Legrain et al. (2002) and Iannetti et al. (2008), it may be that P2 could represent some functional activity equivalent to P3a. However, whether or not the changes in P2 are an effect of P3a temporally overlapping P2, or actually indicate that P2 also represents an orienting response is so far undecided (Lorenz and Garcia-Larrea, 2003).

Although it is impossible for the N2P2 to represent the entire processing and cognitive appreciation of pain, it does correspond to the arrival of the A∂ fibers based on their conduction speed, and changes in its amplitude correspond to perceived intensity rather than stimulus intensity. However, there is no such potential seen under normal circumstances for the C fibers even though there is a reporting of the second pain (Bromm et al., 1983, Bromm and Lorenz, 1998). This is despite the fact that the population density of the C fibers in the skin is much larger than that of the A∂ receptors so it

might be expected that the size of the afferent impulses would be large. Bromm et al. (1983) demonstrated that, if the A∂ fibers were blocked, an ultra late potential at about 900 ms was seen and correlated with the conduction speed of C fibers, and this potential has the same morphology and topology of the A∂associated N2P2 (Magerl et al., 1999). When the A∂ fibers were blocked, the A∂-related N2P2 disappeared along with the perception of the first pinprick. This has been reproduced in every study that has been done aimed at understanding this unique phenomenon.

Reasons for this dissociation have been suggested. The early theories about pain, discussed above, held that there was a spinal interaction between the two afferent fibers such that the activation of A∂- transmission of C afferent volleys. However, if this is true, a second pain corresponding to the conduction time of the C fibers should not be able to be felt, and yet it is. Kobayashi (2009) produced further evidence that C fiber volleys are not suppressed by Aδ by showing that afferent inputs from both fibers were measurable in the thalamus at the latency corresponding to their conductance speeds. Thus, the interaction theory is no longer considered viable (Mouraux and Plaghki, 2006).

Another hypothesis is that the jitter of the ultra-late potential arising from C fiber properties and individual differences are so large that a potential cannot be visualized by averaging trials. This seemed like a good explanation as even though the C fiber receptors have a lower threshold than the A∂ receptors and are therefore more easily excited, they require more spatial and temporal summation than do the A∂ receptors, making the timing of their afferent volleys less synchronized. Bromm and Treede (1987) found that the

intra- and inter-individual peak latency standard deviation was 150 ms, which would cause significant jitter, and thus the peak could be 'smeared' when the epochs were averaged together. When they accounted for the jitter in subjects with an Aδ blockade, a clear ultra-late potential with topography and morphology of the A∂ N2P2 was seen. However, when accounting for jitter in subjects with no A∂ blockade, there was no ultra-late potential seen, demonstrating that jitter is not responsible for the absence of the C fiber ultra-late potential when A∂and C are simultaneously stimulated.

Because the N2P2 resulting from A∂ excitation is similar in morphology and topology to the ultra-late potential (Bragard et al., 1996, Opsommer et al., 2003, Mouraux et al., 2004) visualized when Aδ fibers are blocked, it is a possibility that they result from the same cortical generator (Bromm and Treede, 1987, Mouraux et al., 2004, Mouraux and Plaghki, 2006). Thus, it was proposed by several researchers that the reason the ultra-late potential was not visualized without A∂ suppression was that the generator was in a refractory period (Mouraux et al., 2004). By applying laser stimuli in quick succession Mouraux et al. (2004) were able to demonstrate that regardless of the inter-stimulus interval a clear N2P2 corresponding to A∂ conduction speed was visible. Therefore, the N2P2 generator must have a very short refractory period and this explanation was refuted. The reason for the behavior of the C fiber ultra-late potential remains unclear.

Laser studies in animals

There have been a few pain-related studies using laser heat stimuli in non-human animals, but they have been to verify that the animal is a suitable model for studying human nociception (Beydoun et al., 1997), as opposed to being for the knowledge about the species itself. Perhaps not surprisingly, the monkey pain system, including nociceptive fiber properties, and LEP source generators are similar enough to the human system that they are useful for more invasive anatomical and neurophysiological studies that might be directly translated to the human (Beydoun et al., 1997, Baumgartner et al., 2006). Ultra-late C fiber potentials can also be induced by CO_2 lasers in monkeys after A∂blockade, as they are in humans (Baumgartner et al., 2006), and C fiber thresholds and central coding for heat pain in monkeys is strikingly similar to those in the human (Tillman et al., 1995). Cats also appear to be good models for studying the neuroanatomy, structure and function of the pain system, because they correspond well to the human (Kalliomaki et al., 1993b).

In rats, nociceptive stimuli are processed differently than in humans and other studied animals. The nociceptive fibers in rats are in lamina II and V as opposed to I and V in other studied mammals, and the response properties of the C fibers are different in the different laminae (Schouenborg, 1984) in the rat. The processing of these stimuli in SI is done within the *same* column as other somatosensory stimuli, which is very different from other animals, where there are modality-specific areas in SI. The decoding of pain versus tactile stimuli then must rely on cortical laminar processing as tactile and

nociceptive fibers terminate in different laminae (Jaw et al., 2009). Research in the rat also suggests that it is C fibers that mediate reflexive, nocifensive behavior, such as lifting the leg and kicking. When blocked with capsasin, the nocifensive behaviors are extinguished, but what might be considered to be affective behavior, as opposed to reflexive (like kicking or turning the head toward the injured area), is preserved (Fan et al., 2009). Whether or not this is a primary role for C fibers in other animals was not discussed. What should not be and cannot be assumed is that the affective experience of pain in mice is less significant than other animals because the somatosensory portion of the nociceptive system is organized in a different way. It must be kept in mind that bird neuroanatomy is quite different to mammalian, and yet they have homologous cortical regions and abilities that match mammalian abilities, or surpass them.

Despite these dissimilarities between humans and rats, the medial pathway of the pain system, the region that codes for the affective aspect of pain, is very similar. Both humans and rats have a medial thalamic projection to the ACC (Hsu and Shyu, 1997), and the ACC is found to have similar response properties and contribute significantly to the pain experience in both species. The ACC in the rat has large non-somatotopically arranged nociceptive receptive fields (Hsu and Shyu, 1997, Shyu et al., 2003) that are not suited to sensory discriminative tasks, just as in humans. Though it is impossible to say that the ACC plays the same sensory awareness function in pain processing as in humans, and thus conclude that the experiences are the same, the EEG responses of the ACC to painful stimuli are the same in rats and humans. In humans, pain-related anxiety, or anticipation (as opposed to

general anxiety (Arntz and de Jong, 1993) is well known to increase the subjective perception of pain independently of the stimulus intensity (Warbrick et al., 2006). Wang et al. (2008) demonstrated that anticipation of pain in rats enhances the behavioral response and that this effect is mediated through the ACC. They demonstrated that in the anticipation condition the ACC is activated disproportionately to the other nociceptive-related cortical areas, which corresponds well to human data (Peyron et al., 2000).

The ACC in the rat also contributes to learning and attention to pain (Hsu and Shyu, 1997, Johansen et al., 2001, Kung et al., 2003) as it does in humans (Peyron et al., 2000). Damage to the ACC in rats that have been trained to associate a conditioned stimulus (CS) such as a tone, to an unconditioned laser stimulus (US) disrupts the response to the CS (Johansen et al., 2001). ACC lesions also prevent the pairing of the CS and the US. These results support the role of the human ACC in learning and memory in pain, but also indicate that the ACC in the rat could mediate the pain experience in the rat in a manner similar to that of humans.

The ERPs in response to noxious stimuli in the rat deserves some comment because of the interesting differences compared to the human. When electrical stimuli are used a large vertex potential, and a potential originating from SI are seen (van Oostrom et al., 2005, Stienen et al., 2006, van Oostrom et al., 2007). When fentanyl (a powerful opiate) is administered, the amplitude of the vertex potential is reduced, whereas the SEP from SI is not significantly changed (van Oostrom et al., 2007). In addition, fear behavior is reduced in a dose response fashion with fentanyl (Stienen et al., 2006). Fentanyl also has no effect on SEPs from non-noxious mechanical

stimuli recorded from either SI or the vertex suggesting that the vertex potential from painful stimuli is nociceptive-specific, as it is reduced in the presence of fentanyl. However, the potentials recorded in the above studies were at latencies of about 15ms, which is too fast to correspond to Aδ afferent impulses. This makes interpreting these results difficult.

When lasers are used, the results are also different compared to the human. The LEP in the rat has two components, one with a mean peak latency of about 66ms latency, and the second at about 319 ms (Kalliomaki et al., 1993a). (Kalliomaki, (1993a) also used mechanical stimuli and did not find peak latencies as short as those studied by Oostrom and Stienen (2007). These components are both positive and correspond to the conduction times of Aδ and C fibers, respectively, meaning that the ultra-late potential not visible in the human LEP without an Aδ block, is visible in the rat LEP. The components are differentially sensitive to morphine as well. In humans, the amplitude of the entire complex is reduced by analgesics, whereas in the rat, only the second component representing the arrival of the C fiber afferents are reduced (Kalliomaki et al., 1993a, Kalliomaki et al., 1998, Tsai et al., 2004). Kuo (2005) also recorded potentials directly from the ACC that had mean peak latencies of about 300ms and found that these potentials were also sensitive to morphine.

Mechanical ERP and LEP in rats respond differently to anesthetics, which would not be predicted, as anesthetics are cortical depressants. Under the effects of pentobarbital, LEPs were abolished but the ERP from mechanical stimuli were actually increased (Shaw et al., 2001). Regarding the LEP, its second component was the first to reappear, although in light stages

of anesthesia its polarity was reversed. The morphology and response of the LEP and SEP in rats is then very different to that in humans, so it is not appropriate to compare them to the human LEP, even though the rat is used as a model for pain.

EEG research in animals not for biomedical reasons

There are limited numbers of pain-related EEG studies with non-human animals as subjects outside of a biomedical context, i.e., designed for understanding the neural processes of a specific species rather than exploring its suitability as a model for a human process. Most have looked only at frequency changes, rather than evoked potentials, in response to a surgical stimulus, although a small portion has used electric stimuli to generate an SEP. One of the largest sets of applied EEG studies focuses on understanding the frequency responses to different anesthetic agents in horses, and to painful stimuli such as castration under anesthetics. While horses may seem a surprising species to be the focus of a relatively intensive study program, their susceptibility to die under anesthesia makes identifying a method of depth of anesthesia under surgery clinically important.

The EEG frequency parameters that are commonly measured in EEG studies of horses originated from human anesthetists' methods of assessing anesthetic depth. The most common measures originally employed represent the frequency at which a certain percentage of the power of the signal lies (the spectral edge). So an SF80 would measure the frequency at which 80% of the EEG spectral power lies (Tonner and Bein, 2006). Tonner and Bein (2006)

suggest that these parameters are not sufficient to monitor cortical activity, or depth of anesthesia, and that they miss even large changes in power shifts from high to low frequencies. In other words, the SF50, SF80 and SF95 are not likely to be affected significantly, or consistently, by a shift in power from alpha to theta (Tonner and Bein, 2006).

The effects of different anesthetic and analgesic agents on the EEG spectrum during anesthesia have been consistent. The studies, though done by different groups, used very similar drug protocols, as well as spectral measurements, and therefore direct comparison is possible. In many of these studies the mean peak latency of the auditory evoked potential was also measured (Johnson and Taylor, 1997, 1999, Johnson et al., 2003) with the assumption that increasing CNS depression would increase mean peak latency, but peak latency changes, unlike spectral shifts, were not consistent. All studies show that increasing anesthetic depth decreases spectral edge frequency (SEF; SF80 or SF95) demonstrating an increasing power of the lower frequency bands, but SF50 was not changed (Otto and Short, 1991, Johnson et al., 1994, Johnson, 1996, Johnson and Taylor, 1998, 1999, Johnson et al., 2000b, a). This may be because most of the power of the equine (and human) EEG resides within the lower frequency bands represented mainly by SF50, and so a redistribution of power within these lower frequencies would not affect the SF50. The SF80 or SF95 would encompass some of the higher frequency bands such as beta and gamma, and the power of beta was shown by Otto and Short (1991) to be significantly reduced during anesthesia. SF50 would not detect this decrease in power. One interesting point to note is that in one study by Johnson and Taylor (1999)

ketamine, the anesthetic under study, is a dissociative anesthetic, and therefore may not have been predicted to change SEF significantly as it did because it is not a strong central nervous system depressant. The addition of opiate analgesics and/or benzodiazepine anxiolytics further decreased SF95 below that of the maintenance anesthetic (Johnson and Taylor, 1997, Johnson et al., 2003). Conclusions drawn are that newer anesthetics have less effect, though still measurable, on SEF than do older agents, and that it may be possible to objectively correlate clinical signs of anesthetic depths in horses with EEG power spectrum analysis. However, this is not the same as identifying anesthetic depth objectively using these analyses.

Particularly relevant to this research, the research groups began to focus on identifying a unique frequency response to painful stimuli such as castration, under anesthetics. The results from these studies are not consistent. Surgical manipulations, other than castration, increased the SF50 and the SF80 (Otto et al., 1996) in both soft tissue and orthopedic surgery, whereas during arthroscopic surgery there was an increasing trend, not reaching statistical significance (Miller et al., 1995). Ekstrom et al. (1993) compared surgical (castration) stimulation to non-surgical controls under two different anesthetic conditions finding no significant change in SF50 or SF80 between surgical and non-surgical cases. When castration was used as the stimulus there was no significant change in any of the spectral measures (Murrell et al., 2003, Haga and Dolvik, 2005), but Murrell et al. (2003) did find that the *total* power increased during the castration. Some of the inconsistent results may be due to the use of different anesthetic protocols. For example, Haga and Dolvik (2005) used opiate and α2 agonists before

anesthetic induction, whereas Murrell et al. (2003) used acepromazine. In addition, the number of animals varied between studies, and the animals were clinical cases, meaning it is difficult to control for inter-individual variation between breeds, sizes, and ages of animals, and at least age has been shown by Johnson et al. (2005a) to affect spectral response.

Changes in these same spectral measurements to painful surgical stimulation have also been studied in larger animals such as sheep (Jongman et al., 2000, Johnson et al., 2005a, Johnson et al., 2005b) and deer (Johnson et al., 2005b). Jongman et al. (2000) castrated and tail docked conscious un-medicated lambs and found that the total power and SF95 increased during the surgery, whereas Johnson et al. (2005a) found that only SF50 increased, not the SF95 in anesthetized lambs during castration. The important difference between the two studies, and likely to explain the different result, is that Jongman et al. (2000) did not anesthetize the lambs and so they consciously experienced a surgical procedure. An intense stimulus such as surgery on a conscious subject almost certainly induces an alpha blockade (Berger, 1929) and other EEG changes such as a high gamma increase (Ohara et al., 2004b) explaining the SF95 increase in these subjects.

Evoked potential studies in animals

The arrival to the United States of Bovine Spongiform Encephalopathy (BSE), a disease that cannot be diagnosed pre-mortem, made studying evoked potentials in cattle potentially important. It may have been a method of diagnosing the disease and thus preventing the unnecessary sacrifice of healthy cattle and sheep suffering from the related spongiform

encephalopathy, Scrapie. Once normal potentials were characterized, somatosensory potentials may have been able to be used to test the patency of the CNS ante mortem. Auditory evoked potentials (AEP) were evoked in dairy cows and their morphology was found to be analogous to the human brainstem auditory evoked potentials (Strain et al., 1989b, Dondi et al., 2003). Visual evoked potentials (VEP) have also been recorded from cows (Strain et al., 1987, Strain et al., 1989a, Strain et al., 1990) and used by Strain et al. (1989a) to diagnose a steer with central blindness. Strain's research team also found that both the AEP and the VEP in neonates were within mature ranges for latency and amplitude and suggested that this maturity may be due to the advanced development of ruminants at birth. The use of SEP to diagnose BSE was never realized. SEPs in cattle, illustrated in Figure II-2, have also been recorded and the inter- individual variability in amplitude and latency were found to be similar to that of other species (Strain et al., 1992). The SEP in Figure II-2 were recorded using electrical stimulation applied to the ulnar nerve in the foreleg (top trace) and tibial nerve in the leg (lower trace).

SEP studies have also been done in dogs (Uzuka et al., 1997) and sheep (Morris et al., 1997). In anesthetized dogs electric shock stimuli were given laterally and bilaterally resulting in a reliable SEP that increased in amplitude, with no latency changes in the bilateral condition (Uzuka et al., 1997). Interestingly, even though it is not possible to currently compare human and animal potentials due to different relationships between skull and cortices, the dog SEP's morphology is closer to the human than the SEP from a sheep with stimulation of the same nerve. The SEP of dog and human

are shown in Figure II-3. However, it should be noted that Uzuka et al. (1997) do not specify a baseline for the dog SEP. SEP in sheep were also studied to find a marker of acute pain, and it was found that the amplitude of the SEP increased with intensity of stimulus (Morris et al., 1997). Morris et al. (1997) never mentioned if the stimulus was time locked to the EEG machine, if there was an electro-occulogram used for future removal of blink artifact, or if individual thresholds were decided before the SEP experiment. In Figure II-3, the morphology is similar but reversed, and that is likely because in human cognitive science convention reverses the polarities of the y- axis.

EEG placement of electrodes in large animals

The placement of electrodes on large animals has not been consistent. Some researchers have managed to get as many as 18 electrodes on a horse's forehead (Williams et al., 2008). In humans, an electrode arrangement, or montage, called the international 10-20 is traditionally used in EEG studies (Niedermeyer and Lopes da Silva, 2005). In the international 10-20 systems, first letters stand for location, e.g., central © or parietal (P). The second, smaller case letter, z, stands for a location on the midline. Therefore, electrode Cz would be central and midline. This electrode nomenclature has been used in large animal EEG studies, but little explanation is given for this rationale. The cranial bones of the larger animals do not share the same relationship to the brain and to each other as they do in primates (Lakshminarasimhan, 1975a). Thus, the usefulness of naming these

electrodes in a homologous manner may be questioned.

Lakshminarasimhan (1975) mapped the bovine cortex, and published a description of sulci and gyri aimed at defining functional areas homologous with primates' cortical areas. This proved very difficult but the following conclusions were reached. The sulci tend to be sagittally, instead of coronally oriented, as they are in humans. The coronal sulcus is functionally equivalent to the central sulcus in humans but in the human, presumably to increased encephalization, it has moved much more caudally. There is a much smaller prefrontal cortex in a bovine compared to the human and the bovine insula is exposed laterally rather than shielded by the parietal operculum. The auditory cortex is also very large in comparison to the human encompassing a large part of the sylvian gyrus and fissure. The occipital cortex, as well as the association cortices is much smaller. Figure II-4 (a sagittal section of the skull of a cow) illustrates where the gyri and sulci are, and what their assumed functions are, but it is not yet a precise science in the bovine. Figure II-4 is an extrapolation compiled from three different sources (University, Lakshminarasimhan, 1975a, Strain 2007) and is only a suggestion.

Due to the large sinus cavities in large animals, electrodes can be placed as can be seen below on a very limited area. The area of the skull that looks like it would be best suited for electrode placement due to the proximity of the brain to the surface of the skull appears to be mostly auditory cortex. However, because of how little is known it is presently only of interest, rather than actual significance in interpreting the results.

Conclusion

EEG in humans has been immensely successful at illuminating the cortical and cognitive processing in the human cortex. The ethical importance, and scientific complexity of pain processing, has made it a focus for many researchers. Some progress towards understanding the pain pathway, its relationship with nociception and somatosensory perception has been made in humans using lasers and EEG. Although the experience of pain is not likely to be represented solely by a cortical potential as temporarily brief as 300 ms, study of the laser-evoked potential has yielded much information about pain processing. Some studies in non-human animals have been done using this methodology, but rarely for species-specific interest, and very rarely in large animals. Unfortunately, previous large animal research has not been standardized regarding preparation and electrode placement, making extrapolating from collective studies difficult. However, the research has suggested that acquiring potentials from large animals is possible. The research presented in this dissertation aims to further basic knowledge of bovine cortical processing, and begins to search for an objective neurophysiological marker of pain.

Figures for Chapter II

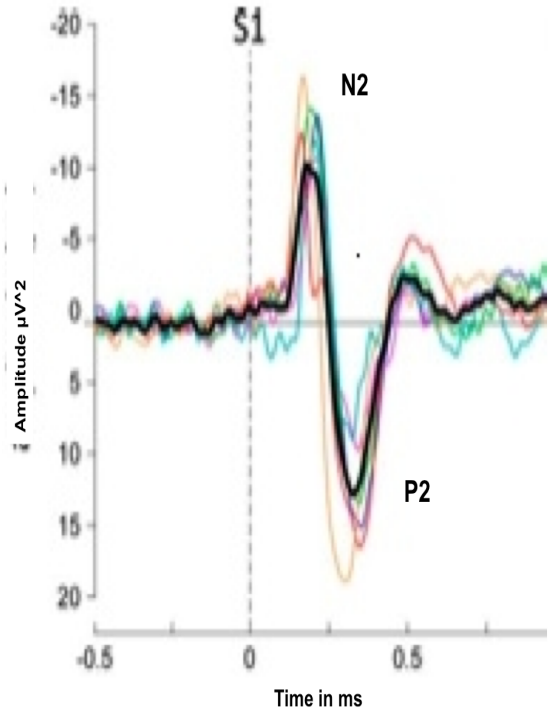

Figure II-1. A grand average of human N2P2/ laser evoked potential (black line) from Ohara (2004)

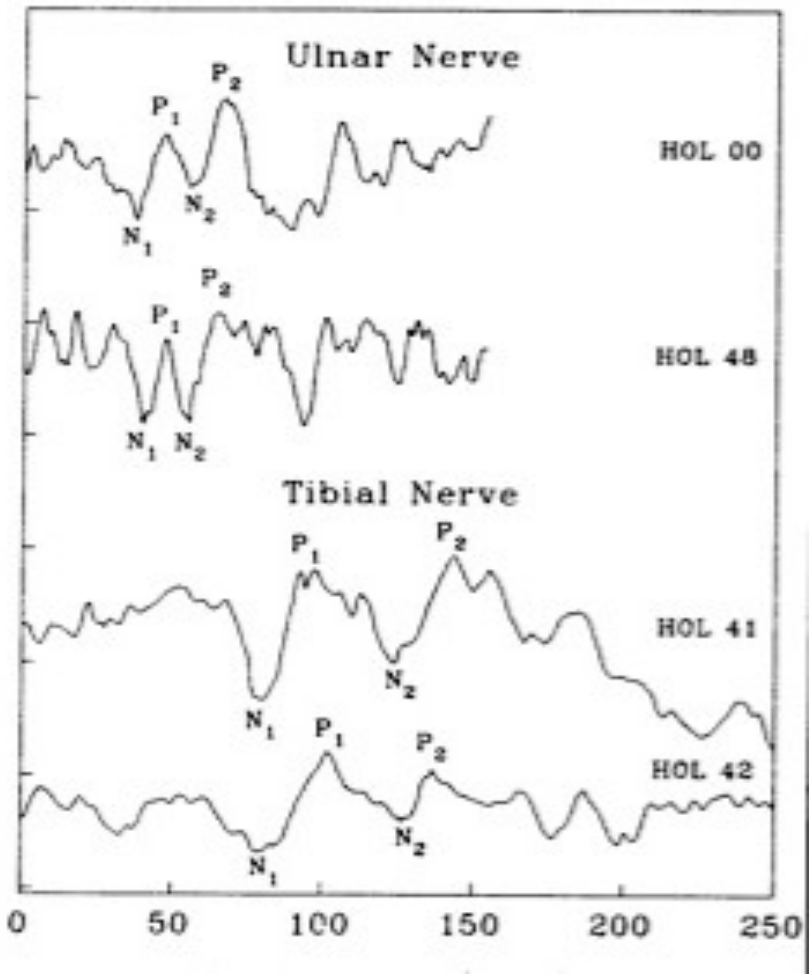

Figure II-2 Somatosensory potential from dairy cows using electrical stimulation on ulnar and tibial nerves. (Strain et al. (2002))

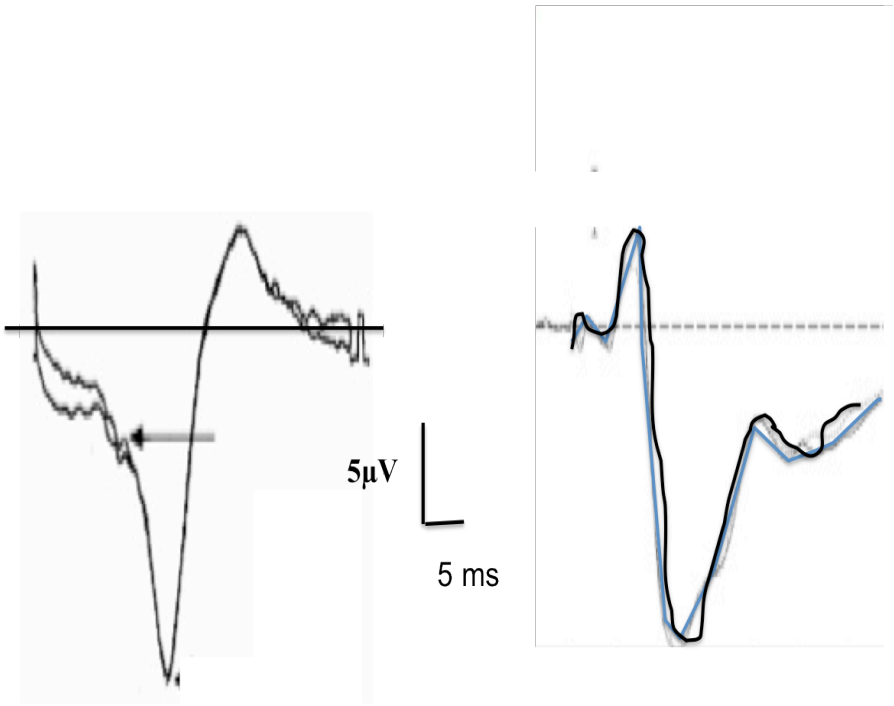

Figure II-3 Grand averages over all subjects somatosensory potentials (SEP) generated by electric stimuli on tibial nerve of 1) canine SEP on left and 2) human SEP on right. Notice that they share similar morphology. The polarities in the canine SEP figure are reversed as compared to the human SEP because it is convention in human cognitive science to reverse positive/negative on the y-axis. From Usaka et al.(1997), canine, and Warbrick (2007), human. Human SEP on left, canine SEP on right.

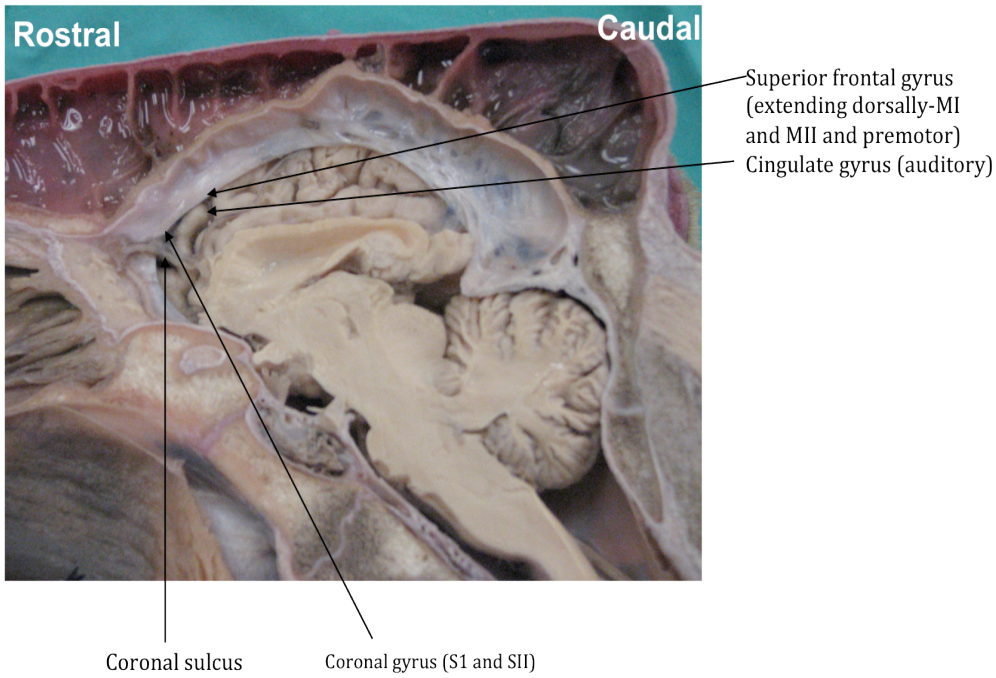

Figure II-4. Diagram of bovine *brain in situ* (medial/sagittal section). Photo by Strain (Strain, 2007). Location of sulci and gyri are extrapolated from cortical map (Lakshminarasimhan, 1975), and ovine brain maps from University of Michigan Brain Biodiversity Bank.

Chapter III: A Proposed Methodology for Standardizing the EEG Recording and Processing in Cattle (Bos taurus)

Abstract

There are limited EEG-based publications related to cognitive processes of cattle. Additionally, unlike EEG recorded on human subjects, the data from cattle that have been reported are not based on a common method of placing and identifying electrodes. Compared to the human skull, the bovine skull is much thicker and larger. Also, the brain is smaller. Accordingly, the areas of the bovine skull that are likely to provide the most useful information lie directly between and slightly above the eyes. We propose using this area and labeling sections using the geometric quadrant method of right to left and top to bottom with Roman numerals (I, II and III, IV, respectively). This results in odd numbering of the right side of the head and even numbers for the left, which is consistent with the system used by human researchers. We also report methods of preparing the animal, attaching the electrodes, and signal processing adaptations that we have found to facilitate the recording and processing of EEG in cattle.

Introduction

Electroencephalography (EEG) has been used clinically and for cognitive research in humans following Hans Berger's discovery that the

electrical potential from the brain could be detected on the human scalp (Berger, 1929). Since then, researchers studying human EEG have developed and standardized the methodology for all stages of EEG collection, from preparation of the subject, choice of electrode and electrode placement, to signal processing. Therefore, human EEG data results are reliably comparable across similar research reports and topics.

A standard methodology has not been developed in the study of EEG for large animals, in particular for cattle, although its use in cattle is increasing for clinical and research purposes. The purpose of this paper is to give (1) a brief overview of the standardizations in human EEG collection and signal processing, (2) a proposed process of adapting the human methods to bovine electroencephalography, (3) a discussion of how recording and interpreting the bovine and human EEG deviate, and (4) to share some experiences encountered in association with the development of our proposed standard methodology.

Human EEG collection and signal processing

Scalp preparation and electrodes

The standardized method for scalp preparation for EEG collection in humans has been developed to ensure adequate electrical conduction between scalp and electrodes required to acquire good data. The electrode sites are scrubbed with a standard cleansing scrub, e.g., NuPrep ® (Weaver and Co., Aurora, CO) prior to scrubbing the site with alcohol. An electrically conductive gel creates an electrical contact between scalp and electrode with a goal of achieving impedance less than 5KOhms (Picton et al., 2000, Niedermeyer and Lopes da Silva, 2005). Silver-Silver Chloride (AgAgCl) electrodes are most

commonly used because of their properties, such as low resistance. They are also the best electrodes for recording slow potentials. (For additional information on properties of electrode material, electrode placements and naming of electrodes see Niedermeyer et al., 2005.)

The standardized electrode placement in human EEG studies is known as the International 10-20 montage (Niedermeyer and Lopes da Silva, 2005). The 10-20 term has to do with the electrode placement in relation to anatomical markers on the skull. A full description of the montage and its nomenclature can be found in *Electroencephalography* (Niedermeyer and da Silva, 2005). This montage contains 21 or more electrodes, usually sewn into a standardized EEG skullcap to ensure that electrode placement relative to cranial anatomical features remains constant across subjects. Electro-occulogram (EOG) electrodes, placed above and below the eyes, are used to identify blink artifacts. EOG allows for an objective method of distinguishing between measured cortical potential and the artifacts caused by blinking.

The close spatial relationship of the human skull relative to the brain allows electrical recording from almost the entire surface of the cortex. Therefore, up to 200 electrodes can be used in recording EEG from humans. This affords the collection of large quantities of data, which allows for the analysis of cortical interactions during task- related experiments. For example, it is possible to measure the frequency changes in right and left motor cortex in preparation for, and prior to, muscle movement (Palva et al., 2005b)

The electrodes in the human 10-20 montage are named Tn, Fn, Pn, and On where the capital letter represents a specific cortex under the electrode. For example, electrodes with a T prefix would lie over the temporal lobe. The letter

n identifies a given position and represents left (odd numbers), and right (even numbers) hemispheres. While it is true that the electrical potential recorded from any electrode represents the spatial summation of signals over the entire cortex, there is an identifiable topological pattern of frequencies that remains consistent in a resting state, or non-task oriented, across subjects (Buckner et al., 2008). For example the alpha frequency is measured most strongly over the occipital cortex. The standardized montage allows researchers to associate data derived from particular electrodes to specific brain regions and activities. The large number of standardly placed and named electrodes has also allowed signal source algorithms to be developed enabling researchers to locate the cortical generator associated with the measured EEG feature.

Recording and signal parameters

Recording parameters, such as sampling frequency and filter bandwidth, in human cognitive studies using EEG, are often the same in studies with common aims. The signal is typically sampled at 256 Hz. Filter type and parameters (band pass, corner frequency, roll off gradient) during collection vary, but a common band pass range is 0.1-100 Hz. Filter parameters for offline filtering are chosen depending on the experiment. For example, the laser-evoked potential (LEP) in humans has theta as its dominant frequency. This means the low pass filters can be set as low as 8 Hz, excluding signal frequencies that might overwhelm the potential. Data are visually inspected and data with blink artifacts, confirmed by comparison to the EOG, and data with voltage excursions +/- 100μV from the baseline are then discarded. Independent component analysis (ICA) can also used to mitigate blink artifacts allowing the preservation of otherwise discarded data. ICA is related to principal component

analysis (PCA) that statistically identifies main sources of variance in the signal without bias because it is purely data driven. For more information on PCA and ICA uses in EEG signal processing see Keyser and Tenke, (2003)

Adaptations of human EEG methods to the study of large animals

The aim of this paper was to develop a method that could be widely used without specialized EEG equipment that allows future cognitive studies on cattle to be comparable across different labs. The areas considered important to standardize were animal restraint, scalp preparation, electrode placement and identification, and initial signal processing techniques.

Restraint

Because the cortical response to the somatosensory stimuli could confound the recorded results, ideally the animal subject would not experience any extraneous somatosensory stimuli, i.e., being touched or held, during the EEG. Allowing cattle freedom of movement within a stall, as Williams (2008) did with horses is preferred, but if event related data are being recorded, as opposed to continuous EEG recording, the animal typically needs to be standing in one place instead of moving around a stall. As much cognitive research involves recording event related data, the animal typically needs to be standing in an enclosed area such as a cow tie stall. In our study, the stall was constructed from metal piping about three quarters the length of the cow and

twice as wide, allowing her some lateral freedom of movement, ability to lie down and drink, but also allows her to be tied loosely to the front of the stall. A stall is suggested instead of full body restraint, such as a crush (also referred to as a chute), which would provide a constant somatosensory stimulus. Occasionally the head will need to be restrained to stop electrodes being pulled loose, but this only need be momentary.

Scalp preparation, affixing electrodes and signal acquisition system choice

We initially assumed that bare skin would be necessary. We used a thyoglycolate-based depilating cream (Nair®; Church & Dwight Co., N.J) to remove hair from electrode sites. We found that this allowed the electrodes to move too easily, introducing noise to the recording. We then decided that hair should be clipped closely, not removed entirely. An effective cleaning protocol was developed based on the preparation of the human scalp. Electrode sites were cleaned with liquid hand soap on a cotton pad, thoroughly rinsed and dried, and then cleaned with NuPrep® (Kappa Medical LLC, Prescott, AZ). Finally, electrode sites were scrubbed with rubbing alcohol. This removed an adequate amount of grease and hair dirt allowing good electrical conduction between scalp and electrode.

Several electrode pastes were tried. The slightly adhesive Ten20® (Kappa Medical LLC, Prescott, AZ) paste was used unsuccessfully for our needs because it allowed the electrode to slip from the head. Williams et al. (2008) and Strain et al. (1992) used Collodion® (Fisher Scientific, CA) that has

been used in human sleep studies. However, based on overt behavioral responses, the cows apparently found the smell of collodion aversive. Also, it never held the electrodes in place through head shaking. Elefix® (Nihon Kohden Int., Japan) is a non-toxic, adhesive paste used in sleep and infant studies. We found it held the electrodes firmly in place, and was slow to melt from the cow's body temperature. Elefix was therefore determined to be the best paste for our use.

Initially 5 mm AgAgCl post electrodes (Biopac, CA) were unsuccessfully used. The problem encountered was lack of electrical contact between scalp and electrode causing inconsistent signal recording. We chose Grass® AgAgCl cup electrodes with 30 mm 10 mm cup electrodes (Grass® Technologies) in conjunction with Stens Biofeedback (Stens Biofeedback Inc., CA) cables with Elefix®. This afforded successful signal acquisition.

The finalized methodology for electrode attachment is as follows. A small (pea sized) amount of Elefix® was placed on each electrode site and allowed to reach body temperature. A similar sized amount was pushed into the cup of the electrode. The cup electrode was then placed on the head firmly until Elefix® was visible around the edges of the electrode and was visible at the top of the cup electrode. We found this electrode application method to be expedient and simple, allowing rapid preparation of the cow with minimal hand movement around the cow's face, which causes signs of agitation in some cows.

EEG acquisition device

Four different EEG acquisition systems were tried, but we found the portable Bluetooth EEG biofeedback machine from Stens Biofeedback to be the best. It was chosen for its price, portability, and that it did not require the acquisition device to be physically connected to the computer. The Nexxus 10® (Stens Biofeedback, CA) was chosen because of its size, portability, price, and superior signal acquisition and signal processing capabilities. It has ten recording channels, four of which can be used for EEG, and six of which can be used for physiological measurements.

Electrode placement and nomenclature

Placement of electrodes

In comparison to human EEG studies, the number of electrode sites available is reduced in large animals because of the extensive cranial sinuses and bone in the dorsal skull. The extent of this limited area is illustrated in Figure III-1. For this reason, a limited number of electrodes can be used. In order to record over both hemispheres, and both ventrally and dorsally, four electrodes evenly spaced were suggested. This number is in sharp contrast to the possible number of electrodes used for EEG in humans.

As we were not able to locate a bovine cortical map illustrating cortex/skull relationships, the cortices over which each electrode lay could not be clearly defined. The Nexus 10® acquisition device has the capability for four EEG channels. We decided initially to capture as much data as the four channels would allow. Accordingly, we decided to split the recording area into

quarters. This means that there is cortical activity recorded from (II) dorsal right hemisphere, (I) dorsal left hemisphere, (IV) ventral right hemisphere and (III) ventral left hemisphere (see Figure III-1).

An electrode position allowing the strongest signal with the fewest artifacts from movement, as well as ease of placement, was established for each of the four electrodes. The final position of the electrodes we decided on is illustrated in Figure III-2 and Figure III-3 and is described as follows. A line between the medial canthi is drawn (intercanthal line; IC) and the distance from the midline of the IC to 2.5 cm laterally either side of the midline was marked. Lines from these lateral marks were followed 3 cm dorsally (bilaterally) and the ventral electrodes were placed at these points. The dorsal electrodes were placed 15cm on the same parallel lines from the IC.

The linked ear reference electrodes were placed on the distal nose, and the ground electrode was placed just caudal to the poll. The position of the ground ideally should be electrically neutral, and therefore not over muscle etc. This position is free from muscle tissue, even though there are tendon attachments onto the vertebrae. In addition, because of the large boney protuberance (poll), there is little chance that cortical potentials will be recorded.

Use of EOG

EOG recordings are used in human EEG recordings in order to identify blink artifacts and have been used by Williams et al. (2008) in the study of horses. However, we found that the EOG electrodes placed dorsally and rostrally about the eye did not stay in place, causing artifacts from electrode movement, and made identification of blinks versus other artifacts difficult. In

addition, the data appeared redundant, and electrodes around the eye also appeared to irritate the cow as indicated by increased head movement, causing additional movement artifacts. We therefore determined that using an EOG was not useful in meeting our objective, particularly as it required using one of the available EEG channels. Theoretically, it should have been possible to use the ventral recording electrodes as the EOG in addition to recording electrodes, because of their proximity to the large ocular muscles. This possibility was analyzed using ICA, but no ICA components reliably identified blink artifacts. Although blinking cannot be lateralized, occasionally the EEG and EOG signal only showed a blink occurring on one side or the other. The reason for this is unclear, but it is possible this is due to the corneal/retinal potential that can affect polarity of the signal when there are horizontal eye movements (Oster and Stern, 1980).

Naming the electrodes

Importance of anatomy in choice of name and electrode montage

Electrode nomenclature in human EEG studies is based on knowledge of over which cortex the electrodes lie. The human skull and its anatomical relationship to particular cortices are well known in humans, but this is not the case in large animals. The large animal skull is extremely different than a human's, and the encephalization of the frontal lobe in humans has pushed the coronal sulcus caudally (Lakshminarasimhan, 1975b). This changes the orientation of the brain in the skull, even if the human and large animal skull were comparable. Electrodes cannot be assumed to lie over the same cortices as they do in humans, which implies that electrode names need to be selected

independently of what appears to be analogous, although superficial, locations.

Previously, some authors have used between four (Strain et al., 1992) and nineteen electrodes (Williams et al., 2008) in large animal EEG studies (Strain et al., 1992, Williams et al., 2008). Others have used as few as one recording electrode (Jongman et al., 2000). In some cases these investigators of large animals have used human EEG nomenclature to identify electrodes. For example, the most ventral electrodes have been identified as frontal (F), and electrodes either on the poll, as the ground, or most dorsally have been identified as occipital (O). As the cortices/skull relationship has not been established, we developed a nomenclature that is independent of underlying cortical structure. This allows data interpretation free of assumption of underlying cortical structures. There are two channels available for recording EEG on the Nexxus 10®, AB and CD, each with two electrodes per channel. AB channel (with two electrodes) was placed dorsally. The manufacturer labeled each individual electrode 1 or 2. In accordance with human standards, electrodes labeled with odd numbers were placed on the right hemisphere, and even numbered electrodes were placed on the left hemisphere. Rather than using the letters to identify channels we decided to use Roman numerals. This resulted in the two electrodes constituting channel AB labeled I and II, and placed dorsally. Ventral electrodes, stemming from cable CD, were labeled III and IV and were placed ventrally as described above. This system follows the human convention of using odd numbered electrodes over the right hemisphere, and even numbered electrodes over the left hemisphere. In conclusion, electrode I records the dorsal left hemisphere, electrode II records the dorsal right hemisphere, electrode III records the ventral left hemisphere, and electrode

IV records the ventral right hemisphere of the cortex. A linked ear reference system was used and was placed below the noseband of the halter to avoid the halter interfering with the electrode contact, although head shape variation occasionally required the recording electrodes to be above the noseband. This small difference is unimportant for the reference function as long as there was no cortical activity to be recorded. We placed the ground electrode just dorsal to the poll on the neck. The anatomy in this area supports minimal muscle, and largely the large, electrically inert, nuchal ligament.

Signal processing

Initially an attempt was made to process data according to human data processing standards. EEG was recorded at 256 Hz sampling rate and band pass filtered on line between 0.1-40 Hz. Epochs were then band pass filtered within a range relevant to subsequent analysis. For example, if changes only in the lower frequencies (delta, theta) were of interest, the low pass filter was set at 10 Hz. The data were then visually inspected. Epochs with excursions +/- 100μV about the baseline or containing blink artifacts were discarded. Power spectra were estimated for each frequency band using a multi-tapering method to achieve a frequency resolution of 0.25 Hz. The spectra had high variance with a range of 100 μV2 to 2,636 μV2. This was considered to be an unacceptable variation, possibly indicating that there was too much noise in the data.

A commonly occurring artifact in our dataset not commonly discussed in human literature was determined to be an eye roll and is illustrated in Figure III-4. Eye role data were visually identified and discarded, and the power

spectra were re-estimated with the same frequency resolution. The range of spectral variance was reduced dramatically with a new range of between 158.48 μV2 and a maximum of 687.2 μV2. However, the variance of the delta frequency band (mean of 223.8 μV2) was still high in comparison to the human delta power frequency band (around 40 μV2) in the waking condition. Therefore, in order to further reduce the variance caused by artifact, the data were reprocessed but wavelet denoised, after band pass filtering using biorthogonal 6.8 level 4, [bior6.8; for more information on wavelet choice and scale see Compo and Torrance (1998), and Mallat, (1992)] with soft-thresholding using standard deviation of coefficients at individual levels. Level 4 was chosen because levels one to four of the bior6.8 approximate each frequency band of interest. This can be determined in Matlab®, or by establishing the Fourier periods of interest in the signal. This reduced the range of the spectral variance further to 84.7 μV^2 from 479.3 μV^2. Wavelet denoising appears to be useful in processing bovine data and has been highly recommended in the processing of human-event related data (Hu et al., 2011).

Verification of acquisition of neurological data

The large animal has only a small cranial surface area that is relatively close to the brain due to large cranial sinuses. In addition, a thick brain case and an undetermined directional orientation of the brain create doubt that EEG might be recorded at all. It was therefore considered important to verify that neurological data were being recorded as a way of validating the proposed standardized EEG methodology. EEG was recorded in the dark and then in the

presence of a spot light. This was aimed at establishing the change in spectral baseline conditions requiring varying levels of primary visual processing. The results of this experiment are given in Chapter IV. Data from the research detailed in Chapter IV demonstrated that this technique allowed the recording of bovine cortical potentials.

Discussion

Previous large animal EEG studies

Literature on EEG in large animals is scarce, and mostly directed towards equine EEG. EEG have been recorded in sheep (Bell and Itabisashi, 1973, Ong et al., 1997a, b), horses (Ekstrom et al., 1993, Johnson et al., 1994, Otto et al., 1996, Johnson and Taylor, 1998, Murrell et al., 2003, Haga and Dolvik, 2005, Johnson, 2006, Williams et al., 2008), deer (Johnson et al., 2005b), and cattle (Bell and Itabisashi, 1973, Strain et al., 1986b, Strain et al., 1989a, Strain et al., 1992, Bergamasco et al., 2011). EEG has been used to identify effects of varying anesthetic agents on horses (Johnson, 1996, Johnson et al., 2000b, 2003), and to determine the depth of anesthetic in this species (Johnson et al., 1993). EEG has also been used with an aim of identifying parameters specific to nociception caused by castration in the anesthetized horse (Murrell et al., 2003). EEG has been used to study the different stages of sleep in horses (Williams et al., 2008). Johnson et al. (2005) examined the benefits of various anesthetics in the use of antler removal in anesthetized deer using EEG parameters (Johnson et al., 2005b). EEG has been used clinically in cattle to

identify central blindness of a steer with a brain abscess (Strain et al., 1987), and to study narcolepsy in a bull (Strain et al., 1984). EEG has also been used clinically to investigate a suspected case of listeria (Strain et al., 1990). EEG has been used less frequently in cognitive, rather than clinical, studies in ruminants. Ong et al., (1999 a, b) used frequency analysis to examine the effects of somatosensory pain in sheep and showed that there was a significant difference in the frequency spectra between the increasing levels of electrical shock stimuli. However, these results were contradictory. Strain et al. (1986, 1980, & 1992) used EEG on conscious cattle to detect event related potentials resulting from electrical stimulation of the legs, but made no inferences regarding the use of these potentials for inferring nociceptive or pain perception. Bell and Itabisashi (1973) studied the spectra of sheep and goat EEG during and around cudding with the purpose of identifying EEG characteristics predicting the onset of rumination. More recently, Bergamasco (2011) studied the effects of analgesia on the EEG of calves if administered before castration and found that there was significant suppression of alpha immediately post-stimulus. None of these studies were directed at identifying an ERP, which has been an important methodology in the study of pain and other cognition in humans. Chapter V presents my investigation of an evoked laser potential in cattle.

Previously used restraint and electrode type

A standardized method of restraint, electrode type, scalp preparation and electrode placement have not been previously proposed. Previous reports

include techniques that have varied with the author, the aims of the research and the species. Restraint is important in the interpretation of the EEG because if there is physical restraint there will be a cortical response to the somatosensory stimuli in addition to a response to any experimental stimuli. Strain et al. (1980,1986 & 1992) restrained cattle on a tilt table as opposed to chemical restraint or anesthetic, although other authors have allowed the animal free movement in a stall (Williams et al., 2008). We propose that restraint to be as minimal to the extent possible. Electrode type, including substrate, indwelling, surface or subcutaneous, has varied. Ong et al. (1997, a, b) and Bell and Itabiashi (1973) used surgically implanted electrodes. Strain et al. (1980, 1986 & 1992) used subcutaneous electrodes, and Williams et al. (2008) used surface cup electrodes. Most authors have used AgAgCl electrodes, but Williams et al. (2008) used Au electrodes. We propose standardly using AgAgCl cup electrodes, unless there are compelling reasons dictated by study aims to use alternative substrates.

Use of species-specific bandwidths for future studies

In humans there is a large interindividual variability in the normatively defined rectangular frequency bandwidths (Takatsuki, 2005, Tenke and Kayser, 2005), and it has been suggested by Keyser and Tenke (2003) that these may not be appropriate for use in all cognitive studies measuring frequency as a dependent variable. In addition to, the problems associated with using human normative bandwidths in cattle, species-specific bandwidth ranges (Kuhlman, 1978) have been identified. Each species has different sensory acuities, or even different senses, and because frequencies

relate to underlying cortical processes, it is reasonable to believe that there may be species-specific frequency bandwidths. It will be important to define appropriate frequency bandwidths in the bovine EEG if future cognitive studies are to be done. One possible way of doing this would be to use PCA as Keyser and Tenke (2003) suggested doing with human frequency bands.

As discussed in the following Chapter, principal components analysis (PCA) on real data yielded negative components, which do not have biological meaning in the frequency domain. It is possible that the PCA solution in this case was not stable. Because of the small amount of data used in bovine EEG, PCA might not be the most appropriate method for identifying peak functional frequencies. Non-negative matrix decomposition may be an appropriate alternative method because it does not allow negative components. It is strongly recommended that this methodology be pursued.

Conclusion

The collection of EEG data is expensive and very time intensive. We propose a method to standardize the collection and processing of bovine EEG data, but it could be useful in other large animal species such as horses and sheep. Neuro-scientists using EEG in the study of humans have established standards for positioning and naming of electrodes and other aspects of data collection and analyses. Investigators using EEG of large animals have yet to establish similar standards, which of course makes it difficult to make comparisons across publications. Thus, we have proposed a standard method to be used in labeling electrodes. Hopefully it will achieve the desired ends, while

standing independently from human protocol where it might confound interpretation when researching EEG in cattle. Additionally, we have described procedures that we have found to facilitate the attachment of electrodes and the recording of EEG. Ultimately, we contend that it would be beneficial for researchers to follow an established and agreed upon method of recording EEG data in cattle. Finally, we suggest that it could ultimately be beneficial for researchers of a given species to share EEG data, after publication, in a common database available to others for comparison and further analyses.

Figures for chapter III

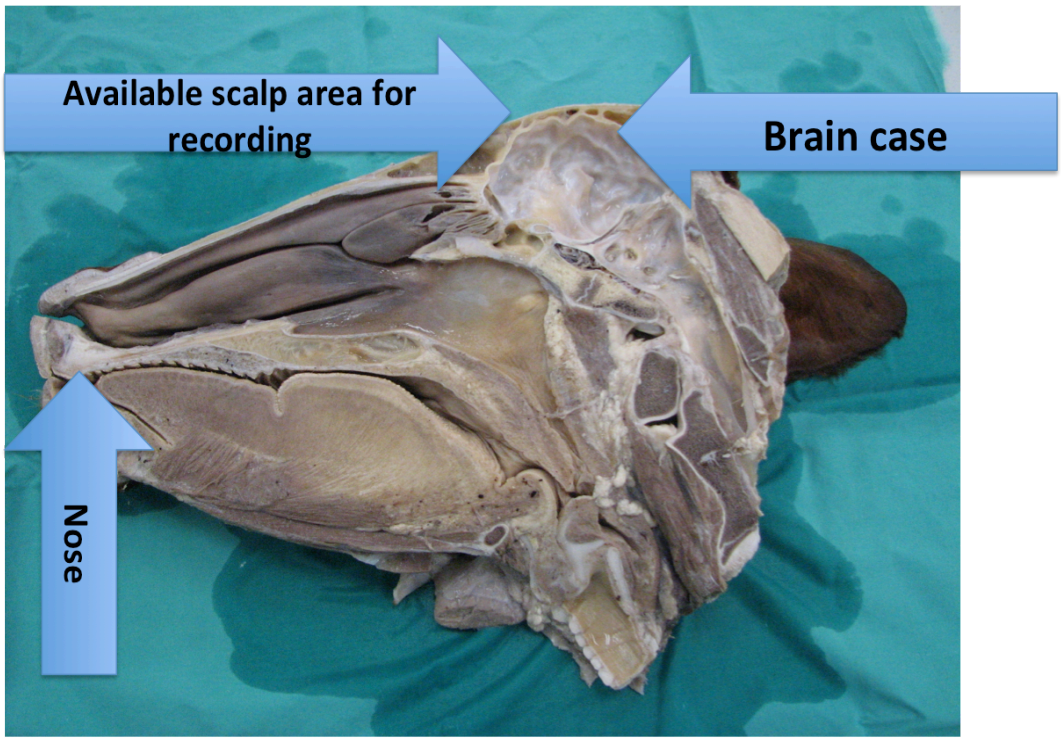

Figure III-1 Sagittal section of bovine skull demonstrating limited recording area on bovine head for electrodes

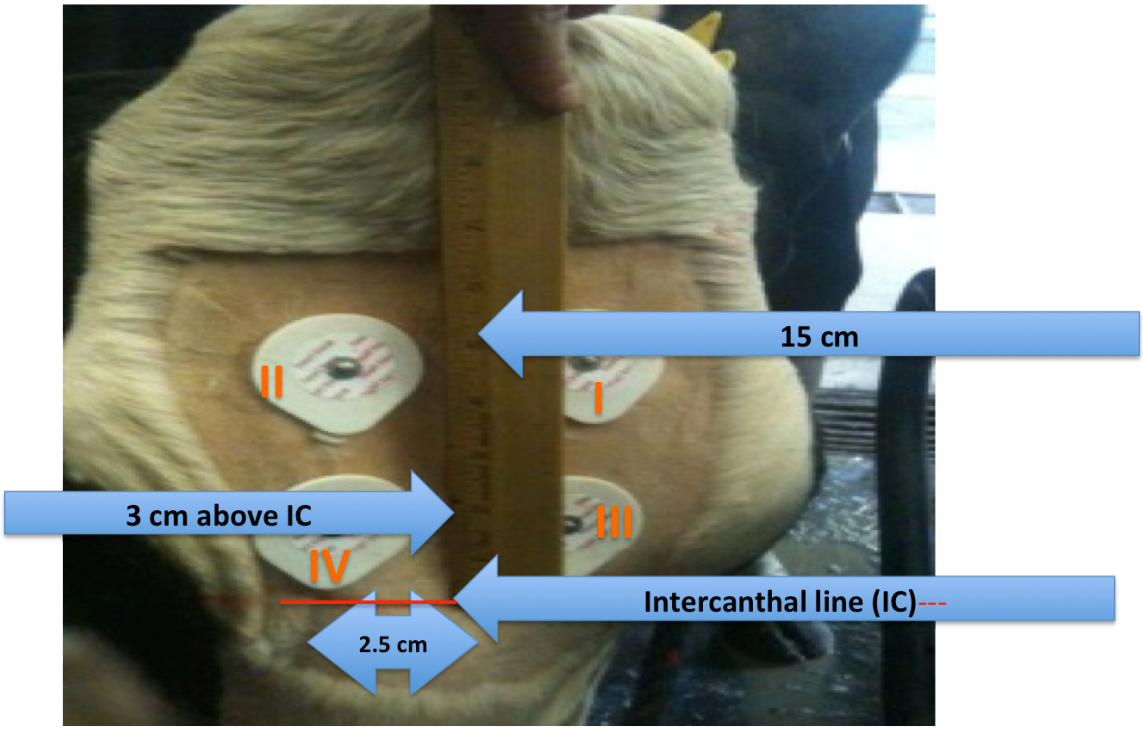

Figure III-2 Electrode placement. All electrodes are placed in relation to intercanthal line (IC). As the location for euthanasia by captive bolt is marked in reference to the eyes, it might assumed the brain location related to the skull is related to the IC. If small (10 mm) electrodes are used allowing a more precise cortical location recording, they should be placed on the AgAgCL snap region of the ECG electrodes mentioned in the text

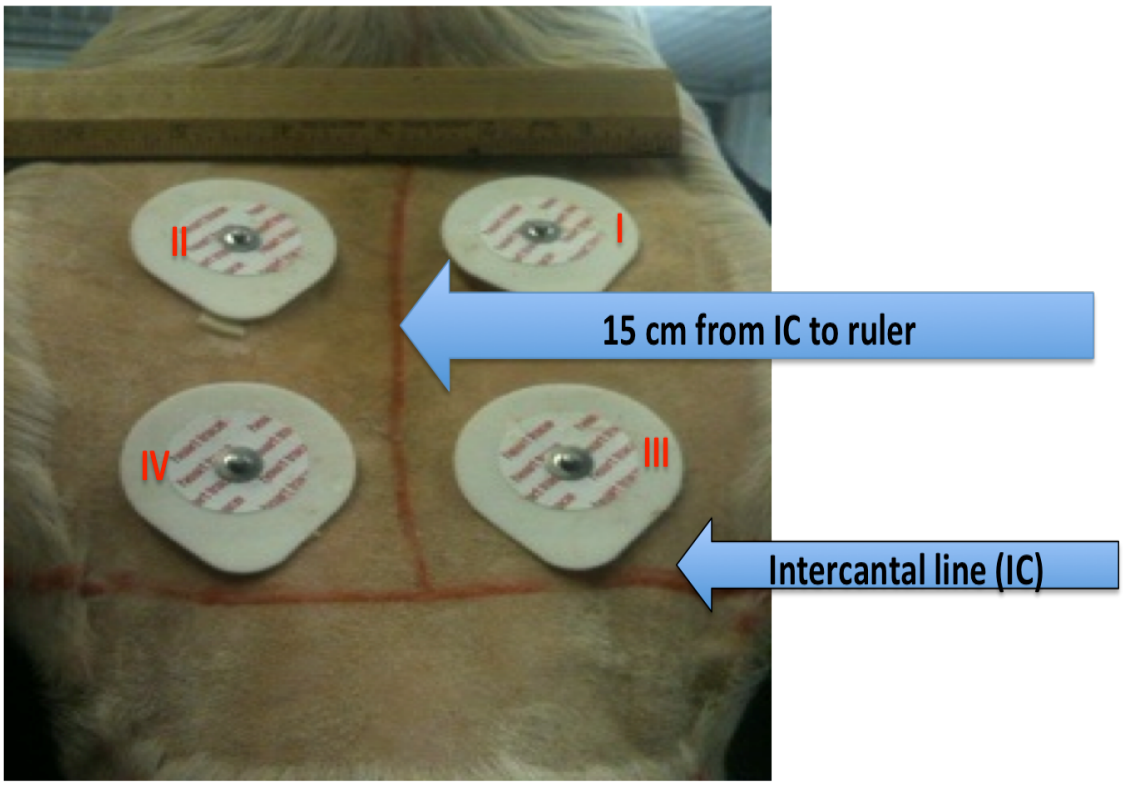

Figure III-3 Electrode placement

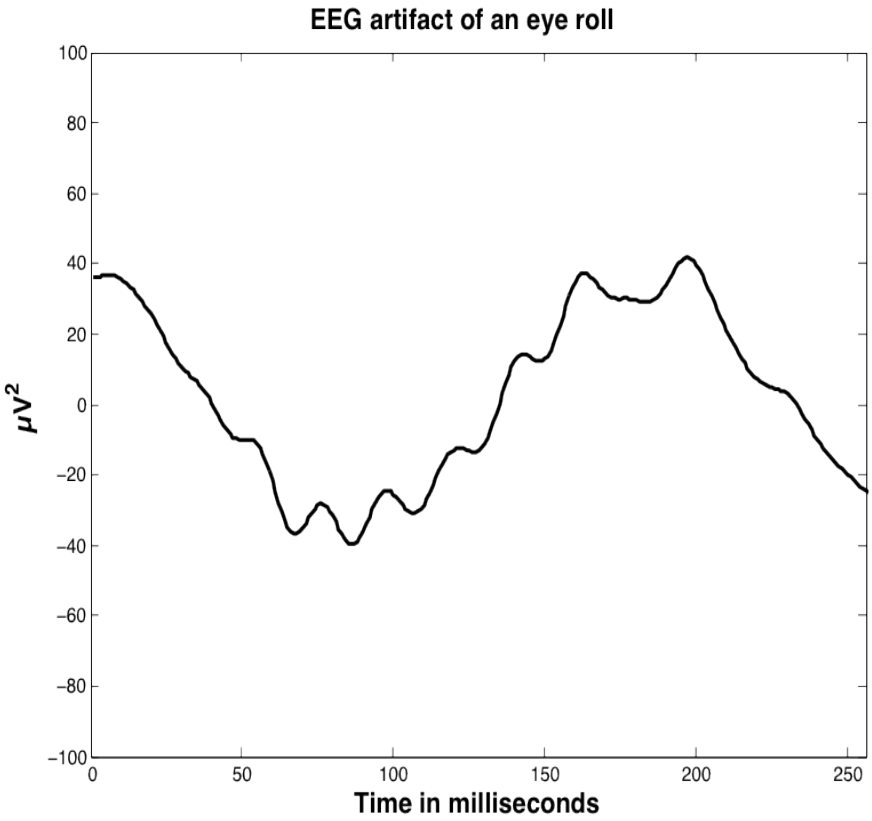

Figure III-4 Bovine eye roll artifact. A figure of a two second epoch from continuously recorded EEG data averaged from all electrodes. If human data rejection criteria are used these would not be rejected, but their power, which can be as high as 900 μV^2 bias the spectral estimates.

Chapter IV: Changes in spectral baseline EEG in cattle during conditions requiring varying levels of primary visual processing

Abstract

EEG recordings were made using conscious, quietly standing Holstein cows (n=6) to determine if cows exhibit shifts in spectral power consistent with those observed in humans. Specifically, we determined that alpha power (8 to 12Hz) in the bovine EEG during darkened (0.4 candela room) versus lighted conditions (1,000,000 candle spotlight). These data were Fourier transformed, and normative delta, theta and alpha frequency band powers were subjected to two-tailed paired t-test. In an attempt to identify species-specific functional frequencies, the spectral power estimates were subjected to principal component analysis. The results demonstrated that Delta (1 to 4 Hz), Theta (5 to 7 Hz) and Alpha 1 (8 to 12 Hz) power increased significantly ($P<.01$) during the darkened room as compared to the spot light condition that is directionally consistent with the human literature. These initial results verify EEG as a viable metric to assess cognitive processes in cattle, and demonstrate that the bovine cortex has similar frequency responses to varying conditions of visual cortical stimulation, as does a human visual cortex. This research is a first step in establishing foundational information about EEG data in cattle, determining that baseline EEG data of cattle are comparable to that of humans.

Introduction

EEG has been successfully used in humans to study mental states, such as arousal, and the cortical processing of specific stimuli. EEG's success in supporting the advancement of cognitive science has been, in part, underpinned by the understanding of human EEG parameters, e.g., the scalp frequency distribution in the resting subject, and its responses to simple stimuli. This foundational information has not been documented in animals, particularly in large animals such as cattle. If the nascent field of cognitive research in animals is to progress as has human cognitive studies, this knowledge and documentation is needed.

Apart from providing data on cognitive states and processing of stimuli, the use of EEG in humans has also provided evidence that there are specific frequencies underlying particular cognitive functions, although there is not a one-to-one mapping. For example, delta (0.1 to 4Hz) has been associated with conscious processing of mental tasks (Harmony et al., 1996), and theta (5 to 7Hz) is related to cognitive and memory performance (Klimesch, 1999, Bernat et al., 2007). The alpha rhythm (8 to 12 Hz) has been the most widely studied frequency. Although it was originally thought to be related to the cortical activity of a brain at rest, 'the idling brain', (Klimesch, 1996, Pfurtscheller et al., 1996) the discovery of three alpha sub bands, each related to differing higher functions, indicate that cortical functions associated with alpha are much more diverse and complicated than first thought.

EEG recorded continuously while conditions are changed or tasks are

performed has allowed a thorough documentation of human EEG parameters such as normative power of specific frequency bands (predominant frequency, etc.). This has aided the advancement of human cognitive science. Hans Berger (1929) was the first contributor to this literature, when he discovered that he could measure cortical potentials on the scalp, that the alpha rhythm is the dominant frequency in the human EEG and that its power decreased, or desynchronized, in the eyes open condition (Berger, 1929, Millett, 2001). Conversely, Berger also found that there was an alpha power increase, or synchronization, particularly over the occipital cortex when the eyes were closed. Since this original work, it has been found that there is synchronization of all the EEG frequencies in the eyes closed position (Klimesch, 1999, Barry et al., 2007). Berger's original protocol involved collecting EEG data in both eyelid conditions. This approach is still used today to collect baseline data before human cognitive studies are begun.

There is a large literature of cognitive studies in humans using EEG, but comparatively few concerning animals have been aimed at furthering species-specific knowledge. Those animal studies are typically aimed at studying the processing of somatosensory stimuli, and this is true in both small and large animals (Strain et al., 1992, Ong et al., 1997a, Murrell et al., 2003, Meij et al., 2006, van Oostrom et al., 2009). There is a small literature on EEG in dogs (Dumenko and Kozlov, 2003), cattle (Ruckebusch and Bell, 1970) and horses (Williams et al., 2008) aimed at studying stages of sleep. However a review of the literature resulted in no documented baseline data on animals for directional frequency changes comparable to that developed through study of human EEG across eyelid

conditions.

The aim of this research was to analyze EEG data recorded from quietly standing, conscious cows in two conditions designed to obtain analogous primary visual cortical stimulation and suppression to the eyelid conditions in humans, and to compare the frequency response to that reported in humans. It was hypothesized that the cortical frequency responses in the cow would have the same directional changes as in humans across analogous conditions. The alpha frequency, in particular, was of interest because alpha is the predominant frequency in human EEG and shows the largest frequency synchronization in response to eyes-closed conditions, i.e., conditions requiring no primary visual cortex processing. A secondary goal was to provide information on EEG parameter changes in continuous EEG with respect to frequency, that could provide a first foundational step for future cognitive studies in large animals in general, and cattle in particular. Cattle were chosen as subjects, in part, because they are underrepresented as a species in the literature, although their population size is very large.

The goal of using the eyelid condition in humans to collect baseline EEG is to excite the primary visual cortex when the eyes are open, and suppress the cortex when the eyes are closed. When the eyes are open the primary visual cortex, over which alpha is predominant, is stimulated, suppressing the alpha frequency. This well documented phenomenon is termed the 'alpha blockade' (Neuper et al., 2006), and can be readily seen in time in the EEG recording display. As animals cannot open and close their eyes on command it was necessary to develop a method that achieved the same goals of

opening and closing eyes. Putting the animals in almost total darkness, and then exposing them to a bright spotlight was considered to be an acceptable analogy for changing eyelid condition because it achieved the aim of eyes open/eyes closed by visual cortical stimulation in the bright light and conversely, cortical suppression in the dark. Allowing the cows to stand comfortably in a stall, minimizing noise and physical contact was considered a practical method of achieving a relaxed condition in a large animal, and avoiding other sensory stimuli that could confound the results. A spotlight was chosen over turning on the lights to ensure that there could be only one visual stimulus, i.e., the light.

Animals, Materials and Methods

Animal Subjects

Ten heifers between one and two years of age at the Central Maryland Research and Education Center, which houses the University of Maryland's dairy herd in Clarksville, MD, were chosen on the basis of age and temperament. Animals that were not easily handled and not halter broken were not feasible research subjects. All procedures were in compliance with, and approved by the University of Maryland Institutional Animal Care and Use Committee.

Preparation and electrode placement and data acquisition

The forehead, dorsal poll, and distal nose were closely clipped using fine clippers. The electrode sites were washed with hand soap applied to a cotton pad, then rinsed well with water and thoroughly dried with paper towels. The dried areas were then scrubbed with NuPrep® before alcohol swabs were rubbed over the electrode sites. Electrode choice and placement was consistent with the procedures in Chapter III, except that only the dorsal electrodes were used.

EEG data were acquired on a Stens® Biofeedback Nexus 10 system (San Rafael, CA), read at 256Hz and band pass filtered between 0.1-30Hz. Elefix® (Nehon Kohden, Japan) gel was used to affix the electrodes to the head. All recordings occurred between 4:30 and 5:45 p.m. in December and January 2011. A companion cow was tied adjacent to each subject cow to alleviate any distress at being separated from the herd (Herskin et al., 2007).

Once confirmation was made that there was a good electrical conduction between scalp and electrode, through visual inspection of the EEG, the barn lights were turned off. Black plastic had previously been taped over windows to reduce light. EEG was continuously recorded for a minimum of 4 minutes and a maximum of 10 minutes. The spot light (Power Pro craft 1 million candle) was turned on and directed at the cow's face (not into her eyes,) and EEG was again continuously recorded from 4 to 10 minutes. If the cow showed signs of discomfort from the light, it was redirected. The spot light was then turned off and the overhead fluorescent lights were turned on. If the cow was still standing quietly EEG data were recorded for as long as the

cow remained tractable. Data were recorded in fluorescent condition from only five cows.

Blink rate and cow tractability were recorded during both conditions. Tractability was measured subjectively using scores between one and three, three being the most compliant, one the least. These data were used for regression analysis.

Signal processing

The data were band-passed filtered offline from 1.5-20Hz and then wavelet denoised using biorthagonal6.8 wavelet at level 4. The data were then broken into two-second epochs using a program specifically written (Long and Wang, 2013) for this study in Matlab®. The epochs were visually inspected and epochs with excursions in excess of +/-100μV from baseline were rejected. If the excursion was identified by morphology as an eye blink artifact, and was +/-60μV, the epoch was also rejected. Epochs containing eye-roll artifacts were excluded (see Chapter III for more details about eye-roll artifacts). The epochs were concatenated and a power spectrum with a frequency resolution of 0.25 Hz was estimated for each data set from each cow using a multi-tapered method. The total power of each bandwidth (delta (0.25 to 4.5 Hz), theta (4.75 to 7.5 Hz), alpha to 12 Hz) was calculated and for statistical analysis, the data were log transformed to meet the requirement for normality.

Data were reprocessed strictly in accordance with normative human data processing methods, which do not document eye-roll, or unique

amplitude criteria for eye blink artifacts. This was done for post-hoc comparison to data processed in a species-specific way for informative purposes.

Statistical analysis

A paired t-test was performed between light and dark conditions for each bandwidth using SPSS® v. 18. A linear regression was performed involving regressing the cow tractability score and blink number on the power of each frequency band in light and dark conditions separately.

Results and discussion

There was a significant (P<. 05) evoked response desynchronization (ERD) in alpha between dark and light conditions, as hypothesized based on the human data (21μ2 to 13μ2). This is illustrated in Figure IV-1. This was considered a critical finding, confirming the similarities in primary visual cortical functions between species. In addition, the fact that there was a significant frequency shift, according to the hypothesis, it was explicit evidence that cortical activity was in fact being recorded.

In humans it was more recently discovered that delta, theta and alpha all undergo ERD in response to increased primary visual cortex stimulation (Barry et al., 2007). All powers analyzed in the bovine EEG also demonstrated significant (P<.05) ERD in response to increased primary visual cortical stimulation.

Alpha power

A significant alpha synchronization is measured in humans in the eyes closed condition and it is most pronounced over the occipital cortex. This was hypothesized given the high evolutionary conservation of the mammalian brain structure and cortical functioning, with the exception of cortical activity driven by specialized species-specific senses, and major network connectivity (Deacon, 1990). Barry et al. (2007) hypothesized that evolutionarily older brain structures and networks, such as the reticular activating system and thalamic-cortical connectivity, mediate mechanisms driving the alpha power changes in these conditions in humans. This means there would be no requirement for a specialized species-specific network that might exist in the human but not in the cow, further supporting the validity of the hypothesis.

However, the limitations of large animal cranial anatomy preclude electrodes being placed over the bovine occipital cortex, and it was considered a possibility that if alpha synchronization occurred, it would not be able to be measured. As hypothesized, synchronization did occur and was able to be measured despite electrode location. This finding suggests that the alpha frequency is related to visual processing in the cow and that the mechanisms for alpha synchronization and desynchronization induced by changes in visual cortex stimulation are similar to that in humans.

Although it is possible that the mechanisms that drive alpha synchronization in conditions of little cortical stimulation are the same in the cow and human, there is not enough evidence, based on this study, to suggest

that the alpha frequency in the bovine EEG is related to all functions to which it is related in humans. The alpha frequency in humans has been the subject of intense study yielding much information about its role in complex cortical processing. For example, three different alpha bands have been identified in humans, each related to different cognitive functions, including memory (Klimesch, 1996, Doppelmayr et al., 2002). No cognitive studies have yet been done in the cow that might suggest any other alpha-related cognitive function apart from primary visual cortical stimulation. However, it may not be unreasonable to hypothesize that alpha might be associated with processes such as memory and attention in all or most mammals, including the cow, as these are necessary processes for independent life.

Theta

A significant desynchronization in theta power is seen in humans during the eyes open condition (Barry et al., 2007). This was also measured in the bovine EEG. However, there is little literature regarding the mechanisms of the theta ERD in humans, making it difficult to postulate that similar mechanisms are responsible for this ERD in the bovine ERD. However, even though the putative role of theta has not been extensively reviewed in human cognition, a brief review of the known cognitive functions associated with the theta band is interesting

Cognitively, theta has been associated with orienting and memory download in mice. In humans, theta has recently been reported to be associated with movement and planning in the sensorimotor cortex

(Cruikshank et al., 2011). This seems contradictory to the hypotheses forwarded by Klimesch (1999) that in lower mammals the theta band is the dominant frequency and that its bandwidth is wider than in humans; from 4 to 13 Hz (Klimesch, 1999). However, Klimesch does not define lower mammals. Takatsuki et al. (2005) defined the murine theta band as 4 to 12 Hz, which overlaps with the alpha range in humans. Theta was not the dominant frequency in the bovine EEG in either condition and so the findings in this paper may or may not agree with Klimesh's findings, although I'm reluctant to classify the bovine species as 'lower' due to their sophisticated social cognitive ability.

Delta

The delta power decreased significantly in the spotlight condition as hypothesized. What was not expected, however, was that delta, as opposed to alpha, appears to be the prominent frequency in both conditions in the cow. As alpha globally dominates the human EEG, this difference between the two species suggests that delta may be associated with different cognitive processes in the cow than in the human, but it is not currently possible, based on these data, to speculate about what those cognitive processes may be.

The delta frequency in humans is less well studied than alpha (Knyazev, 2012) but there is a small literature regarding normative measurements and underlying associated cognitive functions. The power of the low (0.1-1.5 Hz) delta frequency in awake infants can be as high as $2500\mu V^2$ (Knyazev, 2012); whereas, in adults it appears to be much lower.

By the age of six this delta power begins to normalize to an adult range. Barry et al. (2007) have reported delta band power in awake adults as low as $40\mu V^2$. EEG studies done in adults during sleep have found powers of around 500 μV^2 (Munch et al., 2004), but delta is expected to dominate the EEG in adults during sleep (Knyazev, 2012).

In the awake adult, the delta rhythm appears to be divided into at least two operational sub-frequency bands, such that frequencies below 1 Hz in humans may be related to different cognitive functions than those above 1 Hz, and may have different cortical sources than those above 1 Hz (Niedermeyer and Lopes da Silva, 2005), although it is not clear if the cortical sources of the delta frequency in sleep and wakefulness are the same (Knyazev, 2012). Delta power is increased in conditions of pain and fatigue (Knyazev, 2012), and in states of anxiety (Knyazev, 2011). The functions associated with the delta band frequency, such as sleep, motivational states such as hunger, fatigue and pain, are not higher functions; thus, it has been suggested that the delta band frequency is primitive and its oscillations correspond only to motivational and homeostatic states (Knyazev, 2012).

However, delta frequency band power increases have been associated with more complicated cognitive processes such as identifying the face of a loved one (Basar et al., 2008) and anxious pondering related to problem solving (Harmony et al., 1996). Delta power has also been found to increase in verbal or spatial working memory tasks when the subject's answer is incorrect (Fernandez et al., 2000). The source of this delta oscillation occurring largely above 1.5 Hz appears to be from the frontal cortex, which is

the seat of reason and problem solving. These and other findings suggest that the delta rhythm in awake adults is complex and important in higher cognitive functions in humans. This literature suggests that there are many underlying cortical functions related to the delta frequency in humans, which in turn suggests that this possibly might also be the case in other mammals.

The underlying related functions of the delta rhythm, apart from sleep, have not been widely studied in non-human animals. The delta frequency is the predominant frequency in lower vertebrates, such as reptiles during the active state (Knyazev, 2012). In rats delta band power increases during food reward, and decreases during food craving, in the reward center of the brain (Fu et al., 2008), supporting Knyazev's (2012) hypothesis that the delta band is related evolutionarily to reward systems. A pain study in sheep also found that the delta frequency power, as well as that of the entire spectrum, increases in response to pain (Ong et al., 1997b). In contrast, Jongman et al. (2000) found that this delta power decreased in the presence of pain rather than increased as Knyuvez (2012) suggested. In four-week-old lambs, the EEG appears to be dominated by a high delta power band ($3332\mu V^2$) of 2-3.9 Hz (Jongman et al., 2000), which may correspond to the high delta power seen in human infants. In cattle, sleep has been investigated and found that they do not appear to have the same stages of sleep as humans and therefore their delta frequency power may not be the same during sleep (Bell, 1972). As results appear to be contradictory, in order to make meaningful inferences from the data, the normative value and the meaning of the high power of the delta frequency band in cows is deserving of further investigation. However, delta has not been investigated specifically as an

outcome variable in large animals. It is possible that delta frequency in cattle will turn out to be as important to bovine cognitive science as alpha is to human cognitive science.

As discussed in Chapter III, many artifacts could account for some of the high delta power. In addition to those mentioned in Chapter III, further possible artifacts were considered and investigated. Ruminal, respiratory and cardiac potentials were measured and ruled out as contributing significantly to the EEG signal using independent component analysis (ICA). Further investigation into the origin of the high power of the delta frequency and an appropriate definition of its bandwidth is necessary. No significant explanation of delta variance was provided by regression with blink number and behavior scores. The underlying associated functions and properties of the bovine delta band deserve further study.

Principal Component Analysis (PCA) and use of species-specific bandwidths

PCA is a statistical, data driven, non-parametric technique that identifies linear components in the data that account for data variance. A scree plot was used to identify eigenvectors above one, suggesting significance, but the first largest eight were extracted for plotting. Eight were chosen in order to allow for the possibility that delta and alpha had subands that might be revealed. The factor scores for these components were plotted against frequency as Keyser and Tenke (2003) did. These are illustrated in Figure IV-2. Component 1 was assigned to represent the delta frequency, and

component 3 was considered to represent theta. The frequency resolution of the spectra needs to be increased in order to determine if there are more than one component that might explain the variance explained by component 1. The PCA yielded a negative frequency of high significance, but there is no biological explanation for a negative component and it is planned to use non-negative matrix decomposition in the future as a method to identify species specific frequency bands.

Conclusion

Although it was hoped that this research would have provided data sufficient to be used as a baseline in further cognitive studies in cattle, it could not. In order to use the bovine continuous EEG in the dark and light conditions as baseline data for cognitive studies, its properties, particularly properties of delta, need to be better understood. For example, an explanation for the large delta frequency variance, whether artifact or neurophysiological in origin, will be needed. It is also important to define the functional frequency bands with respect to the cow, rather than the human. Once these questions are answered, further cognitive research in cattle can be pursued. One avenue of research that might be of immediate interest is to ascertain what frequencies are particular related to cognitive processes.

Figures for Chapter IV

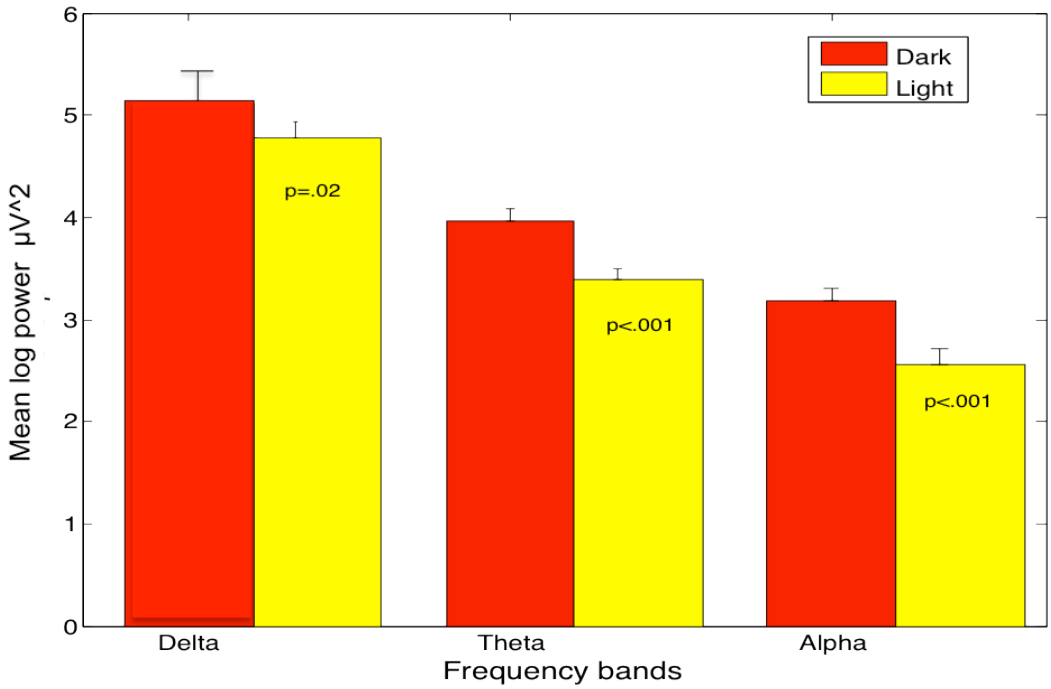

Figure IV-1 Mean log powers of delta, theta, and alpha changes between dark (red) and light (yellow) conditions. Delta is the most predominant frequency but all frequencies were reduced significantly in the light condition.

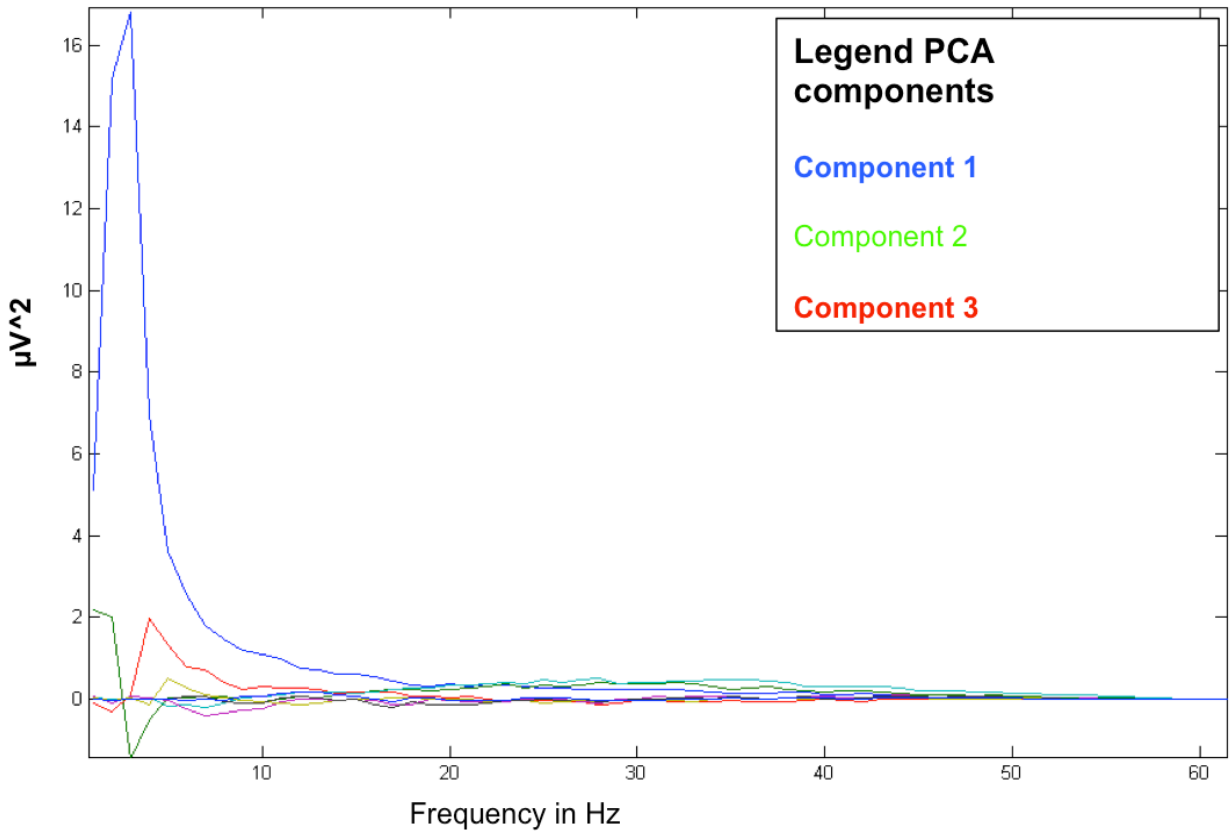

Figure IV-2 Principal component analysis components of power spectra from both light and dark conditions and from all cows. Component number is given by color in the legend. The factor scores were plotted against frequency to suggest biological explanations for particular components. Although eight components were extracted, this figure suggests that fewer is sufficient. Component 1 is likely to represent delta (.1-4 Hz), and 3 may represent theta (5-7 Hz). The extraction of a negative frequency component has no biological meaning, which is why non-negative matrix factorization should be considered.

Chapter V. Noxious laser stimuli elicit laser-evoked potential (LEP) in the bovine species

Abstract

A bovine laser evoked potential (LEP) was measured. The LEP was found to have quantitative features, such as amplitude and latency post-stimulus, that changed in significant ways corresponding to the intensity of the cow's negative behavioral reaction to the stimulus. EEG was recorded continuously from five conscious, standing adult Holstein dairy cows that were halter restrained. Laser pulses of varying intensities (30 to 160J/cm^2) were applied to the shoulders in blocks of fifteen to twenty pulses. The laser stimuli were administered to different locations within blocks of 15-20 stimuli, alternating between left and right shoulders. Importantly, the measured bovine LEP shared qualitative and quantitative features with the human LEP, particularly regarding amplitude responses known to correlate with subjective pain perception as measured by self-report. Because cows are non-verbal subjects, a surrogate of self -report of stimulus perception was used. Behavioral responses, established by previous authors, to indicate pain or discomfort in cattle in response to laser stimuli were used to estimate how noxious she found the stimuli to be. These results provide strong evidence that cortical measures of nociceptive, and potentially painful, stimuli can be measured in cattle. Additionally, we contend that this LEP-based methodology has potential as an objective neurophysiological correlate of measuring

distress or discomfort to painful stimuli in the cow and other animal species.

Introduction

In humans, neurophysiological research has identified the laser-evoked potential (LEP) as a useful tool for studying nociception and, by extension, pain (Chen et al., 1979, Arendt-Nielsen and Bjerring, 1988, Arendt-Nielsen, 1994, Bromm and Lorenz, 1998). The LEP has proven useful for testing the patency of the pain pathway (Bromm and Treede, 1987), and has been used as a method of assessing the efficacy of analgesics (Bromm et al., 1988, Beydoun et al., 1997, Garcia-Larrea, 2006). Researchers have additionally demonstrated that there are LEP features, the magnitude of which correlate to a human subject's perception of pain. This indicates that the LEP may be a correlate of the underlying mechanism of pain perception (Bromm, 1985, Bromm and Lorenz, 1998, Iannetti et al., 2004, Ohara et al., 2004c).

Prior to attempting to record the LEP, we established a priori what criteria must be met in order to classify a measured potential as an LEP from a non-human species. We decided that a bovine LEP must have at least one quantitative feature, such as peak amplitude and peak latency that are not modulated by stimulus intensity, but by behavior that signals increasing discomfort. The necessary and sufficient criteria we established to meet the definition were:

1. Occurs at the correct latency corresponding to the reported Aδ conduction speed (10-30m/s),
2. Is affected by conditions not directly related to stimulus intensity (i.e., a,

attention, etc.), and

3. Is abolished or diminished when there is no behavioral indication that the stimulus was perceived.

Crucially, in order for a bovine LEP to be used to study pain processing, the LEP should be shown to correspond to a measure of the affective component of pain perception. Prior to our investigation, we could identify no EEG studies based on lasers as nociceptive stimuli in large animals, although there have been studies of pain in cattle using lasers alone (Veissier et al., 2000, Herskin et al., 2003). However, in total, when compared to the literature on human pain perception, relatively little work has been done on bovine cognition, and basically no neuro-physiological work related to pain perception.

In order to study pain processing and perception in the cow, human study methodology was adapted to evoke and measure a bovine LEP. We hypothesized that a bovine LEP would:

1. Be measurable,

2. Have features that significantly correlate with perception of stimulus rather than stimulus intensity, and

3. Not be present, or would be significantly diminished, when cows showed no behavioral evidence of perception to the stimulus.

Material and methods

Animal subjects

Five lactating Holstein cows, ranging in age from two to three years old, were selected as subjects based on tractability and quiet temperament. The cows were from the herd housed at the Central Maryland Research and Education Center, University of Maryland, Clarksville, MD. Pairs of cows were used and restrained as described as described in Chapter III. Cows showing overt distress in response to handling and/or laser treatment were returned to the herd and not used in the experiment.

An area of the research barn was created to ensure compliance with health and safety requirements promulgated by the University of Maryland's Department of Environmental Safety (UMDES) for the use of lasers (i.e., reflective surfaces were covered, the experimental area was physically separated from the rest of the barn, and a standardized radiation hazard sign was taped to the outside of the 'room'). People participating in research were trained by UMDES in laser safety. Protective glasses and other safety procedures were followed. A portable air conditioning unit cooled and maintained a moderate ambient temperature in the test area. The research protocol and conduct of the studies were reviewed and monitored by the University of Maryland Institutional Animal Care and Use Committee.

Electrode placement and equipment

Subject cows were prepared for EEG collection according to the method detailed in Chapter III. The location of the seven Silver-silver-chloride Grass® 10 mm electrodes (with 30 cm cables) are illustrated in Figure III-1, and described in Chapter III. In addition to the equipment detailed in Chapter III, an addition Stern Technologies device (Nexus trigger interface®; NTI) was used to facilitate the time locking between the laser and the Nexus 10®. The laser was modified to generate a transistor-transistor-logic (TTL) signal for the NTI to capture and overlay onto the digital EEG data.

Laser stimuli

A 30cm^2 area of the left and right shoulders of each subject cow were clipped with surgical clippers and covered in a thick layer of aloe gel, which is standard human clinical practice, particularly when dealing with dark pigmented skin. Laser heat stimuli, 20ms pulse width, 6mm diameter, were generated with an Nd: YAG (neodymium-doped yttrium aluminum garnet) Harmony® laser (1064nm). Stimuli were delivered in blocks of between 15 and 20 stimuli on the shoulder in varying locations, alternating between left and right sides between each block for a maximum of 200 stimuli with inter-stimulus intervals (ISI) intended to be irregular and longer than 5 seconds.

Laser energy was chosen on the basis of a behavioral score assigned to the previous trial with the aim of achieving a behavioral response indicating the stimulus was nociceptive (See Table V-1 for the method of assigning

behavioral scores per trial.), and yet did not cause distress. The first stimulus intensity was always at the lowest energy level, 30/cm^2. If there was no response, the energy level was increased by 10J/cm^2 for each stimulus until a behavior score of 2-3 was elicited. If the behavioral response indicated that the cow would become distressed if the energy intensity was continued, the laser energy was lowered by 20J /cm^2 before the next stimulus. If the cow did not tolerate the stimuli, or became overtly distressed (intentional attempts to evade the laser) before 200 stimuli were applied, the session was terminated and the cow immediately returned to the herd.

Observations and records

The energy level of the laser and behavioral response of the cow to each stimulus were recorded. The cow's behavior was given a score of 0 to 3 based on a method proposed by Herskin et al. (2003). (See Table V-1 for details about behavioral scoring). Laser energy intensity was also recorded for each trial. Laser intensity was classified as high or low for statistical analysis after each session. The median of the range of applied energies was used to identify 'low' energy level (energies below the median), and 'high' energies (above the median). The energy level classification was independent of the behavior score. Those who scored the behavior were blind to the laser energy level.

Signal recording and processing

Continuous EEG was sampled at 256 Hz, and band pass filtered online from 1 to 40Hz. The code written by Long and Wang, (2013), (see Chapter III), was used for all of the following signal processing except for the statistics. Data were band passed filtered with a digital finite impulse response (FIR) filter between 3 and 15 Hz, and wavelet denoised [biorthogonal 6.8 (bior6.8) wavelet at level four], epoched from 500 ms pre-stimulus to 1500ms post-stimulus, linear de-trended, baseline corrected and visually inspected. Epochs were accepted or rejected based on criteria described in Chapter III.

Data from individual electrodes were time averaged to visualize the laser-evoked potential (LEP). This paper reports only data derived from electrodes contralateral to the stimulus, unless stated otherwise, because they were the most salient. Data were continuously wavelet transformed (CWT) epoch by epoch with a complex Morlet wavelet (1.1-5) to obtain phase information. Phase -locking factors for each electrode for each frequency band were calculated to test for phase locking. Wavelet phase and cross coherence were calculated using code by Grinstead (2002).

Power spectra for frequency bands of interest were estimated for four equal time periods of 500 ms, for (1) each individual epoch for each electrode, for (2) each cow, and (3) for each behavioral/perceptual category, high or low behavioral score (HBS or LBS). The method used was a multi-taper (Percival, 1993). The time bins were 500 ms pre- stimulus, 0-500 ms post-stimulus, 500-1000 ms post-stimulus, and 1000-1500 ms post-stimulus.

The total energy of each bin-band was calculated and log transformed before statistical analysis. This procedure was also done for data from high and low energy laser conditions.

Statistical analysis

Time averaged LEP

A bootstrap method (personal communication, Jonathan Simon) in Matlab® was used to estimate peak latencies and peak amplitudes, maximum and minimum, occurring between 300 and 600 ms post-stimulus. A trimmed mean method (Streiner, 2000, Gleiss et al., 2011) was used to identify and remove outliers of peak amplitude estimates. SPSS® v. 20 was used for a two-tailed, paired t-test with ($P<.05$) to test for significant differences between peak latencies and peak amplitudes between behavioral categories (HBS, LBS), and laser intensity conditions. Figure V-1 illustrates a grand averaged LEP from both HBS and LBS trials.

Frequency and phase

A paired two-tailed t-test ($P<.05$) was used to test for frequency changes in the three frequency bands, between the four time bins and between HBS and LBS trials. A post- hoc Tukey test was used to control for type I error resulting from the multiple comparisons in the frequency data set.

The phase angles derived as above were used to calculate the phase

locking factor (PLF) for each electrode, for each frequency, and for each time bin. A modified Rayleigh test (P<.005) (CircStat® Matlab toolbox) was used to test PLF for significant phase locking. Representations of the potential in the time-frequency plane make visualization of LEP's frequency and phase responses, quantified in discrete time bins for statistical purposes, more intuitive. Two methods that can also be used to test for statistically meaningful measures in the time-frequency plane between electrodes were used. In the first, the signal was Gabor filtered and the magnitude of the complex phase angle *(w(t))* was plotted (Sinkkonen et al., 1995), yielding a visualization of the oscillatory envelope illustrated in Figure V-2 The second was testing the significance (P<.05) of the wavelet cross coherence of phase and frequency against a against a background of white noise from AR(1) model using a Monte Carlo method. Software for the wavelet cross coherence calculations were provided in Matlab® by Grinstead (Grinstead, 2002).

Results

LEP peak amplitudes

In this report amplitude is used when referring to the positive potential, peak magnitude is used when referring to either the peak maximum or minimum (negative) potential. The maximum peak amplitude was significantly (P=.02) greater for the HBS (4.28μV+/-2.4) condition compared to the LBS category (2.08 μV, +/-.8) when electrode data were collectively analyzed. However, when left and right hemisphere electrodes are analyzed in

pairs, only the maximum peak amplitudes of the right hemisphere (electrodes II & IV) showed significant differences (P=. 04) between HBS and LBS. See Table V-3 for the maximum and minimum magnitudes of the LEP for both behavioral conditions.

Peak latencies

LEP were identified for HBS and LBS, and illustrated in Figure V-1. The LEP were visibly different between behavioral categories. The LBS LEP appears to lag behind the LEP from HBS. The differences in peak latencies (the time at which the signal reaches its maximum amplitude) between high behavior score (HBS) and LBS were significant for the time average of all four electrodes (P=. 005), and for electrodes over the right hemisphere (II & IV) (P=. 04), but not over the left hemisphere. Significantly, there was no significant difference in peak latencies or peak amplitudes between high and low laser energy conditions, those conditions that are independent from the behavioral/perceptual response. This is in accordance with human results.

Frequency

The underlying cortical functional significance of the herein reported phase and frequency responses cannot be inferred without knowing the spatial relationship between the skull, electrodes, and cortices. This does not reduce any significance in the findings, but merely means that we cannot comment on which cortices are exhibiting evoked response desynchronization (ERD), or

evoked response synchronization (ERS). The frequency responses for individual electrodes are illustrated in Figure V-3. Frequency responses were similar across all electrodes, particularly in the theta band, where there was a generalized ERD; followed by a marked ERS by 1000 ms. Theta underlies the human LEP so this is not surprising. There also seemed to be a general alpha ERS, peaking by 1500 ms, but not all the alpha responses met significance criteria. All electrodes showed a qualitative ERS in delta by 1500ms, although it only reached significance in electrodes II and IV. There was significant (P<. 05) ERS in theta and alpha for electrode I post-stimulus. For electrode II (right hemisphere, dorsally), an ERD in delta and theta occurred immediately post-stimulus, with the power increasing to above pre-stimulus levels by the end of 1500 ms. ERS in alpha was also evident post- stimulus in electrode II. Figure V-4 and Figure V-5 are time frequency representations of the LEP from each electrode as labeled in the figures.

Although in Figure V-3 there appears to be an ERD in delta in electrode I, it did not reach statistical significance in the post-hoc Tukey test, (left hemisphere, ventrally). Frequency response in electrode IV was similar to its dorsal partner, II, but only an ERD in theta immediately post-stimulus reached statistical significance. The right hemisphere appears to have the strongest responses, whether in latency, frequency or phase. No significant (P< .05) ERD or ERS were noted in any of the comparisons across time bins and electrode pairs in low behavioral score trials (LBS).

Phase

The Rayleigh r-statistic [for more information on circular statistics see Fisher, (1995)] reached significance for synchronization (P<. 005), particularly in the theta band during the first 500 ms post-stimulus in all electrodes, and from 1000 to 1500 ms post- stimulus. This underlies the theta ERS observed in the frequency analysis in the latter part of this epoch. Significant (P< .05) phase synchronization and desynchronization across the four time bins in each electrode in the HBS trials were noted. Only electrodes II and IV showed phase locking in the LBS trials immediately after stimulus. This is consistent with the frequency changes

Scalograms of the wavelet cross frequency and phase coherence are illustrated in Figure V-6 and Figure V-7. Figure V-6 illustrates coherences between ventral and dorsal electrodes. Figure V-7 illustrates coherences between right and left hemispheres. The color represents frequency coherence between electrodes, (red is high coherence) and the arrows represent phase differences between signals across time. The arrows that point right signify phase coherence, or synchronization between electrodes. Arrows pointing upward signify a 90-degree phase lag between signals, and arrows pointing down signify a -90 degree phase lag between signals. Zones outlined in black signify areas where wavelet coherence is significant (P<.05) compared to white noise. The 'cones' in the middle of the figures signify data points that are (outside the cone), and are not (inside the cone), affected by edge effects etc. Although there appears to be significant coherence in other higher frequencies, there is significant coherence around 5 to 7 Hz

between both dorsal and ventral electrodes in HBS trials, at around 500 ms post-stimulus (coinciding with the LEP). These are only measuring evoked coherency as they were done on time averaged epochs.

Discussion

We measured a bovine evoked response potential that satisfied the *a priori* definition of an LEP thus confirming that it is possible to elicit and measure a bovine LEP. Furthermore, this LEP not only had latency, amplitude and frequency properties observed in the human LEP, but also was correlated with behavioral scores indicating each cow's individual degree of awareness of noxious stimuli. As discussed previously, we interpreted the behavior score as indication of the degree of individual aversion to the stimuli. Therefore, in this discussion, instead of using the phrase 'epochs recorded when there was a high behavior score', we will use 'aversive', and referring to trials in the LBS category we will use 'not aversive'. It follows then, that a high behavior score indicates that the stimulus was aversive, or painful. If there was little or no reaction to the stimulus it was assumed the cows had not felt it, or had not been distressed by it. This was considered a legitimate surrogate of human self-report of 'painful' or 'not painful.'

Amplitudes and Latencies

The bovine LEP peak magnitudes and latencies were shown to correlate to the degree she appeared to find the stimuli aversive, but not with stimulus

intensity, in the way that would be expected based on extant human literature. It might be noted that the magnitude of the peaks in the bovine LEP are much smaller than those typically found in the human LEP (by up to +/-20 μV). It is improbable that the relatively small magnitudes reflect any significant difference in nociception and pain perception between bovine and human. Human LEP magnitude is variable depending on site location, even though perception is not reported to change (Truini et al., 2005). Differential distribution of Aδ neurons may allow for poorly innervated skin regions.

Fewer Aδ fibers increase the time needed for a sufficient number fibers to become excited enough to cause a synchronized afferent volley. Afferent signals not temporally synchronized will reduce the magnitude of the time-averaged potential's amplitude (Handy, 2005). Variation in skin thickness may also have contributed to small potentials (Arendt-Nielsen and Bjerring, 1988).

In humans, anxiety significantly increases pain perception, and consequently, the size of the LEP (Arntz and de Jong, 1993, Warbrick et al., 2006). Conversely, distraction can abolish the human LEP, along with perception, given the same stimulus intensity (Ohara et al., 2004a, Del Percio et al., 2006). There was a large inter-individual and intra-individual (even during one session) variation in attention subjectively assessed by noting that the cow might be focused on the laser, possibly anticipating the next stimulus, and then be suddenly distracted by an unplanned environmental stimulus. One trial with high -energy intensity might evoke a two or three behavioral score, but the next trial with unchanged energy went unnoticed. Uncontrolled and immeasurable states of anxiety and distraction would have

modulated the peak magnitudes in undocumented ways. In short, interspecies magnitude differences do not necessarily reflect perceptual differences.

Frequency and phase

In humans, an acute stimulus causes cortical activity and connectivity to abruptly change, resulting in measurable frequency and phase differences pre- and post-stimulus (Palva et al., 2005a). The functional significance of increased and decreased power or phase coherence [cortices acting in synchrony demonstrating connectivity (Thatcher et al., 2009, Palva et al., 2010)] is not always easy to interpret, but does indicate underlying cortical functions (Pfurtscheller and Andrew, 1999), including binding (Meador et al., 2002). Binding is important for creating the feeling of continuous perception from discretely sampled sensory data, and is continuing question in cognitive science (Roskies, 1999). The presented bovine LEP contains significant phase locked frequency changes, and ERD and ERS, which is more a measure of a change in the background cortical activity. This directly implies, by analogy, a more widespread cortical connectivity, leading to the conclusion that the bovine LEP results from functional frequency changes underlying the local and global cortical activity involved in the processing and perception of the unpleasant stimuli.

The wavelet cross-coherences illustrated in Figure V-6, Figure V-7, is a method of depicting mutual changes in frequency and phase between electrodes over time, in response to the stimuli. For reasons discussed in Chapter VI, although there are regions of significance where expected, these

calculations, in this instance, cannot be used to statistically prove relevant cortical activity uniquely in response to the stimulus. However, the coherence patterns are compelling.

One of the most important aspects of the human N2P2 is that it is largely endogenous (Siedenberg and Treede, 1996). That is, cognitive factors such as attention, distraction, anxiety, and arousal, rather than stimulus properties, such as energy intensity, affect the amplitude; the N2P2 'reports' the magnitude of subjective stimulus perception. It has been elegantly shown that the N2P2 is part of the class of somatosensory potentials, and is more closely related to saliency (Mouraux and Iannetti, 2009a), and thus non-nociceptive specific. However, the N2P2 cannot be generated without an intact nociceptive system (Bromm and Treede, 1991, Arendt-Nielsen et al., 1999), is modulated by analgesics (Bromm, 1985, Arendt-Nielsen and Bjerring, 1988, Bromm et al., 1988, Arendt-Nielsen, 1994), and is not measured when there is no conscious (as determined by self report) perception. It is the cognitive, conscious perception of the intensity of the stimulus that is relevant to the individual's experience. If something is not salient, it is not experienced, regardless of the valence. This is critical in the affective aspect of nociception leading to pain and potential suffering. If this were not so, a subject would invariably report an increasing amount of pain with increasing intensity of stimulus and these two factors have been shown to be independent. Thus, that an LEP is measured in the bovine EEG in response to laser stimuli, and that the variance of its latencies and magnitudes are in part explained by the individual's behavior, considered to represent the degree of averseness perceived by the cow, is a significant finding.

Conclusion

We have identified and quantified the first known bovine LEP and shown that it meets the *a priori* definition derived from the responsive features of the human LEP. Namely that (1) it is similar in characteristics and latency to the N2P2, (2) that its parameters are sensitive to the behavioral measures of distress, rather than stimulus properties, and further, (3) it is suppressed or abolished when the cow does not show signs of discomfort or perception of the administered stimulus. There are differences between the human N2P2 and the bovine LEP, namely the peak magnitude latency is sensitive to perception but there are critical similarities between LEP features correlated with perception. Until a bovine cortical map is completed, the functional meaning of phase locking and cortical synchronization or desynchronization in response to stimuli is not possible to say. Mammalian cortical connections are largely preserved across evolution, and therefore it is unlikely that these measured frequency and phase changes are due to completely different networks and mechanisms than they are in humans. This research supports the validity of the idea that if particular features of the human LEP correlate with pain perception, and the bovine and human LEP share these features, that, by analogy, cows also find these laser heat stimuli as noxious, and

Figures for Chapter V

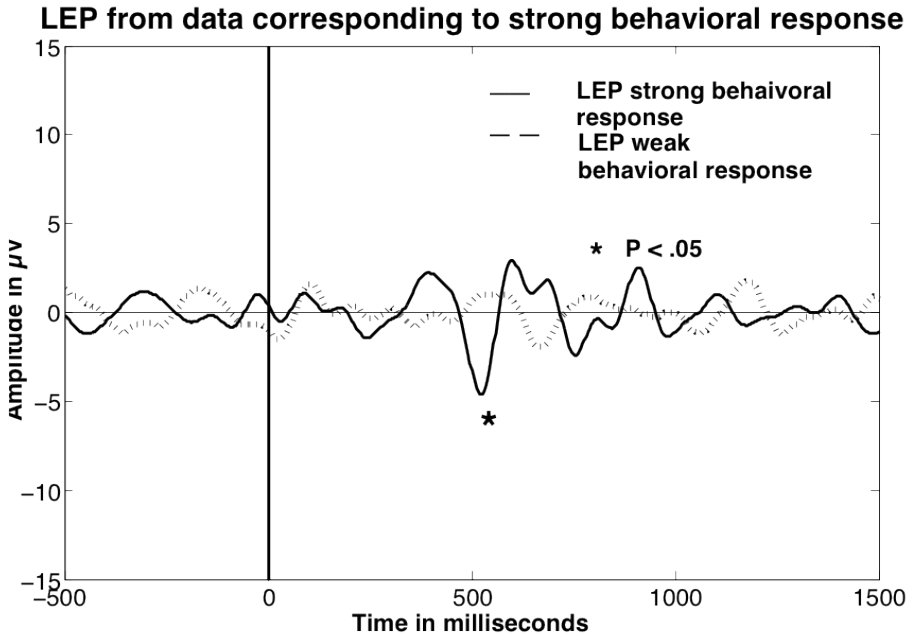

Figure V-1 Bovine laser evoked potential from trials that were perceived as nociceptive vs. not perceived as nociceptive as assessed by behavior. Grand average bovine laser evoked potential from five subjects. The peak amplitude is significantly

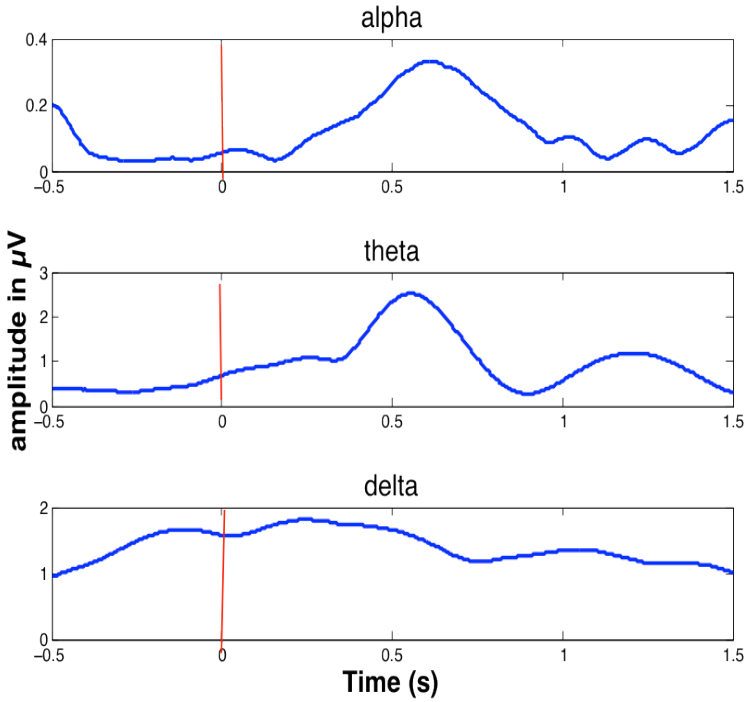

Figure V-2. Oscillatory envelope for combined data from all cows and electrodes perceived trials. These waveforms are not sensitive to phase. It is a useful method of visualizing a potential.

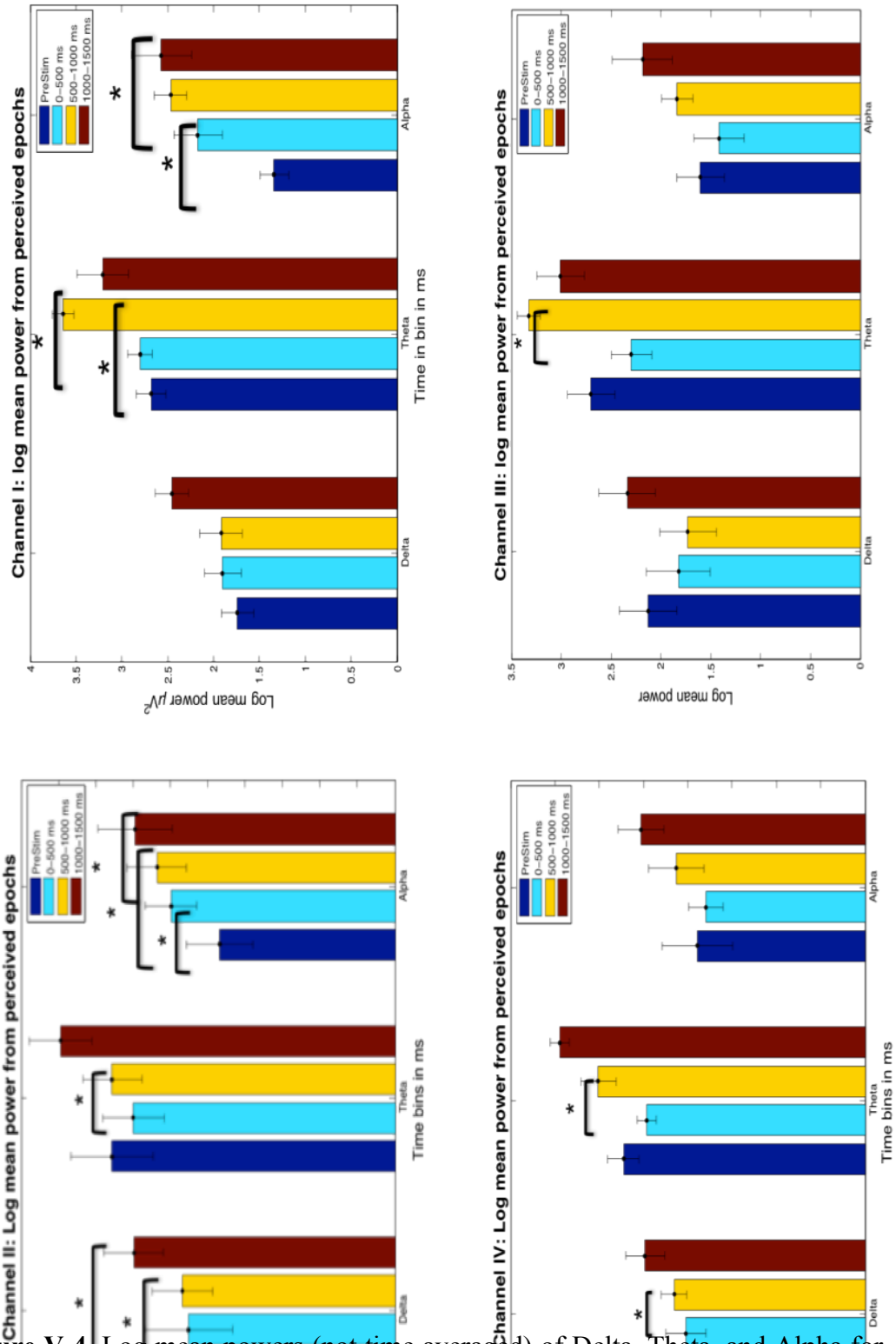

Figure V-4. Log mean powers (not time averaged) of Delta, Theta, and Alpha for each electrode, I, II, III, IV, for each time bin. The dorsal electrodes show the most prominent induced and evoked power changes. (P<.05 =*) Dorsal quadrants show most prominent frequency responses. Theta shows evoked response desynchronization in all quadrants immediately post stimulus. There is an ERS in alpha by 1500 ms post stimulus, although not to significance in the ventral

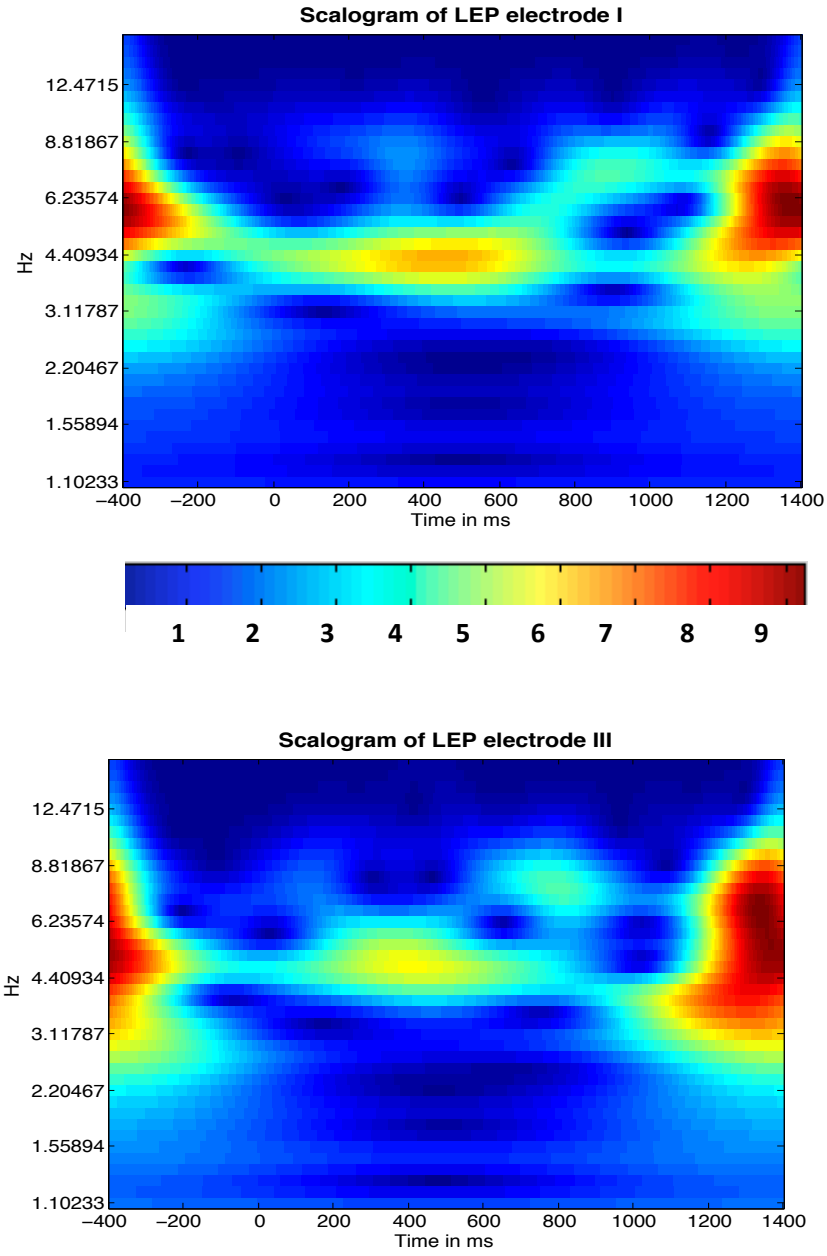

Figure V-4 Time-frequency plot of left hemisphere (I and III) in HBS trials. The red indicates high power in the related frequency band. Color legend is in log μV^2

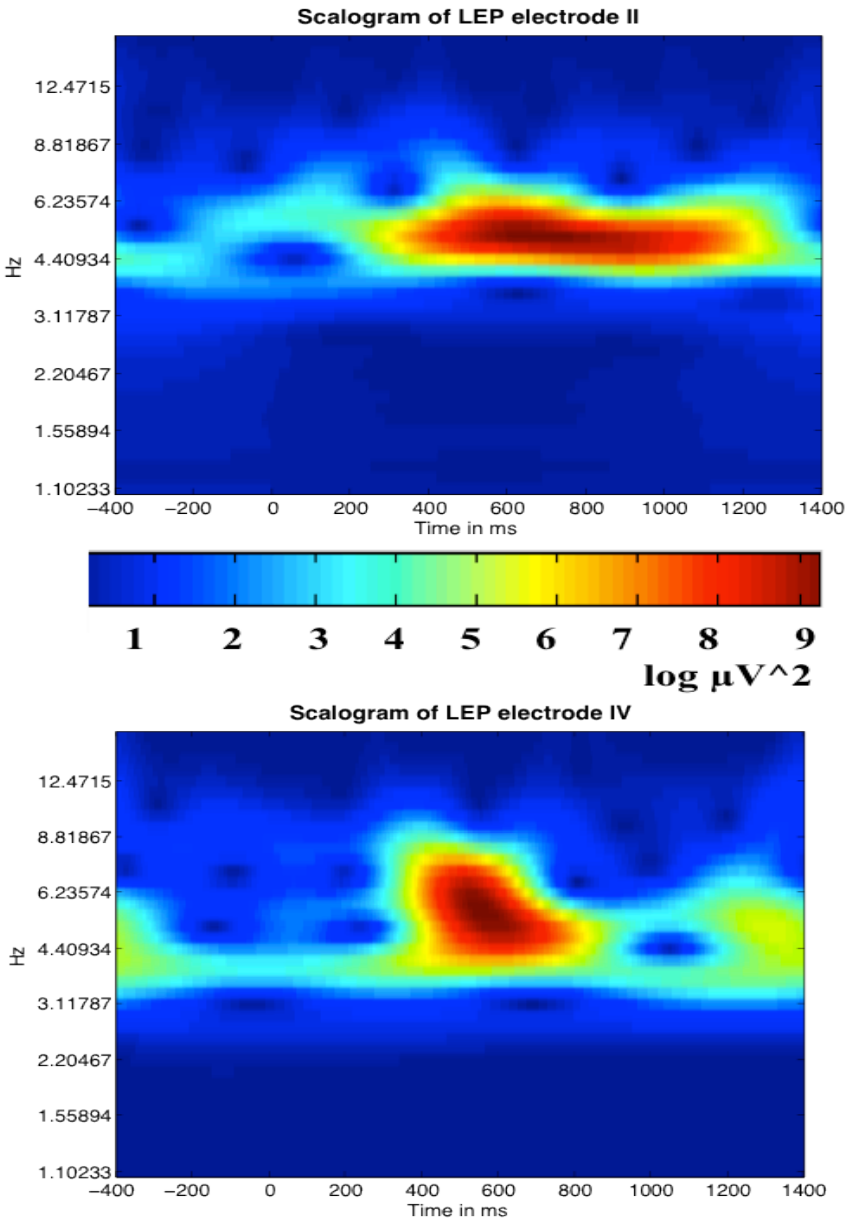

Figure V-5.Time frequency representation of laser evoked potential in HBS trials from electrodes II & IV (right hemisphere)

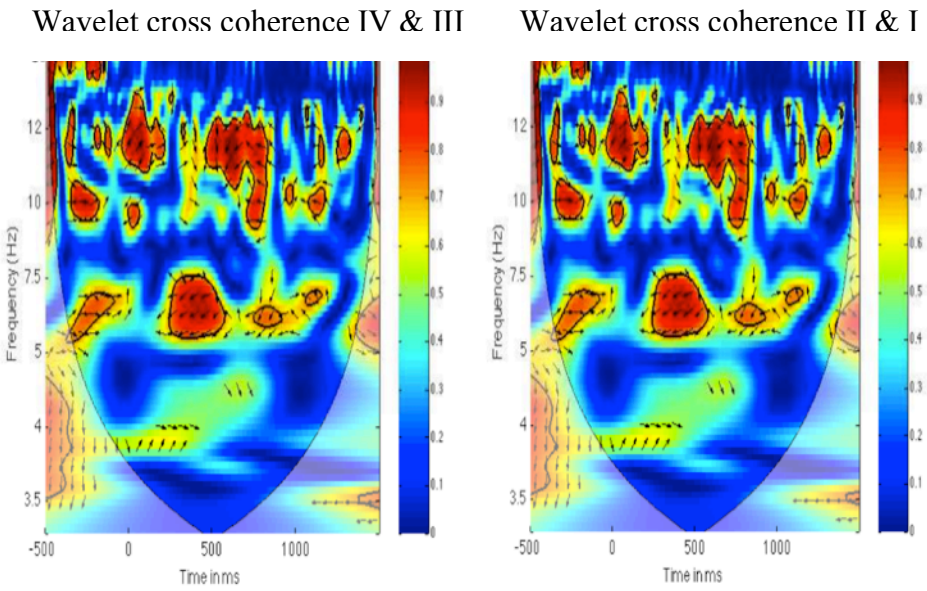

Figure V-6. Wavelet cross-coherence in phase and frequency between dorsal and ventral electrode pairs. Electrodes IV & III (ventral on left), and electrodes II & I (dorsal on right). Color legend :in frequency coherence coefficient (1 is complete frequency coherence, 0 is no frequency coherence). Arrows represent phase locking. Aarons pointing right indicate phase locking. Up is 90 degree difference, down is -90 degrees, and left is not in phase.

Wavelet cross coherence II & IV Wavelet cross coherence I

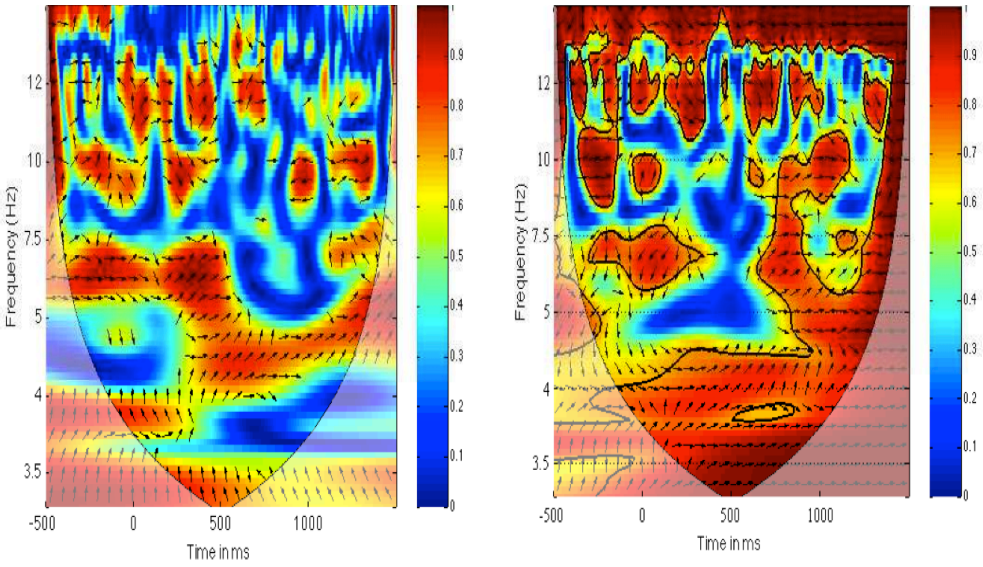

Figure V-7 Wavelet cross-coherence of right and left hemispheres (left and right panels respectively). Darker color is closer to total coherence. Blue is almost no coherence between frequencies.

Table V-1. Description of behavioral scoring. If more than one behavior that would have been scored as 2 was observed for any trial, the trial's behavior score was considered a 3 if exhibited together. Vocalization was considered unacceptable but none were recorded. High behavior score was 2 to 3, low behavior score was 0 to1.

Score	**0**	**1**	**2**	**3**
Behavior				
No reflexive response	X			
Twitching of ear, tail or shoulder muscle		X		
Stomping foot		X		
Flicking tail as if swatting flies, stomping foot, and large muscle twitch over shoulder			X	
Intentionally moving away from stimulus, looking at stimulus site, kicking			X	X

Table V-2. Peak magnitude latencies of bovine laser evoked potentials in ms of perceived (P), and unperceived trials (UP). C= data from electrodes contralateral to stimuli. (I, II, III, IV are electrode numbers). * P< .05. At the bottom of the table are peak latencies for the LEP in high (HE) vs. low (LE) laser intensity conditions.

	Latency of maximum amplitude (ms)	***Latency of minimum Amplitude (ms)***
P (C all electrodes)	545.48	497.87*
UP (C all electrodes)	527.04	533.04*
P (C, I & III)	538.14	466.26
UP (C, I & III)	536.48	522.34
P (C, II & IV)	546.1	479.97*
UP (C, II & IV)	536.48	522.34*
HE (C all electrodes)	500.52	573.93
LE (C all electrodes)	517.18	558.55

Table V- 3. Maximum and minimum amplitudes at peak latencies from table above in perceived (P), unperceived (UP), high energy (HE), and low energy (LE), conditions in μV. P< .05 *

	Maximum peak (μV)	*Minimum peak (μV)*
P (C all electrodes)	4.2 *	-1.91
UP (C all electrodes)	2.1 *	-1.33
P (C, I & III)	2	-2.14
UP (C, I & III)	2.89	-3.11
P (C, II & IV)	3.57*	-1.95
UP (C, II & IV)	1.4*	-1.0
HE (C all electrodes)	1.72	-1.8
LE (C all electrodes)	1.9	-1.85

Chapter VI. Comparison of the Bovine LEP and the Human LEP: Consideration of the causes of variation, similarities and differences

Abstract

We present the first known report of a bovine LEP (Chapter V). The LEP shows some obvious similarities to those reported for humans, which we feel should not be dismissed. However, compared to a human LEP, we determined that the bovine LEP also showed major differences, both temporally and in magnitude. The purpose of this paper is to discuss some of the potential causes of these differences. Signal noise is viewed as one possibility and considered to be responsible for at least some of the differences. However, of more interest is the possibility that the differences represent variation in bovine versus human cortical processes. The possibility that the bovine reported LEP is solely an artifact is also considered herein. However, supporting evidence is presented that the reported bovine LEP is, in fact, cortical-based and therefore due to cognitive, anatomical, and/or inter-individual differences. Therefore, we also present what we anticipate is at least a small step forward in understanding inter-species perceptual qualia.

Biological factors underlying time and frequency measures, and the human/bovine differences

Researchers in humans have previously determined that ERPs are generally sensitive to environmental and movement artifacts (Picton, 2002). Thus, the possibility that the potential was of artifactual origin had to be considered, and, in fact, the initial analysis, following human data processing standards, did not yield an LEP. An artificial neural network (ANN) was built in Matlab® and used as a pattern detector. This was considered to be an objective method testing for real differences between epochs resulting from perceived trials versus epochs resulting from trials that were not perceived. The ANN correctly categorized 88% of the epochs into perceived versus not perceived. Therefore, it was concluded that the data did likely include an LEP, but that it was being overwhelmed by noise.

The cortical activity underlying a human LEP has certain frequency and phase signatures that would not be seen in a signal generated solely by artifact, rather than underlying neurological processes. Confirmation that bovine LEP are of cortical origin was methodically achieved by ticking off a list of differential features that are necessary to confirm a neurological process, much like the diagnosis of a complex medical disease. These include (1) comparison of temporal features such as peak latency, to other somatosensory potentials, including the NI, (2) phase and frequency shifts that were biologically plausible when measured relative to the stimulus onset, and, (3) looking for evidence that the LEP features were consistent with endogenous potential, i.e., that temporal and frequency features of the bovine

LEP were not sensitive to stimulus intensity, but were sensitive to observed degree of perception.

In humans, delta and theta underlie the LEP. These are low frequencies, and so the band pass was narrowed, excluding higher frequencies that also contained high amplitude noise. The signal was then wavelet denoised to eliminate low frequency noise by setting small coefficients to zero. These processing steps yielded a small potential at around 500 ms. However, the question remained as to whether or not the observed potential at 500 ms was the result of an artifact of a reflexive response to the stimulus, for example a blink or muscle twitch, or was cortical.

Comparison of candidate-LEP to non-nociceptive somatosensory potential Auditory evoked potential

The original plan of research in the determination of a bovine LEP proposed to evoke a mechanical, non-noxious, somatosensory potential. The device was to be named a tapper and time-locked to the EEG acquisition device. The plan was to administer a tap of known pressure to the shoulder of a subject cow to provide data resulting from non-nociceptive mechanical stimuli to compare statistically, if possible, to the LEP. After considerable investment in time and money involving three or more specially built devices, this idea had to be abandoned. Problems associated with a coincident trigger noise at stimulus application could not be abated in spite of the best efforts of a number of experts.

Instead of using the planned tapping device and recording

somatosensory-based EEG data, as an alternative, an auditory evoked potential (AEP) was recorded in three cows. The sound stimuli were generated from noise made by the laser when firing. (Later, when recording EEG for the LEP, the laser's speaker was disabled to remove this noise as a factor.)

It was hypothesized that properties of the AEP should approximate the N1 (Hyde, 1997) in humans. It was also hypothesized that the time from stimulus to peak amplitude should be shorter in the AEP than the LEP because the AEP and NI are exogenous potentials, meaning that they are evoked even when the stimulus is not consciously perceived and are sensitive to stimulus intensity.

Method of determination of AEP

The laser was fired near the cow (as a mock laser stimulus), but the laser beam was not applied to her shoulder. The laser was held approximately 1 meter from the cow's ear. Continuous EEG was recorded. Three cows were used for recording AEP, with approximately 200 'beeps' recorded per cow. The data were collected and processed as described in Chapters III and V, and the time-averaged epochs were examined for potentials that could represent the AEP (Figure VI-1). The AEP were easily identified and extracted. These AEPs provide further evidence that neural signals are being acquired. Their peak latencies and peak amplitudes were approximated using the bootstrap method described in Chapter V. Although visually the peak latencies of the AEP and LEP are different, paired sample t-tests were made which verified this difference ($P<.05$). Thus, the temporal properties of

the candidate LEP and AEP varied by stimulus modality in the hypothesized way. This lends support to a contention that the measured LEP was cortical.

Comparison of the bovine and human N1

In humans the EEG recordings in association with any somatosensory stimulus is usually preceded temporally by a smaller potential that has been identified as the N1 (Figure VI-2). This smaller potential occurs at around 150 ms post-stimulus in humans and is correlated with stimulus properties alone (Hyde, 1997). The N1 amplitude increases with increasing stimulus intensity and conversely decreases with smaller stimulus intensities. The N1 is a non-cognitive potential and is considered to give no information about conscious stimulus perception (Palva et al., 2005) It was thus hypothesized that, in cattle, an NI was likely to exist in both perceptual categories, i.e., It was not clear that an N1 existed in data presented from all cows, but in at least three cows, a large potential occurred at around 200ms. The absence of an NI in any individual's EEG might be attributed to large artifacts generated by reflexive muscle movement immediately following the stimulus. These artifacts were overwhelming the NI. The peak minimum and maximum latencies and peak amplitudes for this candidate NI potential were estimated using the procedure for the LEP point estimates reported in Chapter V and subjected to two-tailed student t test.

Peak amplitudes did not change significantly (P<. 05) between behavioral conditions (based on low and high behavioral scores) and there were no significant differences between laser energy conditions. The

candidate NI is illustrated in Figure VI-2. The lack of correlation between peak amplitude and stimulus intensity could be explained by (1) excess post stimulus artifactual noise, (2) misclassification of trial as perceived or not perceived, or, (3) misclassification or wrongly recorded laser intensity. The fact that a potential occurs at the expected latency of NI strongly suggests that it exists. This potential was different to the candidate LEP in that its peak latency is shorter. Importantly, however, the candidate LEP occurred in a time frame considered to contain largely endogenous processes, and was modulated not by stimulus intensity, but by degree of perception.

Peak latencies of the LEP relative to stimulus-hemisphere relationship

Somatosensory stimuli are generally processed contra-laterally to the stimulus site. This directly implies that there should be a small, but real, difference in peak latencies between LEP when measured contra-laterally as compared to ipsilaterally to the stimulus site. There was no statistical significance in latency differences, but there was an average of 18 ms difference between peak latencies. There is no obvious non-biological physical explanation for this time lag. The comparisons given above providing some evidence for a neurological explanation for the candidate LEP.

Biological explanations for phase and frequency response

When neurons are operating in synchrony, their post-synaptic potentials, the potentials that are measured at the scalp surface, are also synchronized. The power of the predominant frequency underlying the potential will increase in power because there will be an increase in power localized in time. This is reflected in frequency changes underlying the ERPs and frequency changes induced by the stimulus.

When cortices globally synchronize, they will begin oscillating in increased synchrony at the frequency specific to the underling task or process. These power changes in a specific frequency may not be time-locked to the stimulus, but by synchronizing they are generating potentials at the same time, resulting in phase locking. Artifacts in a signal are not likely to demonstrate phase-locking because noise is generally considered to be stationary, with a constant mean and variance.

Conversely, some cortices and neuronal networks may be disrupted by particular stimuli, such as opening ones eyes. This disruption in network synchrony is reflected by a decrease in power of a specific frequency, and a shift or desynchronization in phases. A sudden decrease in a specific frequency's power relative to a stimulus could not be explained by movement or reflex because any movement generates power, not decreases it.

Explanation for differences

There seems little compelling evidence to attribute the measured bovine LEP to anything but underlying cortical activity. However, both cortical and non-cortical factors probably explain some of the main differences between human LEP and bovine LEP. The fact that peak latency is modulated by perceptual condition is interesting because it is relatively constant across conditions in humans.

Neuroanatomical differences between human and cow might underlie the differences, but further studies would be needed to confirm these propositions. Evoked response potentials do not result from unique cortical generators (Handy, 2005). They are a product of temporal and spatial summation of electrical potentials measured by the electrode from many possible cortical generators. The N2P2 is certainly generated from a complex network of generators (Garcia-Larrea et al., 2003). That this is the case is evident in the literature where intracranial electrodes are used to monitor multiple cortical generators of interest. For example, the N2P2 can be measured directly with intracortical electrodes in human patients in the anterior cingulate cortex (Rios et al., 1999), the insula (Iannetti et al., 2005), the amygdala (Liu et al., 2010) and the primary somatosensory cortices (Bushnell et al., 1999, Babiloni et al., 2004, Ohara et al., 2004a, Ohara et al., 2004b, Ohara et al., 2004c, Kuo, 2005). If the bovine generators differ, or are connected in different paths, latencies could vary. Other neuroanatomical explanations might exist. For example, in order to record a cortical potential with EEG, the pyramidal neurons generating the post-synaptic potentials

measured by the electrodes must be perpendicular to the electrode (Picton et al., 2000, Handy, 2005, Niedermeyer and Lopes da Silva, 2005). Whether or not this necessary condition is met for all the bovine LEP generators is not known, but may be a reason that the bovine LEP latency appears to be affected by level of perception. The bovine insula is not covered by cortex as is the human (Lakshminarasimhan, 1975b), and the brain is at a different orientation to the skull than humans due to the increased encephalization of humans over evolution (Lakshminarasimhan, 1975a). If the insula is, in fact, at a different orientation to the electrodes than it is to scalp electrodes in the human, it is possible that latencies and morphology could vary.

It is possible that inter-individual variation contributed to the latency differences. In humans, there is significant intra-individual variation of individual peak latencies, causing significant jitter (Hu et al., 2011), even within one test session. When there is jitter, individual potentials overlap, distorting amplitude and latency estimates. The general latency variance in bovine LEPs has not been quantified as it has in humans, and this sample only included six heifers.

Distribution of Aδ receptors and species-specific differences in threshold variation could affect peak latency. Laser stimuli uniquely excite nociceptors, but as a result of their individual threshold variances, a large synchronized afferent volley of electrical potentials such as occurs in an electrical stimulation of A-β (Treede et al., 1988) cannot be evoked in such magnitudes. The human LEP is exclusively related to the excitation of Aδ receptors (Treede et al., 1999). If the receptor density is low on the cow shoulder, as it is in the more distal parts of the human body (Truini et al., 2005),

a synchronized afferent volley is even more unlikely, thus contributing to differences in latencies in perceptual conditions. This is because, depending on where the laser was aimed, achieving an excitation threshold could take longer even though the stimuli were clearly perceived as noxious.

In human EEG, it has been recently possible to measure LEP amplitudes and latencies in individual epochs using techniques such as regression (Hu et al., 2011), matching pursuits (Ohara et al., 2004c), and other matrix-factorization methods such as blind source separation (Sutherland and Tang, 2006, Mouraux and Iannetti, 2009b), ICA (Arruda et al., 1996), PCA, and wavelet thresholding (Mouraux and Plaghki, 2004). This ability has enhanced analysis of human LEP, and allowed for more sophisticated experimental designs that can overcome inter-trial variation (Chen et al., 2007). These techniques have not yet been applied to bovine data, and some cannot be, due to the small number of electrodes used. However, if some of these techniques are applied to improve artifact rejection and allow individual epoch measurements, interaction or confounding might be discovered to explain the latency dependence on apparent the.

Although it is unlikely, due to the conservation of neocortical connections across evolution, that the insula is not critical for the generation of the bovine LEP, it might be that other structures are recruited in the bovine brain that are not in the human brain. The cortical structures involved in coding for the affective aspect of pain can vary across species. For example, in the rat, the primary somatosensory cortex is involved in hedonic coding of pain, whereas in carnivores and primates this cortex only seems to code for somatosensory aspects of pain (Sewards and Sewards, 2002), i.e., the

detecting of the 'what' and the 'where.' None of the above has been studied in the cow, and it is therefore difficult to present a valid explanation of why latencies are affected by perception as measured by behavioral response in the cow, and not the human.

Modulation of peak magnitudes

The critical functions attention and anxiety play in modulating peak amplitudes of a human LEP have been discussed in Chapter II and V. As the N2P2 amplitude is significantly modulated by cognitive states, mental tasks, anxiety, expectation and attention, spatially and with mental imagery, it would be useful to be able to test if this is the case in the bovine LEP. These factors are not possible to introduce, or control for, in any meaningful way when dealing with nonverbal subjects such as children or animals. One cannot ask a child or an animal to imagine, calculate or focus on a spatial location. An individual cows' state (as a result of environment) and trait (as a result of genetic expression), anxiety levels (Vossen et al., 2006), expectations, or lack of arousal cannot be assessed, although any of those factors will influence both the laser intensity needed to elicit a behavioral response, and the size of the LEP. In particular, fear and state anxiety are important affective modulators of human N2P2s (Kenntner-Mabiala and Pauli, 2005). Although using these affective and attentive states as conditions in bovine ERP protocols may not be viable, it may one day be possible that the anxiety or arousal levels of the cow might be inferred by EEG measurements, much as it can be in humans.

Central habituation to frequently repetitive stimuli, with constant inter-

stimulus intervals, even if they are nociceptive, may explain the small magnitudes, as it explains the slow deprecation of the somatosensory vertex potential in man with short (less than 900 ms) inter-stimulus intervals (Bromm and Treede, 1987, Mouraux, 2005). Central habituation, which can be thought of as a lack of novel stimuli, is said to occur when three conditions are satisfied (Thompson and Spencer, 1966). The first is that the amplitude decrease over time should follow a negative exponential function of the number of stimuli. Second, the amplitude should recover when a change of stimulus is inserted into a train of repetitive stimuli, and third, if the habituated stimulus is then re- presented, the amplitude of the potential related to the stimuli should again attain the original amplitude. It was not possible to confirm any of these conditions as recording time was short, driven only by the cows' tolerance to the stimuli and discomfort of being away from the herd. It was, however, noted that the stimulus intensity had to be altered frequently either due to lack of response, or too much response, requiring a period of at least three minutes to reset the time locking mechanism of the laser and EEG. Although no objective record of this was kept, it appeared that after a long period of rest, the cow was again more sensitive to the stimuli than before the recording break. It is therefore possible that there was central habituation occurring, which would account for smaller amplitudes over time.

Frequency responses in humans vs. bovine LEP

After laser stimuli, in humans there is an ERS between 1 to 5 Hz in the first 500 ms post-laser stimulus (Mouraux et al., 2003) at the vertex.

From here, it will be implied, unless stated otherwise, that any result reported in humans refers to the vertex electrode. In the bovine LEP, for electrode I the most significant ERS occurred not in theta (5 to 7 Hz), but delta (1 to 4 Hz), qualitatively appeared to have an ERS. In humans, there is alpha ERD post stimulus. This occurred, insignificantly, in electrodes III and IV, but, in contradiction to the human findings, there was significant ERS in the dorsal electrodes. This could have been a result of the low alpha power pre-stimulus in the perceived conditions, rather than an absence of ERD. In humans, alpha ERD in all sub-bands is significant pre-stimulus when there is anxiety and anticipation of a painful stimulus (Babiloni et al., 2003). It is quite likely that the cows were anxious and in some cases, they clearly anticipated the stimulus, which might explain why perceived trials have such a low alpha power pre-stimulus.

Increased anxiety increases the likelihood that there would be conscious adverse perception, which would be recorded as a perceived trial. The confound of the emotional states, stimulus energy level, and perception in these cows means it is difficult to say confidently that these results are contradictory to human results, or whether they would agree, if analogous mental states could be compared. When the human subject is distracted, they do not tend to report that the stimulus is painful, even if the energy is held constant. In that case, the alpha ERD is substantially, or completely abolished (Ohara et al., 2004a). In humans, an ERD in the alpha band might indicate a change of focus or attention in particular cortical regions (Klimesch et al., 1998, Laufs et al., 2003). The study in Chapter IV provides support that there are underlying similarities between human and

bovine cortex, at least in respect to primary sensory processing. It might be hypothesized, then, that the generalized alpha ERD seen in response to laser stimuli in the cow could be representing a change in cortical readiness, alertness, or orientation towards the noxious stimulus.

The argument that cows consciously perceive stimuli based on LEP

The argument presented herein is that the LEP when correlated with the behavior indicates that cows are aware of noxious stimuli, and find them aversive or painful. Their behavior indicates that their individual subjective mental experience is negative. We administered a laser stimulus that can be described as comparable to what the typical human would experience when a pin-prick is administered. And the cows exhibited both behavioral responses and recorded LEP responses that were measureable. Cattle that are used for food production, both dairy and beef, are subjected to experiences including castration, dehorning, branding, etc. These practices are administered to adult animals in some instances, and typically done without anesthesia.

In the case of the laser stimulus it is a noxious stimulus. While we currently do not have data associated with positive stimuli, we extrapolate (in part from reports on human ERP), that the bovine species has similar capacities.

If the stimulus was salient, then it was felt, regardless of the underlying neural networks that were recruited to produce the potential. It was shown that the stimulus was felt, via behavioral reaction, and the magnitude of the behavioral reaction correlated with features of the endogenous LEP. This

is compelling evidence that cows find painful stimuli distressing or aversive.

Conclusion

Large differences between human and bovine LEPs were determined, but herein we present evidence that this is likely the result of either cognitive or neuroanatomical differences. A conclusion that a bovine LEP exists and reflects a negative perception of the stimulus was reached. However, which of these explanations regarding inter-species LEP differences cannot be known without further study into bovine neuroanatomy and cognition. These differences may or may not have real consequences for bovine perception. Without the knowledge-base available for human cognitive science, i.e., accurate cortical maps, an understanding of cognitive roles specific cortices play, cortical connectedness, and source localization, the functional significance these differences must remain unknown, but deserving of further study.

Figures for Chapter VI

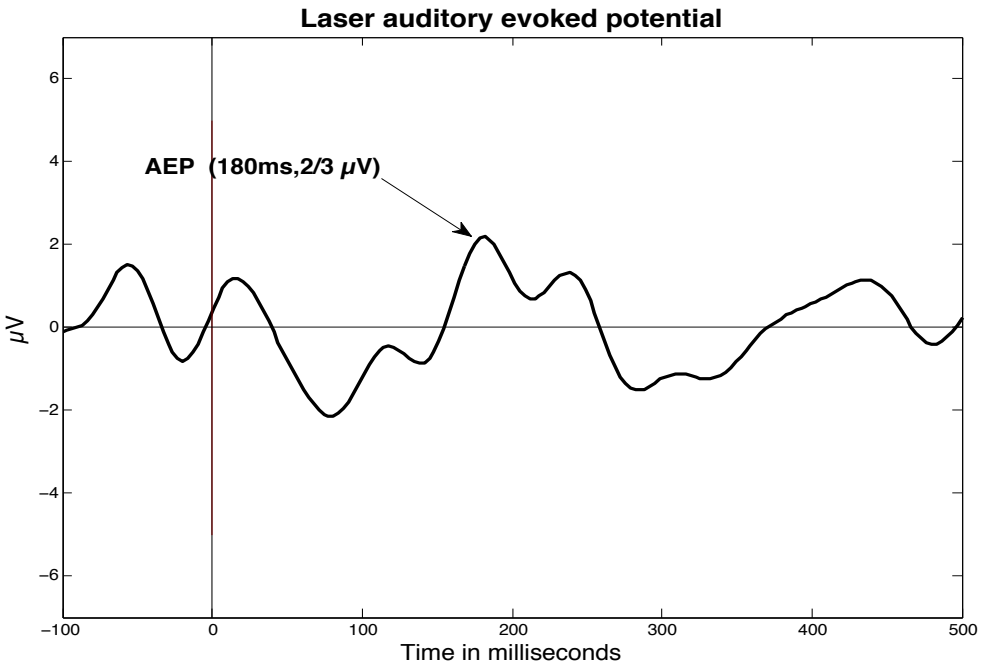

Figure VI-1. An auditory evoked potential from 3 cows. Notice the peak latency is different than that of the laser evoked potential

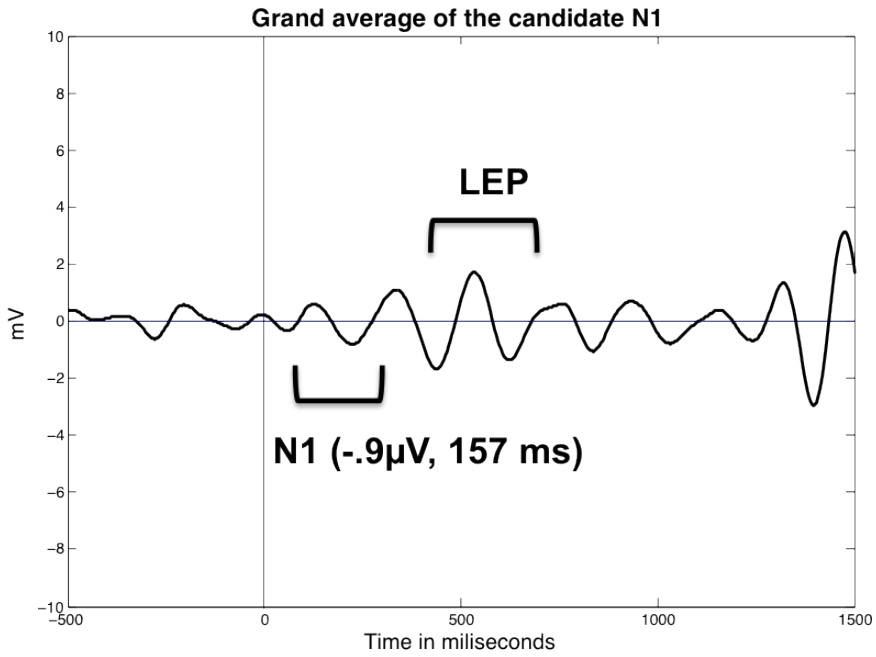

Figure VI-2. Plot of possible NI from a grand average across all electrodes contra-lateral to stimulus. The grand average laser evoked potential is marked for reference

Chapter VII. Discussion

This dissertation aimed to study pain in cattle with nociceptive laser stimuli and EEG. There is little known about the cognitive processes of the bovine cortex with only a handful of EEG studies having been done in research and clinical settings. Before attempting to collect data for an LEP study, a brief pilot study was done to ensure that what was being recorded was, in fact, cortical potentials. The bovine skull has large air filled sinuses and thick bone, and therefore, an initial concern was that cortical potentials could not be recorded with scalp electrodes. The initial pilot study aimed to measure frequency changes in continuous EEG in varying conditions of primary visual cortex stimulation. It was modeled on the eyes open/eyes closed procedure done in humans before data collection to ensure that electrodes are placed properly and that there is a reasonable signal. The pilot study revealed some surprising results, namely that the delta frequency band in the cow EEG has a much higher power than that of a waking human EEG. This led to further questions about bovine cognition and appropriate signal processing methods for bovine EEG. As the pilot study confirmed that all the frequencies in the bovine EEG was significantly decreased in conditions of primary visual cortex stimulation, the nociceptive study proceeded. However, coincidentally with preparing for the nociceptive study, a standardized methodology of recording and processing bovine EEG was developed. This discussion will be laid out according to the order of the studies that were done, concluding with the successful recording of a bovine LEP.

The initial aim of this dissertation was to investigate cortical processing

of nociceptive stimuli in cattle, and look for some quantitative method of objectively measuring pain in this species. Cows were chosen as the subjects for several reasons, both purely scientific, and for ethical reasons. About one billion cattle are used in the US every year in agriculture, and they are subjected to procedures that would be considered painful in humans, but not, in general, provided with either anesthetic or analgesic. Due to the number of animals used yearly, there is a significant ethical cost in continuing these policies if the bovine cortex is processing pain in a manner similar to the human cortex. In addition to ethical considerations, bovine cortical processing is of academic interest. A review or base of literature describing a normative bovine EEG does not exist, as it does it does for the human EEG. There is a long history of studying human EEG, beginning as early as 1929 when Hans Berger first discovered that cortical potentials could be measured from the scalp. From then on, human EEG has been used successfully as a method in cognitive science to study mental states such as arousal, how the human cortex processes speech, the degree of top down or bottom up processing involved in particular somatosensory stimuli, and much more. For thirty years researchers have used EEG and laser stimuli as a method to study (1) how pain is processed in the human cortex, i.e., which cortices support the perception of certain aspects of pain, (2) what factors affect the perception of noxious stimuli, (3) how effective analgesics are, and (4) dissecting the interaction of saliency of a stimulus, and the perception of it being nociceptive.

As discussed in the introduction and literature review of this dissertation, pain is a complex entity. Its perception is subjective, making it

difficult to objectively assess. It requires global cortical processing, and includes at least two significant aspects that are experienced when pain is perceived; a somatosensory component, and an affective, hedonic component that humans report as what constitutes 'suffering'. In fact, if the ACC is damaged, the human subjects do not tend to experience the negative emotions that accompany a nociceptive or painful stimulus. Although it is an important area of study for clinical reasons, particularly in non-verbal patients, and in the research field, it must be stated that, due to its subjective and personal nature, an individual's subjective experience may never be objectively measured. However, it may be possible to discover neurocorrelates of the subjective intensity that can aid in objectively assessing experience of pain, both human and animal.

Study of frequency changes in the bovine EEG in response to varying conditions of primary visual cortex stimulation

While the results of these studies vary, and in some instances demonstrate that the frequency changes in the bovine EEG in response to pain are similar, or even homologous over time (cortical generators excluded), there has never been the equivalent of Hans Berger's work, which was so foundational to using EEG in cognitive science in humans. Namely, describing the conscious resting EEG of a cow in time and frequency, frequency distribution over the scalp, and responses to cortical changes prompted by simple stimuli such as bright lights and total darkness. Hans Berger reported his findings, with little interpretation, except for the possible

meaning of the alpha scalp distribution, namely that it is highest in power over the visual cortex. Foundational knowledge, such as relationship between the distribution of frequencies over the scalp and cortical generators, which would eventually lead to the beginning of understanding cortical functional frequencies and the role that they may play in cognition from simple somatosensory perception to binding, has not been developed for the bovine EEG, or any large animal EEG of which I am aware. Furthermore, the type of foundational knowledge that exists for rat and primate EEG is largely established for biomedical purposes, rather than for species-specific interest.

In addition, many of the earlier studies in sheep and cattle, and those currently done in the biomedical arena, used indwelling electrodes. The signal to noise ratio for these EEG data were ideal, and allowed monitoring of specific cortices, but are not practical for use in the clinical setting, and do not allow large animal EEG studies to be widely done. Technically, this methodology is called electrocorticography (EcOG). While allowing for low noise data, it does not very well represent the cortical potentials that are measured on the scalp. Potentials measured on the scalp include temporal and spatial summations of post -synaptic potentials, and represent both volume conduction and changes in distant cortical oscillations. In humans, detailed cortical maps, knowledge of cortical interactions and underlying functional frequencies, and results from other neuroimaging methods mean that EcOG and scalp recordings can be better interpreted. Regarding the cow, there is no current cortical map or knowledge of cortical connectivity. Nor is there an understanding of the underlying functions that measured frequency or temporal changes represent. It is, therefore, difficult to interpret scalp -

recorded potentials in relation to ECoG data. The gaps in knowledge described above served as a primary motivation to measure EEG in cows in varying conditions that would cause a varying degree of primary cortical stimulation. It was hypothesized that if we were able to measure cortical potentials, the EEG frequencies would show a directional power change when the cow was in absolute darkness (low cortical stimulation), and then exposed to bright light (high cortical stimulation). It was further hypothesized that these changes would be identical or very similar to those found in the human EEG when cortical stimulation is low, and then increased by opening the eyes. The results confirmed the hypotheses, we also found some surprising results that as of yet, remain to be explained, and have been discussed in Chapters V and VI. This study, however, is important because it provides original results and one of the first foundational building blocks that will be important in future bovine cognitive studies.

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Drnec, Kim	PhD Candidate, University of Maryland, College Park

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Royal Veterinary College, University of London	BVetMed, MRCVS	1992	Veterinary Medicine
Royal College of Veterinary Surgeons	Certificate in animal welfare, ethics and law	1999	Ethics, Law and Animal Welfare Assessment
Johns Hopkins University School of Public Health	MPH	2002	Bioethics/environmental health/biostatistics

A. Work Experience

1992 Researcher, Overseas Development Agency, WHO

1992-1994 Elm Cottage Veterinary Centre, Torquay, UK.
Veterinary internal medicine
Official Veterinary Surgeon for Devon Abattoir

1994-1996 Veterinary Officer for Genus Breeding, Milton Keynes, UK.
Responsible for compliance with Ministry of Agriculture, Fishery and Foods regulations
Developed the first fixed-time, artificial insemination program for horses.
Commissioned the research at the Royal Veterinary College and analyzed results to create the program for Genus Breeding.

1996-2000 Veterinarian and Director with Minister Maneka Gandhi charity in India serving Old Delhi.

Taught animal welfare at a local private high school. Also served as the veterinarian for the Old Delhi Society for the Protection of Animals

2000-2002 Johns Hopkins University, Dept. of Environmental Health; OSHA Graduate Assistantship.

2008-2009 Instructor for American College for Applied Sciences, Crescent City, Florida
Taught online course in Veterinary Psycopharmacology in M.Sc. Program (4 semesters); 5-10 students per semester

Present Doctoral Candidate, University of Maryland, Neuro &Cognitive Sciences (NACS) Program,
Department of Animal Sciences (PhD anticipated 5/2013)

Co-Instructor:
ANSC453 (Animal Welfare & Bioethics): 2008-present.
HONR238F (Roles of Applied &Cognitive Ethology in Animal Welfare): 2008-present.

2009-present Co- Investigator: EEG and Nociception in Cattle ($265,000; USDA AFRI Grant No. 2009 65120-05791)

B. Scientific society memberships
American Society of Animal Science (ASAS)
Society for Neuroscience (SFN)
International Society for Applied Ethology (ISAE)
Sigma Xi
MRCVS (Member of the Royal College of Veterinary Surgeons)/British Veterinary Association (BVA)

C. Publications and Presentations
Drnec, K. 1996 Fixed Time Breeding in Mares, British Equine Veterinary Association (Speaker Series)
Drnec, K. 1996 Crestar Fixed Time AI in Cattle (Speaker Series)
Drnec, K. 1996 Invited Lecture Cambridge University, Reproduction
Drnec, K. 1997 Artificial Insemination in Horses. Vet Rec. 140(2):52
Drnec, K. 1998 Vitamin D Deficiency in a Shar Pei. Drnec K. Vet Rec. 143(9):260.
Drnec. K. 2002 Pet Dog Epidemiology as a Sentinel for Environmental Health Diseases.
Goldberg, A. and Drnec, K. 2003 Alternatives: Refinement, Reduction and Replacement of Animal Use in the Life Sciences. Chapter 22 Handbook of Laboratory Science. 2nd ed. Hau, J.& Van Hoosier, G.(eds.)
Drnec, K. The non-invasive use of pet dogs in Reducing, Refining, or Replacing animal tests for regulatory testing. J.Appl Anim Welfare Sci.
Kim Drnec, Brad Hatfield, K.S. Schwartzkopf-Genswein and W.R. Stricklin. 2008

EEG and Nociceptive Processing in Cattle. Universities Federation for Animal Welfare. Conference on Recent Advances in Animal Welfare Science. **Birmingham. (Abstract).**

Kim Drnec, W.R. Stricklin. 2010 Changes in Spectral Baseline EEG in Cattle During Conditions Requiring Varying Levels of Primary Visual Processing. Poster for 44th International Society for Applied Ethology. conference, Uppsala Sweden

Drnec, K., Stricklin, W.R. 2011 EEG and Nociceptive Processing in Cattle for USDA-NIFA Washington DC (poster)