Investigating the electrical response of the brain of the domestic chicken (*Gallus gallus domesticus*) to nociception through the use of depth electroencephalography (dEEG)

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Abstract

Nociception is an unavoidable side effect of many routine management and clinical procedures in animals. Electroencephalography (EEG) has previously been used to investigate the effect of nociception on mammalian brain activity. This study aimed to develop a method of assessing the avian response to nociception through depth electroencephalography (dEEG) of brain regions believed to be involved in central pain processing. Two groups of chickens were used in this study to investigate two brain regions, the rostral hyperpallium apicale (HA) and the caudomedial nidopallium (NCM). These regions were chosen due to the afferent and efferent projections they receive from the sensory thalamus and their previous implication in pain processing. Subjects were anaesthetised, and a concentric needle electrode was inserted into the brain to record the electrical activity in response to a number of stimuli. These stimuli included one non-painful, somatosensory stimulus, and four nociceptive stimuli (mechanical, thermal, feather removal and electrical). The dEEG data was then run through a spectral analyser which generated the median frequency (F50), spectral edge frequency (F95) and total power (P_{TOT}). Inspection of these variables determined that within the HA there were two populations of birds, therefore these birds were treated as separate groups in the analysis (hHA and lHA).

It was seen that spectral characteristics of the three groups investigated differed significantly, indicating differences in activity and function. The response to stimulation was seen to be significantly different between these brain regions. Following stimulation, the hHA was seen to have a significantly lower percentage of baseline spectral edge frequency and median frequency compared to the NCM and lHA. In response to stimulation the activity of the NCM and lHA remained constant and showed no distinguishable response, while the hHA was more variable. The hHA was much more variable. Although there was no consistent response to stimulation, there was a significant decrease in total power following electrical stimulation in the hHA.

This study presents a number of interesting findings and demonstrates that different regions of the brain respond in differing ways to stimulation. The findings suggest that the hyperpallium apicale may respond to nociceptive stimulation, however further work is required to distinguish this. The presence of two populations within the HA group suggests that recordings were taken from two distinct brain regions, one of which displayed comparatively higher sensitivity to nociceptive stimulation. Elucidation of this brain region and further research into the response to nociception is required to further understand the

response of the avian brain to pain. For future studies, the development of more precise methods will be required to enable more accurate recording of the activity occurring throughout the avian brain.

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Abbreviations

ACC – Anterior cingulate cortex

EEG – Electroencephalogram

dEEG – Depth electroencephalogram

FFT – Fast Fourier transformation

F50 – Median frequency

F95 – Spectral edge frequency

hHA –'High' Hyperpallium apicale

lHA – 'Low' Hyperpallium apicale

NCM – Caudomedial nidopallium

P_{TOT} – Total Power

S1 – Primary somatosensory cortex

S2 – Secondary somatosensory cortex

1. Literature review

1.1. Introduction

The assessment of pain is an important issue in the treatment and welfare of animals. It is likely that the experience of pain differs between species due to vast differences between animals. Mammalian pain is well understood due to similarities in brain structure between humans and other mammals, yet pain in bird species remains an area of limited understanding. It is difficult to study pain in animals, as it is an inherently subjective experience and animals lack the ability to verbally express such experiences. Therefore, pain must be assessed through studying physiological and behavioural responses to nociceptive stimuli. Electrophysiological recordings of the brain have been used in mammals to assess the brains response to pain, but this has proven difficult in birds. Successfully recording the electrical activity of the avian brain may provide a means of investigating the pain associated with potentially painful treatments and procedures, and could also be used to test the efficacy of analgesic drugs used on birds. This knowledge is important in order to maintain and improve the welfare of avian species in clinical, agricultural and domestic environments. While it has been shown that birds are capable of experiencing pain, the extent of this experience and the central brain regions involved in the integration of avian pain remain unclear, making electrophysiological investigations difficult. The effect of pain on the avian brain and the use of electrophysiological techniques to measure pain shall be investigated. The comparative anatomy of the avian and mammalian brain, the physiology of pain in avian species and the response of the brain to pain shall be thoroughly reviewed.

1.2. Animal welfare

Animal welfare refers to the physical and emotional state of an animal, which results from the inputs of both its external and internal environment (Mellor, Patterson-Kane, & Stafford, 2009). It is the responsibility of those who care for animals to maintain their welfare to an acceptable level. Under the New Zealand Animal Welfare Act (1999), in order to maintain animal welfare, it is suggested that animals should be provided with the ‘Five Freedoms’. An important aspect of animal welfare is freedom from pain. In order to maintain animal welfare, it is important to manage pain and keep it to a minimum ("Animal Welfare Act," 1999). While an animal’s welfare cannot be measured directly, it can be assessed using a number of indicators (Mellor et al., 2009). However, difficulties arise in trying to quantitatively assess pain in animals and differences between animals may mean that the perception of pain varies

between animal groups (Le Bars, Gozariu, & Cadden, 2001). Pain in animals can be assessed through monitoring physiological and behavioural responses to nociceptive stimuli (Woolley & Gentle, 1987). In mammals, pain is well studied due to similarities between the brain structure of humans and other mammals. However, the avian brain structure differs significantly from that of mammals, making studying pain in birds more difficult.

1.3. Pain

1.3.1. Transmission and integration of nociceptive information

In order to fully understand the processing of pain, one must first have knowledge surrounding the transmission and integration of nociceptive information and pain. Pain is defined as a sensory or emotional experience associated with real or potential injuries (Almeida, Roizenblatt, & Tufik, 2004). While pain involves the emotional and conscious processing of noxious stimulation, nociception is the processing of noxious stimuli that occurs in both the peripheral and central nervous system (Criado, 2010). Pain occurs due to the processing of nociceptive information by higher brain centres, whereas nociception can occur in the absence of pain (Criado, 2010). The first step in the transmission of nociceptive information is the activation of peripheral sensory receptors known as nociceptors (Almeida et al., 2004). These are high threshold receptors which will generally only respond to a stimulus that is intense enough to be considered noxious (Basbaum, Bautista, Scherrer, & Julius, 2009). When these receptors are activated, an action potential is propagated along afferent fibres which convey noxious information to the dorsal horn of the spinal cord (Almeida et al., 2004; Doubell, Mannion, & Woolf, 1999). Within the spinal cord, these primary afferents synapse with the intrinsic neurons of the dorsal horn (Doubell et al., 1999). The intrinsic neurons of the dorsal horn are responsible for the integration of afferent and efferent nociceptive stimuli and therefore have an effect on the modulation of the nociceptive signal (Almeida et al., 2004). From the dorsal horn, these intrinsic neurons transmit the nociceptive signal to supraspinal structures such as the brain stem and thalamus (Almeida et al., 2004). From the brain stem and thalamus, nociceptive information is distributed to the cortical brain structures involved in the processing and integration of nociception and pain (Basbaum et al., 2009). While there is no single brain region for the processing of pain, a group of brain regions are involved in the processing of nociceptive information and are thus involved in the perception of pain (Apkarian, Bushnell, Treede, & Zubieta, 2005; Basbaum et al., 2009). While these regions have been studied in mammals, the regions of the avian brain involved in nociceptive processing remain unknown.

1.3.2. Brain regions involved in pain

In the mammalian brain, there is a group of cortical and subcortical structures that are activated in response to noxious stimulation (Legrain, Iannetti, Plaghki, & Mouraux, 2011). These structures are known as the 'pain matrix' and are believed to be involved in the integration of nociceptive stimuli and perception of pain (Legrain et al., 2011; Mouraux, Diukova, Lee, Wise, & Iannetti, 2011). While a number of areas are implicated in the pain matrix, the most common that are seen to be activated in response to nociception are the primary and secondary somatosensory cortices (S1 & S2), the cingulate cortex and the insular cortex (Legrain et al., 2011). The anterior cingulate cortex (ACC) is highly implicated in pain perception due to its activity in response to noxious stimulation (Derbyshire et al., 1997). The ACC is believed to be mediate the emotional response to pain due to its involvement in emotion (Vogt, 2005). The insular cortex is also implicated in pain processing and the intensity of its activation is correlated to the intensity of stimulation, leading to the belief that the insular cortex plays a role in determining the intensity of pain (Derbyshire et al., 1997). The insular cortex receives reciprocal inputs from a number of other areas of the pain matrix which means it is well situated to integrate both emotional and sensory aspects of pain (Derbyshire et al., 1997). Along with these regions, the somatosensory cortices are also often implicated in the integration of noxious stimuli (Derbyshire et al., 1997). These regions receive inputs from the thalamus which provides information required for their roles in sensory processing (Medina & Reiner, 2000). The primary somatosensory cortex (S1) receives inputs from regions of the thalamus that contain nociceptive neurons which provide nociceptive information to the somatosensory cortex (Bushnell et al., 1999). Studies have shown that destruction of the somatosensory cortex causes an impaired ability to localise pain without affecting the emotional aspect of pain, implicating the somatosensory cortices in the sensory component and localisation of pain (Vogt, 2005). These regions make up the mammalian pain matrix, while the regions of the avian brain implicated in pain processing are less well known. This is due to differences in the structure of the avian brain when compared to the mammalian brain.

1.4. Avian brain structure

The avian brain differs from the mammalian brain in regards to the organisation of the forebrain, with the notable difference being that the avian telencephalon lacks a laminated cortex (Güntürkün, 2005). While the organisation of the basal ganglia is similar between birds and mammals, the organisation of the forebrain is much more variable (Güntürkün, 2005). The mammalian forebrain contains a laminated neocortex, while the avian forebrain lacks such

layered organisation and contains an enlarged pallium (Figure 1). The avian forebrain is characterised by fields of cells with nuclear clusters and a thin overlying cortex, rather than a laminar arrangement like that seen in mammals (Benowitz, 1980). This initially led researchers to believe that the avian brain was a primitive structure and that birds were incapable of performing advanced cognitive functions (Jarvis et al., 2005). It has only been in the past few decades, through advances in technology, that the complexity of the avian brain has been realised (Reiner, Perkel, Mello, & Jarvis, 2004). It has been found that, despite anatomical differences in brain structure, the avian brain contains complex regions homologous to those seen in the mammalian brain (Jarvis et al., 2005; Reiner et al., 2004). The avian brain has been found to be capable of performing functions similar to, and often exceeding, those of the mammalian brain despite these morphological differences (Jarvis et al., 2005). Included in these functions is believed to be the ability of birds to perceive painful stimuli (Machin, 2005).

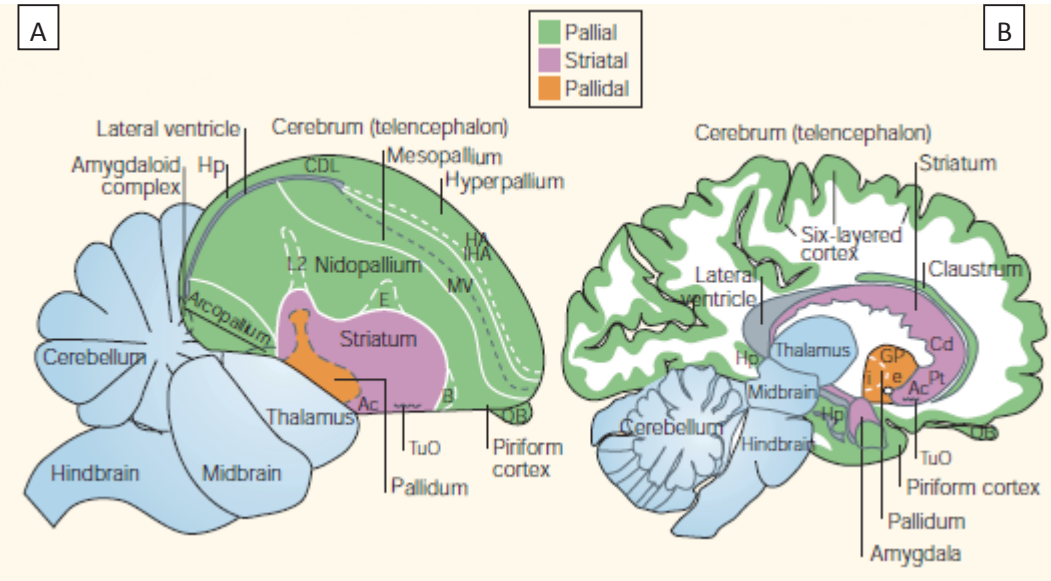

Figure 1. Comparative view of the avian (Zebra Finch, A) and mammalian (Human, B) brain to illustrate the differences in the structure and organisation of the brain (From Jarvis *et al*., 2005).

1.4.1. Avian pain centres

While regions of the mammalian brain have been strongly implicated in the integration of pain, the homologous regions of the avian brain are yet to be determined. The mechanisms required for nociception are present in avian species, from nociceptors to a behavioural response to nociceptive stimuli (Gentle, 1992). This would indicate that a central region, or regions, of the avian brain must exist in order to integrate nociceptive stimuli (Kuenzel, 2007). While the avian brain differs from the mammalian brain in its structure, a number of homologues exist

between the two (Csillag & Montagnese, 2005; Harris, 2015; Medina & Reiner, 2000). For example, the expression of genes in the embryonic telencephalon has suggested the homology of the avian hyperpallium with the medial, superior parts of the mammalian neocortex (Medina & Reiner, 2000). The connections of the hyperpallium suggest that it contains a primary visual and primary somatosensory-somatomotor area that are likely to be homologous with their mammalian counterparts (Medina & Reiner, 2000). It is possible that homologues in the avian brain play a similar role in pain processing as the mammalian pain matrix, therefore identifying these homologues may help identify avian brain regions responsible for nociceptive processing (Jarvis et al., 2005). Some studies have indicated that the avian nidopallium is the corresponding structure to the mammalian pain centres (Lierz & Korbel, 2012). Other studies have found sensory inputs from the body to brain regions such as the rostral hyperpallium apicale (HA) and caudomedial nidopallium (NCM) which may suggest a role for these regions in pain integration (Kuenzel, 2007). The sensory projections of the body to these brain regions are shown in Figure 2. These regions receive inputs from the thalamic nuclei, similar to the pain regions seen in the mammalian brain (Kuenzel, 2007).

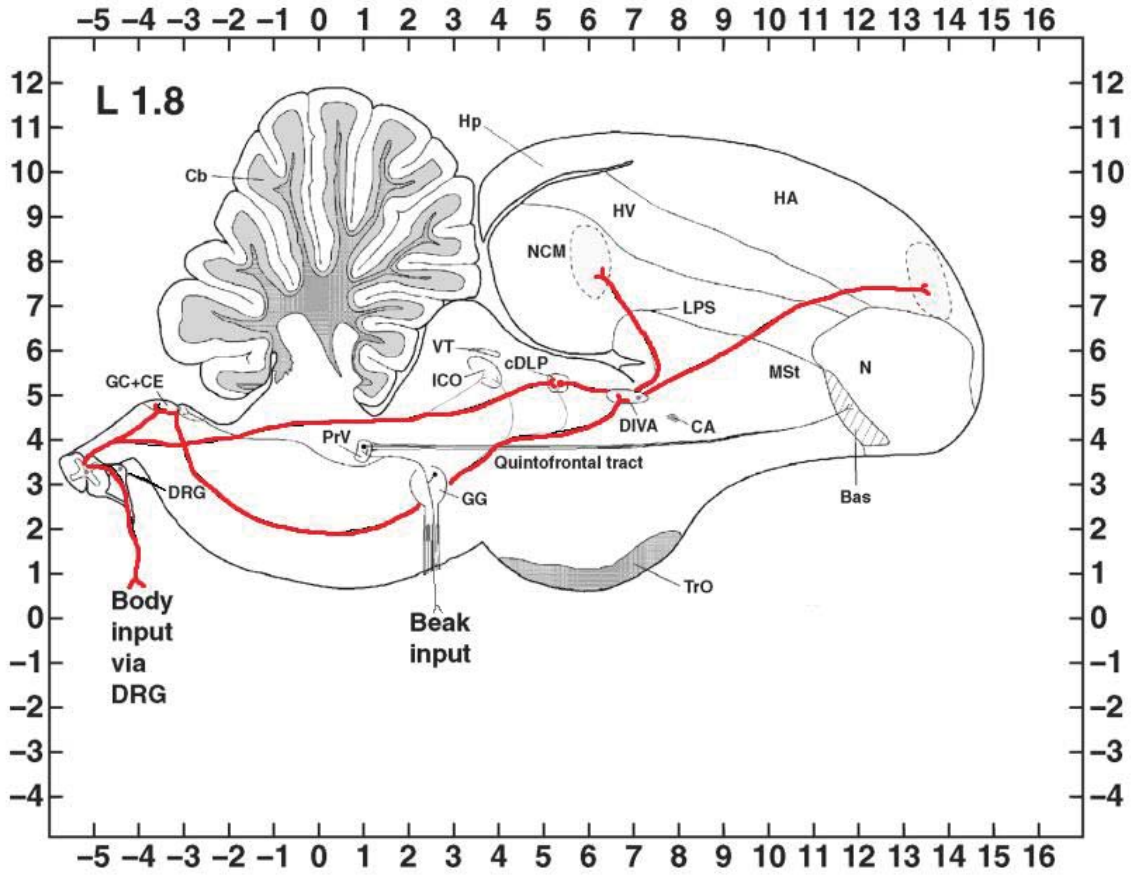

Figure 2. Sagittal section of the avian brain showing sensory inputs from the body throughout the brain (red). Sensory inputs from the body enter through the dorsal root ganglia (DRG), through the thalamic nuclei (DIVA and cDLP) to the hyperpallium apicale (HA) and the caudomedial nidopallium (NCM). (Adapted from Kuenzel (2007).

1.4.2. Hyperpallium

The avian hyperpallium is a region of the brain that receives visual, auditory and somatosensory inputs from the thalamus (Reiner, Yamamoto, & Karten, 2005). Unlike the mammalian neocortex, the hyperpallium is non-layered and has a columnar organisation (Montiel & Molnár, 2013). Despite the differences in organisation, the hyperpallium has been found to contain regions that resemble the mammalian visual, somatosensory, and somatomotor areas (V1, S1 & M1) (Medina & Reiner, 2000). The hyperpallium contains 2 separate sensory areas; the larger, caudal, primary visual area (V1) and the smaller, rostral somatosensory hyperpallium (S1) (Reiner et al., 2005). The rostral hyperpallium receives somatosensory inputs from the ventral tier of the thalamus, much like the mammalian S1 (Medina & Reiner, 2000). The avian S1 receives inputs from the nucleus dorsalis intermedius ventralis anterior (DIVA) and the caudal nucleus dorsolateralis posterior (cDLP) of the thalamus (Csillag & Montagnese, 2005; Wild & Williams, 2000). These constitute the afferent projections to the hyperpallium, while the efferent projections from the avian S1 resemble those of the mammalian S1 (Medina & Reiner, 2000). Efferent projections from the hyperpallium include targets such as the DIVA, the basal ganglia and the reticular formation of the brain stem (Medina & Reiner, 2000; Wild & Williams, 1999). High threshold sensory neurons present in the mammalian S1 respond to painful stimuli, and it is likely that this is the case in the avian S1 also (Legrain et al., 2011). The role of the avian S1 in pain remains to be seen, but it is likely, due to its similarities in connections and sensory functions to the mammalian S1, that it will play a role in central pain processing.

The hyperpallium is a non-layered structure that has a columnar 'pseudolayered' organisation (Montiel & Molnár, 2013). Rather than cortices, the avian pallial territory is characterised by the organisation of nuclear cell masses, and this is seen in the hyperpallium (Suárez, Dávila, Real, Guirado, & Medina, 2006). The hyperpallium can be arranged into four distinct regions (Srivastava & Gaur, 2013). These four regions are the hyperpallium apicale (HA), interstitial nucleus of the hyperpallium apicale (IHA), hyperpallium intercalatum (HI) and the hyperpallium densocellulare (HD) (Montiel & Molnár, 2013). Of these, the HA is the most superficial region and is a broad layer of medium sized stellate cells which sits above a thin granule layer of cells, the interstitial nucleus of the hyperpallium apicale (IHA) (Montiel & Molnár, 2013). The IHA is the major target for projections into the hyperpallium from the thalamus and receives somatosensory inputs from the DIVA (Wild & Williams, 1999, 2000). The IHA projects to the HA, which is the major region for outputs from the hyperpallium, including somatosensory pathways (Güntürkün, Verhoye, De Groof, & Van der Linden, 2013). The IHA is difficult to

distinguish from the HA based on morphology alone and this has led to some researchers combining the two into an HA complex (Güntürkün et al., 2013). Due to the involvement of the HA complex in somatosensory processing and the reciprocal sensory inputs it receives from the thalamus, it is a prime target for investigations of avian pain integration.

1.4.3. Nidopallium

Another region of the avian brain which has been implicated in nociceptive processing is the nidopallium (Kuenzel, 2007). The nidopallium is one of the four major sub-divisions of the avian pallium (Jarvis et al., 2005). It has been found to contain nuclei that receive direct visual, auditory and somatosensory input from the thalamus (Karten, 1969; Karten, Hodos, Nauta, & Revzin, 1973). The nidopallium is believed to play a role in sensory processing similar to that of the layer 4 neurons in the sensory cortex of mammals (Karten, 1969). A number of studies have since reinforced the belief that both the hyperpallium and the nidopallium play roles in the processing of sensory information and motor control (Bonke, Scheich, & Langner, 1979; Güntürkün, 1991, 1996; Heil & Scheich, 1985). The avian nidopallium has been suggested as the corresponding structure to the mammalian pain centres (Lierz & Korbel, 2012). The nidopallium contains three major cell groups which receive sensory input from the thalamus (Reiner et al., 2004). The entopallium receives visual input from the thalamus, Field L receives auditory input from the thalamus and the nucleus basorostralis pallii receives trigeminal input from the sensory nucleus of the pons (Reiner et al., 2004). Of these cell groups, the field L cells are considered to be the region in the NCM (Reiner et al., 2004). The NCM also receives inputs from the DIVA of the thalamus which convey sensory inputs about the body from the dorsal root ganglia (Kuenzel, 2007). The NCM is particularly implicated in the avian auditory circuit and processing of species specific sounds and has also been implicated in pain integration (Kuenzel, 2007; Reiner et al., 2004). The field L cells of the NCM receive input from the nucleus ovoidalis of the thalamus (Wild, Karten, & Frost, 1993). Due to the sensory inputs into the NCM from the sensory thalamus, this region has been suggested as a region involved in pain perception and integration of nociceptive information (Kuenzel, 2007).

1.5. Brain response to pain

1.5.1. Electrophysiological recording

Through the normal activity and function of the brain, fluctuating electrical potentials are produced and can be recorded in order to give an indication of the underlying brain function (Markand, 2003). There are a variety of methods which are used in order to record such activity (Sanei, 2013). Electroencephalography (EEG) allows us to record the spontaneous

electrical changes that are occurring throughout the brain (da Silva, 1991). This method involves using from electrodes placed on the surface of the scalp to record the fluctuating potentials from within the brain (Teplan, 2002). Along with electroencephalography, other methods can also be used to record these electrical changes of the brain such as electrocorticography (ECoG), which records from electrodes in contact with the surface of the cortex, and depth electroencephalography (dEEG) which uses depth electrodes in order to record the electrical activity of deep brain structures from within the brain (Arroyo & Lesser, 1999; Quesney & Nicholas, 1999). Depth electroencephalography (dEEG) is thought to provide a more precise indication of the areas from which electrical activity arises when compared to recordings from the scalp or cortical surface which cover a considerable portion of the brain (Arroyo & Lesser, 1999). The electrical activity which occurs in the brain can be studied to infer a number of things about the underlying brain function (Markand, 2003). Surface recordings have been used to monitor anaesthetic depth and responses to nociception, while depth electroencephalography has mainly been used to assess the onset of seizures in epilepsy (Behrens et al., 1994; Murrell et al., 2003; Otto, 2008; Spencer, Spencer, Williamson, & Mattson, 1990). The use of dEEG gives a more precise localisation of seizure origin than EEG using surface electrodes (Spencer et al., 1990). Depth electrodes have been used to study the response to pain in the human brain and have been used to localise the source of evoked potentials following nociceptive stimulation (Frot, Garcia-Larrea, Guénot, & Mauguière, 2001). However, the effect of nociception on the brain has not been monitored using dEEG in animal models, instead such studies have mainly employed the use of scalp surface electrodes (Gibson et al., 2009; McIlhone, Beausoleil, Mellor, Mitchinson, & Johnson, 2011; Murrell et al., 2003). Such scalp electrodes give an indication of electrical activity which occurs over a large area of brain, however they will only pick up activity produced by large groups of active neurons (Teplan, 2002). Depth electrodes have been seen to detect electrical activity that is not detectable at the surface and may therefore be useful at monitoring changes in the brain that aren't seen by surface recordings alone (Cooper, Winter, Crow, & Walter, 1965; Teplan, 2002). For this reason, dEEG may provide new insights into the response of the brain to nociception by detecting changes that have previously gone unseen.

1.5.2. Electrical changes in response to nociception

Through the use of EEG it has been seen that changes in the electrical activity of the mammalian brain occur in response to nociception (Gibson, Johnson, Stafford, Mitchinson, & Mellor, 2007; Murrell & Johnson, 2006; Murrell et al., 2003). Through the mathematical process of Fast Fourier Transformation (FFT), raw EEG data can be transformed from the time

domain to the frequency domain (Murrell & Johnson, 2006). This produces a power spectrum (Figure 3) and generates numerical variables which can then be used to provide a quantitative tool to assess changes in EEG activity (Murrell & Johnson, 2006). Such variables include: the total power (P_{TOT}), which is the total area under the frequency spectrum curve; the median frequency (F50), the frequency below which 50% of the total power is located; and the spectral edge frequency (F95), the frequency below which 95% of the power is located. Following noxious stimulation in mammals there is generally an activation of the brain and an increase in high frequency activity causing an increase in F50 and F95, and a decrease in P_{TOT} (Gibson et al., 2009). The changes in the EEG have been used to assess the response of the brain to noxious stimuli in a number of mammalian species (Gibson et al., 2007; Murrell et al., 2003; Murrell et al., 2005)

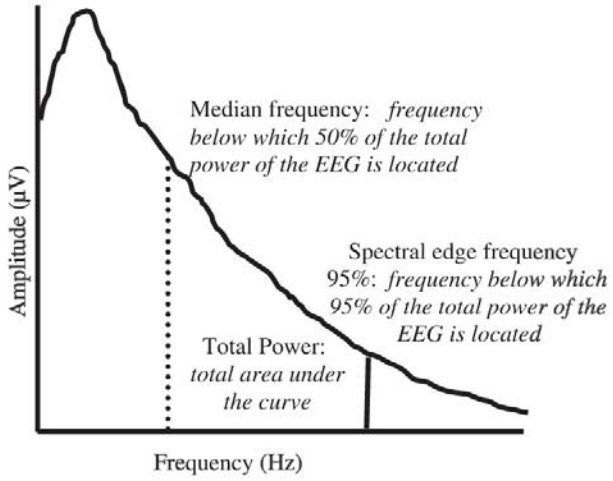

Figure 3. Representation of the power spectrum produced by Fast Fourier Transformation (FFT). The dashed line represents median frequency (F50), the solid line represents spectral edge frequency (F95) and the area under the curve gives the total power (From Murrell & Johnson, 2006)

1.5.3. Mammalian response to nociception

There has been a number of studies that have investigated the EEG following nociceptive stimulation in mammals (Bergamasco et al., 2011; Gibson et al., 2007; Murrell et al., 2003). Most commonly, the response to nociception is a change to high frequency, low amplitude EEG, resulting in an increase in median frequency and spectral edge frequency, while total power decreases (Gibson et al., 2007; Johnson et al., 2009; Murrell et al., 2003). For example, following the scoop dehorning of cattle, there was a significant increase in both the median frequency and spectral edge frequency (Gibson et al., 2007). This change in EEG was seen to be

eliminated when the subjects received an analgesic ring-block (Gibson et al., 2007). This study also found a decrease in the total power of the EEG following dehorning. These responses are similar to those seen in a number of previous studies. Castration of ponies caused an increase in median frequency, while castration of lambs produced an increase in both median frequency and spectral edge frequency and velvet removal in deer caused an increase in median and spectral edge frequency and a decrease in total power (Johnson et al., 2009; Johnson, Wilson, Woodbury, & Caulkett, 2005; Murrell et al., 2003). A study investigating the castration of conscious calves also found that there was an increase in higher frequencies following castration when compared to baseline values (Bergamasco et al., 2011).

While this change to high frequency, low amplitude EEG is most common, there may instead be a change to low frequency, high amplitude activity. For example, a study by Diesch, Mellor, Johnson, and Lentle (2009), demonstrated decreases in the median and spectral edge frequencies of the EEG of rat pups in response to tail clamping. While the EEG response to pain may differ slightly between species in mammals, it has been shown that identifiable changes occur in the EEG in response to nociceptive stimulation.

1.5.4. Avian response to nociception

Avian pain is believed to be analogous to pain felt by mammals (Machin, 2005). It was originally believed that birds could not experience pain due to the perceived simplicity of their nervous system (Lierz & Korbel, 2012). Birds often do not show behavioural signs of pain in obvious ways, however, a number of studies have shown through behavioural and physiological measurements that birds do feel pain (Gentle, 1992; Gentle & Hunter, 1991; Woolley & Gentle, 1987). Studies on the chicken have found behavioural responses to pain in the form of active avoidance or through passive immobility (Woolley & Gentle, 1987). These findings have been supported in a number of studies showing withdrawal responses of birds to a noxious stimulus (Geelen et al., 2013; Guzman, Drazenovich, Olsen, Willits, & Paul-Murphy, 2014). The majority of studies have shown behavioural responses to pain, while the neurological response to pain has not been thoroughly investigated.

There is a quantifiable change in the EEG power spectrum due to the cortical processing of nociceptive stimuli in mammalian species. Due to the similarities between avian pain and mammalian pain, it is believed that the avian brain would also respond to such stimuli with a change in electrical activity (Machin, 2005). Only a limited number of studies have previously investigated the avian EEG response to nociceptive stimulation (Gentle & Hunter, 1991; McIlhone et al., 2011; Woolley & Gentle, 1987). Early studies found a change in EEG activity

following nociceptive stimulation, however this change was likely due to a reduction in motor output or reduced vigilance seen during tonic immobility (Gentle & Hunter, 1991). There appeared to be no response in the EEG recordings that indicated an acute response to pain like that seen in the mammalian EEG (Gentle & Hunter, 1991). A similar study found that no change was seen in the EEG recordings of hens in response to nociceptive stimulation (Woolley & Gentle, 1987). A more recent study also investigated whether nociceptive stimuli cause a change in the EEG of the chicken (McIlhone et al., 2011). It was found that, following electrical stimulation, a change was seen in the EEG recording but no change was seen following stimulation by other nociceptive stimuli. It is unclear whether this finding was due to the nociceptive stimulation, as electricity causes non-specific activation of neurons that may result in increased brain activity (McIlhone et al., 2011). It is likely that difficulties in detecting changes in brain activity are due to the structure of the avian central nervous system. A lack of knowledge regarding the central brain regions of the avian brain associated with pain integration may make it difficult to record EEG responses like those seen in mammals.

1.6. Study objectives

Avian pain is an area of importance in terms of animal welfare. It is clear that compared to mammalian pain, avian pain is not very well understood. Due to the analogous nature of mammalian and avian pain, a central brain response to pain is expected in avian species. While the mammalian brain has been found to respond to nociceptive stimuli through electroencephalogram readings, such findings are yet to be seen in the avian brain. This is most likely due to the differing anatomy of the avian brain from that of mammals. In order to record a response of nociceptive stimulation on the brain, a different method is likely to be required. The objective of this study is to investigate the response of the avian brain to nociception through the use of dEEG to target brain regions thought to be involved in the central processing of pain in birds, the rostral hyperpallium apicale (HA) and the caudomedial nidopallium (NCM). Through recording the electrical activity from these brain regions, this study will investigate whether the avian dEEG will change in response to stimulation. The findings of this investigation will help to distinguish the brain regions responsible for pain integration in avian species and may also provide a means to more effectively test avian pain and could be used to determine the efficacy of analgesics. Such studies are important in order to improve the limited understanding surrounding the avian response to pain and central integration of nociceptive stimuli in the avian brain. This has particular importance in avian welfare in order to more effectively prevent pain during husbandry procedures and in the treatment of birds.

2. Material and Methods.

2.1. Animals/General care

This study used two groups of chickens for the two brain regions investigated. A set of 10 chickens were used for the hyperpallium apicale (HA), and 10 for the caudomedial nidopallium (NCM). Chickens were male white broiler chickens aged between 5-6 weeks and weighing between 1.99 and 3.67 kg. Animals were sourced from the Massey University Poultry Research and Feed Processing Unit. The subjects were kept at the poultry unit in ground pens with free access to mash and water. On the day of testing, chickens were transported from the poultry unit to the IVABS neurophysiology lab at Massey University. All treatments in this study were approved by the Massey University Animal Ethics Committee (MUAEC) and every effort was taken to minimise negative impacts on the subjects, in accordance with the MUAEC code of ethical conduct for the use of live animals for research, teaching and testing.

2.2. Preparation of study

2.2.1. Determination of brain regions

The rostral hyperpallium, in particular the hyperpallium apicale (HA), and the caudomedial nidopallium (NCM) were targeted in this study. The position of these regions in the avian brain was determined through the use of the stereotaxic atlases of the chickens brain (Kuenzel & Masson, 1988; Van Tienhoven & Juhasz, 1962). Through the use of these stereotaxic atlases, coordinates for these brain regions were determined in relation to a zero point. The co-ordinates for these regions are given in Table 1.

Table 1. The co-ordinates determined for the location of the brain regions to be targeted. Distances given are relative to a specified zero point.

	Rostral Distance (mm)	**Lateral Distance (mm)**	**Dorsal Distance (mm)**
Rostral Hyperpallium Apicale	13.8	1.5	7.0
Caudomedial Nidopallium	6.0	3.5	7.0

These coordinates were used to insert an electrode into the brain in order to record the electrical activity from these regions.

2.2.2. Stereotaxic positioning

The electrode placement was determined by the coordinates given in the previous section. In order to place the electrode into these regions, the head was first positioned in the stereotaxic apparatus. The stereotaxic apparatus used was the KOPF model 900 small animal stereotaxic instrument with 957 ear bars (David Kopf Instruments, Tujunga, CA, USA) and the head was positioned as outlined in "A Stereotaxic Atlas of the Brain of the Chick (*Gallus domesticus)*" (Kuenzel & Masson, 1988). In order to establish the correct orientation of the bird in the apparatus, the ear bars were positioned into the external auditory canals. The bars were inserted into the most posterior region of the auditory canals. Once the bars were inserted into the posterior, ventral region of the canal, the head was centralised in the apparatus with 12.2 ± 0.45 mm between the ear bars. The angle of the head was then adjusted in order to ensure the correct orientation, with the plane of the skull parallel to the surface of the apparatus. In this position, the bregma of the skull was anterior to the ear bars. This could be determined when an incision was made to expose the calvarium. The correct positioning of the head in the apparatus is shown in Figure 4.

Figure 4. The correct positioning of the head in the stereotaxic apparatus. The ear bars are inserted into the ventro-posterior aspect of the auditory canals, the head is centralised in the apparatus and the plane of the skull is parallel to the surface of the apparatus

2.3. Experimental procedure

2.3.1. Anaesthesia

Anaesthesia was induced using halothane vaporised in oxygen, delivered through a facemask. Halothane was delivered at a rate of 0.25–3% in 2–4 $Lmin^{-1}$ oxygen and was maintained until the righting reflex was lost. In order to desensitise the larynx, 0.1–0.2 mL lignocaine local anaesthetic

was applied to the back of the throat. Subjects were then intubated using an endotracheal tube between 3.5–4.5 mm diameter. Anaesthesia was maintained using a t-piece non-rebreathing anaesthetic circuit and subjects were kept ventilated using an intermittent positive pressure ventilation (IPPV) system. Anaesthesia was monitored using an anaesthetic monitor which recorded inspired and end-tidal gas concentrations, respiration rate, oxygen saturation, heart rate and temperature. End tidal halothane was monitored as an indicator of anaesthetic depth and was maintained between 0.9–1.2%. Chickens were placed on a heated water blanket (Gaymar T-pump & MUL-T-PAD; Gaymar Industries Inc, Orchard Park, NY, USA) maintained at approx. 38°C in order to limit heat loss during the experiment. Throughout the course of anaesthesia, body temperature was monitored through a rectal thermometer. Temperature was recorded and mean temperature was seen to be stable throughout anaesthesia (41 ± 0.2 °C). Once anaesthesia was stable, the chicken was then placed in the stereotaxic apparatus as outlined in *Stereotaxic Positioning.*

2.3.2. Electrode placement

Following anaesthesia and positioning of the subject in the stereotaxic apparatus, a concentric needle electrode was positioned into the brain in order to begin recording of the electrical brain activity. In order to do this, the epidermis was cut along the midline of the dorsal skull, exposing the calvarium. Once the calvarium was exposed, it could be determined if the lateral bregma was anterior to the ear bars. This indicated correct positioning of the head in the apparatus. The angle of the head in the apparatus was adjusted in order to ensure that it was parallel to the base of the apparatus. A centred needle in the stereotaxic apparatus was used to mark the entry point of the drill using Indian ink. Positioning of this entry point was determined as outlined in Section 2.2.1. A small hole was drilled in the skull using a 50/60 Hz, Series CC drill (Foredom Electric Co, Bethel, CT, USA) to allow clear entry of the electrode into the brain. The stereotaxic apparatus was used to insert a TECA elite disposable concentric needle electrode (Natus Neurology Incorporated, Middleton, WI, USA) into the brain in one of the two brain regions being studied. Due to differences in the size and positioning of the head, the depth at which the needle needed to be positioned was difficult to determine. In order to get the correct depth of the electrode into the brain, the needle was visually positioned at the surface of the brain and inserted to a depth of 2 mm for the HA and 3 mm for the NCM. The height of the electrode above the zero point was recorded with the average being 11.36 mm ± 0.42 mm for the HA, and 9.74 mm ± 0.24 mm for the NCM. Once the electrode was positioned, recording of the electrical activity from within the brain region began.

2.3.3. Recording electrical brain activity

Electrical activity was recorded in chart recording software (LabChart 7.2.5, ADinstruments, Dunedin, New Zealand). The electrode was connected to a break out box which was itself connected to a biological signal amplifier at a signal gain of 1000 at frequencies between 0.1 Hz–0.5 kHz (Iso-Dam isolated biological amplifier, World Precision Instruments Inc, Sarasota, FL, USA). This amplifier was connected to an analogue-to-digital converter (Powerlab 16/30, ADinstruments, Dunedin, New Zealand) which digitised the inputs from the amplifier and displayed and recorded them on an Apple computer in the chart recording software.

2.3.4. Treatments

The recording was first left to stabilise in order to eliminate any background interference and to produce a consistent recording. Once the electrical recording was stable, baseline activity was recorded for 15 minutes. After these 15 minutes, the first stimulus was applied. This was a non-painful somatosensory stimulus applied to the pad of the foot for 5 seconds using a pair of haemostat forceps. Following stimulation, 10 minutes of electrical activity was then recorded. The non-painful stimulation was the first performed to ensure there was no sensitisation which may occur following nociceptive stimulation. Following this recording, the first of four nociceptive stimuli was applied. This was either a mechanical, thermal or feather removal stimulus applied in a randomised order. Mechanical stimulation was applied by a toe clamp applied for 5 seconds to the middle tarsal. Thermal stimulation was applied using a copper rod heated in a Techne TE-10A Tempette water bath (Bibby Scientific Limited, Staffordshire, UK) set to 90°C. The temperature of the rod was checked prior to application using a Dick Smith electronics digital thermometer to ensure the temperature was at 55°C. This was applied to the skin beneath the wing for 5 seconds. Feather pluck stimulus was performed by the removal of a feather from the base of the wing. Lastly, an electrical stimulus was applied using an S48 Stimulator (Astro-Med Inc, West Warwick, RI, USA) which delivered a 50 V, 50 Hz stimulus for 2 seconds. The electrical activity was recorded during these stimulations and for the 10 minutes following before application of the next stimulus. This was repeated for all four stimuli, with the electrical stimulus being the final manipulation. Following these manipulations, subjects were euthanased by an overdose of intravenous sodium pentabarbitone.

Indian ink was injected into the brain in order to determine the position of the electrode within the brain and to determine the accuracy of the brain coordinates (Appendix A). A Hamilton syringe was centred in the stereotaxic apparatus and used to deliver 1 µl of black Indian ink into the brain at the coordinates previously specified. The brain was then removed from the skull

and soaked in 10% buffered formalin for fixation. Following fixation, the brain was sliced and the position of the ink identified (Figure 5). This gave an indication whether the electrode placement into the specified brain regions was correct. Through this method, it was determined that the electrode was being inserted into the correct brain regions and therefore that recordings were being taken from the targeted brain region.

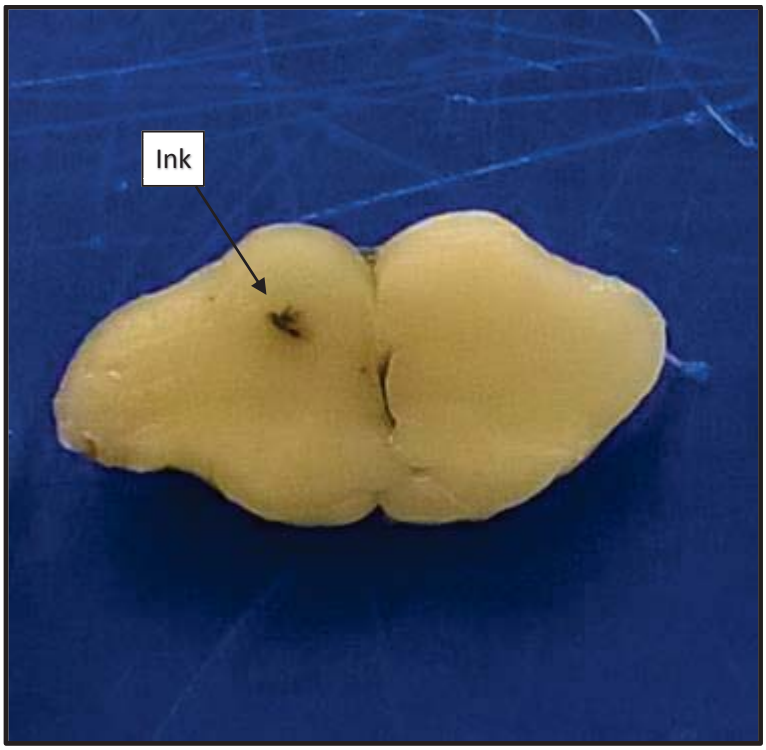

Figure 5. An example of a brain slice showing ink injected into the correct area for the hyperpallium apicale (HA).

2.4. Analysis

2.4.1. Analysis of electrical activity

Following recording, the data was stored on a personal Apple computer and analysed following completion of the experiments. The recordings were visually inspected, in order to remove any artefacts from the recordings that were not caused by the activity of the brain. Any artefacts of considerably high or low amplitude were eliminated, as well as periods of burst suppression, where high-voltage periods of activity are alternated by periods of low activity (Ching, Purdon, Vijayan, Kopell, & Brown, 2012). The recordings were converted to text and run through a custom written software programme (Spectral analyser, C.B. Johnson, 2002) which performed Fast Fourier Transformation (FFT) on the recordings. This programme analysed 1 second blocks

of the recordings and produced a frequency spectrum, which represents the contribution of each frequency to the power of the electrical recording. Analysis of the electrical activity in the brain was based on the variables produced through this transformation. The variables produced by the spectral analyser software were: total power (P_{TOT}), the total area under the frequency spectrum; the median frequency (F50), the frequency under which 50% of the total power is located; and spectral edge frequency (F95), the frequency under which 95% of the total power is located.

In order to examine the changes which occur within the brain in response to nociceptive stimuli, the means for each of these variables were calculated for two 30 second periods prior to stimulation and for four consecutive 30 second periods following the application of each stimulus. The means prior to stimulation were calculated for the periods -60 to -30 seconds and -120 to -90 seconds, while the means following stimulation were generated for four consecutive 30 second blocks from 10–130 seconds after stimulation (Figure 6). The period of stimulation was eliminated from these means in order to avoid contamination of movement artefacts or activity from the stimulus itself.

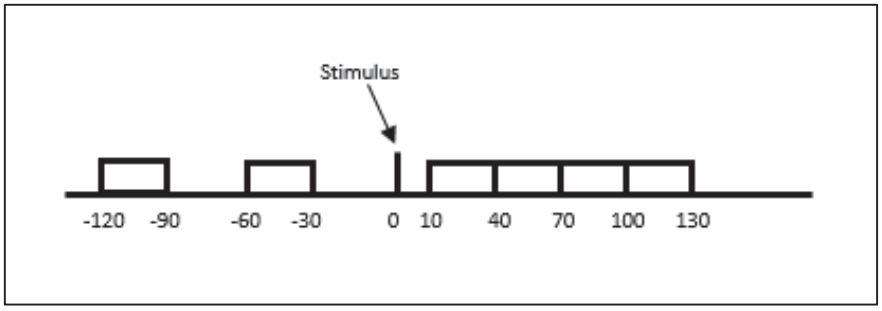

Figure 6. A diagram representing the time periods used to generate the percent changes prior to and following stimulation.

The mean values -60 to -30 seconds prior to stimulation were designated the baseline values to reflect the activity that occurs in the absence of external stimulation. These values were standardised to 100% and used to convert the other values to a percentage change from baseline. This was done to eliminate any skew caused by differing values between subjects. The percentage change was calculated by dividing each mean value (-120 to -90, 10 to 40, 40 to 70, 70 to 100, 100 to 130) by the baseline mean (-60 to -30) and multiplying by 100. The percentage change generated by the two periods prior to stimulation therefore represented the pre-treatment mean, while the other percentages represent the response to stimulation. Through this process of standardisation, any differences between individual subjects were eliminated. The percentages of baseline calculated here were used in the statistical analysis.

2.4.2. Populations of the HA

The preliminary investigation of the Fourier variables revealed two population of subjects from the HA. Within the HA, these populations could be divided based upon considerable differences in their total power. While there was seen to be differences in F50 and F95, the P_{TOT} was the most distinguishable difference. Five birds were found to have large, highly variable and unstable total powers, while five were found to have low, stable total powers. Such variability was not seen in the recordings of the NCM. The recordings from the NCM found a consistently low, stable total power similar to the 'low' population seen in the HA. Due to the distinct differences between these groups, it was decided that each group would be treated as an independent brain region. While this was not intended at the beginning of the study, a post-hoc decision was made following the preliminary investigation of the data. This was decided as the difference was believed to be due to recording occurring from two distinct regions within the hyperpallium group, through variation in the positioning of the electrode, although there is not clear evidence that this is the case (Appendix A). Therefore, the data analysis involved three separate brain regions: the high hyperpallium apicale (hHA), characterised by a high total power (5 birds); the low hyperpallium apicale (lHA), characterised by a low and stable total power (5 birds); and the caudomedial nidopallium (NCM) (9 birds). These brain regions were compared to determine if there was a difference in their response to stimulation.

2.4.3. Unstandardised data analysis

Prior to analysis, the raw, unstandardised data was analysed. The mean F50, F95 and P_{TOT} of the baseline period (-60 to -30 seconds prior to stimulation) was used to assess the activity occurring throughout the recording. This was to determine if the baseline EEG variables were changing throughout the course of the recording, or whether the baseline activity remained constant. This would determine whether a single baseline would be used to represent every stimulus, or whether baseline values should be calculated separately for each stimulus. In order to analyse this, a generalised linear model was used to assess the effect of order and brain region on the F50, F95 and P_{TOT} of the baseline means. The order indicated the position of the baseline period in the recording, with "1" being the baseline prior to the 1st stimulus applied and "5" being prior to the last stimulus applied etc. This therefore gave an indication of the change in activity over the time of the recording. For the Unstandardised baseline data, the generalised linear model for the analysis included the fixed effects of order and brain region, and the random effect of bird within brain region, to account for repeated measures from the same individual. Least squared means were calculated and compared for these

effects and for interactions between these effects. Where there was a significant overall or interaction effect, P values were manually adjusted for multiple comparisons by multiplying by the number of within-treatment comparisons. Only adjusted P values <0.05 were considered significant. Based upon the results outlined in Section 3.1, it was decided not to use a single baseline value, but rather to use the baseline mean calculated prior to each individual stimulus as the pre-treatment baseline. These pre-treatment means would then be used to investigate the change in activity following stimulation.

2.4.4. Statistical analysis

The changes in F50, F95 and P_{TOT} for each variable (calculated as percentages of pre-treatment baseline) were subjected to analysis of variance using SAS® 9.4 statistical analysis software (SAS Institute Inc, Cary, NC, USA). A generalised linear model was used to assess the difference between brain regions and the change in F50, F95 and P_{TOT} following stimulation. The general linear model included the fixed effects of brain region, stimulus, time and the random effect of bird within brain region to account for repeated measures from the same individual. Least squared means were calculated for these effects and for interactions between these effects. Where there was a significant overall or interaction effect, this was investigated through post hoc comparison of the least squared means. P values were manually adjusted for multiple comparisons by multiplying by the number of within-treatment comparisons. Only adjusted P values <0.05 were considered significant.

A generalised linear model was also used to assess individual time points to analyse the effect of stimulus and brain region on F50, F95 and P_{TOT}. For each time period (one pre-treatment and four after stimulation) the generalised linear model for the analysis included the fixed effects of stimulus and brain region, and the random effect of bird within brain region, to account for repeated measures from the same individual. Least squared means were calculated for these effects and for interactions between these effects. Where there was a significant overall or interaction effect, P values were manually adjusted for multiple comparisons by multiplying by the number of within-treatment comparisons. Only adjusted P values <0.05 were considered significant.

Analysis was also performed on data for each individual stimulus using a generalised linear model. Mean F50, F95 and P_{TOT} calculated for four consecutive 30 second blocks from 10 seconds until 130 seconds following stimulation were compared to the pre-treatment mean. For each stimulus, the generalised linear model included the fixed effects of brain region and time, with the random effect of bird within brain region to account for repeated measures

from the same individual. Where an overall effect was found, P values were manually adjusted for multiple comparisons by multiplying by the number of within-treatment comparisons. Only adjusted P values <0.05 were considered significant.

3. Results

3.1. Analyses of unstandardised baseline data

There were significant differences in the unstandardised data between the brain regions (Table 2). The order of baseline period was seen to have a significant effect on F95 and P_{TOT}, while it did not affect F50 (Table 2). There was no interaction effect of brain region and order on either F50 or F95, however there was a significant effect on P_{TOT} (Table 2).

Table 2. Results of ANOVA of unstandardised dEEG data looking at the effects of brain region, order and their interaction on the changes in F50, F95 and P_{TOT} of the chick dEEG

	Brain Region		**Order**		**Brain region*order**	
	F value	**P value**	**F value**	**P value**	**F value**	**P value**
F50	17.95	*<.0001**	2.34	*0.0645*	1.01	*0.4366*
F95	37.21	*<.0001**	4.00	*0.0059**	1.05	*0.4109*
P_{TOT}	125.58	*<.0001**	11.16	*<.0001**	13.52	*<.0001**

Total power was significantly different between all brain regions, with the hHA (LS mean ± SE: 184.28 ± 2.13) higher than both the lHA (LS mean ± SE: 36.31 ± 2.07, t=49.81, p<.0001) and the NCM (LS mean ± SE: NCM= 64.40 ± 1.57, t=45.32, p<.0001), while the NCM was higher than the lHA (T=10.83, p=<.0001). While order was seen to have an effect on F95 and P_{TOT}, only P_{TOT} was seen to have an interactive effect of brain region and order (Table 2). The P_{TOT} of the hHA was seen to decrease significantly with order (Figure 7). The P_{TOT} of the lHA and NCM did not differ based on their order (Figure 7).

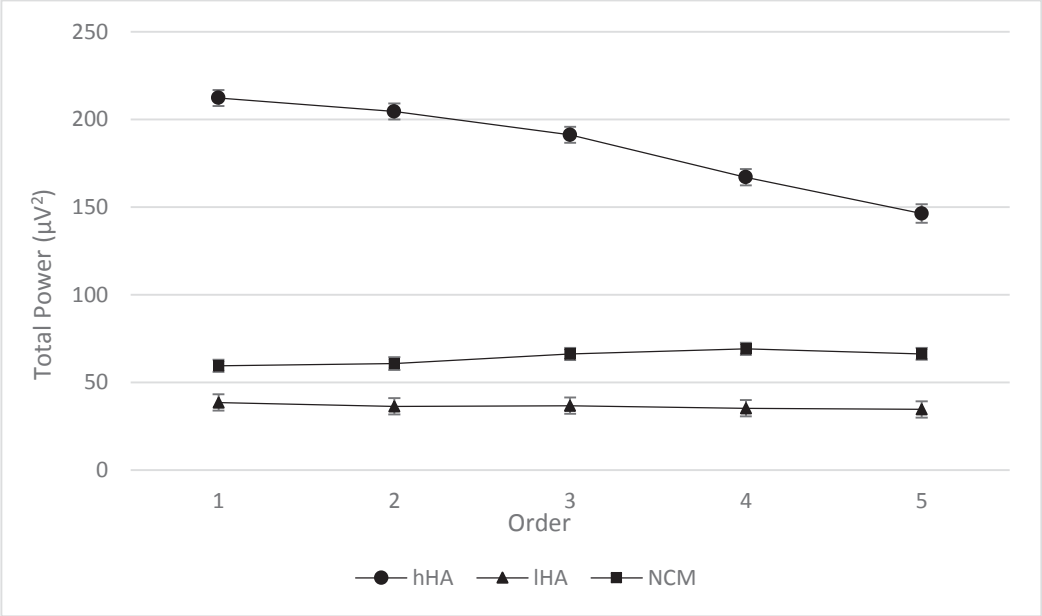

Figure 7. Unstandardised total power of the chick dEEG (least squared means ± SE) for each pre-treatment baseline period by order. All brain regions were significantly different at all times ($p<0.05$).

F50 differed significantly between brain regions, with the hHA (LS mean ± SE: hHA= 4.58 ± 0.088) lower than both the lHA (LS mean ± SE: lHA= 6.89 ± 0.085, t=18.85, p<.0001) and the NCM (LS mean ± SE: NCM=8.87 ± 0.065, t=39.27, p<.0001). The lHA was also significantly lower than the NCM (t=18.45, p<.0001; Figure 8). Order had no effect on F50 within any of the brain regions studied (Figure 8).

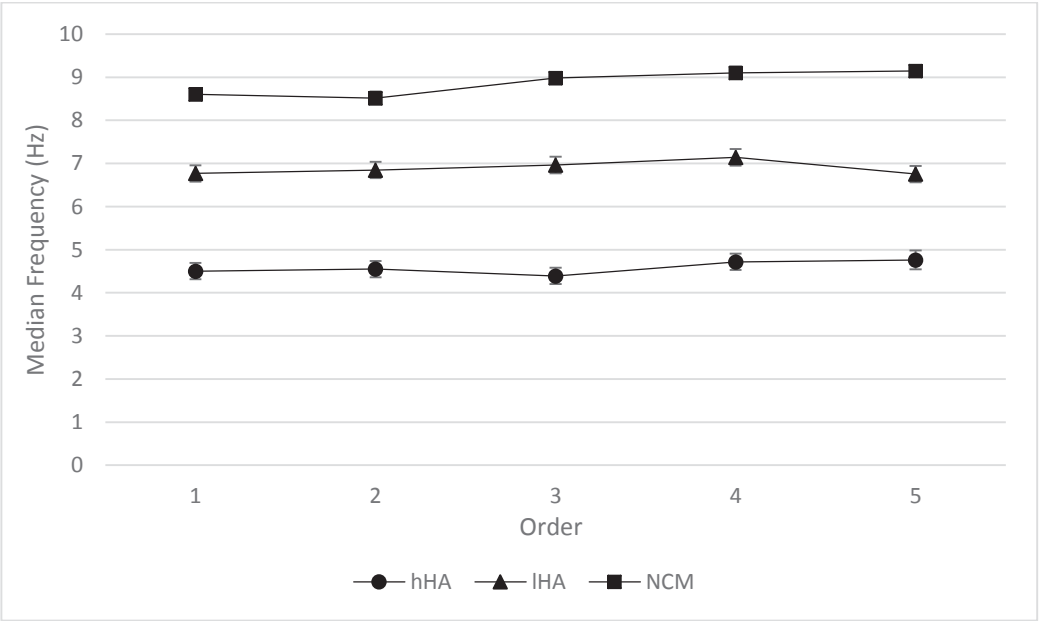

Figure 8. Unstandardised F50 of the chick dEEG (least squared means ± SE) for each pre-treatment baseline period by order. All brain regions were significantly different at all times ($p<0.05$).

F95 also differed significantly between the brain regions studied (Table 2). The F95 of the hHA (LS mean ± SE: 20.19 ± 0.091) was lower than that of the lHA (LS means ± SE: 24.32 ± 0.088, t=32.68, p<.0001) and the NCM (LS mean ± SE: 25.65 ± 0.067, t=48.54, p<.0001). The lHA F95 was also lower than the NCM (t=12.08, p<.0001). There was an effect of order on the F95, with the baseline prior to the second stimulus applied (LS mean ± SE: 23.14 ± 0.10) being lower than that prior to the fourth (LS mean ± SE: 23.59 ± 0.10, t=3.00, p=0.040) and fifth (LS mean ± SE: 23.63 ± 0.11, t=3.21, p=0.021) stimulus applications. However, there was no interaction between brain region and order on F95, with no differences in F95 seen at any point within any brain region (Figure 9).

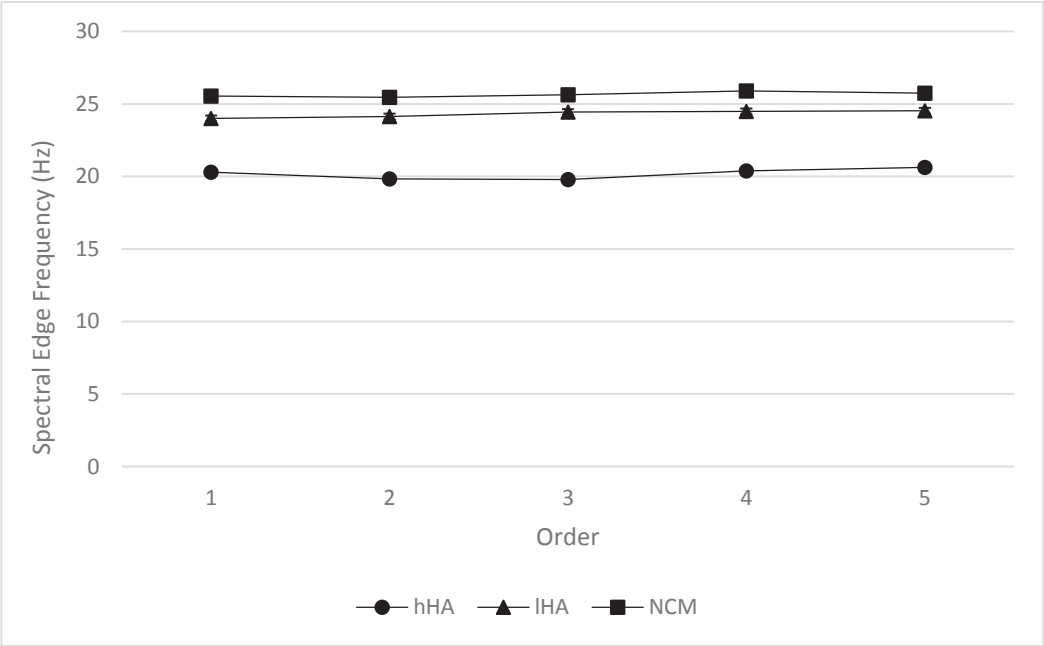

Figure 9. Unstandardised F95 of the chick dEEG (least squared means ± SE) for each pre-treatment baseline period by order. All brain regions were significantly different at all times (p<0.05).

3.2. Overall repeated measures model of standardised data

There was a significant effect of brain region on F50 and F95, but not on P_{TOT} (Table 3). The mean F50 of the hHA (LS mean ± SE: 98.76 ±0.47) was significantly lower than the NCM (LS mean ± SE: 100.83 ± 0.35, t=3.52, p=0.0015) while no other brain regions differed. The mean F95 of the hHA (LS mean ± SE: 98.64 ± 0.16) was also significantly lower than both the lHA (LS mean ± SE: 99.94 ± 0.16, t=5.67, p<.0001) and the NCM (LS mean ± SE: 100.16 ± 0.12, t=7.39, p<.0001) while the lHA and NCM did not differ from each other.

There was a significant effect of stimulus on F95 and P_{TOT}, while there was no effect on F50 (Table 3). Mean F95 surrounding thermal stimulation (LS mean ± SE: 99.04 ± 0.19) was significantly lower than mechanical (LS mean ± SE: 99.84 ± 0.19, t=2.96, p=0.033) stimulation, while no other differences were seen between stimuli. The mean P_{TOT} surrounding thermal stimulation (LS mean ± SE: 102.58 ± 0.61) was significantly higher than all other stimuli (LS means ± SE: Electrical 98.19 ± 0.65, t=4.94, p=0.001; Feather 98.04 ± 0.61, t=5.26, p=0.001; Mechanical 98.70 ± 0.62, t=4.46, p=0.001; Non-painful 99.48 ± 0.62, t=3.58, p=0.004).

There was a significant interaction effect of brain region and stimulus on F50 and P_{TOT}, but not F95 (Table 3). The mean F50 surrounding the electrical stimulus in the lHA (LS mean ± SE: 104.57 ± 1.03) was significantly higher than in the hHA (LS mean ± SE: 98.34 ± 1.18, t=3.99, p=0.0045) and the NCM (LS mean ± SE: 100.09 ± 0.77, t=3.50, p=0.023). The mean F50 surrounding the electrical stimulus was also significantly higher than the other stimuli of the lHA (LS means ± SE: Feather 98.11 ± 1.03, t=4.45, p=0.0045; Mechanical 99.14 ± 1.03, t=3.74, p=0.009; Non-painful 99.15 ± 1.03, t=3.73, p=0.009; Thermal 98.84 ± 1.03, t=3.95, p=0.0045). The mean P_{TOT} surrounding the thermal stimulus of the hHA (LS mean ± SE: 104.60 ± 1.15) was significantly greater than the electrical (LS mean ± SE: 95.58 ± 1.31, t=5.18, p=0.0045), feather (LS mean ± SE: 97.81 ± 1.15, t=4.19, p=0.0045) and mechanical (LS mean ± SE: 98.81 ± 1.15, t=3.57, p=0.018) stimuli of the hHA. However, the mean P_{TOT} of the hHA thermal did not differ from either the lHA or NCM. The mean P_{TOT} surrounding the thermal stimulus of the lHA (LS mean ± SE: 103.11 ± 1.15) differed from the feather (LS mean ± SE: 97.12 ± 1.15, t=3.69, p=0.014), mechanical (LS mean ± SE: 97.55 ± 1.15, t=3.43, p=0.032), and non-painful (LS mean ± SE: 97.60 ± 1.15, t=3.40, p=0.036) stimuli of the lHA.

There was no effect of time on F50, F95 or P_{TOT} (Table 3). There was no interaction effect of stimulus by time, brain region by time, or brain region by stimulus by time on F50, F95 or P_{TOT} (Table 3).

Table 3. Results of ANOVA of standardised dEEG data looking at the effects of brain region, stimulus, time and their interactions on the changes in F50, F95 and Ptot of the chick dEEG

	Brain Region		Stimulus		Time		Brain region*stimulus		Stimulus*time		Brain region*time		Brain region*stimulus*time	
	F value	**P value**	**F value**	**P value**	**F value**	**P value**	**F value**	**P value**	**F value**	**P value**	**F value**	**P value**	**F value**	**P value**
F50	4.01	*0.039**	1.83	*0.12*	0.96	*0.43*	3.78	*0.0003**	0.36	*0.99*	0.65	*0.74*	0.38	*0.99*
F95	6.66	*0.008**	2.83	*0.0246**	1.25	*0.29*	1.43	*0.18*	0.30	*0.99*	1.20	*0.30*	0.54	*0.98*
Ptot	0.51	*0.61*	9.23	*<.0001**	0.68	*0.6070*	3.37	*0.0009**	0.30	*0.9964*	0.92	*0.50*	0.77	*0.81*

3.3. Analyses of individual time points

Table 4. Results of ANOVA of standardised dEEG data looking at the effects of brain region, stimulus and their interaction on the changes in F50, F95 and P_{TOT} of the chick dEEG at each time point

	Brain Region		Stimulus		Brain region*stimulus	
	F value	**P value**	**F value**	**P value**	**F value**	**P value**
Pre-treatment						
F50	0.06	*0.95*	0.63	*0.64*	0.69	*0.70*
F95	0.79	*0.47*	1.11	*0.36*	0.76	*0.64*
P_{TOT}	1.62	*0.23*	0.96	*0.44*	0.64	*0.74*
10–40secs						
F50	3.25	*0.07*	0.43	*0.97*	0.47	*0.87*
F95	5.49	*0.015**	0.28	*0.89*	0.65	*0.74*
P_{TOT}	0.58	*0.57*	2.36	*0.063*	2.00	*0.062*
40–70sec						
F50	4.21	*0.034**	1.42	*0.24*	1.45	*0.20*
F95	8.09	*0.0037**	1.53	*0.21*	0.64	*0.74*
P_{TOT}	0..70	*0.51*	2.34	*0.066*	1.43	*0.20*
70–100secs						
F50	3.70	*0.048**	0.47	*0.76*	1.49	*0.18*
F95	6.94	*0.0068**	0.96	*0.44*	0.70	*0.69*
P_{TOT}	2.98	*0.080*	2.51	*0.051*	0.86	*0.56*
100–130secs						
F50	1.54	*0.24*	0.29	*0.88*	1.01	*0.44*
F95	4.18	*0.035**	0.19	*0.94*	0.52	*0.83*
P_{TOT}	0.35	*0.71*	1.26	*0.30*	0.89	*0.53*

3.3.1. Pre-treatment

There were no difference between brain regions in the pre-treatment means for F50, F95 or P_{TOT} (Table 4). There was also no overall effect of stimulus on these means, and no interaction effect of brain region and stimulus (Table 4).

3.3.2. 10–40 seconds following stimulation

In the first time period following stimulation, there was no difference in mean F50 or P_{TOT} between the brain regions studied (Table 4). There was, however, a significant difference in mean F95 between brain regions in the 10–40 seconds following stimulation (Table 4). The mean F95 of the hHA did not differ from the lHA (t=2.13, p=0.11), however, it was significantly lower than the NCM (t=3.00, p=0.012; Figure 10). The activity of the lHA did not differ from the NCM following stimulation (Figure 10). There was no overall effect of stimulus on these means, and no interaction effect of brain region and stimulus (Table 4).

3.3.3. 40–70 seconds following stimulation

In the 40–70 seconds following stimulation, there were significant differences in both the mean F50 and F95 between the brain regions studied (Table 4). Mean F50 of the hHA was significantly lower than NCM (t=2.69, p=0.0273), while there were no significant differences between the hHA and the lHA (t=1.71 p=0.2742), or the lHA and the NCM (t=0.80, p=0.43; Figure 11). The mean F95 of the hHA was significantly lower than the lHA (t=4.28, p<0.0001) and the NCM (t=5.79, p=<.0001), while the lHA and NCM did not differ from each other (t=1.05, p=0.30; Figure 10). There were no significant differences between the brain regions in mean P_{TOT} 40–70 seconds after stimulation (Table 4). There was no overall effect of stimulus on these means, and no interaction effect of brain region and stimulus (Table 4).

3.3.4. 70–100 second following stimulation

In the 70–100 seconds following stimulation, there was a significant effect of brain region on mean F50 and F95 (Table 4). However upon investigation, the mean F50 of the hHA was not significantly different from the NCM (t=2.32, p=0.0715), or the lHA (t=1.23, p=0.6721), and there was no significant difference in mean F50 between the lHA and the NCM (t=0.97, p=0.3365; Figure 11). The mean F95 of the hHA 70–100 seconds after stimulation was significantly different from the lHA (t=2.84, p=0.0184) and the NCM (t=3.36, p=0.0040), while there was no difference between the lHA and the NCM (t=0.20, p=0.8387; Figure 10). There were no significant differences in the mean P_{TOT} of these brain regions (Table 4). There was no overall effect of stimulus on these means, and no interaction effect of brain region and stimulus (Table 4).

3.3.5. 100–130 seconds following stimulation

In the 100–130 seconds following stimulation, there was a significant difference in F95 between brain regions (Table 4). The mean F95 of the hHA was lower than that of the NCM (t=2.82, p=0.0064; Figure 10). There was no significant difference in mean F95 between the hHA and the lHA (t=2.37, p=0.063) or the lHA and the NCM (t=0.19, p=0.8502; Figure 10). There was no significant difference between brain regions in mean F50 or P_{TOT} (Table 4). There was no overall effect of stimulus on these means, and no interaction effect of brain region and stimulus (Table 4).

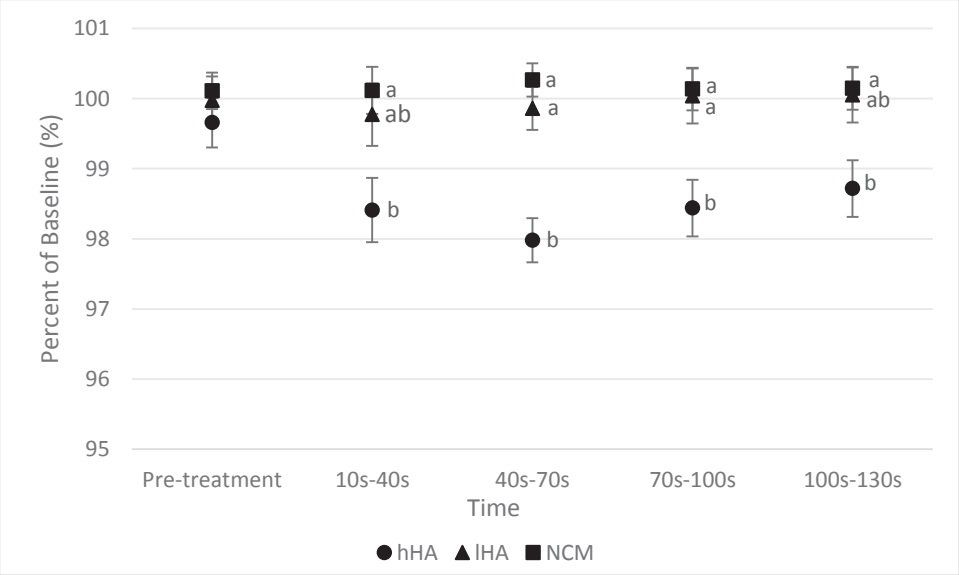

Figure 10. Standardised F95 of the chick dEEG (least squared means ± SE) for all brain regions investigated in response to all stimuli, calculated for each time period. Different letters indicate significant differences between brain regions within the same time period (p<0.05).

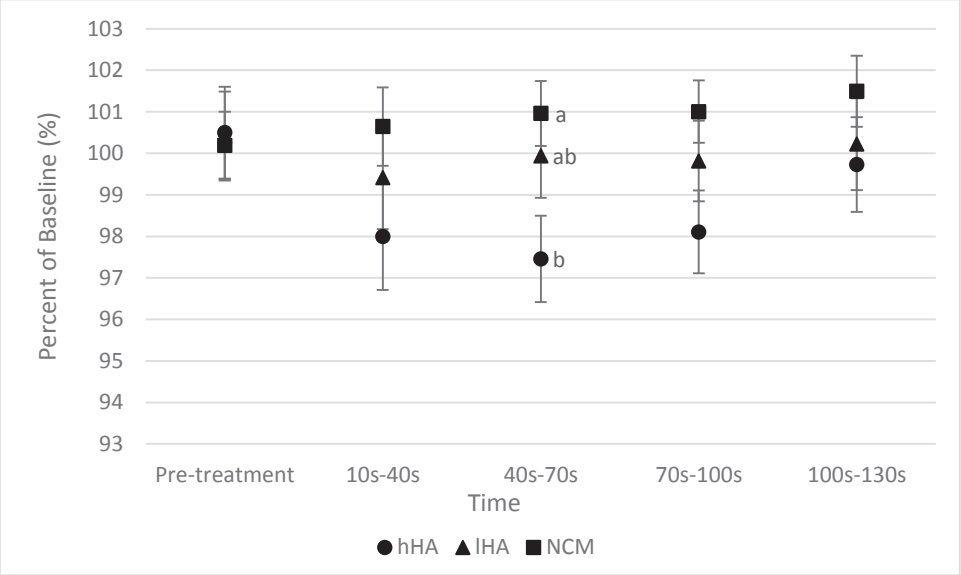

Figure 11. Standardised F50 of the chick dEEG (least squared means ± SE) for all brain regions investigated in response to all stimuli, calculated for each time period. Different letters indicate significant differences between brain regions within the same time period ($p<0.05$).

3.4. Analyses of individual stimuli

Table 5. Results of ANOVA of standardised dEEG data looking at the effects of brain region, time and their interaction on the changes in F50, F95 and P_{TOT} of the chick dEEG for all stimuli

	Brain Region		Time		Brain region*time	
	F value	**P value**	**F value**	**P value**	**F value**	**P value**
Non-painful						
F50	0.26	*0.78*	0.51	*0.73*	0.24	*0.98*
F95	1.06	*0.37*	0.34	*0.85*	1.13	*0.36*
P_{TOT}	1.97	*0.17*	0.30	*0.88*	1.03	*0.42*
Mechanical						
F50	0.45	*0.65*	1.95	*0.11*	1.55	*0.16*
F95	0.40	*0.68*	1.07	*0.38*	1.79	*0.10*
P_{TOT}	0.40	*0.68*	0.42	*0.80*	0.71	*0.68*
Thermal						
F50	3.25	*0.065*	0.89	*0.47*	0.70	*0.69*
F95	8.72	*0.0028**	1.75	*0.15*	1.44	*0.20*
P_{TOT}	1.12	*0.35*	0.17	*0.95*	1.79	*0.095*
Feather						
F50	0.37	*0.69*	0.41	*0.80*	1.36	*0.23*
F95	0.45	*0.65*	1.66	*0.17*	1.46	*0.19*
P_{TOT}	0.39	*0.69*	0.12	*0.98*	3.23	*0.0038**
Electrical						
F50	3.78	*0.047**	0.67	*0.61*	0.62	*0.76*
F95	4.31	*0.033**	0.60	*0.67*	1.15	*0.34*
P_{TOT}	1.17	*0.34*	2.11	*0.091*	2.24	*0.037**

3.4.1. Non-painful stimulation

Following non-painful stimulation there was no overall effect of time or brain region on mean F50, F95, or P_{TOT} (Table 5). There was also no interaction effect of brain region by time on mean F50, F95, or P_{TOT} following non-painful stimulation (Table 5).

3.4.2. Mechanical stimulation

There was no overall effect of time or brain region on mean F50, F95, or P_{TOT} following the application of the mechanical stimulus (Table 5). There was also no interaction effect of brain region by time on mean F50, F95 or P_{TOT} following mechanical stimulation (Table 5).

3.4.3. Thermal stimulation

There was no overall effect of time on mean F50, F95 or P_{TOT} following the application of the thermal stimulus (Table 5). There was also no interaction effect of brain region by time on mean F50, F95 or P_{TOT} following thermal stimulation (Table 5). There was an overall effect of brain region on the F95 following thermal stimulation (Table 5). All brain regions differed significantly from one another in mean F95 surrounding thermal stimulation, with the hHA (LS mean ± SE: 97.77 ± 0.21) being lower than the lHA (LS mean ± SE: 99.21 ± 0.21, t=4.91, p=<.0001) and the NCM (LS mean ± SE: 100.13 ± 0.16, t=9.07, p=<.0001), and the lHA was lower than the NCM (t=3.51, p=0.0024). However, there was no interaction effect between brain region and time (Table 5). There was no overall effect of brain region on mean F50 or P_{TOT} (Table 5).

3.4.4. Feather removal

There was no overall effect of time or brain region on mean F50, F95 or P_{TOT} following feather removal (Table 5). There was also no interaction effect of brain region by time on mean F50 or F95 following feather removal (Table 5). There was an interaction effect of brain region by time on P_{TOT} following feather removal (Table 5). However, when this effect was investigated, there were no significant differences of interest caused by feather removal.

3.4.5. Electrical stimulation

There was no effect of time on the mean F50, F95 or P_{TOT} following application of the electrical stimulus (Table 5). There was also no effect on brain region by time on mean F50 or F95 (Table 5). There were significant differences between the brain regions in both mean F50 and F95 (Table 5). The F50 of the lHA (LS mean ± SE: 104.57 ± 0.75) was greater than both the hHA (LS mean ± SE: 98.27 ± 0.84, t=5.62, p<.0001) and the NCM (LS mean ± SE: 100.09 ± 0.56, t=4.81, p<.0001). There was no difference in mean F50 between the hHA and the NCM (t=1.81,

p=0.22). The mean F95 of the hHA (LS mean ± SE: 98.33 ± 0.23) was reduced compared to the lHA (LS mean ± SE: 100.22 ± 0.21. t=6.04, p<.0001) and the NCM (LS mean ± SE: 100.32 ± 0.16, t=7.10, p<.0001), while there was no difference between the lHA and the NCM (t=0.38, p=0.70). There was an interaction effect of brain region by time on P_{TOT} following stimulation (Table 5). In the hHA, mean total power was significantly lower than the pre-treatment mean at 10-40 seconds following stimulation (t=3.44, p=0.049; Figure 12).

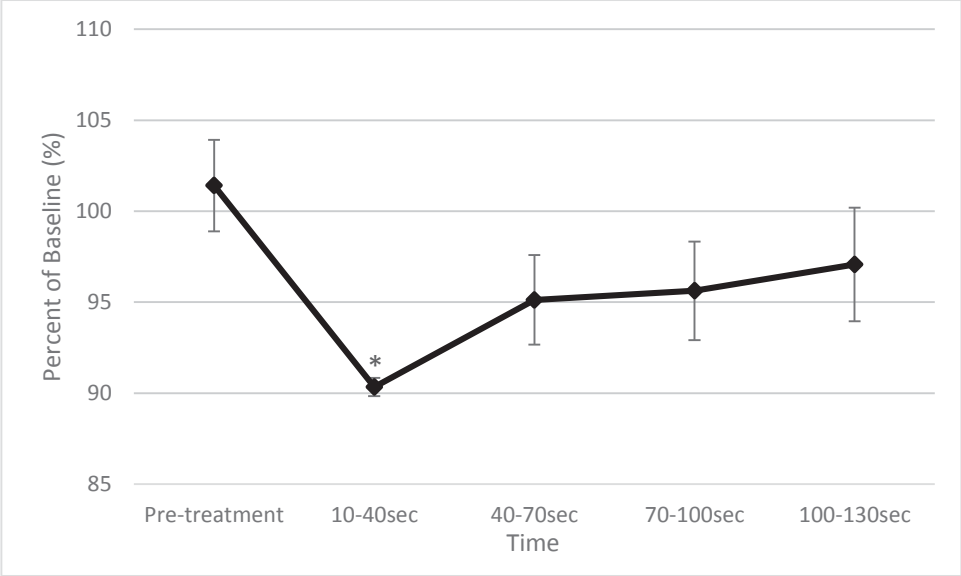

Figure 12. Standardised P_{TOT} of the chick dEEG (least squared means ± SE) of the hHA from the brain region by time interaction of the electrical stimulus. The * represents a significant change from the pre-treatment value prior to stimulation (p<0.05).

4. Discussion

4.1. Aims of the study

The aims of this study were to investigate the response of the avian brain to nociception through the use of dEEG to target brain regions thought to be involved in the central processing of pain. This was done by recording the electrical activity from these brain regions before and after application of a range of different stimuli. The rostral HA and NCM were targeted due to their implication in the processing of avian pain, and homologies in structure and function with mammalian pain structures. Firstly, the raw, unstandardised dEEG data was analysed to investigate the change in the recording over time, and to identify any differences between the brain regions studied. The data was then standardised to a percentage of baseline mean and analysed in order to assess the change in activity that occurs in response to stimulation. This was done by assessing individual time points prior to and following stimulation, and also by assessing the response of the brain to individual stimuli. The mean F50, F95 and P_{TOT} for four consecutive 30 second blocks were used to investigate the change from a pre-treatment mean value. This investigation aimed to improve the knowledge of the avian response to pain and to determine if the avian brain shows identifiable, consistent responses to stimulation.

4.2. Findings

The findings of this study indicate that the activity occurring throughout the avian brain is considerably variable between brain regions. The change in the activity of distinct brain regions following stimulation was found to differ. However, there were no consistent responses seen in any brain region studied in response to nociceptive or innocuous stimulation. Lack of a single consistent change meant that no overall trend could be identified and attributed to the avian brains response to nociceptive stimulation. This study has, however, provided a number of interesting insights into the activity of the avian brain.

4.2.1. Analysis of unstandardised data

Analysis of the unstandardised data indicates that the brain regions targeted have distinctly different characteristics of activity, but that this activity is generally consistent within each of these brain regions. Each region was distinctly different from others in terms of its baseline F50, F95 and P_{TOT}. The hHA was seen to be lower than both the lHA and NCM in its F50 and F95, while higher in its P_{TOT}. Lower frequencies contribute more to the P_{TOT} of the recording than higher frequencies, which would suggest that the hHA is more dominated by lower

frequency activity when compared to the other brain regions (Teplan, 2002). Conversely, the NCM and lHA had a lower P_{TOT} and higher F50 and F95, suggesting higher frequencies are more dominant in the NCM and lHA compared to the hHA. The results of this analysis suggests that the three groups investigated in this study reflect distinct brain regions based upon the significant differences seen in their spectral characteristics. As such, the decision to split the HA data into two experimental groups reflecting two distinct brain regions is believed to be correct.

As the recordings were conducted over an extended time period, it was thought possible that activity within each brain region may have varied in a temporal manner. Subjects were kept under anaesthesia for over an hour and as such, there may have been changes in EEG activity occurring throughout the treatment period. In order to investigate this, the activity of target brain regions over the course of the recording was analysed. For the most part, activity remained constant for the entire period of the recording, with no statistically significant increases or decreases seen in the F50 or F95 of any of the brain regions. Therefore, the length of the recording and time spent under anaesthesia was concluded to have had no significant effect on these variables. In contrast, the P_{TOT} of the hHA was seen decline significantly over the length of the recording, for reasons which are unclear. It is possible that anaesthesia caused this decrease in the P_{TOT} of the recording as anaesthesia has been shown to alter the recording of the electrical activity of the brain. Increasing depth of anaesthesia has been associated with a slight, and inconsistent, increase in the amplitude of the EEG, leading to an increase in total power (Otto & Short, 1991; Teplan, 2002). Therefore the observed decrease in total power may reflect the level of anaesthesia decreasing over the course of the recording. High concentrations of halothane have also been seen to reduce F50 (McIlhone, Beausoleil, Johnson, & Mellor, 2014). However, in this study, the F50 of the hHA remained constant throughout the entire recording and end tidal halothane concentrations were monitored and kept stable throughout anaesthesia. While the reason for the change in the P_{TOT} of the hHA is unknown this variability was accounted for in the analysis by calculating a new pre-treatment mean prior to each stimulus application.

4.2.2. Analyses of individual time points

For each time period, the mean F50, F95 and P_{TOT} were compared between brain regions, between stimuli and analysed for an interaction effect of brain region and stimuli. The overall repeated measures analysis of the complete data set found significant differences between brain regions, as well as a stimulus effect and an interaction effect of brain region and stimulus. The analysis of individual time points provides insights into the time frame of these

effects. Significant differences between the brain regions were seen at a number of time points following stimulation.

The activity of the lHA and NCM were not seen to differ at any time prior to, or following the application of the stimuli. However, the change in activity of the hHA following stimulation differed from the change seen in both the lHA and NCM. The P_{TOT} remained constant between these brain regions, and there were no differences in P_{TOT} at any time following stimulation. However, the F50 and F95 differed between brain regions. In the pre-treatment period, there was no difference in the activity of any brain region. In the absence of a stimulus, the change from baseline did not differ between any of these groups for any of the variables investigated. This would suggest that the underlying brain activity, in the absence of an applied stimulus, is consistent and that the change in brain activity does not differ significantly throughout these brain regions in the absence of external stimulation. Following stimulation, F95 in the hHA was significantly lower than that of the NCM. This difference was present from 10–130 seconds following stimulation. F95 of the hHA was also lower than the lHA from 40–100 seconds following stimulation. A similar response was seen in F50 where the hHA was significantly lower than the NCM in the 40–70 seconds following stimulation. While these results do not clearly indicate the response of these brain regions to stimulation, they do suggest that the response differs between regions. The hHA may show a decrease in its F50 and F95 following stimulation, whereas the hHA and NCM may instead show a slight increase or no change at all. While these results suggest a significant difference in response between brain regions investigated, they lack information about the direction or specific details of this response. As such, no definite conclusion can be made as to the exact characteristics of each brain region in response to stimulation and further work will be necessary in order to determine this. The response of these regions to stimulation may differ with the type of stimulus applied, as this analysis investigated all stimuli combined.

4.2.3. Analyses of individual stimuli

The aims of these analyses were to investigate the electrical response of the avian brain to different types of stimuli. Stimulation was applied either as a non-painful, somatosensory stimulus, or as one of four nociceptive stimuli. The response of the dEEG was studied following the application of the stimulus to assess the response of the brain to nociception. The overall repeated measures analysis of the full data set revealed a difference between brain regions, and these analyses provide a means to investigate this based upon the stimulus applied. In addition, these analyses allowed a closer investigation of the response of these brain regions following stimulation. It was seen that there were significant differences between brain

regions for some stimuli. There were no consistent changes seen in response to nociceptive stimulation; however, some changes in the dEEG following nociceptive stimulation were seen. The response to stimulation differed between the brain regions investigated. The NCM and lHA showed no response to stimulation and while the hHA did not show consistent changes in response to nociceptive stimulation, it did appear responsive to some stimuli.

Significant differences between brain regions were found when individual stimuli were analysed separately. In the analysis of thermal stimulus, it was seen that F95 was significantly different between all brain regions. Additionally, both mean F50 and F95 differed significantly between brain regions following electrical stimulus. It is possible that the differences seen for the thermal and electrical stimuli are responsible for the difference between brain regions seen in the analyses of individual time points. However, the absence of a brain area by time interaction effect makes this unlikely. These differences were present at all time points prior to and following stimulation so do not reflect a response to stimulation and weren't of great interest in this study. The activity within each brain region was consistent in both the pre- and post- treatment time groups of the thermal and electrical stimuli.

There were no consistent responses to stimulation seen in any of the brain regions studied. The NCM and lHA showed no changes in any dEEG variables following the application of any stimulus. The hHA on the other hand, did show a response to electrical stimulation, while not responding to any other stimulus. The P_{TOT} of the hHA was seen to undergo a significant decrease 10–40 seconds after the application of the electrical stimulus. There was no change in P_{TOT} of the hHA in response to any other stimulus and no change was seen in F50 or F95 of the hHA in response to any stimulus.

4.3. Implications of these findings

4.3.1. Variation in activity throughout the brain

Based upon the differences seen in the FFT variables between brain regions, the spectral characteristics of these regions differ significantly, and therefore activity occurring within these regions is also considerably different. The hHA exhibits lower F50 and F95, and higher P_{TOT} which would suggest that lower frequencies are more dominant in this region compared to the lHA and NCM where higher frequency, low amplitude brain waves are likely dominant (Teplan, 2002). The oscillatory activity of the brain is related to the functional state of the system and can therefore indicate the activity of the system (Ploner, Gross, Timmermann, Pollok, & Schnitzler, 2006). A state of high amplitude behaviour is believed to represent an idle state of the brain, while low amplitudes indicate increased activation and excitability (Ploner et

al., 2006). Therefore, the high P_{TOT} and low F50 and F95 of the hHA may be due to an idle state of this brain region at the time of recording. The spectral characteristics of the NCM and lHA may reflect a more active state of these brain regions due to the greater presence of higher frequency activity (Ploner et al., 2006). Anaesthesia and sleep are known to affect the oscillatory activity of the brain, with deeper sleep resulting in a change to slow frequencies (Steriade, McCormick, & Sejnowski, 1993). These differences could reflect the variable effect of anaesthesia occurring throughout the brain or may reflect differences in function between these regions. In any case, the activity occurring throughout the brain is seen to be highly variable which is of interest in terms of the function and activity of these brain regions.

Given the discrepancy between hHa and lHA data, it would appear that they represent distinct brain regions which respond differently to stimulation. It is believed that the recordings from the HA were taken from two different brain regions based upon the differences in the unstandardised data and the response to stimulation. This is believed to be due to variable positioning of the electrodes. Steps were taken to ensure accuracy of the electrode placement, but variation in subject size and positioning in the stereotaxic frame resulted in some variability of the electrode placement within the HA. The size of the bird will have an effect on brain size, which will affect the accuracy of the co-ordinates given for the brain regions targeted (Kuenzel & Masson, 1988). This will in turn affect the consistency of the positioning of the electrode within the target area. Recording from different brain regions will result in inconsistent recordings due to the differences in structure and function that occur throughout the brain (Alivisatos et al., 2012). Therefore, recording from different structures would have an obvious effect on the dEEG recording. This was seen in this study in both the unstandardised data, and the standardised data. While centimetre changes in the positioning of scalp electrodes will not cause much change in recording, even slight changes in the positioning of intracerebral or cortical electrodes can cause a difference (Cooper et al., 1965). Through the use of intracerebral electrode, a spacing between electrodes of 1 mm was found to produce markedly different results (Cooper et al., 1965). Therefore accuracy of the electrode placement is important in order to consistently record from the same brain region. It is due to these reasons that the differences seen in the HA recordings were believed to be due to the recording being taken from separate, distinct regions of the brain. This would explain the distinct differences seen in the unstandardised data from these regions and also the differences seen in the response to stimulation.

4.3.2. Differing response of the brain to stimulation

These findings suggest that a standard stimulus has significantly different effects on the activity occurring throughout different regions of the brain. Variation in response to stimulation would be expected based upon the differences in structure and function that occur throughout the avian brain. Structures within the mammalian brain are also seen to vary in their response to stimulation, with a number of structures activated in response to nociception (Derbyshire et al., 1997; Mouraux et al., 2011). The brain regions investigated in the current study differ in both their structure and function. Both the rostral HA and NCM receive sensory inputs about the body from the thalamus, however they have different roles in sensory processing (Kuenzel, 2007; Reiner et al., 2004; Reiner et al., 2005). The rostral HA is a small somatosensory region which receives somatosensory inputs from the ventral tier of the thalamus (Reiner et al., 2005). It plays a role in the processing of somatosensory information from the body (Medina & Reiner, 2000). The NCM also receives sensory information from the thalamus, however it has been largely implicated in the avian auditory circuit and processing of auditory information (Karten, 1969; Reiner et al., 2004). The differences in the function and activity of these brain regions are supported by the differences in their spectral characteristics seen in this study. Based upon the separate roles of these regions in sensory processing, a differing response would be expected to stimulation. The type of stimulation may also affect the response of these regions due to their differences in function. The majority of stimuli used in this study had a somatosensory component which may explain the difference in responses seen between the hHA and the NCM. The rostral HA, being predominantly somatosensory, is likely to respond to such somatosensory inputs, while the NCM may not respond as strongly to somatosensory stimuli. Instead, the NCM may show a greater response to auditory stimuli (Medina & Reiner, 2000; Reiner et al., 2004).

4.3.3. Response of the brain to nociceptive stimulation

The results of this study indicate that neither the NCM nor the lHA respond to nociceptive stimulation, while there is some suggestion of a response to nociception in the hHA. However, there were no consistent, identifiable responses seen following nociceptive stimulation in any of the brain regions studied. This is consistent with previous studies investigating the response of the avian brain to nociception which also failed to find a response (McIlhone et al., 2011; Woolley & Gentle, 1987). McIlhone et al. (2011) investigated the cortical EEG responses of anaesthetised chickens to thermal, electrical and mechanical noxious stimuli using sub-cutaneous electrodes and failed to find a consistent response to any noxious stimuli. An earlier study on conscious chickens also failed to find a nociceptive response to a 50°C thermal

stimulus or a comb pinch (Woolley & Gentle, 1987). However, one particular study did find a change in the EEG from a low frequency/high amplitude state to a high frequency/low amplitude state in response to feather removal (Gentle & Hunter, 1991). However, the changes in this study were attributed to a reduction in motor output or reduced vigilance following stimulation and were not necessarily thought to reflect processing of nociceptive stimuli (Gentle & Hunter, 1991). While the present study did not see a consistent response to nociception, there hHA did respond to electrical stimulation, with a decrease seen in P_{TOT}. This is a response that has not previously been reported in studies of the avian brain to nociception, however, McIlhone et al. (2011) did find a small yet significant increase in F50 in response to electrical stimulation, which may suggest a similar change in activity following electrical stimulation. In mammalian studies of pain, a decrease in total power is commonly seen following nociceptive stimulation (Gibson et al., 2007; Johnson et al., 2009; Murrell & Johnson, 2006). A decrease in total power in the 30 seconds following electrical stimulation has also been seen in mammalian studies (Murrell, Mitchinson, Waters, & Johnson, 2007). This response is comparable to the response seen to electrical stimulation in the hHA in this study. Therefore, while the response of the hHA to electrical stimulation differs from previous work in birds, it is consistent with a number of mammalian studies of nociception.

While this study saw a response of the hHA to electrical stimulation, it did not find a significant response to the other noxious stimuli. There were no responses seen to mechanical or thermal stimulation, nor to feather removal. However, the lack of response to these stimuli does not necessarily suggest a lack of response of this brain region to nociception, as different noxious stimuli have been seen to cause differing responses in the EEG (Murrell et al., 2007). Nociceptive stimuli, such as thermal, mechanical or electrical stimuli, cause qualitatively different sensations of pain and as such are associated with a quantitatively different response in the EEG (Arndt & Klement, 1991; Murrell et al., 2007). Murrell et al. (2007) investigated the comparative effects of thermal, mechanical and electrical stimulation on the EEG of rats. It was demonstrated that the response to these stimuli was quantitatively different. For example, electrical stimulation caused an increase in F50 and a decrease in total power, thermal caused a lesser increase in F50 and no change in total power, and mechanical only caused a slight decrease in total power (Murrell et al., 2007). Based on this, it would be expected that the response of the dEEG to different stimuli will vary, which is what was seen in the present study. The different responses seen to these different stimuli may be due to the different sensations evoked by different stimuli, rather than solely due to their nociceptive properties.

The brain regions investigated did not display any significant change in the dEEG as a result of non-painful stimulation. Studies investigating the response of the brain to both painful and innocuous stimuli have found differences in the response of the brain to these stimuli (Frot et al., 2001; Kunde & Treede, 1993). Therefore, a different response would be expected to a non-painful stimulus than a nociceptive stimulus. However, in this study the response of the brain regions to the non-painful stimulus did not differ from that of the nociceptive stimuli, except for the P_{TOT} following electrical stimulation. The difference seen in response to the non-painful and electrical stimuli may be due to the nociceptive aspect of the electrical stimulus, however, the lack of a difference between the non-painful stimulus and the other nociceptive stimuli suggest that this is not the case. These other stimuli may not be considered noxious, or the brain regions targeted may not be sensitive in discriminating nociceptive information of these stimuli. A response to electrical stimulation may be seen as it is likely to cause non-specific activation of neurons, leading to a generalised brain response that may not be related to nociceptive processing (McIlhone et al., 2011). Electrical stimulation has been previously investigated in mammalian models with a decrease in total power reported, similar to that seen in this study (Murrell et al., 2007). Therefore, it is possible that this represents a response to nociception. If electrical stimulation does cause non-specific activation of the brain then it is of interest that a response was not seen in the lHA or NCM. The fact that a response to electrical stimulation is only seen in the hHA and not the other brain regions would suggest that this is not merely a response to the non-specific activation of neurons. Instead, the response of the hHA may reflect a response to nociceptive stimulation and may represent the processing of nociceptive stimulation within this brain region. A decrease in P_{TOT} would suggest a change to lower amplitude oscillations, an indication of increased activation (Steriade et al., 1993). As such, this change seen in response to electrical stimulation may reflect an increase in the activation and excitability of the hHA in response to nociceptive stimulation.

4.3.4. The effect of noxious stimulation on the mammalian EEG

The results of this study in chickens differed from those seen in mammalian studies investigating the response to nociceptive stimulation. A number of studies have investigated changes in the cortical EEG following nociceptive stimulation using the minimal anaesthesia model. Most commonly, the response to pain is a change to high frequency, low amplitude EEG, resulting in an increase in F50 and F95, while P_{TOT} decreases known as EEG arousal (Gibson et al., 2007; Johnson et al., 2009; Murrell et al., 2003; Otto & Mally, 2003). While this change to high frequency, low amplitude EEG is most common, there may instead be a change to low frequency, high amplitude activity. For example, a study investigating EEG responses to

tail clamping in anaesthetised rat pups identified a decrease in F50 and F95 following stimulus application (Diesch et al., 2009). This change to slow frequencies is known as paradoxical arousal and resembles EEG patterns during anaesthesia (Otto & Mally, 2003). Experimental studies have shown that either arousal or paradoxical arousal can occur in response to nociceptive stimulation and are dependent on the intensity of stimulation and depth of anaesthesia (Otto & Mally, 2003). Deeper levels of anaesthesia, and an increase in stimuli intensity appear to predispose EEG arousal responses as opposed to paradoxical arousal responses (Otto & Mally, 2003).

The findings of the present study differed from those seen in mammals. There was no single identifiable change seen in the dEEG in response to nociceptive stimulation in the chicken. While the change in activity differed between brain regions, the exact response remains unknown. It appeared that the F95 differed following stimulation. F95 has commonly been seen to increase following nociceptive stimulation in mammalian studies of pain, however, no change in F95 was seen in response to any of the stimuli in this study (Gibson et al., 2007; Johnson et al., 2009). An increase in F50 is commonly associated with nociception in mammals and this differs from what was found in the present study, with no clear increase or decrease seen in F50 following stimulation (Gibson et al., 2007; Murrell et al., 2003). The present study saw a significant change in P_{TOT} in the hHA following electrical stimulation, which is similar to the decrease in the P_{TOT} of the EEG seen in mammalian studies (Johnson, Wilson, et al., 2005). However, this was the only response seen to stimulation, for the most part, there was no response seen following stimulation. The results of this study differ from those that have previously been seen in studies of both the avian and mammalian response to pain.

4.4. Possible reasons for the differences seen

This study differed from previous works which have investigated the electrical response of the brain to nociceptive stimulation. The majority of studies have investigated the response of the mammalian brain to nociception, while few have investigated the response of the avian brain. The change in P_{TOT} following electrical stimulation differs from previous avian works, while the lack of change in any other variable differs from mammalian studies. There are a number of possible reasons why the responses seen in this study may differ from that of previous works. These include differences in the anatomy of the brain between animal groups, the method of EEG recording and the positioning of the electrodes. Potential reasons for differences found in the present study are discussed in detail below.

4.4.1. Brain anatomy

Birds and mammals differ markedly in the organisation of their forebrains, with the notable difference being that the avian telencephalon lacks a laminated cortex (Güntürkün, 2005). It was originally believed that birds would only display instinctive behaviours due to their lack of a neocortex like that seen in mammals (Jarvis et al., 2005). It has since been proven that lamination is not required for the performance of higher cognitive functions, due to the fact that the non-laminated portions of the avian brain can perform the same functions as the mammalian brain (Güntürkün, 2005). The avian telencephalon histologically resembles the mammalian basal ganglia and not the neocortex (Reiner et al., 2005). This is in contrast to the mammalian brain where the cerebral cortex is enlarged relative to the underlying structures (Benzo, 1986; Sherwood, Klandorf, & Yancey, 2012). However, recent studies have found that the avian telencephalon contains only a small basal ganglia with a large pallial territory (Reiner et al., 2005). The organisation of the basal ganglia is very similar between mammals and birds, whereas the organisation of the pallium is much more variable (Güntürkün, 2005). While the mammalian cortex is organised in a laminar arrangement, the avian telencephalon is characterized by fields of cells with nuclear clusters and a thin overlying cortex (Benowitz, 1980). Despite the morphological differences between the mammalian and avian brain, evidence suggests that the majority of the avian telencephalon is comparable to structures in the mammalian cerebral cortex and is capable of performing functions similar to those in mammals (Benowitz, 1980; Jarvis et al., 2005). The internal portions of the cerebral hemispheres are enlarged in the avian brain, whereas the surface structures of the cerebrum are enlarged in mammals (Benzo, 1986). These differences in brain structure may contribute to the differences we see in the EEG responses of the brain to noxious stimulation between mammals and birds.

Studies of the mammalian brain have shown that there are a variety of cortical and subcortical structures throughout the brain that are activated in response to nociceptive stimulation (Derbyshire et al., 1997; Mouraux et al., 2011). These structures have since been named the pain matrix and are responsible for the integration of nociceptive stimuli and the perception of pain (Legrain et al., 2011). The magnitude of response within this matrix is representative of the intensity and unpleasantness that is perceived of a noxious stimuli (Legrain et al., 2011). In this way, the pain matrix can be used as an indicator of pain intensity, with the response of these brain regions differing depending on the strength and type of stimuli. There is extensive literature that demonstrates neuronal activation of these structures following acute experimental pain in mammals (Prichep, John, Howard, Merkin, & Hiesiger, 2011). Due to the

multiple regions involved in pain, a stimulus will have widespread effects on brain activity, with a general change in cortical functioning, rather than a change in localised regions of the brain (Ploner et al., 2006). In response to painful stimuli, studies have shown that there is a suppression of oscillations throughout the cerebral cortex (Mouraux, Guerit, & Plaghki, 2003; Ohara, Crone, Weiss, & Lenz, 2004). Therefore, pain causes a widespread increase in excitability and activation of cortical systems throughout the mammalian brain (Ploner et al., 2006). It is perhaps due to this widespread change in cortical function that the change in the mammalian brain has been seen in response to nociception using surface-recorded EEG, while no such changes have been identified in avian studies.

As well as differences in anatomy, there are also differences in the arrangement of neurons between the avian and mammalian brain (Butler, Manger, Lindahl, & Århem, 2005). The mammalian neocortex is composed of complex horizontal layers, and cortical columns of vertical pyramidal cells (Butler et al., 2005). This is in contrast to the pallial regions of the avian brain, in particular the hyperpallium and dorsal ventricular ridge, where the cells are mostly organised in nuclear clusters (Butler et al., 2005). The dendritic trees of the avian brain are star shaped and non-segregated (Butler et al., 2005). These dendrites project in multiple dimensions and also receive inputs from a variety of directions (Butler et al., 2005). It is perhaps due to this multi-dimensional signal transfer that the electrical changes recorded by the EEG are less able to be localised in the avian brain. The EEG detects dipoles which run through the layers of the cortex and is less able to separate the signal which arises from multiple inputs (Apkarian et al., 2005). During synaptic excitation, local current flows are produced on the dendrites of pyramidal cells and the EEG measures the differences in electrical potential between the soma and dendrites of these neurons (Teplan, 2002). It may be due to the differences in cell architecture that there have been difficulties in detecting a change in the electrical brain activity of birds in response to noxious stimulation, with the EEG less able to differentiate changes occurring in the avian brain due to the multi-directionality of the neuronal connections (Butler et al., 2005). This may account for the lack of response seen in this study, and previous studies of the avian response to nociception. However it seems clear that the dEEG was able to pick up changes in brain activity due to the differences seen between the brain regions following stimulation in this study.

4.4.2. Depth electroencephalography (dEEG)

This study used depth electroencephalography to monitor the electrical activity of the brain. This was done through the use of concentric needle electrodes, to monitor the electrical activity that was occurring in deep brain structures as opposed to the electrical activity that

can be recorded from the scalp. It is likely that the use of dEEG will cause differences in the recordings obtained when compared to scalp EEG (Cooper et al., 1965). Depth electroencephalography has been widely used in studies of epilepsy and seizure localisation (Behrens et al., 1994; Spencer et al., 1990; Sperling & O'Connor, 1989). A few studies have investigated the response of the brain to nociception through the use of depth electrodes (Frot et al., 2001; Frot & Mauguière, 1999). These studies have been used to localise the source of the response to pain in humans using depth electrodes (Frot et al., 2001). However, these methods have not been used to investigate the response to pain in animal models. Instead, these animal studies have mostly employed the use of scalp surface electrodes (Bergamasco et al., 2011; Johnson, Stafford, et al., 2005; McIlhone et al., 2011; Murrell et al., 2003). This difference in recording technique is likely to affect the outcome of the recordings. Studies investigating the onset of epilepsy seizures have found that depth electrodes are better at localising the origin of seizures than subdermal or scalp electrodes (Spencer et al., 1990). While scalp recordings are a good indication of electrical activity over a wide area of the brain, some electrical activity is lost during transduction and scalp electrodes will only pick up the electrical activity generated by large groups of active neurons (Teplan, 2002). Between these neural generators and the scalp electrodes, the signal must pass between layers of skin, skull and several other layers (Teplan, 2002). Weaker electrical activity is unable to pass through these barriers and is therefore often not seen from scalp recordings (Teplan, 2002). Depth electrodes record electrical activity without these barriers and may therefore detect the weaker activity that is not be seen from the scalp (Cooper et al., 1965). In response to sensory stimulation, cortical recording has been seen to increase by ~200 μV, an increase which was not seen through scalp recordings and in the same study, the intrinsic beta activity of the brain was not recorded by scalp electrodes, while it was detected by intracerebral and cortical electrodes (Cooper et al., 1965). There are differences between intracerebral recordings and scalp recordings, with the amplitude of recordings at cortical level generally about two to three times that of the recordings from the scalp (Cooper et al., 1965). Therefore, the recordings we see in dEEG are likely to differ from what we see in scalp EEG. This is a possible reason why the results of this study differ compared to previous studies investigating nociceptive effects on the EEG.

4.4.3. Intensity of stimulation

It is possible that this study did not find a consistent response to nociception because the stimuli applied may not have been intense enough to elicit a noticeable response in the dEEG of the subjects. Previous studies have used stimuli that are painful and that cause considerable

tissue damage, such as castration or scoop dehorning (Gibson et al., 2007; Murrell et al., 2003). Whereas the present study attempted to use painful, but non-damaging stimuli to maintain the welfare of subjects. Due to this, it is possible that the stimuli chosen were below the threshold needed to activate nociceptors and elicit a response of the central nervous system. However, these stimuli were chosen as they were believed to be noxious enough to elicit a response as shown in mammalian studies, and so this seems unlikely. The intensity of stimulation also affects the response of the brain, with paradoxical arousal seen in response to less intense stimuli (Otto & Mally, 2003). This may account for differences seen in the present study from previous works.

The non-painful stimulus used in this study was chosen as it was believed not to be painful and could therefore be used to differentiate the response to a nociceptive stimulus from an innocuous stimulus. This was to ensure the response that was seen was due to the noxious component of the stimulus, and not just due to a sensory aspect. Previous studies have investigated the response of the brain to both painful and non-painful stimulation (Frot et al., 2001; Kunde & Treede, 1993). These studies found a differing response of the brain to these stimuli which suggests a non-painful stimulus would cause a different response of the brain than nociceptive stimuli. Therefore, this stimulus is useful in order to differentiate a central brain response to nociception from a response to innocuous stimulation.

The mechanical stimuli was a toe clamp applied to the middle metatarsal. A toe or tail clamp stimulus has been previously used as a nociceptive stimulus (Eger et al., 1988; Moeser, Blikslager, & Swanson, 2008). Chickens have also been found to have a withdrawal response to toe clamps which would indicate that this stimulus is strong enough to activate the central nervous system (McIlhone et al., 2011). Therefore, this stimulus is likely to cause a response of the EEG and as such was included in this study.

The thermal stimulus used in this study was the application of a 55–60°C metal rod to the skin below the wing. The temperature used was believed to be hot enough to be noxious, but not hot enough to cause damage to the skin (Woolley and Gentle 1987). Thermal stimuli have been used in studies testing analgesic efficiency in avian species (Geelen et al., 2013). The withdrawal temperature of parrots was investigated and it was found that the mean temperature which caused a foot withdrawal ranged from 38.6–47.8°C (Geelen et al., 2013). This suggests that a thermal stimulus becomes noxious around these temperatures. The stimulus used in the present study was in excess of these temperatures (55–60°C) and so there is little doubt that it would be considered nociceptive. The temperature of the rod was

monitored right up until application to the skin to ensure that the temperature was maintained until application. Therefore, it is believed that this stimulus would induce a response of the brain due to its noxious characteristics.

Previous studies have used feather removal as a means of noxious stimulation in chickens (Gentle & Hunter, 1991). The removal of feathers has been shown to cause both a behavioural and physiological response, indicating that it is perceived as a painful stimulus (Gentle & Hunter, 1991). Plucking of feathers is even considered more painful than cutting the skin of birds (Lumeij & Deenik, 2003). It has been shown that a feather pluck increases heart rate considerably and that this is eliminated following the administration of an analgesic (Lumeij & Deenik, 2003). This suggests that feather removal is indeed a nociceptive stimulus. Feathers are anchored in tissues that are heavily innervated by mechanically sensitive fibres and nerves (Gentle & Hunter, 1991). High threshold pressure receptors have also been found to be associated with the feather follicle in the chicken (Holloway, Trouth, Wright, & Keyser, 1980). The force required to remove feathers is in excess of that required to activate nociceptors and the removal of feathers results in tissue damage (Gentle & Hunter, 1991). As such, it is believed that feather removal will have nociceptive consequences and is considered painful.

The electrical stimulus used in this study was a 50 V/50 Hz stimulus applied to the leg for 2 seconds. Such a stimulus has previously been used in pain models of the chicken and rat (McIlhone et al., 2011; Murrell et al., 2007). In studies of the rat EEG, this stimulus was seen to increase F50 significantly compared to baseline (Murrell et al., 2007). This response is conducive with a response of the brain to painful stimulation and as such this stimulus was believed to be nociceptive. However, electrical stimulation will cause non-specific activation of afferent fibres and will result in both noxious and non-noxious inputs into the brain (Murrell et al., 2007). Therefore, the changes seen in the EEG may not reflect nociceptive processing but may just reflect a change due to activation of non-noxious fibres (McIlhone et al., 2011). The response to electrical stimulation is similar to the response to nociceptive stimulation and as such was considered painful and included in the present study.

4.4.4. Anaesthesia

EEG techniques can be used to assess anaesthesia and detectable differences are seen between varying levels of anaesthetic depths and between anaesthetic agents (McIlhone et al., 2014; Murrell, Waters, & Johnson, 2008). The depth of anaesthesia has been shown to alter the recording of the electrical activity of the brain, with an increasing depth of anaesthesia associated with a slight, and inconsistent, increase in the amplitude of the EEG (Otto & Short,

1991). Therefore, any variation in the depth of anaesthesia is likely to result in differences in the EEG recording. The present study aimed to maintain anaesthesia to a consistent level, however, individual differences between subjects will mean that there is uncontrollable variation in the depth of anaesthesia. Electroencephalographic changes during anaesthesia have been described as a shift from high frequency/low amplitude, to low frequency high amplitude (Otto, 2008; Otto & Short, 1991). As low frequencies contribute more to the power, this would cause an increase in the P_{TOT} of the recording (Teplan, 2002). Halothane was used in this study as it has previously been shown to have only a slight effect on the EEG of the chicken (McIlhone et al., 2014). However, increases in the concentration of halothane have been associated with changes in the EEG of chickens. High concentrations of halothane are seen to reduce F50 and increase P_{TOT} (McIlhone et al., 2014). This may explain the differences which occurred between subjects that were seen in this study. However, by converting variables to a percentage of baseline, differences between individuals that may have been due to differing anaesthetic depths were eliminated.

It is possible that the response of the avian brain under anaesthetic will differ to that of the mammalian brain due to different distribution of receptors throughout the central nervous system (Lichtenberger & Ko, 2007). For example, pigeons are seen to have predominantly κ-opioid receptors in the forebrain rather than μ- opioid receptors (Lichtenberger & Ko, 2007). As such, the response of birds to μ- agonists is not the same as that seen in mammals (Lichtenberger & Ko, 2007). Birds also respond differently to anaesthetics due to differences in their physiology and anatomy compared to mammals (Lierz & Korbel, 2012). Halothane exerts its anaesthetic effect through interaction with $GABA_A$ and glycine receptors (Wood, Campagna, Miller, & Forman, 2003). The distribution of these receptors in the avian brain may differ from the mammalian brain, therefore causing a differing response to halothane. However, the distribution of GABA receptors in the avian brain have been shown to be similar in terms of both pharmacological properties and anatomical distribution compared to the mammalian brain (Dietl, Cortes, & Palacios, 1988). As well as this, the response of chickens to halothane anaesthesia is similar to the response of mammals (McIlhone et al., 2014) While the response of the avian brain under anaesthesia may differ to that seen in mammals, similarities in EEG recorded under halothane anaesthesia would suggest that this was not a major factor in this study. The avian response to halothane would suggest that anaesthesia will produce the same effects as in mammalian studies.

4.4.5. Accuracy of electrodes

The concentric needle electrodes used in this study have a small recording area, therefore accuracy was important in order to target the correct region of the avian brain. While scalp electrodes can be positioned to record a much larger area of the brain, these electrodes targeted a considerably smaller area (Cooper et al., 1965). The recording area of the electrodes used in this study was 0.07 mm^2 (Natus Neurology Incorporated, Middleton, WI, USA). This results in a recording hemisphere of 1.07 x 10^{-3} mm^3. While centimetre changes in the positioning of scalp electrodes will not cause much change in recording, even slight changes in the positioning of intracerebral or cortical electrodes can cause a difference in the recording (Cooper et al., 1965). Through the use of intracerebral electrodes, a spacing between electrodes of 1 mm was found to produce a markedly different recording (Cooper et al., 1965). Therefore accuracy of the electrode placement was important in order to consistently record from the same brain region. The differences between this study and those previous studies in mammals may be due to the accuracy of placement. While all efforts were taken to ensure the accuracy of the electrode, variations in bird size and head positioning made it difficult to ensure that all needles were in the same spot, especially to the degree of 0.07 mm^2. Therefore, recordings from the subjects were likely to be from slightly different brain regions. This may contribute to the variation in electrical response that was seen in this study.

4.4.6. Pain in birds

There was no consistent response of the avian brain to nociceptive stimulation. It is therefore a possibility that birds may not have a central brain response to pain. The behavioural and physiological responses to pain that have previously been seen may instead be reflex responses to nociception that are not processed by the brain. This, however, seems unlikely due to the complex behavioural responses that are seen in birds following nociception (Gentle & Hunter, 1991; Woolley & Gentle, 1987). It seems more likely that some of the brain regions targeted in this study did not show a response to pain as they are not involved in pain processing, and that the central brain regions involved in nociceptive processing are located in other brain regions, or that the targeted structures were not recorded from in this study. There did however appear to be a response in the hHA to nociception, which should be further investigated with a larger sample size. It seems unlikely, due to the similarities in the function of the avian brain to the mammalian, that there would be no response to pain. Therefore, further investigation will be required in order to determine if there is an avian response to nociceptive stimulation.

4.5. Limitations to the Study

4.5.1. Variation in subject size

This study had a number of limitations which may have had implications on the results. Firstly, the size of subjects was quite variable. As previously mentioned, the birds used were broiler chickens (*Gallus gallus domesticus*) which are bred for meat and as such have a rapid growth rate (Buzała, Janicki, & Czarnecki, 2015). In this study, chickens used were between the age of 5–6 weeks in order to limit differences in size and brain maturity. However, due to the high growth rate, over the course of one week the variation in size was considerable (1.99–3.67kg). As weight is directly correlated with head size, this variation in subject weight also meant that there were substantial differences in the size of the head (Kuenzel & Masson, 1988). This resulted in considerable variation in the position of the head within the stereotaxic frame. Head size is directly correlated with brain size and thus with this variation in head size, the brain size was also variable (Kuenzel & Masson, 1988). This caused problems with the positioning of the brain in relation to the stereotaxic frame, and the size of the brain in comparison to the coordinates given by the stereotaxic atlas. The stereotaxic atlas of the brain was designed for the use of chicks between 2–3 weeks of age and of considerably smaller size (Kuenzel & Masson, 1988). The preliminary trials of the co-ordinates suggested that they were accurate for the brain regions being targeted. However, any variation in brain size would mean that there would be a discrepancy in the position of the electrodes within the brain. Therefore, the variation between subject sizes means that the accuracy of the electrode placement within the brain was likely to be variable, resulting in recordings being taken place from unintended brain regions. This is likely to be a major influence on the different populations that were seen from within the hyperpallium apicale.

4.5.2. Accuracy of electrode placement

The accuracy of the electrode position within the brain was a major limitation in this study. As previously discussed, due to the small recording area of the concentric needle electrode, even a slight difference in electrode placement is likely to produce a different recording. This was believed to be the reasoning for the two populations seen within the hyperpallium apicale. There were a number of reasons for the inaccuracy of the electrode placement. As discussed, the variation in size of the subjects meant that there was a variation in the position of the head in the stereotaxic frame and location of the brain regions in comparison to the zero point of the stereotaxic frame. Therefore the positioning of the electrode within the brain using the stereotaxic co-ordinates was likely to vary. The depth at which the electrode was positioned was a key factor in determining the accuracy of the electrode. The depth at which the needle

was inserted was the most difficult to gauge. This was due to the differing position of the head in the stereotaxic frame and slight differences in the angle of the head in the frame. The head was aligned in the frame so that it was parallel to the base of the stereotaxic frame, however it was difficult to judge this exactly. A change in the angle of the head would mean that the targeted regions of the brain would have varied in height in relation to the stereotaxic frame. In order to overcome this, it was decided that the electrode would be positioned at the surface of the brain and then inserted 2 mm to reach the HA, and 3 mm to reach the NCM. This was done in an attempt to maintain a consistent depth of the electrode. This worked well in trials; however with live subjects it became difficult as the surface of the brain quickly became obscured by blood. Therefore it was difficult to position the electrode on the brain surface and variation in depth was likely to occur. Future studies employing the use of depth electrode in this way will need to focus on better methods of accurately inserting the electrode within the brain. Suggestions would include developing a more consistent approach to positioning the head in the apparatus, and developing a way to adjust the co-ordinates for the brain regions based upon the individual size of the head/brain.

4.5.3. Sample size

A major limitation of this study was the small sample size that was used. Initially, the HA and NCM were each to be investigated using 10 subjects each. However, throughout the research, these numbers changed, with the HA being separated into two groups and one bird from the NCM being eliminated due to a large amount of burst suppression in the recording. Therefore, each brain region was investigated using either 5 (hHA and lHA) or 9 (NCM) birds. This small sample size may have affected the likelihood of identifying any statistically significant differences between regions. There were a number of variables which appeared to be trending toward a change, however were not considered significant. With a larger sample size, it is possible that this investigation may have found some significant responses following stimulation. In future research, a larger sample size will be necessary in order to have a greater chance of observing a statistically significant response and to increase confidence in these results.

4.5.4. Determination of correct electrode position

In order to determine whether the electrode placement was correct Indian ink was injected into the brain in the same location as the electrode. This was good at giving a general indication of the electrode position, however it was associated with a number of issues. A 1 µl volume was injected into the brain as this was enough ink to be easily identifiable within the formalin fixed brain and small enough to provide a reasonably accurate indication of electrode

placement. However, a 1 µl injection has a volume of 1 mm^3, while the recording hemisphere of the electrode has a radius of 0.07 mm (Natus Neurology Incorporated, Middleton, WI, USA). Therefore, the recording volume of the electrode is 1.07 x 10^{-3} mm^3, considerably smaller than the area indicated by the ink. Therefore, while the ink will give a broad indication as to the electrode placement, it will not be entirely accurate in determining the exact region that was being recorded from. Further research should attempt to improve the method of determining the electrode position in order to better know the exact brain regions that are being targeted. This would have been especially helpful in this study in order to distinguish which brain regions were recorded from in the two distinct populations of the HA. The ink position in the NCM group was quite constant, while the HA was much more variable. This was possibly due to the fact that the HA was further away from the zero point of the stereotaxic frame, meaning that it was more affected by differences in head position and brain size than the NCM. Also, a number of the trials from the HA had no ink located in the brain due to errors in the insertion (Appendix A). Therefore, identifying the electrode placement within the HA was unfortunately problematic, and thus the brain regions of the two populations in the HA remain unknown. If further research is undertaken to investigate the HA in its response to nociception, then more care will be necessary in order to determine the placement of electrodes and the exact region of the brain that is being studied.

4.5.5. Age of the stereotaxic atlas

The age of the stereotaxic atlases used in this study may have been an issue. The main atlas used, "A stereotaxic atlas of the brain of the chick (Gallus domesticus)" was from 1988 and was the most recent that could be found (Kuenzel & Masson, 1988). The other atlas that was used as a secondary reference was from 1962 (Van Tienhoven & Juhasz, 1962). The issue with this is that the understanding of the avian brain has changed considerably in the time since these atlases were produced. During the time of these atlases, the avian brain was believed to be composed almost entirely of an enlarged striatum or basal ganglia (Jarvis et al., 2005; Reiner et al., 2004). However, since this time, the belief that birds had a hyperstriatum has been proven wrong (Güntürkün, 2005; Jarvis et al., 2005). It has since been proven that the avian brain is composed of a small basal ganglia and a large pallium (Reiner et al., 2005). Therefore, in 2005, the Avian Brain Nomenclature Consortium produced new terminology to better represent the avian brain (Jarvis et al., 2005). The regions of the brain that were originally believed to be striatum were renamed to reflect their pallial origin (Jarvis et al., 2005). For example, the hyperstriatum (H) was renamed the hyperpallium (H) and the hyperstriatum accesorium (HA) was renamed the hyperpallium apicale (HA) (Reiner et al., 2004; Reiner et al., 2005). The

neostriatum (N) was renamed the nidopallium, and the caudal medial neostriatum (NCM) became the caudomedial nidopallium (NCM) (Reiner et al., 2004). Therefore the atlas used was using the outdated nomenclature. However, only the names of the brain structures changed, therefore, the region labelled the hyperstriatum accesorium was believed to represent the hyperpallium apicale and the caudal medial neostriatum was believed to represent the caudomedial nidopallium. A more up to date atlas of the chicken brain would be a benefit for further research in order to have a more accurate and modern resource of the avian brain.

4.5.6. Use of alternative avian models

It is possible that the chicken is not the most suitable model for investigations of avian pain. As mentioned, the stereotaxic atlas of the chicken is out of date, while more modern atlases of the avian brain exist. A 3-D atlas of the pigeon brain has been developed, as well as an atlas of the brain of the zebra finch, however due to species differences, these atlases are not likely to be useful for the brain of the chicken (Güntürkün et al., 2013; Nixdorf-Bergweiler & Bischof, 2007). Pigeons are a widely used model of avian neurobiology and anatomy and as such may provide a better model for investigations of avian pain (Güntürkün et al., 2013).The pigeon has been used extensively in neurological research with the majority of understanding of avian neurobiology achieved through the use of pigeons (Güntürkün et al., 2013). The revised nomenclature of the avian brain was based mostly upon the pigeon brain, and an atlas of the pigeon brain remains widely used (Jarvis et al., 2005; Karten et al., 1973; Reiner et al., 2005). They are a widely used model of avian neurobiology and anatomy, and the ascending sensory pathways have been more thoroughly characterized than any other bird (Güntürkün et al., 2013). As such, pigeons may provide a better model for investigations of avian pain than the chicken. The 3-d atlas of the pigeon brain can be used to determine the stereotaxic location of identified neuronal structures (Güntürkün et al., 2013). The atlas can be used to find optimal positioning for electrophysiological recording and as such would be a highly beneficial tool for future research (Güntürkün et al., 2013). A more modern atlas also exists for the brain of the zebra finch. The zebra finch is a popular model system for studying the neuronal mechanisms in a variety of settings, in particular, song learning and behaviour (Nixdorf-Bergweiler & Bischof, 2007). While an atlas of the zebra finch brain exists, this atlas has been set up mainly for use in locating nuclei of the song system (Nixdorf-Bergweiler & Bischof, 2007). Due to this, the zebra finch is less likely to be a useful model of pain than the pigeon, due to the advanced knowledge of the sensory pathways in the pigeon. Future research into this area should consider the use of the pigeon as a model of avian pain due to the depth of knowledge relating to the pigeon nervous system.

4.5.7. Brain regions targeted

This study was limited by the fact that the avian brain regions involved in the integration and perception of pain are unknown. Therefore, the brain regions targeted in this study have an uncertain role in pain processing, and as such may display no response to nociceptive stimulation. The regions of the brain targeted had to be decided based upon their neuronal connections and their comparative structure and function to pain processing areas in the mammalian brain. The hyperpallium apicale (HA) and the caudomedial nidopallium (NCM) have been suggested as possible brain regions involved in the central processing of pain (Kuenzel, 2007; Lierz & Korbel, 2012). However, the role that these regions play in the pain response is unknown and it is therefore possible that their activity may not change in response to nociception. The rostral HA has homologous function to the somatosensory cortices (S1 & S2) of the mammalian brain in terms of somatosensory processing and has strong reciprocal connections with the thalamus (Medina & Reiner, 2000; Reiner et al., 2005). The mammalian S1 and S2 are known to be part of the pain matrix and so the avian S1 was investigated due to its homologous function. However, it is possible that the avian S1 only shares the somatosensory function with its mammalian counterparts and may not contain high threshold sensory neurons which respond to pain. The NCM also has a role in sensory processing. It has largely been associated with auditory processing and has sensory input from the thalamus (Reiner et al., 2004). It is likely that it is implicated in pain response due to its sensory role and connections from the thalamus. Emission topography of the brain has suggested that the nidopallium is the corresponding anatomic structure of birds to the mammalian pain centres (Lierz & Korbel, 2012; Paul-Murphy, Sladky, McCutcheon, Steege, & Converse, 2005). It is possible that the NCM is an auditory region of the brain that does not have a role in nociceptive processing. However, the nidopallium is a large brain region which contains a number of large cell populations and therefore cannot be discounted completely (Reiner et al., 2004). It is possible that other regions of the nidopallium are involved in the processing of nociceptive information, or other regions of the brain entirely. These may include the dorsal ventricular ridge or corticoidea dorsolateralis (CDL), which is a thin, superficial layer of the avian brain which has similarities in its connections to the mammalian cingulate cortex and may therefore share a function in pain perception (Csillag & Montagnese, 2005). The fact that the pain centres of the avian brain are unknown means that the brain regions targeted in this study may have no role in pain processing and that the regions of the brain responsible for this are located elsewhere.

4.5.8. Minimal anaesthesia model

The use of the minimal anaesthesia model for monitoring the electrical changes of the brain in response to nociceptive stimulation has some limitations. Fluctuations in the depth of anaesthesia are likely to affect the recording of the dEEG (Murrell & Johnson, 2006). There are also unexplained differences which often occur between individuals (Murrell et al., 2003). In order to attempt to limit these differences, end-tidal gas concentrations were monitored and were kept constant to ensure anaesthetic depth was consistent. By converting the FFT variables to a percentage of baseline, the influence of any inherent differences between individuals were eliminated (McIlhone et al., 2011). The skew that was caused by differing baseline values was eliminated by standardising these values to 100%. Some studies suggest that the recording of the EEG is not reliable or consistent enough to use as a measure of pain (Norman et al., 2008). However, a number of studies have found consistent responses of the EEG to nociception (Bergamasco et al., 2011; Johnson, Wilson, et al., 2005; Murrell & Johnson, 2006; Murrell et al., 2003; Murrell et al., 2005). The effect of halothane on the EEG of the chicken has been investigated and has been seen to be similar to that seen in mammals (McIlhone et al., 2014). Therefore, the use of the minimal anaesthesia model in this study is not believed to have an effect on the EEG recording.

4.5.9. Contralateral control of the vertebrate brain

An oversight of this study may have been the contralateral control of the vertebrate brain. Contralateral control, where the left side of the body interacts with the right side of the brain and vice-versa, is common throughout vertebrate species (Whitehead & Banihani, 2014). Most motor and sensory fibres in the central nervous system of mammals cross the midline, therefore sensory fibres from the left side of the body enter the right hemisphere of the brain (Banihani, 2010). The presence of contralateral control in avian species was not accounted for in the present study. The recordings of the hyperpallium apicale were taken from the left hemisphere of the brain, while the caudomedial nidopallium was taken from the right hemisphere. The stimulation was applied predominantly to the right hand side of the body. Studies in humans have shown that there is contralateral activation of the primary somatosensory cortex in response to pain (Bingel et al., 2003; Ploner, Schmitz, Freund, & Schnitzler, 1999). Other studies have reported predominantly contralateral responses of the thalamus in response to pain, as well as the secondary somatosensory cortex (Derbyshire, 2000; Peyron, Laurent, & Garcia-Larrea, 2000). Thus, nociceptive stimulation of the left side of the body has been seen to activate central brain regions of the right side of the brain (Bingel et al., 2003). Therefore, the presence or absence of contralateral control in the nociceptive

circuits of the avian nervous system is likely to affect the response of the investigated brain regions to stimulation. The somatosensory hyperpallium in birds receives inputs from the DIVA of the thalamus which are mostly from the contralateral body surface (Medina, 2009). The avian nervous system contains both ipsilateral and contralateral pathways (Nottebohm, 1980). While the somatosensory hyperpallium has been shown to receive contralateral inputs, other regions of the brain have been seen to receive ipsilateral inputs (Nottebohm, 1980). The auditory pathways have been seen to be dominated by contralateral projections when compared to the vocal pathways (Nottebohm, 1980). Regions of the caudomedial nidopallium, such as field L are seen to have ipsilateral projections, so it is possible that any nociceptive projections to this region may also have ipsilateral projections (Nottebohm, 1980). In any case, due to the unknown characteristics of the nociceptive pathways in birds, consideration needs to be taken into account for both possibilities. There are a few ways this could be achieved, the most simple of which would be to apply stimulation to both sides of the body. This would be interesting to see if the response of the brain would vary depending on the location of stimulation. Another method would be to record simultaneously from both hemispheres in order to achieve the same result. However, it may be difficult to ensure that the recordings are both from identical brain regions in the separate hemispheres. Lastly, a more thorough investigation could take place to determine the characteristics of the nociceptive pathways in the avian nervous system. This would help determine the presence of contralateral/ipsilateral pathways in nociceptive processing and therefore inform future studies of the necessary precautions to take.

4.5.10. EEG Analysis

The method of EEG analysis used may not be the best suited for studying the avian brain. In studies of the mammalian response to pain, F50 and total power are of particular use in terms of their response (Murrell & Johnson, 2006). However, there is nothing to suggest that these variables will be useful in avian models. It may be that other variables of the Fourier transform may provide a better indication of the avian response to pain. For example, perhaps variables such as the F80, the frequency below which 80% of the power is located, or F25, the frequency below which 25% of power is located, may provide additional insights into the activity of the avian brain following stimulation. Previous studies have used F80 in the analysis of the mammalian response to nociception, so this is a viable option (Otto & Mally, 2003). However, the use of F50 and P_{TOT} as measures of nociception are well established in mammals. It is likely that these may also be useful parameters for assessing nociception in birds, however it would be of interest to examine such possibilities.

The use of FFT in this study may also be unsuitable. The Fourier transform used in this study produces a power spectrum for 1 second windows of the dEEG recording. The major limitation of the FFT is that the window length will affect the time resolution of an event (Subha, Joseph, Acharya, & Lim, 2010). While a short time window offers better time resolution of the EEG than a larger window, it has a poor frequency resolution and vice versa (Subha et al., 2010). Therefore, the window size for FFT analysis needs to be carefully considered in order to maintain both time and frequency resolution (Subha et al., 2010). A window length of 1 second has previously been utilised and has been effective in assessing the response to pain (Gibson et al., 2009; Murrell et al., 2007). It is believed to be long enough to maintain frequency resolution and short enough to maintain adequate time resolution of changes that are occurring in the EEG. In saying this, there are methods which can be used to resolve the resolution problems seen in FFT (Rao & Bopardikar, 1998). Wavelet transform could be of particular use in assessing the response of the EEG to pain. The benefit of the wavelet transform is that the window size varies, being larger with low frequencies and smaller for high frequencies (Quiroga, 1998). This allows for optimal time-frequency resolution for all frequencies, which overcomes the major downfall of the FFT (Chui, 1992; Strang & Nguyen, 1996). It would be quite interesting to investigate different methods of analysis of the EEG. This may prove useful in assessing the avian response to nociception.

Along with this, the length of the period being investigated may play a part in the outcome of the study. This study investigated 30 second blocks following stimulation. This was to ensure that any change in activity was included in this time frame. However, 30 seconds is quite a long time in terms of brain activity, where changes can occur over milliseconds. Acute changes in brain activity may be drowned out in the 30 second mean. Shorter periods may be useful to determine if there are any responses occurring in a shorter time frame than 30 seconds. Previous studies have employed the use of 30 second blocks to investigate the response to nociception and changes are seen to occur over this time period (Murrell et al., 2007). Other studies have also investigated shorter time frames in order to examine the response of the brain to nociception (Gibson et al., 2007; Johnson et al., 2009). While 30 second periods following stimulation may be useful, it might also be of interest to investigate these changes occurring over shorter time frames.

4.5.11. Alternative methods of assessing brain activity

Methods other than electroencephalography can also be used in order to assess the activity of the brain. Methods such as fMRI and PET scans have previously been used in order to assess the activity of the brain. The use of such methods may provide a more accurate means of

assessing response of the avian brain to pain. Positron emission tomography (PET) is widely used in studies of human pain since the beginning of the 1990s (Peyron, Laurent et al., 2000). PET allows the investigation of underlying brain activity through the measurement of the distribution of radioactive isotopes (Peyron, Laurent et al., 2000). The functional activation of particular brain regions is reflected in an increase in regional cerebral blood flow to these areas. This change in blood flow is detected by PET, giving an indication of the activation of the brain in response to stimulation (Peyron, Laurent et al., 2000). As well as this, functional magnetic resonance imaging (fMRI) has also been used to detect changes in brain activity. This method has been a major neuroimaging tool in cognitive neuroscience since its introduction in the 1980s (Logothetis, 2008). Studies of fMRI investigate changes in the blood oxygenation level dependent (BOLD) signal, which reflects changes in cerebral blood flow and variations in deoxyhemoglobin content (Peyron, Laurent et al., 2000). Through this, the activity of the brain can be assessed. It has been shown that results obtained via fMRI are strongly correlated to those if PET scans and these methods have been used to investigate the response of the brain to pain in both humans and other mammals (Borsook & Becerra, 2011; Oshiro et al., 1998; Peyron, Garcia-Larrea, et al., 2000). Such investigations of pain in humans and mammals have seen the activation of the secondary somatosensory (SII) cortices, insular cortices, and in the anterior cingulate cortex (ACC) (Brooks, Nurmikko, Bimson, Singh, & Roberts, 2002). Studies have also used fMRI in conjunction with EEG in order to better assess cortical pain processing (Christmann, Koeppe, Braus, Ruf, & Flor, 2007). The use of such methods could be a valuable tool for the investigations of the response of the avian brain to pain. Future studies should consider their use either independently, or in conjunction with electrophysiology.

4.5.12. External warming protocol of birds

During general anaesthesia it is common for subjects to develop hypothermia and it is therefore important to provide a means of maintain body temperature (Murison, 2001; Nevarez, 2005). Hypothermia during anaesthesia of avian species can result in life-threatening complications and therefore maintaining body temperature is of importance during prolonged anaesthesia (Boedeker, Carpenter, & Mason, 2005). Even with supplementary heat provided, hypothermia can occur (Boedeker et al., 2005). Hypothermia has been seen to have a significant effect on the EEG spectrum in mammalian studies (Briatore et al., 2013; Levy, 1984). Hypothermia in human subjects was seen to cause an increase in burst suppression, leading to a decrease in total power of the EEG, while causing no effect on spectral edge or average frequency (Levy, 1984). It is therefore a possibility, due to the effect of hypothermia on the EEG, that the temperature of the anaesthetized subjects has had an effect on the results in this study. The method of

supplementary heating used in this study may not have been fully effective and therefore a decrease in temperature, resulting in EEG changes may have occurred. A number of studies have investigated the effectiveness of different warming methods in birds (Rembert, Smith, Hosgood, Marks, & Tully Jr, 2001; van Zeeland, Cardona, & Schoemaker, 2012). It has been shown that over the course of a 60 minute period, no tested warming protocol was able to prevent a decrease in temperature in anesthetized Hispaniolan Amazon parrots (Rembert et al., 2001). However a convective, forced-air warmer, was able to significantly slow this decrease in temperature when compared to a conductive heat blanket and an infrared heat emitter (Rembert et al., 2001). This would suggest that a convective method would have been more effective in this study. However, these findings are contradicted by a similar study which saw that a conductive warming protocol, such as that used in this study, was more effective at maintaining body temperature in pigeons under anesthesia when compared to a convective heating device (van Zeeland et al., 2012). It was suggested that the differences seen between these studies may be due to differences in design of the conductive warming device (van Zeeland et al., 2012). However, no study saw a stop to heat loss in spite of the warming protocol used (Rembert et al., 2001; van Zeeland et al., 2012).. However, temperature was monitored throughout anaesthesia in this study and was not seen to fall below the clinically acceptable body temperature (>38.3 °C) and therefore hypothermia was unlikely to have occurred (Rembert et al., 2001). As such, future studies should be vigilant in ensuring body temperature is maintained to acceptable levels in order to limit the possible effects of hypothermia as it can have a significant impact on the EEG.

5. Conclusions and future work

This work aimed to investigate the response of the avian brain to pain through targeting brain regions that were believed to have a role in the central processing of nociceptive stimuli and the perception of pain. The results provide some interesting insights into the activity of the avian brain and its response to stimulation. It was seen that the brain regions studied differed considerably in their spectral characteristics, indicating a difference in the oscillatory activity occurring throughout the brain. This suggest a difference in both the function and activity of these regions. In response to stimulation, the change in activity of these regions was seen to be significantly different. This suggests that, firstly, neuronal activity differs throughout the avian brain, and secondly, these changes in activity can be identified using electrophysiological techniques. However, the results of this study do not indicate a clear response of any of the brain regions studied to nociceptive stimuli. While the exact response of these regions to stimulation remains to be seen, these results provide promising insights for future work. It appears as though the caudomedial nidopallium (NCM) and the low hyperpallium apicale (lHA) show no response to nociceptive stimulation. This conclusion was reached based on the lack of response seen from these brain regions to nociceptive stimulation and consistency of their dEEG variables following stimulation. In contrast, the high hyperpallium apicale (hHA) did display a change in activity following stimulation. While no consistent response was found, there was seen to be a decrease in total power following electrical stimulation. This response may represent the increased activity of the hHA due to nociceptive processing. It would appear that the hHA has a greater response to stimulation than the other brain regions and is likely to be a promising target for further work into the avian response to pain. Further research will be required in order to determine the extent of the response of the hHA to nociceptive stimulation. The findings of this study suggest that future research should further investigate the response of the rostral hyperpallium apicale to nociceptive stimulation.

The small sample size used to study the hHA likely reduced the statistical power of this study. It would be of interest to investigate this response with a larger sample size to better determine the activity of this brain region following stimulation. In order to do this, future research will need to focus on developing a more accurate method of recording from targeted regions within the brain. This could achieved through assessing the use of alternative avian models, such as the pigeon, and by improving the accuracy of electrode placement. The use of pigeons may prove useful due to the existence of a modern atlas of the brain and greater knowledge surrounding their neuronal anatomy when compared to the chicken. This will help to ensure more consistent electrode placement.

Having not clearly identified a consistent response of the avian brain to pain, future research will need to determine more precisely the regions of the brain that are responsible for nociceptive processing. This study would suggest that the rostral hyperpallium apicale is a region of the brain which should be further studied, while the caudomedial nidopallium is less promising. However, in saying this, the nidopallium is a large brain region which contains a number of large cell populations and therefore cannot be discounted completely. Instead, perhaps different regions of the nidopallium are responsible for nociceptive processing. Along with this, a number of other brain regions may be possible targets for future work. These may include the dorsal ventricular ridge or corticoidea dorsolateralis (CDL) which may be regions of interest for future studies. Another approach may be to target the thalamus in order to determine the populations of the thalamus that contain nociceptive neurons. The projections of the thalamus could then be used to infer the regions of the brain which receive nociceptive inputs and are thus likely to be involved in pain perception.

In conclusion, further research is required in order to improve understanding of avian pain. Determining the regions of the brain involved in nociceptive processing is of particular interest and will help to improve the knowledge of avian pain and will assist in future studies. This study has taken steps in this direction, finding a difference in response between different parts of the avian brain to nociceptive stimulation. This response will need to be further investigated in order to gain a better understanding of what is occurring. The rostral hyperpallium apicale appears to be responsive to stimulation and is therefore of particular interest in ongoing investigations into the avian response to pain. This is important for improving our knowledge of avian pain, and will act to improve treatment and welfare of avian species.

6. References

Alivisatos, A. P., Chun, M., Church, G. M., Greenspan, R. J., Roukes, M. L., & Yuste, R. (2012). The brain activity map project and the challenge of functional connectomics. *Neuron, 74*(6), 970-974.

Almeida, T. F., Roizenblatt, S., & Tufik, S. (2004). Afferent pain pathways: a neuroanatomical review. *Brain research, 1000*(1), 40-56.

Animal Welfare Act (1999).

Apkarian, A. V., Bushnell, M. C., Treede, R. D., & Zubieta, J. K. (2005). Human brain mechanisms of pain perception and regulation in health and disease. *European Journal of Pain, 9*(4), 463-484.

Arndt, J., & Klement, W. (1991). Pain evoked by polymodal stimulation of hand veins in humans. *The Journal of physiology, 440*(1), 467-478.

Arroyo, S., & Lesser, R. (1999). Subdural electrodes. In E. Niedermeyer & F. Lopes da Silva (Eds.), *Electroencephalography: Basic Principles, Clinical Applications, and Related Fields*. USA: Williams & Wilkins.

Banihani, S. M. (2010). Crossing of neuronal pathways: Is it a response to the occurrence of separated parts for the body (limbs, eyes, etc.) during evolution? *Medical hypotheses, 74*(4), 741-745.

Basbaum, A. I., Bautista, D. M., Scherrer, G., & Julius, D. (2009). Cellular and molecular mechanisms of pain. *Cell, 139*(2), 267-284.

Behrens, E., Zentner, J., Van Roost, D., Hufnagel, A., Elger, C. E., & Schramm, J. (1994). Subdural and depth electrodes in the presurgical evaluation of epilepsy. *Acta neurochirurgica, 128*(1-4), 84-87.

Benowitz, L. (1980). Functional organization of the avian telencephalon *Comparative neurology of the telencephalon* (pp. 389-421): Springer.

Benzo, C. (1986). Nervous system *Avian Physiology* (pp. 1-36): Springer.

Bergamasco, L., Coetzee, J., Gehring, R., Murray, L., Song, T., & Mosher, R. (2011). Effect of intravenous sodium salicylate administration prior to castration on plasma cortisol and electroencephalography parameters in calves. *Journal of veterinary pharmacology and therapeutics, 34*(6), 565-576.

Bingel, U., Quante, M., Knab, R., Bromm, B., Weiller, C., & Büchel, C. (2003). Single trial fMRI reveals significant contralateral bias in responses to laser pain within thalamus and somatosensory cortices. *Neuroimage, 18*(3), 740-748.

Boedeker, N. C., Carpenter, J. W., & Mason, D. E. (2005). Comparison of body temperatures of pigeons (Columba livia) anesthetized by three different anesthetic delivery systems. *Journal of Avian Medicine and Surgery, 19*(1), 1-6.

Bonke, D., Scheich, H., & Langner, G. (1979). Responsiveness of units in the auditory neostriatum of the guinea fowl (Numida meleagris) to species-specific calls and synthetic stimuli. *Journal of comparative physiology, 132*(3), 243-255.

Borsook, D., & Becerra, L. (2011). CNS animal fMRI in pain and analgesia. *Neuroscience & Biobehavioral Reviews, 35*(5), 1125-1143.

Briatore, E., Ferrari, F., Pomero, G., Boghi, A., Gozzoli, L., Micciolo, R., . . . Calzolari, S. (2013). EEG findings in cooled asphyxiated newborns and correlation with site and severity of brain damage. *Brain and Development, 35*(5), 420-426.

Brooks, J. C. W., Nurmikko, T. J., Bimson, W. E., Singh, K. D., & Roberts, N. (2002). fMRI of thermal pain: effects of stimulus laterality and attention. *Neuroimage, 15*(2), 293-301.

Bushnell, M., Duncan, G., Hofbauer, R., Ha, B., Chen, J.-I., & Carrier, B. (1999). Pain perception: is there a role for primary somatosensory cortex? *Proceedings of the National Academy of Sciences, 96*(14), 7705-7709.

Butler, A. B., Manger, P. R., Lindahl, B., & Århem, P. (2005). Evolution of the neural basis of consciousness: a bird–mammal comparison. *Bioessays, 27*(9), 923-936.
Buzała, M., Janicki, B., & Czarnecki, R. (2015). Consequences of different growth rates in broiler breeder and layer hens on embryogenesis, metabolism and metabolic rate: A review. *Poultry Science, 94*(4), 728-733. doi: 10.3382/ps/pev015
Ching, S., Purdon, P. L., Vijayan, S., Kopell, N. J., & Brown, E. N. (2012). A neurophysiological–metabolic model for burst suppression. *Proceedings of the National Academy of Sciences, 109*(8), 3095-3100.
Christmann, C., Koeppe, C., Braus, D. F., Ruf, M., & Flor, H. (2007). A simultaneous EEG–fMRI study of painful electric stimulation. *Neuroimage, 34*(4), 1428-1437.
Chui, C. K. (1992). *An introduction to wavelets.* (Vol. 38): Academic Press Inc.
Cooper, R., Winter, A., Crow, H., & Walter, W. G. (1965). Comparison of subcortical, cortical and scalp activity using chronically indwelling electrodes in man. *Electroencephalography and clinical neurophysiology, 18*(3), 217-228.
Criado, A. (2010). Recognition and alleviation of pain in laboratory animals. *Laboratory Animals, 44*(4), 380-380.
Csillag, A., & Montagnese, C. M. (2005). Thalamotelencephalic organization in birds. *Brain research bulletin, 66*(4), 303-310.
da Silva, F. L. (1991). Neural mechanisms underlying brain waves: from neural membranes to networks. *Electroencephalography and clinical neurophysiology, 79*(2), 81-93.
Derbyshire, S. W. (2000). Exploring the pain "neuromatrix". *Current review of pain, 4*(6), 467-477.
Derbyshire, S. W., Jones, A. K., Gyulai, F., Clark, S., Townsend, D., & Firestone, L. L. (1997). Pain processing during three levels of noxious stimulation produces differential patterns of central activity. *Pain, 73*(3), 431-445.
Diesch, T., Mellor, D., Johnson, C., & Lentle, R. (2009). Electroencephalographic responses to tail clamping in anaesthetized rat pups. *Laboratory animals, 43*(3), 224-231.
Dietl, M., Cortes, R., & Palacios, J. (1988). Neurotransmitter receptors in the avian brain. III. GABA-benzodiazepine receptors. *Brain research, 439*(1), 366-371.
Doubell, T. P., Mannion, R. J., & Woolf, C. J. (1999). The dorsal horn: State-dependant sensory processing, plasticity and the generation of pain. . In P. D. Wall & R. Melzack (Eds.), *The Textbook of Pain*. London: Harcourts Publishers.
Eger, E. I., Johnson, B. H., Weiskopf, R. B., Holmes, M. A., Yasuda, N., Targ, A., & Rampil, I. J. (1988). Minimum alveolar concentration of 1-653 and isoflurane in pigs: definition of a supramaximal stimulus. *Anesthesia & Analgesia, 67*(12), 1174-1176.
Frot, M., Garcia-Larrea, L., Guénot, M., & Mauguière, F. (2001). Responses of the supra-sylvian (SII) cortex in humans to painful and innocuous stimuli: A study using intra-cerebral recordings. *Pain, 94*(1), 65-73.
Frot, M., & Mauguière, F. (1999). Timing and spatial distribution of somatosensory responses recorded in the upper bank of the sylvian fissure (SII area) in humans. *Cerebral Cortex, 9*(8), 854-863.
Geelen, S., Guzman, D. S.-M., Souza, M. J., Cox, S., Keuler, N. S., & Paul-Murphy, J. R. (2013). Antinociceptive effects of tramadol hydrochloride after intravenous administration to Hispaniolan Amazon parrots (Amazona ventralis). *American Journal of Veterinary Research, 74*(2), 201-206.
Gentle, M. (1992). Pain in birds. *Animal Welfare, 1*(4), 235-247.
Gentle, M., & Hunter, L. N. (1991). Physiological and behavioural responses associated with feather removal in *Gallus-gallus var domesticus*. *Research in Veterinary Science, 50*(1), 95-101. doi: 10.1016/0034-5288(91)90060-2
Gibson, T., Johnson, C., Murrell, J., Hulls, C., Mitchinson, S., Stafford, K., . . . Mellor, D. (2009). Electroencephalographic responses of halothane-anaesthetised calves to slaughter by

ventral-neck incision without prior stunning. *New Zealand Veterinary Journal, 57*(2), 77-83.
Gibson, T., Johnson, C., Stafford, K., Mitchinson, S., & Mellor, D. (2007). Validation of the acute electroencephalographic responses of calves to noxious stimulus with scoop dehorning. *New Zealand Veterinary Journal, 55*(4), 152-157.
Güntürkün, O. (1991). The functional organization of the avian visual system. *Neural and Behavioural Plasticity*, 92-105.
Güntürkün, O. (1996). Cognitive impairments after lesions of the neostriatum caudolaterale and its thalamic afferent in pigeons: functional similarities to the mammalian prefrontal system? *Journal fur Hirnforschung, 38*(1), 133-143.
Güntürkün, O. (2005). The avian 'prefrontal cortex'and cognition. *Current opinion in neurobiology, 15*(6), 686-693.
Güntürkün, O., Verhoye, M., De Groof, G., & Van der Linden, A. (2013). A 3-dimensional digital atlas of the ascending sensory and the descending motor systems in the pigeon brain. *Brain Structure and Function, 218*(1), 269-281.
Guzman, D. S.-M., Drazenovich, T. L., Olsen, G. H., Willits, N. H., & Paul-Murphy, J. R. (2014). Evaluation of thermal antinociceptive effects after oral administration of tramadol hydrochloride to American kestrels (Falco sparverius). *American Journal of Veterinary Research, 75*(2), 117-123.
Harris, K. D. (2015). Cortical computation in mammals and birds. *Proceedings of the National Academy of Sciences, 112*(11), 3184-3185.
Heil, P., & Scheich, H. (1985). Quantitative analysis and two-dimensional reconstruction of the tonotopic organization of the auditory field L in the chick from 2-deoxyglucose data. *Experimental brain research, 58*(3), 532-543.
Holloway, J. A., Trouth, C. O., Wright, L. E., & Keyser, G. F. (1980). Cutaneous receptive field characteristics of primary afferents and dorsal horn cells in the avian (Gallus domesticus). *Experimental neurology, 68*(3), 477-488.
Jarvis, E. D., Güntürkün, O., Bruce, L., Csillag, A., Karten, H., Kuenzel, W., . . . Shimizu, T. (2005). Avian brains and a new understanding of vertebrate brain evolution. *Nature Reviews Neuroscience, 6*(2), 151-159.
Johnson, C., Stafford, K., Sylvester, S., Ward, R., Mitchinson, S., & Mellor, D. (2005). Effects of age on the electroencephalographic response to castration in lambs anaesthetised using halothane in oxygen. *New Zealand Veterinary Journal, 53*(6), 433-437.
Johnson, C., Sylvester, S. P., Stafford, K. J., Mitchinson, S. L., Ward, R. N., & Mellor, D. J. (2009). Effects of age on the electroencephalographic response to castration in lambs anaesthetized with halothane in oxygen from birth to 6 weeks old. *Veterinary anaesthesia and analgesia, 36*(3), 273-279.
Johnson, C., Wilson, P. R., Woodbury, M. R., & Caulkett, N. A. (2005). Comparison of analgesic techniques for antler removal in halothane-anaesthetized red deer (Cervus elaphus): electroencephalographic responses. *Veterinary anaesthesia and analgesia, 32*(2), 61-71.
Karten, H. J. (1969). The organization of the avian telencephalon and some speculations on the phylogeny of the amniote telencephalon. *Annals of the New York Academy of Sciences, 167*(1), 164-179.
Karten, H. J., Hodos, W., Nauta, W. J., & Revzin, A. M. (1973). Neural connections of the "visual wulst" of the avian telencephalon. Experimental studies in the pigeon (Columba livia) and owl (Speotyto cunicularia). *Journal of Comparative Neurology, 150*(3), 253-277.
Kuenzel, W. J. (2007). Neurobiological basis of sensory perception: Welfare implications of beak trimming. *Poultry Science, 86*(6), 1273-1282.
Kuenzel, W. J., & Masson, M. (1988). A stereotaxic atlas of the brain of the chick (Gallus domesticus).

Kunde, V., & Treede, R.-D. (1993). Topography of middle-latency somatosensory evoked potentials following painful laser stimuli and non-painful electrical stimuli. *Electroencephalography and Clinical Neurophysiology/Evoked Potentials Section, 88*(4), 280-289.

Le Bars, D., Gozariu, M., & Cadden, S. W. (2001). Animal models of nociception. *Pharmacological reviews, 53*(4), 597-652.

Legrain, V., Iannetti, G. D., Plaghki, L., & Mouraux, A. (2011). The pain matrix reloaded: a salience detection system for the body. *Progress in neurobiology, 93*(1), 111-124.

Levy, W. J. (1984). Quantitative analysis of EEG changes during hypothermia. *Anesthesiology, 60*(4), 291-297.

Lichtenberger, M., & Ko, J. (2007). Anesthesia and analgesia for small mammals and birds. *Veterinary Clinics of North America: Exotic Animal Practice, 10*(2), 293-315.

Lierz, M., & Korbel, R. (2012). Anesthesia and analgesia in birds. *Journal of Exotic Pet Medicine, 21*(1), 44-58. doi: http://dx.doi.org/10.1053/j.jepm.2011.11.008

Logothetis, N. K. (2008). What we can do and what we cannot do with fMRI. *Nature, 453*(7197), 869-878.

Lumeij, J., & Deenik, J. (2003). Medetomidine-ketamine and diazepam-ketamine anesthesia in racing pigeons (Columba livia domestica)-a comparative study. *Journal of avian medicine and surgery, 17*(4), 191-196.

Machin, K. L. (2005). *Avian analgesia.* Paper presented at the Seminars in avian and exotic pet medicine.

Markand, O. N. (2003). Electroencephalogram (EEG). In M. J. A. B. Daroff (Ed.), *Encyclopedia of the Neurological Sciences* (pp. 84-94). New York: Academic Press.

McIlhone, A., Beausoleil, N., Mellor, D., Mitchinson, S., & Johnson, C. (2011). Effects of noxious stimulation on the electroencephalogram of the chicken. *10th World Congress of Veterinary Anaesthesia*, 161.

McIlhone, A., Beausoleil, N. J., Johnson, C. B., & Mellor, D. J. (2014). Effects of isoflurane, sevoflurane and methoxyflurane on the electroencephalogram of the chicken. *Veterinary anaesthesia and analgesia*.

Medina, L. (2009). Do Birds and Reptiles Possess Homologues of Mammalian Visual, Somatosensory, and Motor Cortices? In J. H. Kaas (Ed.), *Evolutionary Neuroscience* (pp. 265). Oxford, UK: Elselvier Inc.

Medina, L., & Reiner, A. (2000). Do birds possess homologues of mammalian primary visual, somatosensory and motor cortices? *Trends in neurosciences, 23*(1), 1-12.

Mellor, D., Patterson-Kane, E., & Stafford, K. J. (2009). *The sciences of animal welfare*: John Wiley & Sons.

Moeser, A. J., Blikslager, A. T., & Swanson, C. (2008). Determination of minimum alveolar concentration of sevoflurane in juvenile swine. *Research in veterinary science, 84*(2), 283-285.

Montiel, J. F., & Molnár, Z. (2013). The impact of gene expression analysis on evolving views of avian brain organization. *Journal of Comparative Neurology, 521*(16), 3604-3613.

Mouraux, A., Diukova, A., Lee, M. C., Wise, R. G., & Iannetti, G. D. (2011). A multisensory investigation of the functional significance of the "pain matrix". *Neuroimage, 54*(3), 2237-2249.

Mouraux, A., Guerit, J.-M., & Plaghki, L. (2003). Non-phase locked electroencephalogram (EEG) responses to CO 2 laser skin stimulations may reflect central interactions between Að- and C-fibre afferent volleys. *Clinical neurophysiology, 114*(4), 710-722.

Murison, P. (2001). Prevention and treatment of perioperative hypothermia in animals under 5 kg bodyweight. *In Practice (0263841X), 23*(7).

Murrell, J., & Johnson, C. (2006). Neurophysiological techniques to assess pain in animals. *Journal of Veterinary Pharmacology and Therapeutics, 29*(5), 325-335.

Murrell, J., Johnson, C. B., White, K. L., Taylor, P. M., Haberham, Z. L., & Waterman-Pearson, A. E. (2003). Changes in the EEG during castration in horses and ponies anaesthetized with halothane. *Veterinary Anaesthesia and Analgesia, 30*(3), 138-146.

Murrell, J., Mitchinson, S., Waters, D., & Johnson, C. (2007). Comparative effect of thermal, mechanical, and electrical noxious stimuli on the electroencephalogram of the rat. *British journal of anaesthesia, 98*(3), 366-371.

Murrell, J., Waters, D., & Johnson, C. (2008). Comparative effects of halothane, isoflurane, sevoflurane and desflurane on the electroencephalogram of the rat. *Laboratory animals, 42*(2), 161-170.

Murrell, J., White, K. L., Johnson, C. B., Taylor, P. M., Doherty, T. J., & Waterman-Pearson, A. E. (2005). Investigation of the EEG effects of intravenous lidocaine during halothane anaesthesia in ponies. *Veterinary Anaesthesia and Analgesia, 32*(4), 212-221.

Nevarez, J. G. (2005). *Monitoring during avian and exotic pet anesthesia.* Paper presented at the Seminars in Avian and Exotic Pet Medicine.

Nixdorf-Bergweiler, B. E., & Bischof, H.-J. (2007). A stereotaxic atlas of the brain of the zebra finch, Taeniopygia guttata.

Norman, E., Rosén, I., Vanhatalo, S., Stjernqvist, K., Ökland, O., Fellman, V., & Hellström-Westas, L. (2008). Electroencephalographic response to procedural pain in healthy term newborn infants. *Pediatric research, 64*(4), 429-434.

Nottebohm, F. (1980). Brain pathways for vocal learning in birds *Progress in psychobiology and physiological psychology* (pp. 86-125): Academic Press New York.

Ohara, S., Crone, N., Weiss, N., & Lenz, F. (2004). Attention to a painful cutaneous laser stimulus modulates electrocorticographic event-related desynchronization in humans. *Clinical Neurophysiology, 115*(7), 1641-1652.

Oshiro, Y., Fuijita, N., Tanaka, H., Hirabuki, N., Nakamura, H., & Yoshiya, I. (1998). Functional mapping of pain-related activation with echo-planar MRI: Significance of the SII-insular region. *Neuroreport, 9*(10), 2285-2289.

Otto, K. (2008). EEG power spectrum analysis for monitoring depth of anaesthesia during experimental surgery. *Laboratory animals, 42*(1), 45-61.

Otto, K., & Mally, P. (2003). Noxious stimulation during orthopaedic surgery results in EEG 'arousal'or 'paradoxical arousal'reaction in isoflurane-anaesthetised sheep. *Research in veterinary science, 75*(2), 103-112.

Otto, K., & Short, C. E. (1991). Electroencephalographic power spectrum analysis as a monitor of anesthetic depth in horses. *Veterinary Surgery, 20*(5), 362-371.

Paul-Murphy, J., Sladky, K. K., McCutcheon, R. A., Steege, E. E., & Converse, A. K. (2005). *Using positron emission tomography imaging of the parrot brain to study response to clinical pain.* Paper presented at the Proceedings.

Peyron, R., Garcıa-Larrea, L., Gregoire, M. C., Convers, P., Richard, A., Lavenne, F., . . . Laurent, B. (2000). Parietal and cingulate processes in central pain. A combined positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) study of an unusual case. *Pain, 84*(1), 77-87.

Peyron, R., Laurent, B., & Garcia-Larrea, L. (2000). Functional imaging of brain responses to pain. A review and meta-analysis (2000). *Neurophysiologie Clinique/Clinical Neurophysiology, 30*(5), 263-288.

Ploner, M., Gross, J., Timmermann, L., Pollok, B., & Schnitzler, A. (2006). Pain suppresses spontaneous brain rhythms. *Cerebral cortex, 16*(4), 537-540.

Ploner, M., Schmitz, F., Freund, H.-J., & Schnitzler, A. (1999). Parallel activation of primary and secondary somatosensory cortices in human pain processing. *Journal of Neurophysiology, 81*(6), 3100-3104.

Prichep, L. S., John, E. R., Howard, B., Merkin, H., & Hiesiger, E. M. (2011). Evaluation of the pain matrix using EEG source localization: a feasibility study. *Pain medicine, 12*(8), 1241-1248.

Quesney, L. F., & Nicholas, J. F. (1999). Electrocorticography. In E. Niedermeyer & F. Lopes da Silva (Eds.), *Electroencephalography: Basic Principles, Clinical Applications, and Related Fields*. USA: Williams & Wilkins.

Quiroga, R. Q. (1998). *Quantitative analysis of EEG signals: time-frequency methods and chaos theory.* (PhD), Medical University Lubeck.

Rao, R. M., & Bopardikar, A. S. (1998). *Wavelet transforms: introduction to theory and applications*: Prentice Hall

Reiner, A., Perkel, D. J., Mello, C. V., & Jarvis, E. D. (2004). Songbirds and the revised avian brain nomenclature. *Annals of the New York Academy of Sciences, 1016*(1), 77-108.

Reiner, A., Yamamoto, K., & Karten, H. J. (2005). Organization and evolution of the avian forebrain. *The Anatomical Record Part A: Discoveries in Molecular, Cellular, and Evolutionary Biology, 287*(1), 1080-1102.

Rembert, M. S., Smith, J. A., Hosgood, G., Marks, S. L., & Tully Jr, T. N. (2001). Comparison of traditional thermal support devices with the forced-air warmer system in anesthetized Hispaniolan Amazon parrots (Amazona ventralis). *Journal of Avian Medicine and Surgery, 15*(3), 187-193.

Sanei, S. (2013). Fundamentals of EEG signal processing. *Adaptive Processing of Brain Signals*, 37-44.

Sherwood, L., Klandorf, H., & Yancey, P. (2012). *Animal physiology: from genes to organisms*: Cengage Learning.

Spencer, S. S., Spencer, D. D., Williamson, P. D., & Mattson, R. (1990). Combined depth and subdural electrode investigation in uncontrolled epilepsy. *Neurology, 40*(1), 74-74.

Sperling, M. R., & O'Connor, M. J. (1989). Comparison of depth and subdural electrodes in recording temporal lobe seizures. *Neurology, 39*(11), 1497-1497.

Srivastava, U., & Gaur, P. (2013). Naturally occurring neuronal plasticity in visual wulst of the Baya weaver, Ploceus philippinus (Linnaeus, 1766). *Cell and tissue research, 352*(3), 445-467.

Steriade, M., McCormick, D. A., & Sejnowski, T. J. (1993). Thalamocortical oscillations in the sleeping and aroused brain. *Science, 262*(5134), 679-685.

Strang, G., & Nguyen, T. (1996). *Wavelets and filter banks*: Wellesley-Cambridge Press.

Suárez, J., Dávila, J. C., Real, M. á., Guirado, S., & Medina, L. (2006). Calcium-binding proteins, neuronal nitric oxide synthase, and GABA help to distinguish different pallial areas in the developing and adult chicken. I. Hippocampal formation and hyperpallium. *Journal of Comparative Neurology, 497*(5), 751-771.

Subha, D. P., Joseph, P. K., Acharya, R., & Lim, C. M. (2010). EEG signal analysis: a survey. *Journal of medical systems, 34*(2), 195-212.

Teplan, M. (2002). Fundamentals of EEG measurement. *Measurement science review, 2*(2), 1-11.

Van Tienhoven, A., & Juhasz, L. (1962). The chicken telencephalon, diencephalon and mesencephalon in stereotaxic coordinates. *Journal of Comparative Neurology, 118*(2), 185-197.

van Zeeland, Y. R. A., Cardona, T., & Schoemaker, N. J. (2012). Maintenance of core body temperature in anaesthetised pigeons (Columba livia domestica): A comparison of two thermal devices. *The Veterinary Journal, 194*(3), 429-432.

Vogt, B. A. (2005). Pain and emotion interactions in subregions of the cingulate gyrus. *Nature Reviews Neuroscience, 6*(7), 533-544.

Whitehead, L., & Banihani, S. (2014). The evolution of contralateral control of the body by the brain: Is it a protective mechanism? *Laterality: Asymmetries of Body, Brain and Cognition, 19*(3), 325-339.

Wild, J., Karten, H., & Frost, B. (1993). Connections of the auditory forebrain in the pigeon (Columba livia). *Journal of Comparative Neurology, 337*(1), 32-62.

Wild, J., & Williams, M. (1999). Rostral wulst of passerine birds: II. Intratelencephalic projections to nuclei associated with the auditory and song systems. *Journal of Comparative Neurology, 413*(4), 520-534.

Wild, J., & Williams, M. (2000). Rostral wulst in passerine birds. I. Origin, course, and terminations of an avian pyramidal tract. *Journal of Comparative Neurology, 416*(4), 429-450.

Wood, A. J., Campagna, J. A., Miller, K. W., & Forman, S. A. (2003). Mechanisms of actions of inhaled anesthetics. *New England Journal of Medicine, 348*(21), 2110-2124.

Woolley, S. C., & Gentle, M. J. (1987). Physiological and behavioural-responses in the hen (*Gallus domesticus*) to nociceptive stimulation. *Comparative Biochemistry and Physiology a-Physiology, 88*(1), 27-31. doi: 10.1016/0300-9629(87)90093-4

7. Appendix A

Hyperpallium apicale

The position of the brain regions targeted were determined through the use of the stereotaxic atlas (Figure 13; Figure 15).

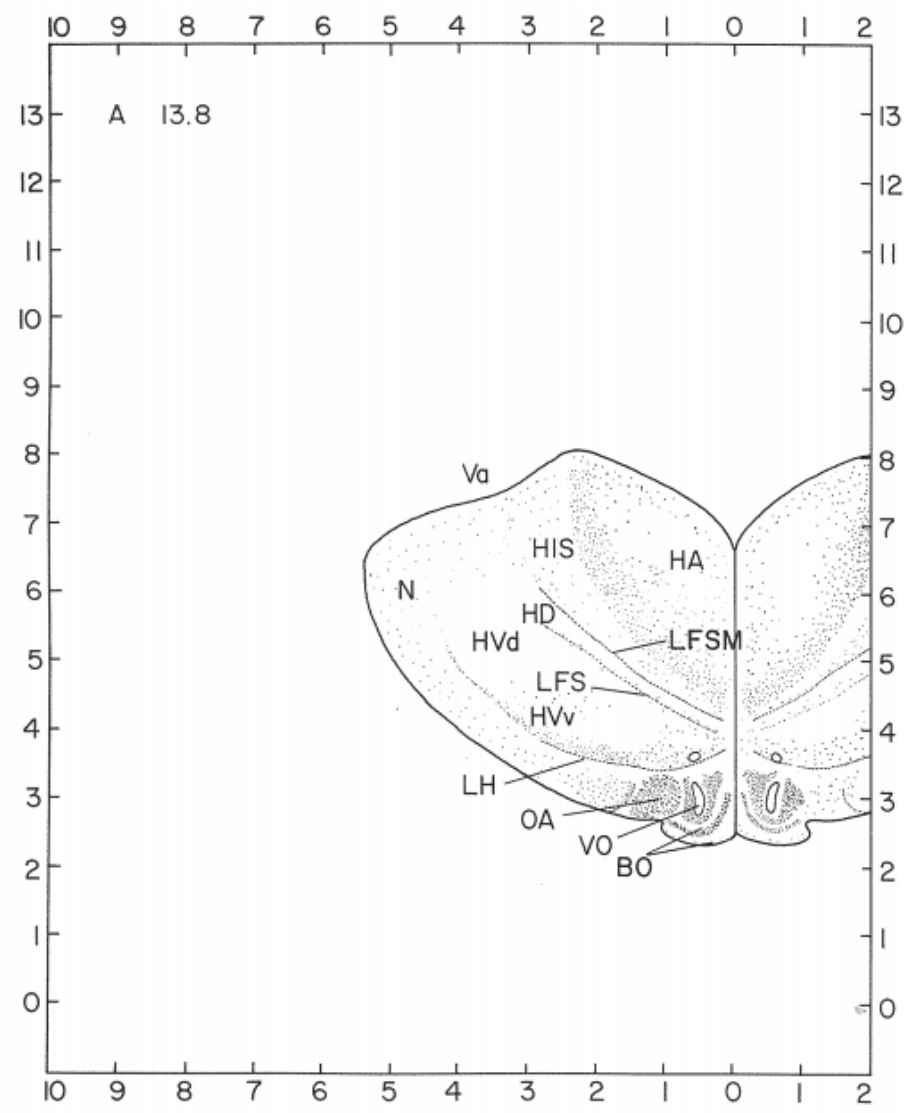

Figure 13. The position of the HA within the brain as indicated by the stereotaxic atlas (From Kuenzel & Masson, 1988)

The location of the ink gives an indication of the electrode placement within the brain (Figure 14). This was used to give a general idea of the electrode placement. Bird #1 did not receive an ink injection and no ink was seen in birds #5 and #6.

Figure 14. Photographs of the brain slices for birds in the HA group showing the position of the ink injection.

Caudomedial Nidopallium

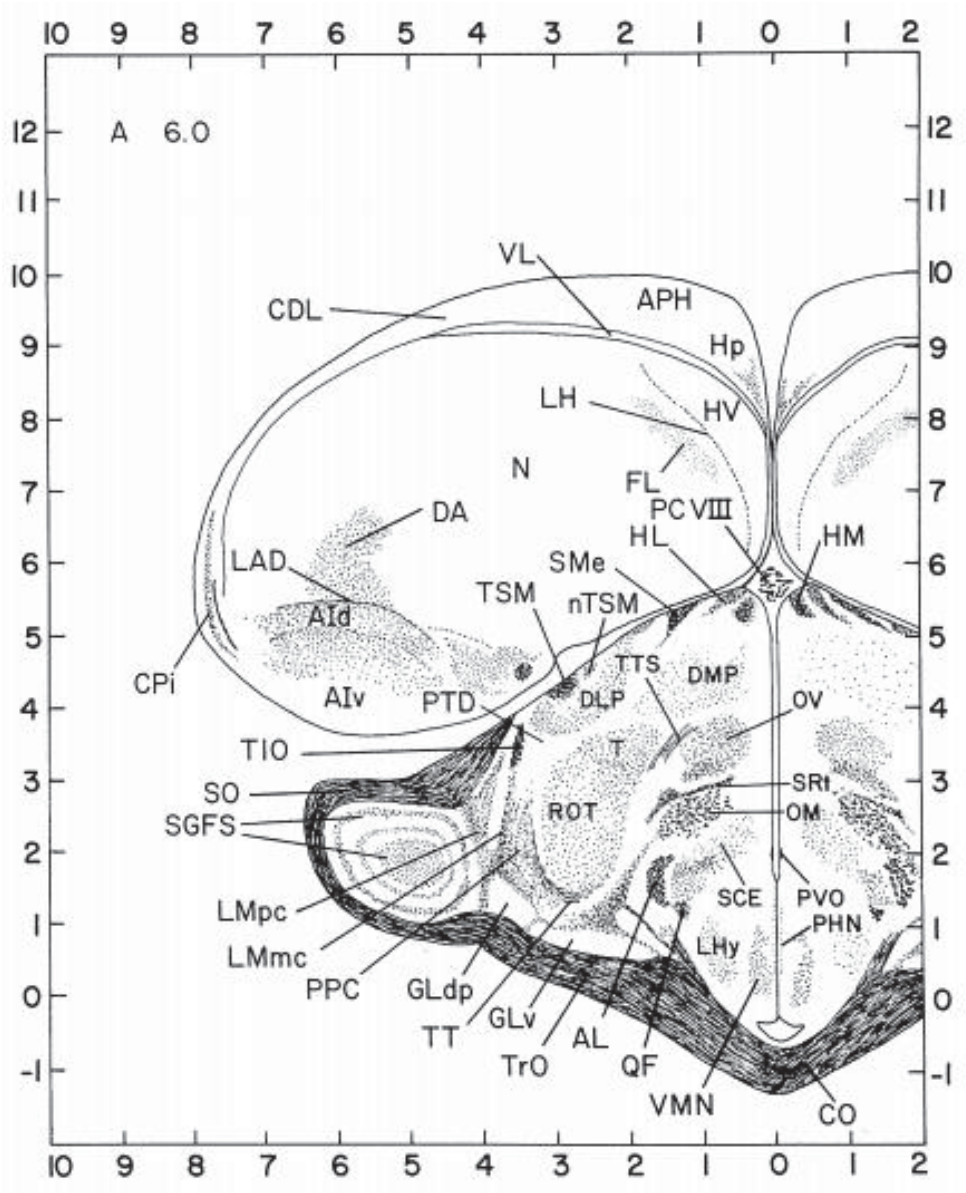

Figure 15. The location of the NCM (N) in the brain, as indicated by the stereotaxic atlas (From Kuenzel & Masson, 1988).

Ink was seen in all birds and was much more consistent that the HA (Figure 16). The ink in bird #7 had entered the lateral ventricle and dispersed, meaning ink placement was unreliable.

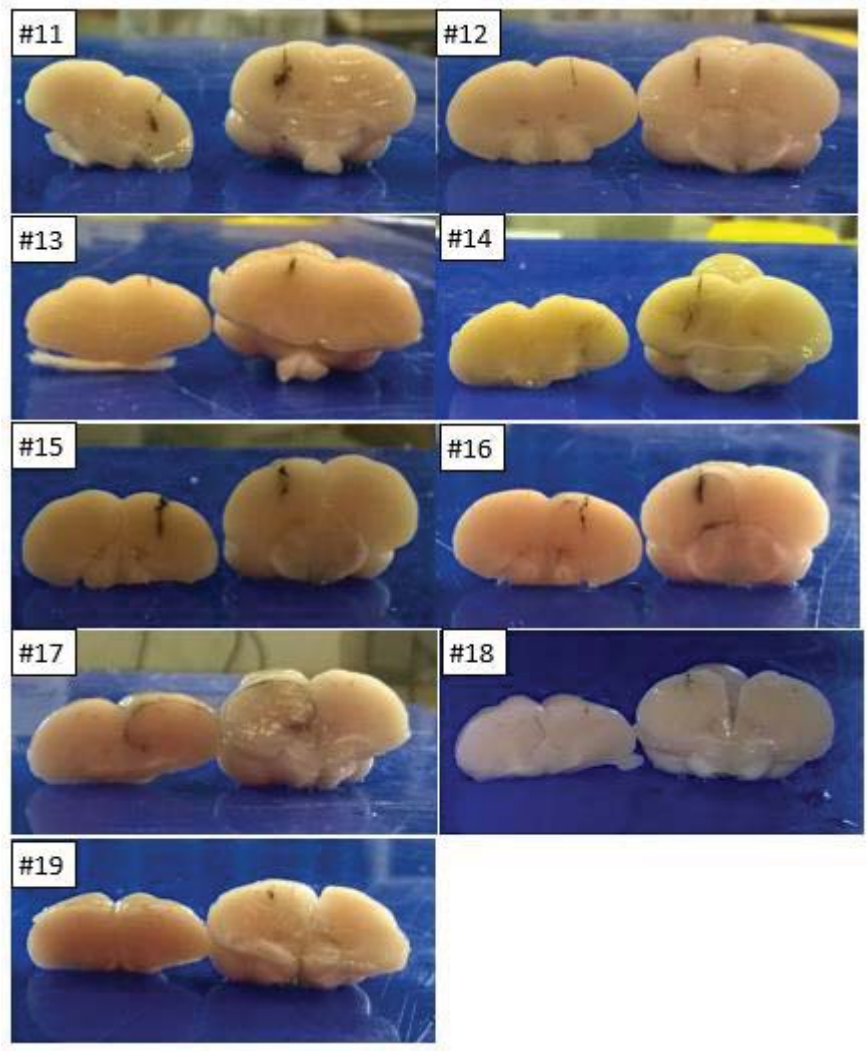

Figure 16. Photographs of the brain slices for birds in the NCM group showing the position of the ink injection.