Neurological Development and the Potential for Conscious Perception after Birth

Comparison between Species and Implications for Animal Welfare

A Thesis Presented in Partial

Fulfilment of the Requirements for the Degree of

Doctor of Philosophy

in

Physiology

Massey University
Palmerston North
New Zealand

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MSc, BSc

2010

Widmung

Ich widme diese Dissertation meiner Familie. Im Besonderen meinem Mann Cedric Priest, meiner Tochter Mackenzie, meinen Eltern Heidrun und Peter Diesch und meinen Grosseltern Liesbeth und Oskar Schienbein, Gertrud Kopp-Diesch und Josef Kesenheimer.

Ihr seid meine ganze Welt. Ohne Euch wäre dieses Unternehmen nicht möglich gewesen. Danke für Eure Liebe, Euer Verständis und Eure Unterstützung.

Dedication

I would like to dedicate this thesis to my family. In particular to my husband Cedric Priest, my daughter Mackenzie, my parents Heidrun and Peter Diesch and my grandparents Liesbeth and Oskar Schienbein, Gertrud Kopp-Diesch and Josef Kesenheimer.

You are my world. Without you this endeavour would not have been possible. Thank you so much for your love, understanding and support.

Ethical Affirmation

As I embark on my career as a scientist I willingly pledge that I will conduct my research and my professional life in a manner that is always above reproach and I will seek to incorporate the body of ethics and moral principles that constitute scientific integrity into all that I do.

I will always strive to ensure that the results of my research and other scientific activities are ultimately beneficial – for animals and humans alike- and that they do not cause any harm.

With this affirmation I pledge to acknowledge and honour the contributions of ethical scientists who have preceded me, to seek the truth and the advancement of knowledge in all my work.

Adapted from

Craig CR, Cather A & Culberson J. (2003). An ethical affirmation for scientists. *Science* **299,** 1982-1983

Acknowledgments

This thesis has been a fantastic, although at times trying, experience. I have learned so much over the last 5 years, on a scientific as well as on a personal basis, and this would not have been possible without the help of a great many special people and organisations.

First, I would like to express my deepest and sincere gratitude to my supervisor Prof David Mellor for his continual guidance and support. His enthusiasm has been infectious and has often saved me from the desire of wanting to give up. I would like to thank him for teaching me that this journey was not only to learn about science and research, but also to learn about life in general. I owe him a lot of gratitude for his financial support, for believing in my abilities, for always being there for me when I had concerns, for being patient with me and for being such a wonderful and passionate person. It was an honour to have him as my mentor.

My special thanks also to my co-supervisor Assoc Prof Craig Johnson for teaching me how to undertake EEG recordings, how to analyse the data and for doing such a great job of explaining to me what all the 'squiggly' lines were all about. Thank you for your support, for your encouragement, financial help and for believing in my abilities.

I am also very grateful for Assoc Prof Roger Lentle (IFNHH) and his assistance with statistical matters, the support he gave unquestioningly and our stimulating conversations.

I would also like to express my gratitude to Dr Laura Bennet and Prof Alastair Gunn (Auckland University) for their collaboration on the literature review, which was the basis for this thesis, and for their encouragement.

Special thanks also to Assoc Prof David Walker (Monash University, Melbourne) for allowing me to join his laboratory on two occasions to undertake blood sample and brain tissue sample analyses. My thanks to Isabella Ciurey, Dr Tamara Yawno and Jan Loose (Monash University, Melbourne) for teaching me extraction and RIA procedures and for their help throughout my stay at Monash University.

My thanks to Drs Paul Chambers and Joanna Murrell for assisting with anaesthesia matters, helpful discussions about planned projects, answering questions regarding anaesthesia and pharmacology and for their support.

Thank you to Jim Battye for his involvement with, his ideas for and helpful comments on the review presented in Chapter 8.

I am particularly grateful for the practical support by Corrin Hulls and Sheryl Mitchinson, without which none of my projects would have been possible.

I would also like to acknowledge the following people for their practical help and support with various parts of my projects: Nicola Bell, Leanne Betteridge, Helene Davesne, David DeAlmeida, Dephine Pernot and Marion Sandrin. Bruce Cann, Cathy Davidson, Dr Troy Gibson, Mike Hogan, Dr Ignancio Lizzaraga, Amanda McIlhone, Dr Vaughan Seed, Prof Kevin Stafford, Neil Ward (IVABS), Dr Sharon Hearne, Phil Pearce and Dr David Simcock (IFNHH), Matthew Levin (IT support; IFNHH), Lee-Anne Hannan, Yvonne Parkes, Christine Ramsay and Kathryn Tulitt (finance office and administration; IFNHH), Karen Pickering and Susan Sims (finance office and administration; Riddet Institute), Barry Evans (Engineering Services), Debbie Chesterfield (SAPU) and the following staff members of the Agricultural Services at Massey University: Byron Taylor, Geoff Warren, Phill Brooks (Tuapaka) and Peter Jessup (Haurongo).

Thank you to Mark Oliver from Auckland University for permission to use his photo of lambs at birth for the purposes of this thesis (Chapter 4).

Funding

I gratefully acknowledge the Agricultural and Marketing Research and Development Trust (AGMARDT) for awarding me a Doctoral Scholarship and for being so patient with me.

I also acknowledge the following organisations for personal, project and travel funding

during the course of my PhD:

Ministry of Agriculture and Forestry, New Zealand
Palmerston North Medical Research Foundation, New Zealand
Institute of Food, Nutrition and Human Health, Massey University, New Zealand
Riddet Institute, Massey University, New Zealand
Animal Welfare Science and Bioethics Centre, Massey University, New Zealand
Geoffrey Gardiner Foundation, Australia
New Zealand Vice Chancellor Committee (Claude McCarthy Travel Grant)
Education New Zealand (New Zealand Study Abroad Award 2005 and 2007)

Work

Thank you to Dr David Bayvel, Dr Cheryl Conner and Dr Kate Littin for providing me with casual work with the Animal Welfare Group at the Ministry of Agriculture and Forestry, NZ.

Personal

Without the support, love and understanding of my parents and family, my husband Cedric Priest and my best friend Nicole Tubach this endeavour would not have been possible. Thank you all from the bottom of my heart.

I would also like to thank my friends in New Zealand and Germany for believing in me, for encouraging me and for cheering me up when it was badly needed:
NZ: Ngaio Beausoleil, Aurelie Castinel, Rene Corner, Amelie Deglaire, Daniel Gray and Abbie McKee, Carly Heatherwick, Sharon Henare, Janina Kuehn, Kate Littin, Megan McGregor, Amanda McIlhone, Anusiah Nicolls and Jeff Devey and Mischa and Paul Walton and Paul Wood.
Germany: Tamara Diesch, Malta Fazzari, Elvira Fehringer, Carmen Haberland, Karsten Janz, Alexandra Schatz, Carmen Stoffel, Sabine Tutzauer and the girls from my former dancing group.

Thank you to Cathrin Klare and family, Kirsty Silcock and Joseph McGehan for helping me chase away the homesickness at my second stay in Melbourne. Special thanks also to Simon Verschaffelt and Amber Hays for allowing me to live with them during the

time of my projects and for being great friends.

Animals

Last, but not least, I would like to acknowledge the animals that involuntarily partook in the experiments outlined in this thesis.

All the experiments presented here have been approved by the Massey University Animal Ethics Committee.

Abstract

In order for animals to experience pain and to suffer from it, they have to be capable of conscious perception. Recent evidence suggests that the fetus is maintained in a sleep-like unconscious state and that conscious perception therefore only occurs after birth. The timing of the onset of conscious perception depends on the maturation of underlying neurological processes and is anticipated to be species dependent. Pain-specific electroencephalographic (EEG) responses of lightly anaesthetised young of three species born at different levels of neurological development were investigated. The results of the present thesis are in agreement with published data on general neurological, EEG and behavioural development. This information, in addition to the present results, has been used to estimate the approximate time of the onset of conscious perception in tammar wallaby joeys, rat pups and newborn lambs.

In wallaby joeys (extremely immature at birth), the EEG remained isoelectric until about 100-120 days of in-pouch age and became continuous by about 150-160 days, with electroencephalographic and behavioural signs of conscious perception apparent by about 160-180 days. In rat pups (immature at birth), the absence of a differentiated EEG suggests that the ability for conscious perception in pups younger than 10-12 days is doubtful. The marginal EEG responses to noxious stimulation in 12-14 day-old pups and the pronounced EEG responses in pups 18-20 days suggest that rats may be capable of conscious perception from 12-14 days onwards. In lambs (mature at birth), full conscious perception is probably not apparent before 5 minutes after birth and may take up to several hours or days to become fully established. Its modulation by the residual neuroinhibitor allopregnanolone, if that occurs, would be highest over the first 12 hours after birth.

Overall, the onset of conscious perception does not seem to follow an "on-off phenomenon", but seems to develop gradually, even in species born neurologically mature. Although conscious perception, and hence pain experience, may be qualitatively different in younger animals, on the basis of the precautionary principle, when significantly invasive procedures are planned, pain relief should be provided from those postnatal ages when pain may first be perceived – i.e. from about 120 days in the tammar wallaby joey, about 10 days in the rat pup and from soon after birth in the lamb.

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Chapter 1

The candidate participated fully in the literature research and in the writing of several review articles which form the basis of major parts of this Chapter *(see Appendix 2 for copies of these publications)*

Mellor DJ, Diesch TJ, Gunn AJ & Bennet L. (2005). The importance of 'awareness' for understanding fetal pain. *Brain Research Reviews* **49,** 455-471.

Mellor DJ & Diesch TJ. (2006). Onset of sentience: The potential for suffering in fetal and newborn farm animals. *Applied Animal Behaviour Science* **100,** 48-57.

Mellor DJ & Diesch TJ. (2007). Birth and hatching: Key events in the onset of awareness in the lamb and chick. *New Zealand Veterinary Journal* **55,** 51-60.

Mellor DJ, Diesch TJ, Gunn AJ & Bennet L. (2008). Fetal "awareness" and "pain": What precautions should be taken to safeguard fetal welfare during experiments? Proceedings of the Sixth World Congress on Alternatives and Animal Use in the Life Sciences, Tokyo, Japan, 2007. *Alternatives to Animal Testing and Experimentation* **14**, 79-83.

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Literature Review

1.1 Introduction

The prevention or alleviation of negative mental states, such as pain, is essential if we want to ensure good animal welfare (Mellor & Reid, 1994). In order for any animal to experience negative mental states and suffer from them, two prerequisites have to be met (Mellor & Diesch, 2006). First, the animal has to be sentient. This means that the animal's nervous system has to be sufficiently developed to relay sensory inputs (i.e. electrical impulses) from the periphery to the higher centres of the brain (i.e. cerebral cortex), where such impulses can then be further processed and interpreted. Second, the animal has to be conscious. Unconscious animals cannot experience negative mental states, such as pain, which of course is one of the main underlying reasons for using general anaesthesia during invasive and potentially noxious procedures.

The developmental stage at which animals become able to suffer therefore depends on *when* during development they become sentient and conscious, i.e. when they are able to consciously perceive sensations.

Newborn and young animals of various species have been used in developmental research for decades. Such research has advanced our understanding of normal and disordered function and has led to vast improvements in health and welfare in newborn domestic, farm and laboratory animals (Mellor & Gregory, 2003). Additionally, such research has vastly improved our knowledge about the neurological development in a variety of species. Nonetheless, we do not yet know when conscious perception emerges and how the timing of its emergence may differ between species.

1.2 Definition of consciousness and conscious perception

Before moving on, it may be prudent to give a brief definition of what is meant here by consciousness and conscious perception, as there are several concepts/definitions of consciousness (Tassi & Muzet, 2001; Zeman, 2001). A definition useful in the present context has been presented by Zeman (2001).

He suggests that there are two key senses of consciousness, namely wakefulness and awareness. Wakefulness is a state of consciousness, while awareness is the content of such consciousness, meaning 'being conscious of something'. The following is an excerpt of Zeman (2001) where he explains these concepts in more detail:

"… consciousness is generally equated with the waking state, and the abilities to perceive, interact and communicate with the environment and with others in an integrated manner which wakefulness normally implies. Consciousness in this sense is a matter of degree: a range of conscious states extends from waking through sleep into coma. Thus we speak of consciousness dwindling, waning, lapsing and recovering, it may be lost, depressed and regained. To be conscious in this state is to be awake, aroused, alert or vigilant.

… When we are conscious in this first sense we are always conscious of something. In its second sense consciousness is the content of experience from moment to moment: what it feels like to be a certain person…"

However, wakefulness and consciousness do not always co-exist. Hence, one can be awake and not aware, as is the case in the vegetative state, but one cannot be aware without being awake (Zeman, 2006).

Conscious perception, also termed explicit perception, can be defined as neural processes which give rise to conscious awareness of sensory stimuli, such as conscious vision (Zeman, 2001). In contrast, implicit perception refers to perception occurring in the absence of conscious experience of the stimulus perceived, such as is the case in blindsight where in the absence of visual awareness a range of visual capabilities can be demonstrated (Zeman, 2001).

Sleep, although being named a state of consciousness by Zeman (2001), is a state of reversible unconsciousness (i.e. one can awaken from sleep). This is in contrast to other unconscious states, such as coma, which may not be reversible.

For the purposes of this thesis we will use the following definitions of states of consciousness and unconsciousness:

Wakefulness

A state that can be either conscious or unconscious and which includes brainstem and thalamic activity. Wakefulness is accompanied by a set of behavioural, physiological and electroencephalographic variables allowing its distinction from other states (see section on sleep-wake physiology).

Consciousness

Is a state of conscious wakefulness during which cortical processing of internal and external stimuli occurs and which includes awareness of such stimuli.

Conscious perception

Sensory information arising from environmental or internal stimuli reaches higher centres of the brain where such information can be further processed and will lead to awareness of these stimuli.

Sleep

A state of reversible unconsciousness characterised by a set of behavioural, physiological and electroencephalographic variables (see section on sleep-wake physiology).

Sleep-like state

An unconscious state resembling sleep behaviourally and physiologically.

1.3 Physiological basis of consciousness

Just as there is debate regarding the definition of consciousness, there are also various concepts for the physiological bases of consciousness. The reader is referred to a recent article by Merker (2007) which questions the prevalent view, adopted here, that the cerebral cortex is the main seat for consciousness. Merker (2007) suggests that "*…brainstem mechanisms are integral to the constitution of the conscious state and that an adequate account of neural mechanisms of conscious function cannot be confined to the thalamocortical complex alone'*. According to this theory, conscious processing is possible even without a cerebral cortex, for example in children born

without a cerebral cortex (anencephaly). However, while structures in the upper brainstem are essential for the production of a conscious state via activation/arousal of the thalamocortical system and associated production of a waking state (see paragraphs below), Merker's idea that the thalamocortical system may not be necessary for consciousness to occur remains an interesting speculation that requires rigorous testing. For instance, his inferences based on pathological states involving lesions or congenital reduction in or absence of cortical or other higher brain structures implicitly assume that the functionality of remaining brain tissues would be unaltered from that present in the operationally integrated entire brain. This may not be correct for two reasons. First, any residual neural tissues in traumatically lesioned or congenitally malformed brain areas evidently have a remarkable capacity for neuroplastic adjustment to reduce functional impairment (Doidge, 2008). Second, during normal brain development the eventual appearance of the functional capacities of the cerebral tissues that are required for the eventual appearance of sentience and consciousness occurs against a neuroanatomical substratum of developing interconnectivity between *all* brain centres, not the potentially neuroplatically adjusted functionality of the structures that remain after traumatic lesioning or congenital malformation.

Underlying physiology of sleep-wake states

There are three states of vigilance that are distinguished from each other by a set of physiological signs, including EEG (electroencephalogram) rhythms, muscular tone and eye movements (Steriade & McCarley, 2005). Wakefulness is characterised by behavioural arousal, an EEG consisting of low-voltage high-frequency waves and voluntary eye-movements. Rapid eye movement (REM) sleep has EEG characteristics indistinguishable from the waking EEG, however, in contrast to the waking state muscular atonia is present and involuntary eye movements occur which are accompanied by spiky PGO (pontogeniculooccipital) potentials (Steriade & McCarley, 2005). In contrast, non-REM sleep, also called slow-wave sleep, is characterised by high-voltage low-frequency waves in the EEG.

The induction and maintenance of wakefulness, REM sleep and non-REM sleep have been attributed to the interactions between multiple wake and sleep-promoting systems widely distributed within the brain from the medulla to the cerebral cortex (see Table 1)

(Steriade & McCarley, 1990; McCormick & Bal, 1997; McCarley, 1999; Steriade, 1999; Szymusiak *et al.*, 2000; McGinty & Szymusiak, 2001; Jones, 2003; Moruzzi & Magoun, 2003; Sakai & Crochet, 2003; Steriade & McCarley, 2005; McCarley, 2007; Szymusiak *et al.*, 2007).

1) Non-REM sleep and wake mechanisms

The current hypothesis of sleep-wake regulation proposes that sleep-promoting structures are located in the preoptic area (POA) of the anterior hypothalamus and wake-promoting neurons are located in the posterior hypothalamus, basal forebrain and mesopontine tegmentum (Sakai & Crochet, 2003).

Sleep-promoting neurons of the POA appear to exert their effects via descending inhibitory modulation of various arousal systems in the posterior hypothalamus and brainstem, in particular the histaminergic neurons of the tuberomammillary nucleus (TMN) (Szymusiak *et al.*, 2007). Thus, electrical stimulation of POA neurons in brain slices was shown to evoke GABA-mediated inhibitory postsynaptic potentials in the TMN, indicating that POA neurons inhibit TMN cell activity during both REM and non-REM sleep (Szymusiak *et al.*, 2007). In addition, the POA is the source of afferents to the dorsal raphe nucleus and the locus coeruleus (LC), both of which exhibit REM-off discharge patterns (see below) (Szymusiak *et al.*, 2007). The POA also receives input from these monoaminergic areas and its GABAergic neurons can be inhibited by noradrenaline and serotonin. Thus, activation of the monoaminergic system has the potential to suppress POA activity during wakefulness (Szymusiak *et al.*, 2007).

As the rostral projections of the posterior hypothalamus include the thalamus and the cerebral cortex, the inhibition of histaminergic neurons in these areas leads to disfacilitation of the thalamus and cerebral cortex (Steriade, 1999). The histaminergic neurons of the posterior hypothalamus also project to the mesopontine cholinergic neurons of the upper brainstem so that inhibition of the histaminergic cells also leads to disfacilitation in the mesopontine cholinergic neurons, which are part of the ascending reticular activating system (Steriade, 1999).

Adenosine and other neuromodulators seem to be involved in sleep regulation

(McCarley, 2007). Adenosine apparently promotes sleep by inhibiting wake-promoting neurons, in particular the cholinergic and non-cholinergic neurons in the basal forebrain, but may also exert excitatory effects on POA sleep-active neurons (Szymusiak *et al.*, 2007). Adenosine concentrations in the extracellular space increase when there is an energy deficit during periods of neuronal activation (Basheer *et al.*, 2004). In particular, elevated levels of extracellular adenosine have been observed in the basal forebrain after prolonged wakefulness and thus seem to underlie increased sleep propensity, increased sleep amount and increased EEG slow-wave activity that occur as a consequence of sustained waking (Basheer *et al.*, 2004; McCarley, 2007).

Presumably during non-REM sleep, when neuronal activation is reduced, extracellular adenosine concentrations in the basal forebrain decrease releasing the inhibition of wake-promoting centres, thereby leading to the inhibition of sleep-promoting centres and wakefulness.

Table 1.1: Brain regions, particular nuclei and associated transmitters involved in the sleep-wake cycle, which all directly or indirectly project to various areas of the cerebral cortex.

(Steriade & McCarley, 1990; McCormick & Bal, 1997; McCarley, 1999; Steriade, 1999; Szymusiak et al., 2000; McGinty & Szymusiak, 2001; Jones, 2003; Moruzzi & Magoun, 2003; Sakai & Crochet, 2003; Steriade & McCarley, 2005; McCarley, 2007; Szymusiak et al., 2007).

Brain area	Nuclei/Area/Neurons	Transmitter
Medulla	*Reticular Formation*	Glutamate GABA
Pons	*Reticular Formation*	Glutamate GABA
	Locus Coeruleus (LC)	Noradrenaline (NA)
Ponto-mesencephalon	*Peribrachial Nuclei* - Pedunculopontine Tegmentum (PPT) - Laterodorsal Tegmentum (LDT)	Acetylcholine (ACh)
Mesencephalon	*Reticular Formation*	Glutamate GABA
	Dorsal Raphe Nucleus (DRN)	Serotonin
	Ventral Mesencephalic Neurons - Substantia Nigra (SN) - Ventral tegmental area (VTA)	Dopamine
Posterior Hypothalamus	*Posterior Hypothalamus*	Glutamate GABA
	Ventrolateral posterior Hypothalamus - Tuberomammillary Nucleus (TMN)	Histamine
	Peri-fornical neurons	Orexin/hypocretin
Anterior Hypothalamus	*Preoptic area (POA)* - Ventrolateral POA (vlPOA) - Median Preoptic Nucleus (MnPN)	GABA Galanin
Basal Forebrain	*Cholinergic*	Acetylcholine (ACh)
	Non-cholinergic	Glutamate GABA
Thalamus	*Midline Nuclei*	Glutamate
	Medial Nuclei	Glutamate
	Intralaminar Nuclei	Glutamate
	Reticularis Neurons	GABA

2) REM sleep

The following description represents a synthesis of the information provided by Steriade & McCarley (2005) and McCarley (2007).

Although forebrain mechanisms have the capacity to modulate REM sleep, the rhythmic generating structures for REM sleep are located in the brainstem. Of particular interest at brainstem level are the neurons of the pontine reticular formation (PRF), which are

hyperpolarized during non-REM sleep and begin to depolarise prior to EEG signs of REM sleep approaching. These neurons then have a high discharge rate during REM sleep and lead to behavioural signs of REM sleep including rapid eye movement, PGO waves and muscle atonia. Neurons in the midbrain reticular formation (MRF) are important for the EEG activation seen during REM sleep.

Apparently, cholinergic influences, in particular REM-on neurons of the laterodorsal and pedunculopontine tegmental nuclei (LDT/PPT), act to increase the excitability of reticular neurons in the brainstem by inhibiting GABAergic neurons, which in turn are inhibitory to reticular brainstem neurons. In contrast, REM-off neurons, located in the raphe nuclei and the locus coeruleus, decrease discharge activity with the approach and onset of REM sleep. Discharge rates of these neurons are highest during waking, decrease in non-REM sleep and nearly cease discharge in REM sleep. It is not apparently known why these neurons cease discharging during REM sleep, but a GABAergic mechanism may be involved.

According to the reciprocal interaction model, REM-on neurons have a positive feedback so that their activity grows. However, this activity gradually excites REM-off neurons, which then inhibit the REM-on neurons terminating the REM episode. In addition, the REM-off neurons are also self-inhibiting and thus as REM-off activity declines the REM-on neurons are released from inhibition beginning a new cycle of REM.

3) Relevance of sleep-wake states for conscious perception

As mentioned above, the waking state is essential for conscious perception/awareness to occur. Therefore, neuronal structures underlying sleep and wake mechanisms also appear to play an important role in the production of reversible unconsciousness, as seen during sleep, and consciousness (Coenen, 1998; Evans, 2003). It can be deduced from this that unless the neurological systems underlying the production of sleep and wakefulness are present, interconnected and functional, conscious perception is also not possible.

1.4 Neurological development and the ontogeny of conscious perception

A detailed description on the neurological development of the tammar wallaby, the rat and the lamb, as representatives of animals born at differing degrees of maturity, will be presented in Chapters 2 to 6.

Overall, the pattern of neurological development is similar in different species of mammals, and the timing of birth during the course of this development determines whether animals are born relatively mature (e.g. sheep, cattle, horses and other ungulates), moderately immature (e.g. mice, rats, cats and dogs) or extremely immature (marsupials). This can be illustrated by looking at the development of brain electrical activity, the electroencephalogram (EEG). Initially, the EEG is absent (isoelectric trace), but intermittent spikes develop and then longer more sustained epochs of EEG activity, which are also separated by isoelectric periods, become apparent (Ellingson & Rose, 1970; Mellor *et al.*, 2005). Subsequently, continuous EEG activity appears, which then differentiates into alternating rapid-eye-movement (REM) and non-REM sleep-like patterns with the later addition of characteristics indicating conscious awareness (Ellingson & Rose, 1970; Mellor *et al.*, 2005). The neurological capacity for consciousness appears to coincide with, or follow, REM-non-REM differentiation, and birth may occur either before or after the capacity for consciousness becomes evident.

In animals born extremely or moderately immature (altricial species such as mice, rats, cats and dogs), REM-non-REM differentiation occurs some time after birth (Ellingson & Rose, 1970). This therefore suggests that these animals will not be sufficiently developed neurologically for sentience or consciousness to be evident *in utero.* Conscious perception is thus anticipated to occur at some stage after birth in these species.

In animals that are born neurologically mature (precocious species such as ungulates, guinea pigs, humans), EEG differentiation into REM-non-REM sleep-like states occurs before birth indicating that, by that stage, the nervous system has developed sufficiently to support sentience. In the sheep it has been observed that late gestation fetuses alternate between two well-defined sleep-like states of unconsciousness which are

similar in appearance to REM and non-REM sleep postnatally (Clewlow *et al.*, 1983). These states apparently account for 95% of fetal EEG activity during each day and hence sleep-like unconsciousness is the predominant fetal state (Dawes, 1988; Szeto, 1992). However, during the remaining 5% of the time the fetus is in a state, which used to be called "wakefulness". During this state a combination of eye movements, vigorous muscular activity, breathing movements, unstable heart rate and REM-like EEG activity can be observed (Ioffe *et al.*, 1980; Szeto, 1992; Mellor *et al.*, 2005). However, this brief and infrequent state usually only occurs as the fetus transitions between the REM and non-REM sleep-like states (Schwab *et al.*, 2000; Mellor *et al.*, 2005). As the characteristics of this transitional state appear to be similar in nature to what has been described in the newborn human infant as sleep-arousal - brief periods of indeterminate or transitional sleep during which the infant is apparently not awake (Gottesmann, 1996; McNamara *et al.*, 2002) - it is suggested that it does not involve conscious waking (Mellor *et al.*, 2005).

Moreover, it appears unlikely that the fetus can be woken up by external stimulation (Mellor *et al.*, 2005). Thus, hypoxia and extreme asphyxia, which would normally induce an arousal response in the postnatal animal, suppress fetal arousal thereby ensuring the survival of the fetus (Bennet *et al.*, 2003; Mellor *et al.*, 2005). Also, potentially painful vibro-acoustic stimulation has been shown to lead to EEG changes similar to those of sleep state transitions rather than arousal to an awake state (Schwab *et al.*, 2000; Mellor *et al.*, 2005).

Thus, although animals that are born neurologically mature have the capacity for sentience during late gestation, conscious perception does not seem likely, as these fetuses are apparently continually asleep during late gestation, i.e. at the stage at which their nervous system would be sufficiently developed and functional to support conscious perception.

Maintenance of fetal sleep-like states before birth in mature fetuses during late gestation

In support of the above conclusions, there is evidence that a variety of neurosuppressive agents are involved in the maintenance of sleep-like states of unconsciousness in the

fetus. Below is a description of what we believe are some of the main neurosuppressors involved. These have been identified according to their established neurosuppressive or somnogenic properties, their presence in the fetal brain and circulation or their supply via the placenta as well as their proven effects on fetal sleep state where such research has been undertaken.

1) Neuroactive steroids – progesterone metabolites

Allopregnanolone (5α-pregnane-3α-ol-20-one) and pregnanolone (5β-pregnane-3α-ol-20-one), both metabolites of the hormone progesterone, which contain a 3α-hydroxyl group, have potent sedative-hypnotic, soporific and anaesthetic effects (Majewska, 1992; Paul & Purdy, 1992; Lancel *et al.*, 1996; Damianisch *et al.*, 2001; Rupprecht, 2003). They exert their effects via the $GABA_A$ receptor by enhancing GABA-mediated inhibitory synaptic events, bringing about increased inhibition in the central nervous system (Majewska, 1992; Paul & Purdy, 1992; Compagnone & Mellon, 2000; Belelli & Lambert, 2005). Like other sedative-hypnotic drugs, such as benzodiazepines and barbiturates, they bind to specific sites on the receptor which brings about allosteric changes that increase GABA-mediated chloride ion conductance (Paul & Purdy, 1992) .

Pregnane metabolites can be synthesised in the CNS from cholesterol *de novo* via enzymatic actions (Majewska, 1992; Lephart, 1993; Mensah-Nyagan *et al.*, 1999; Birzniece *et al.*, 2006). As steroid hormones are lipophilic and easily cross the blood-brain-barrier, cholesterol and progesterone derived from other tissues may also be used in the synthesis of neuroactive steroids (Rupprecht, 2003; Birzniece *et al.*, 2006).

There is evidence that points towards the involvement of these potent neurosuppressive steroid metabolites in the modulation of fetal EEG activity and behavioural state. During pregnancy, the placenta produces large quantities of progesterone (Bassett *et al.*, 1969) which is partially metabolised before entering the fetal circulation (Crossley *et al.*, 2000). Hence, concentrations of allopregnanolone and its precursors pregnenolone and progesterone are elevated during late gestation in both plasma and different brain regions in fetal sheep (Nguyen *et al.*, 2003). It is also not surprising therefore that once the placenta is lost after birth, progesterone and pregnane metabolite concentrations drop markedly (Petratos *et al.*, 2000; Nguyen *et al.*, 2003).

In fetal sheep, $GABA_A$ receptor concentrations reach adult levels by about 120 days of gestation (Crossley *et al.*, 2000) and allopregnanolone may interact with these receptors from mid-gestation (Crossley *et al.*, 2003). In addition, electrocortical activity and fetal behaviour during late gestation are markedly affected by pregnane steroids. Hence, injections of progesterone or its metabolites into the maternal and fetal circulation and fetal cerebral ventricles reduce fetal EEG activity and eye, breathing and body movements (Nicol *et al.*, 1997; Nicol *et al.*, 1998; Nicol *et al.*, 1999; Hirst *et al.*, 2000). In contrast, inhibition of placental production of progesterone and its metabolites or the inhibition of $GABA_A$ receptors has the opposite effect on these parameters (Crossley *et al.*, 1997; Nicol *et al.*, 1999; Nicol *et al.*, 2001).

2) Adenosine and oxygen status

Adenosine is a purinergic messenger molecule acting mainly in excitable tissues where it regulates physiological processes either by reducing metabolic activity or increasing the supply of metabolic substances thereby balancing energy supply and expenditure (Dunwiddie & Masino, 2001).

Adenosine receptors are highly expressed in brain tissue (Dunwiddie & Masino, 2001) and it is here that adenosine has the effects that are of interest in the context of this thesis. Adenosine potently inhibits neural activity by inhibiting the release of neurotransmitters, especially excitatory ones, thereby playing a major role in the modulation of behavioural states (Deckert & Gleiter, 1994; Fredholm, 1995; Portas *et al.*, 1997; Porkka-Heiskanen *et al.*, 2002).

Evidence to support its modulatory effect on behavioural state has come, among others, from pharmacological studies, showing that agonists for adenosine receptors promote sleep (Rainnie *et al.*, 1994; Portas *et al.*, 1997; Alam *et al.*, 1999), whereas antagonists (e.g. caffeine and theophylline) reduce sleep or affect sleep efficiency (Landolt et al., 1995). In addition, extracellular concentrations of adenosine in various areas of the brain increase during wakefulness and decrease during sleep (Strecker *et al.*, 2000; Obal & Krueger, 2003).

Plasma adenosine concentrations have been found to be 2-to 4-fold higher in the sheep and human fetus than the dam (Mentzer *et al.*, 1985; Sawa *et al.*, 1991; Yoneyama *et al.*, 1994; Yoneyama *et al.*, 1996; Yoneyama *et al.*, 2001). The placenta and probably the fetal liver are major sources of adenosine in the fetus (Maguire & Krishnakantha, 1979; Slegel *et al.*, 1988; Ball *et al.*, 1995; Ball *et al.*, 1996). Superimposed on the elevated levels of adenosine present in the fetus, fetal plasma adenosine concentrations fluctuate in response to hypoxaemia or anemia (increase) (Koos & Doany, 1991; Russell *et al.*, 1993; Yoneyama *et al.*, 1994; Yoneyama *et al.*, 1996; Kubonoya & Power, 1997) and hyperoxaemia (decrease) (Mentzer *et al.*, 1985; Sawa *et al.*, 1991; Ball *et al.*, 1995; Ball *et al.*, 1996). In case of hypoxaemia, this serves a protective function, as cerebral metabolism is suppressed and hence neural injury is limited (Hunter *et al.*, 2003).

Adenosine not only affects sleep state and arousal (as defined by electrocortical activity, breathing and eye movements as well as muscular activity) in adult animals, but it also affects them in the fetus. Hence, infusion of adenosine or adenosine analogues into sheep fetuses has been shown to suppress fetal breathing movements, eye movements and the incidence of REM-related EEG activity and to increase the incidence of non-REM-related EEG activity (Szeto & Umans, 1985; Koos & Matsuda, 1990; Chau & Koos, 1999; Koos *et al.*, 2001). In turn, infusions of the non-specific adenosine receptor antagonist theophylline were shown to increase fetal breathing movements and the incidence of REM sleep (Koos & Matsuda, 1990; Avital *et al.*, 1993).

These findings seem to indicate that high adenosine levels present during fetal life contribute to maintaining the fetus in sleep-like unconscious states (Mellor *et al.*, 2005; Mellor & Diesch, 2006).

3) Prostaglandins

Prostaglandin D_2 (PGD_2) is a potent sleep-promoting substance (Ram *et al.*, 1997; Urade & Hayaishi, 2000; Hayaishi & Urade, 2001), intracerebroventricular injection of which induces sleep which is apparently indistinguishable from natural sleep (Ueno *et al.*, 1983; Onoe *et al.*, 1988).

PGD_2 is apparently most effective in promoting sleep following the infusion into the subarachnoid space close to the preoptic area from where it can increase firing rates of sleep-active neurons located in the ventro-lateral preoptic area (VLPO) which, in turn, leads to inhibition of wake-promoting neurons in the tuberomammillary nucleus (TMN) considered to be a wake centre (Hayaishi, 2002). A current theory suggests that the mechanism of action of PGD_2 involves the stimulation of paracrine signalling molecules, such as adenosine (Satho *et al.*, 1996), exciting sleep-active neurons projecting to the VLPO which in turn inhibit neural activity in the TMN via GABAergic mechanisms (Matsumura *et al.*, 1994; Hayaishi, 2002).

PGD_2 is synthesised in the mammalian central nervous system from prostaglandin H_2 via the action of the enzyme prostaglandin D synthase (PGSD) (Urade & Hayaishi, 2000). PGD_2 has been found in the cerebrospinal fluid (CSF) of fetal sheep at 125 and 135 days of gestation, but not at 90 days (Lee *et al.*, 2002). This coincides with the presence of discrete REM and non-REM sleep states, which begin to occur at around 115 days of gestation in the sheep fetus (Clewlow *et al.*, 1983). In addition, intracerebroventricular infusion of the PGDS inhibitor, $SeCl_4$, increased fetal arousal periods as determined by the presence of low amplitude ECoG (electrocorticogram) in combination with EMG (electromyogram) and EOG (electrooculogram) activity. The changes observed in response to $SeCl_4$ infusion were reversed by infusion of PGD_2 (Lee *et al.*, 2002).

Thus, PGD_2 in the fetal brain may play a role in maintaining the fetus in sleep-like states of unconsciousness at a time when the fetal EEG has differentiated into REM-non-REM sleep states.

4) Placental peptide inhibitor and other factors

Placental inhibitor

There is evidence suggesting involvement of the placenta in the maintenance of fetal sleep-like states (Mellor *et al*., 2005). Ventilation of sheep fetuses with 100% oxygen plus umbilical cord occlusion have been shown to stimulate continuous fetal breathing movements and continuous behavioural activity (Baier *et al.*, 1990; Alvaro *et al.*, 1993;

Alvaro *et al.*, 1996; Alvaro *et al.*, 1997). Although such activity may be brought about by removal of neuroinhibitory factors such as adenosine and pregnane steroids, there is evidence that other placental factors may also be involved. The administration of a placental extract, but not extracts of other fetal tissues, brings about suppression of fetal behavioural and breathing activity induced by ventilation and umbilical cord occlusion (Alvaro *et al*., 1993; Alvaro *et al*., 1996). Further studies suggest that this placental factor is probably a peptide produced by the placenta, with a molecular mass between 2.5 and 4.5 kDa (Alvaro *et al*., 1997). However, the presence of such a placental factor has not been proven unequivocally (Kuipers *et al.*, 1992).

Other factors

In addition to the above-mentioned neuroinhibitory substances, there are others that may play a role in maintaining the fetus in sleep-like states of unconsciousness. These may include neuropeptide Y (NPY), corticotropin releasing hormone (CRH), somatostatin, enkephalins, growth hormone and possibly others (Mellor *et al.*, 2005).

5) Warmth, tactile stimulation and buoyancy

That body temperature and sleep are related is well known. We know that on hot days or during a hot bath we become drowsy, while exposure to a cold environment has the opposite effect. This relationship has been tested in a variety of studies looking at the effect of temperature on REM and non-REM sleep and wakefulness (Bach *et al.*, 2002).

Temperature-sensitive neurons in the hypothalamus, especially the preoptic area (POA), have been shown to be involved in both thermoregulation and sleep-wakefulness regulation (McGinty & Szymusiak, 2001). Moreover, a link between thermoregulation and sleep regulation via the POA has been demonstrated in a variety of species where local warming of the POA triggered non-REM sleep or slow wave EEG activity (Roberts & Robinson, 1969; Benedek *et al.*, 1982), whereas local cooling was shown to suppress sleep ((McGinty *et al.*, 1996).

Fetal temperature in humans, sheep and other species is about half a degree higher than that of the mother, allowing a balance between fetal heat production and heat loss via

dissipation to the mother (Adamsons & Towell, 1965; Power, 1989). Thus, the exposure of the fetus to an environment that is only half a degree below its own body temperature may assist in maintaining the fetus in sleep-like states of unconsciousness during late gestation. This is supported by observations where cooling of well-oxygenated fetuses *in utero* via cooling coils elicited behavioural activity, shivering and increased respiration (Gluckman *et al.*, 1983) and EEG changes indicative of arousal (Schwab *et al.*, 1997).

In addition to thermal status, the minimal tactile stimulation present *in utero* due to buffering by amniotic fluid and its buoyancy effects on the fetus may also be involved in maintaining the fetus in sleep-like unconscious states.

6) Summary

Taken together, the above information suggests that the fetus of maturely born animals is sentient during late gestation, but that it is actively maintained in sleep-like unconscious states due to the exposure to a neurosuppressive internal and external environment. If we accept this, then conscious perception would not be possible before birth.

However, some may argue that evidence suggests otherwise. First, studies have shown that the fetus responds to potentially noxious stimuli, such as intrahepatic needling, by withdrawing from the stimulus, increased circulating levels of stress-related hormones and haemodynamic changes (Giannakoulopoulos *et al.*, 1994; Teixeira *et al.*, 1996; Smith *et al.*, 2003). Second, a variety of studies have demonstrated that fetuses are capable of so-called "learning" *in utero* (Mickley *et al.*, 2004), so that prenatal exposure of the fetus to certain stimuli may lead to responsiveness to these stimuli postnatally (Schaal *et al.*, 1995, 1998, 2000; Gruest *et al.*, 2004; Kawai *et al.*, 2004; Mickley *et al.*, 2004; Wells & Hepper, 2006).

These responses may be interpreted to mean that the fetus is capable of conscious perception. However, this is not supported by other evidence. First, the responses to potentially noxious stimulation can be mediated by subcortical and brainstem centres and do not require cortical input (Lloyd-Thomas & Fitzgerald, 1996; Zeman, 2001;

Mellor *et al.*, 2005). Second, fetal "learning" could simply be the neural entrainment to a repeated stimulus which will produce a reflex response to that stimulus (Mellor *et al.*, 2005) or, even if learning is involved, this may nevertheless occur as an implicit (unconscious) process (Mellor & Diesch, 2007) and hence would not refute our hypothesis that the fetus is maintained in unconscious states.

Is conscious perception possible during birth?

It is not very likely that conscious perception would become possible during birth, despite the increasing concentrations of activators of arousal (see below) that will be present at the time.

Fetal states of unconsciousness appear to persist during labour and may even become deeper as labour progresses. Thus, as labour approaches fetal ECoG activity indicates a predominance of high voltage slow wave activity indicative of sleep (Berger *et al.*, 1986; Shinozuka & Nathanielsz, 1998), and fetal motor systems remain largely quiescent (Berger *et al.*, 1986; Hasan & Rigaux, 1991). Some of these effects are evidently independent of hypoxaemia associated with labour contractions (Shinozuka & Nathanielsz, 1998), whereas others are due to them. During strong labour contractions, hypoxaemia-induced elevations in adenosine concentrations would be expected to inhibit fetal EEG activity. Thus, if the resultant hypoxaemia is severe and protracted enough, EEG activity may be almost completely suppressed resulting in an isoelectric EEG (Mallard *et al.*, 1992; Hunter *et al.*, 2003).

Conscious perception after birth

Birth is associated with the rupture of the umbilical cord and hence loss of placental oxygen supply. While breathing does not occur, the EEG will progress towards an isoelectric state, which is reached after about 60 to 90 seconds in lambs (Mallard *et al.*, 1992; Hunter *et al.*, 2003). However, once the newborn breathes successfully this is reversed (Mellor & Diesch, 2006).

In addition, birth is associated with a variety of changes in the internal and external environment of the newborn, including changes in hormone concentrations,

environmental temperature, oxygen concentrations and somatosensory input (Lagercrantz *et al*., 1992). These, in addition to the removal of neuroinhibitory factors, may all be involved in promoting the activation of conscious perception in the neurologically mature newborn.

1) Removal of neuroinhibitory factors

The rupture of the umbilical cord at birth is associated with the loss of the placental supply of oxygen and an associated increase in carbon dioxide tensions in the newborn, both of which will help stimulate the onset of breathing (Mellor & Gregory, 2003). This, in addition to the loss of the major source of adenosine would lead to a rapid decrease of circulating and central adenosine concentrations due to its apparently short half life (Moser *et al.*, 1989). This in turn would reduce the inhibition of the cerebro-cortical function exerted by high concentrations of adenosine before and during birth (Mellor & Diesch, 2006).

Due to the severance of the umbilical cord at birth, the placenta, which is the main source for allopregnanolone, pregnanolone and their precursors as well as the peptide inhibitor(s), is lost. Thus, a reduction in the levels of these factors may play a passive role in the arousal of the newborn, by reducing the neurosuppressive effect, thereby allowing activators of arousal to exert their arousing effects (see below).

Could there be modulation of conscious perception by continuing production of neuroinhibitors after birth?

A recent study by Johnson *et al.* (2009) seems to suggest that conscious perception may be different in newborn lambs compared to that of older lambs. They investigated the EEG responses to castration in lightly anaesthetised lambs and found that lambs at few days after birth showed a reduced response to castration compared to the responses of older animals.

Although concentrations of pregnane metabolites will decline after birth due to the loss of the placenta, plasma concentrations are still elevated three days after birth (Nguyen *et al.*, 2003). It is therefore possible that these neuroactive steroids continue to exert

neurosuppressive effects, despite the presence of the various activators that begin to operate around the time of birth (see below).

Endogenous and exogenous opioids may also play a modulatory role. Plasma β-endorphin-like immunoreactivity has been shown to increase prior to labour in the lamb fetus (Hennessy *et al.*, 1982) and plasma β-endorphin concentrations of human infants are elevated for the first hours after birth (Facchinetti *et al.*, 1982). In addition, the concentration of β-endorphin in human colostrum is elevated compared to transitional and mature human milk (Zanardo *et al.*, 2001) and opioid peptides derived from caseins in colostrum or milk, such as β-casomorphin, may also exert opioidergic properties if they are absorbed into the circulation and reach the CNS (Teschemacher *et al.*, 1997; Sun *et al.*, 2003; Teschemacher, 2003; Silva & Malcata, 2005).

Whether the modulatory effects of such factors would translate into a delay in the onset of conscious perception or a possible modulation of conscious perception leading to qualitative differences in conscious perception in newborn compared to older animals, is not known at present.

2) Activators of brain function

Neuroactive steroids – 17β-oestradiol

17β-oestradiol is a neuroactive steroid that exerts rapid excitatory effects in a variety of brain areas (Wong *et al.*, 1996; McEwen & Alves, 1999; Woolley, 1999; McEwen, 2002). Among other mechanisms this seems to be partly due to the induction of short-term facilitation of glutaminergic synaptic transmission (Wong *et al.*, 1996). In addition, oestrogens have been shown to affect the GABAergic system via their long-term genomic actions, affecting the level and turnover of GABA and the activity of GABA-related enzymes (Mansky *et al.*, 1982; Duvilanski *et al.*, 1983; Schumacher *et al.*, 1989), as well as regulating the confirmation, synthesis and turnover of $GABA_A$ receptors (Hamon *et al.*, 1983; Schumacher *et al.*, 1989; Wong *et al.*, 1996).

That 17β-oestradiol may be involved in arousal of the newborn after birth is supported by the observation that injections of this hormone lead to behavioural arousal in inactive

lambs delivered prematurely but close to the time of normal birth (Mellor *et al.*, 1972). In addition, fetal 17β-oestradiol concentrations are low during pregnancy, but increase as birth approaches (Challis & Patrick, 1981), thereby exposing fetal neural tissue to increasing concentrations of 17β-oestradiol around the time of birth.

Catecholamines

During the birth process the fetus is exposed to a variety of stressors including strong tactile stimulation (and possibly nociceptor stimulation) during the passage through the birth canal and exposure to a cold *extra-uterine* environment. This is reflected in a significant rise in plasma catecholamine levels during and after labour, of which 85% are represented by noradrenaline (Lagercrantz, 1994, 1996). Some may cross the blood-brain-barrier (BBB) to exert an activating effect. In addition, arousal around the time of birth may occur due to activation of the locus coeruleus (Lagercrantz, 1996). The locus coeruleus forms part of the ascending reticular activating system (ARAS) (Jones, 2003) and extends noradrenaline-releasing neurons throughout the brain including the cerebral cortex, brain stem and spinal cord (Moore & Bloom, 1979; Lagercrantz, 1996; Berridge & Waterhouse, 2003; Jones, 2003). The locus coeruleus has major roles in stimulating and maintaining arousal and state-dependent cognitive processes (Svensson, 1987; Berridge & Waterhouse, 2003; Jones, 2003), and it is very responsive to a variety of stimuli including noxious stimulation, non-noxious cutaneous sensory stimulation, hypoxia and hypercapnia (Svensson, 1987; Tang *et al*., 2000). Thus, the strong tactile stimulation during the passage through the birth canal as well as transient periods of hypoxaemia and hypercapnia, which are commonly associated with labour contractions, and the severing of the umbilical cord at birth before breathing has started, would all be strong stimuli to contribute to cortical activation thereby assisting in the onset of arousal soon after birth (Mellor & Gregory, 2003; Mellor & Diesch, 2006, 2007).

Sensory stimulation

At birth, the newborn animal is exposed to a variety of sensory stimuli, which will elicit impulse barrages in the somatosensory system of the animal and these are likely to be arousing. First, the newborn will usually be exposed to air temperatures below those present *in utero* leading to activation of cutaneous thermoreceptors, which presents a

strong stimulus for arousal and breathing (Mellor & Gregory, 2003; Mellor & Diesch, 2006). Second, tactile stimulation during the birth process by the cervix and vagina as well as after birth by maternal licking apparently help to initiate arousal and breathing soon after birth (Mellor & Gregory, 2003; Mellor & Diesch, 2006).

Summary on the onset of conscious perception

Due to the link between wakefulness and consciousness, conscious perception would not be expected to occur before the age at which the EEG differentiates into REM-non-REM sleep and wake states, as underlying neurological structures and their interconnections would not be sufficiently mature and functionally competent to support conscious perception. However, even if underlying neurological structures are mature and functional, the first appearance of conscious perception may be delayed by the exposure to environmental factors that suppress it, as seen in the neurologically mature late-gestation fetus. Thus, it is concluded from the above analysis that conscious perception is not present until after birth in both altricial and precocial mammals. In addition, even in maturely born animals the onset of conscious perception after birth may be delayed by residual neurosuppressors or may be modulated by these factors until their levels would fall below that required for significant suppression to occur.

Although arousal is observed in the newborn and is associated with behaviour that would be indicative of conscious perception, such as teat seeking and other contact with the dam, it is not entirely certain that conscious perception is involved. First, increased arousal may not necessarily include conscious perception, because, it is possible to be awake but unconscious (Zeman, 2001; Mellor *et al.*, 2005). Second, even animals that are born extremely immature, such as marsupial pouch young, have the capacity to orient towards and attach to the nipple in the absence of higher brain structures that are necessary for conscious perception to occur.

Additionally, although sleep behaviours such as muscle twitches and eye movements are observed in newborn rats for example (Frank & Heller, 2003; Blumberg *et al.*, 2005; Seelke *et al.*, 2005), this is not necessarily indicative of fully established sleep-wake cycles and therefore the presence of conscious perception. Studies in chronically decerebrate cats have shown that sleep-wake cyclicity is maintained, but that this

consists merely of postural and motor components of sleep-wake behaviours in the absence of a functional thalamocortical system (Moruzzi, 1972).

Thus, although conscious perception seems unlikely before and during birth, it is not known when after birth animals become capable of conscious perception and how the timing of that onset differs according to maturity at birth.

1.5 Purpose of thesis

The purpose of this thesis was to assess when after birth animals born with varying degrees of neurological maturity gain the capacity for conscious perception and to investigate whether modulation of conscious perception by residual concentrations of *in utero* neuroinhibitors may be possible in precocial species where conscious perception appears to occur soon after birth. The results will have important implications for newborn animal welfare and will be discussed in detail in Chapter 7 of this thesis.

Below follows an outline of and background information on the methods used to assess the capacity for conscious perception in the present studies.

1.6 Using pain perception to investigate conscious perception

Experiments outlined in the present thesis employ responsiveness to noxious stimulation as a means to investigate conscious perception. Hence here follows a definition of pain as well as a brief summary of the underlying physiology.

What is pain?

According to the International Association for the Study of Pain (IASP), pain can be defined as follows:

"An unpleasant sensory or emotional experience associated with real or potential tissue damage, or described in terms of such damage."

This definition takes into consideration that pain is a complex sensory modality which

is accompanied by affective, motivational and cognitive aspects (Almeida *et al.*, 2004).

Underlying physiology

It is beyond the scope of this thesis to present an in depth account on pain physiology and the reader is referred to the comprehensive review by Millan (1999) for a more complete introduction to the topic.

Noxious insults to peripheral tissues activate nociceptors. These are free nerve endings representing the distal part of first-order afferent neurons, and respond to extreme mechanical, chemical or thermal stimuli (Basbaum & Jessell, 2000). There are two main types of pain. First, sharp fast pain, which is transmitted by small myelinated Aδ fibres and triggers withdrawal reactions, and second, dull, burning or longer lasting pain, which is transmitted via unmyelinated C fibres (Basbaum & Jessell, 2000; Livingston & Chambers, 2000; Almeida *et al.*, 2004). In addition, nerve endings may be exposed to chemicals released from damaged cells and these inflammatory mediators may lead to direct excitation of nociceptors or may increase the sensitivity of these to subsequent noxious stimulation (Livingston & Chambers, 2000).

The impulses generated by activation of nociceptors are transmitted to the superficial layers of the dorsal horn of the spinal cord, the marginal layer (lamina I) and the substantia gelatinosa (lamina II). From there the information is carried to the brain via six major ascending pathways (Millan, 1999; Basbaum & Jessell, 2000; Almeida *et al.*, 2004) including

- the *spinothalamic tract* (axons of neurons of laminae I and V-VII of dorsal horn, projecting to contralateral side of spinal cord and terminating in the thalamus),
- the *spinoreticular tract* (axons of neurons of laminae VII and VIII, terminating in reticular formation and thalamus),
- the *spinomesencephalic tract* (axons of neurons of laminae I and V, projecting to the mesencephalic reticular formation and periaqueductal grey matter),
- the *cervicothalamic tract* (axons of neurons of laminae III and IV, projecting to nuclei in the midbrain and the thalamus),
- the spinoparabrachial tract (axons of neurons of laminae I and II, projecting to the

parabrachial nuclei) and

the *spinohypothalamic tract* (axons of neurons of laminae I, V, and VIII, projecting to the hypothalamus and other supraspinal autonomic control centres).

In addition, connections are made with other areas, including the ventral horn of the spinal cord so that reflex arcs are established leading to withdrawal responses and other related phenomena including cardiovascular changes (Livingston & Chambers, 2000).

Thalamic nuclei are the main relay station for nociceptive information, where such information is processed before being relayed to various areas of the cerebral cortex. The thalamus appears to encode information concerning the type, temporal patterns, intensity and topographic localisation of the painful stimulus and specific nuclei of the thalamus seem to play distinct, but complimentary, functions in integrating the sensory-discriminative and the affective-cognitive components of pain (Millan, 1999; Almeida *et al.*, 2004). Two systems, the medial and lateral systems, act in parallel to project nociceptive information to three important cortical areas, namely the primary and secondary somatosensory cortices as well as the anterior cingulate cortex (Millan, 1999; Almeida *et al.*, 2004). The lateral system, involving specific thalamic nuclei projecting to somatosensory cortices I and II, appears to be associated with the sensory-discriminative component of nociception, while the medial system, with less defined projections from the medial region of the thalamus to the cortex including the limbic structures and the anterior insular cortex, appears to be associated with the motivational-affective component of pain (Almeida *et al*., 2004).

An important aspect of pain physiology is the ability of the central nervous system to modulate the perception of pain, either via spinal cord or via descending control by higher centres, particularly brainstem mechanisms involving the periaqueductal grey, raphe nuclei and locus coeruleus (Stamford, 1995). These control systems exert their effects by reducing or modulating ascending noxious information thereby reducing the level of pain perceived (Livingston & Chambers, 2000).

Traditional measures of pain

A variety of means are traditionally employed in measuring pain in non-verbal subjects

such as infants and animals. These include autonomic reflexes such as heart rate and blood pressure changes, respiratory rate and pattern, sweating, piloerection and gut motility; stress hormone concentrations including adrenaline, cortisol and beta-endorphin; and behavioural changes (Livingston & Chambers, 2000). However, none of these indicators is a reliable means to measure pain, as they can be induced and modified by stress-related endocrine activity, drugs or external physical effects, including those experienced when trying to obtain the samples in question (Livingston & Chambers, 2000). In addition, these measures cannot tell us about the perception of pain as they are related to activation of subcortical structures (Livingston & Chambers, 2000).

The function of the central nervous system in response to noxious stimulation is now increasingly assessed by modern techniques, such as electroencephalography (EEG), magnetoencephalography (MEG), and functional imaging techniques such as positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) (Murrell & Johnson, 2006). Although PET and fMRI are now becoming more accessible, the spatiotemporal resolution of EEG and MEG are apparently still superior (Kakigi *et al.*, 2005; Murrell & Johnson, 2006).

Using the electroencephalogram to investigate pain perception

1) The electroencephalogram (EEG)

Ionic currents generated by biochemical processes at the cellular level generate the electrical activity of the brain (Schaul, 1998). The potentials recorded from the cortex and the scalp are generated within the cortex itself by convoluted dipole layers of pyramidal neurons in the cortical grey matter (Creutzfeld, 1974; Schaul, 1998). It is not action potentials (APs) that are responsible for the EEG, but postsynaptic potentials (PSPs), which are longer in duration than action potentials (AP 1-2msec, PSP 10-250msec; Lopes da Silva, 1987) and involve a larger membrane surface allowing for both temporal and spatial summation (Schaul, 1998). During a PSP current flows in the extracellular space between the membranes of pyramidal and glial cells. This creates a field potential around the neuronal membrane which in turn is recorded by the EEG (Schaul, 1998). The electrical field generated by layers of pyramidal neurons is referred

to as an "open field" and the field potential around an open field has been found to decay inversely with the distance from the generator and thus can be viewed from almost any distance in a volume conductor (i.e. the scalp) (Schaul, 1998). Although lower brain centres such as the thalamus and brain stem also generate field potentials (so-called closed fields), they are not large enough to be detected by scalp electrodes and thus do not play a part in the generation of EEG waves (Schaul, 1998).

Detailed statistical analysis of the changes in the frequency components of the EEG have been made possible by the development of the Fast Fourier Transform (Cooley & Tukey, 1965; Johnson, 2003). The Fast Fourier Transform converts a time domain signal into the frequency domain, the output resembling a histogram, the so-called power spectrum (Johnson, 2003). The power spectrum can further be analysed to provide a variety of variables upon which statistical analysis is possible, including the median frequency, spectral edge frequency and total EEG power (Johnson, 2003).

The *median frequency* measures the central location of the power spectrum giving the frequency below which 50% of the power spectrum resides (50^{th} percentile). The *spectral edge frequency* measures the highest frequencies in the power spectrum and indicates the frequency below which 95% of the power spectrum resides (95^{th} percentile) (Johnson, 2003; Tonner & Bein, 2006). *Total power* is the overall area under the power spectrum curve, and usually decreases as median frequency and spectral edge frequency increase and *vice versa* (Johnson, 2003).

2) Cortical representation of pain

For many years it had been doubted that the cortex was involved in pain perception (Kenshalo & Willis, 1991; Treede *et al.*, 1999). Instead, it was believed that pain was the only sensation that was derived from thalamic rather than cortical activity (Treede *et al.*, 1999; Treede *et al.*, 2000). Thanks to the advances in imaging technologies such as PET and fMRI our knowledge about nociceptive areas in the cerebral cortex has now dramatically increased (Jones *et al.*, 1991; Treede *et al.*, 2000). PET studies showed that acute heat pain in humans led to activation of the cingulate cortex (Talbot *et al.*, 1991). However, there is disagreement as to whether or not the somatosensory cortex was also activated. Despite this disagreement, the results showed that the cerebral cortex was

involved in pain perception and the emphasis in the current dispute has thus shifted to the question of what specific functions the cortical areas subserve in pain perception (Treede *et al.*, 1999). As Treede *et al.* (1999) suggest, it appears that sensory, affective and other dimensions of pain may be processed in parallel by different parts of the nociceptive system (see section above on underlying physiology).

3) Pain assessment using the minimal anaesthesia model

Recently it has been possible to correlate different electroencephalographic variables with different aspects of central nervous system function (Johnson, 2003). One of those aspects is nociception. It is therefore possible to use the EEG for pain assessment, at least in controlled studies (Murrell & Johnson, 2006).

There are similarities in the EEG response to potentially noxious stimulation in conscious and anaesthetised animals (Ong *et al.*, 1997; Murrell *et al.*, 2003) and in conscious humans (Chen et al., 1989). Therefore, the minimal anaesthesia model was developed and tested. This model allows investigation of changes in EEG spectral responses to noxious stimulation in animals anaesthetised with halothane (Murrell *et al.*, 2003; Johnson *et al.*, 2005a; Johnson *et al.*, 2005b). Halothane is used in the model as it is not considered to have antinociceptive properties and appears to cause less depression of the cerebral cortex than other anaesthetic agents (Murrell & Johnson, 2006; Murrell *et al.*, 2008). Indeed, studies have shown that 'light' general anaesthesia with halothane only leads to partial depression of the cerebral cortex (Johnson & Taylor, 1998). Advantages of this model are that the use of anaesthetised animals is a humane way of investigating nociceptive processes and that contamination of EEG records by movement artefact is minimised.

1.7 Experimental outline

Chapter 2

The onset of conscious perception in mammals that are neurologically extremely immature at birth was considered by investigating the pouch young of one marsupial species. Thus, electroencephalographic responsiveness to a noxious stimulus of lightly

anaesthetised in-pouch tammar wallaby joeys (*Macropus eugenii eugenii*) aged between 95 and 260 days was measured and the results were interpreted in light of general neurological, behavioural and sleep-wake cycle development.

Chapter 3

The onset of conscious perception in mammals that are neurologically moderately immature at birth was considered by investigating the young of one such species. Thus, electroencephalographic responsiveness to a noxious stimulus of lightly anaesthetised rat pups (*Rattus norvegicus*) aged between 5 and 21 days was measured and the results were interpreted in light of general neurological, behavioural and sleep-wake cycle development.

Chapters 4, 5 and 6

The capacity for conscious perception and the potential for modulation of conscious perception by residual concentrations of the *in utero* neuroinhibitors allopregnanolone/pregnanolone in mammalian young that are neurologically mature at birth were investigated. The animal model used here was the newborn and young sheep (*Ovis ovis*).

1) Chapter 4

The development of the electrical activity of the brain in lambs over the first two days after birth was assessed. This was done to determine whether changes occurred in the EEG of an animal species born neurologically mature over the first few minutes and days after birth. Such changes, if present, may indicate neurological development beyond birth associated with exposure to a new environment or the potential for modulation of consciousness perception for minutes or even days after birth.

2) Chapter 5

Changes in the plasma and brain concentrations of the neuroinhibitory steroid hormone allopregnanolone and its precursors progesterone and pregnenolone were measured in newborn lambs aged from within the first 12 hours to 9 days after birth. This was done

to assess the potential for modulation of conscious perception via the well-established anaesthetic, sedative/hypnotic and analgesic actions of allopregnanolone.

3) Chapter 6

Electroencephalographic responsiveness to noxious stimulation in lambs at 4-24 hours or 7-11 days after birth was investigated during intravenous infusions of the neuroinhibitory steroid hormone pregnanolone or the $GABA_A$ receptor antagonist picrotoxin. Again, this was done to assess the capacity of residual concentrations of *in utero* neuroinhibitors to modulate conscious perception for the first day after birth. Although the experimental study described in this chapter is presented as the last study in this thesis, it was in fact the first to be undertaken by the candidate. The study design was very complex, making it difficult to interpret the data. Hence, although the chapter makes brief statements on the possible meaning and significance of the results, it discusses the lessons learnt and the possible ways outcomes could be improved were the study to be repeated.

Chapter 7

This chapter consists of a general discussion of the overall findings of Chapters 2 to 6. In addition, suggestions for future research and a short discussion on experimental limitations are presented. Animal welfare implications of the results of the present thesis are also discussed.

Retrospective note

At the outset of this PhD programme the appearance of consciousness after birth was the initial focus of the planned studies. However, during the course of the work it became evident that the hoped-for ability to comment on the onset of conscious perception after birth using the data of the present studies would not be possible. This was because the animals were anaesthetised, and this, together with the fact that they were studied at different postnatal ages, meant that potentially different effects of anaesthesia at different ages could not be definitively distinguished from neurological developmental changes.

Therefore, as the data by themselves were insufficient to comment on the onset of consciousness, the candidate additionally used information already available in the literature, namely information on EEG and behavioural development, to give an indication of the onset of conscious perception. While, if based solely on the data of the present studies, the conclusions drawn should be more conservative to reflect the actual findings and methodological limitations, the use of this additional information on neurological and behavioural development allowed wider interpretation regarding the onset of consciousness. Caution was nevertheless exercised in such interpretation and only tentative suggestions have been made throughout the thesis.

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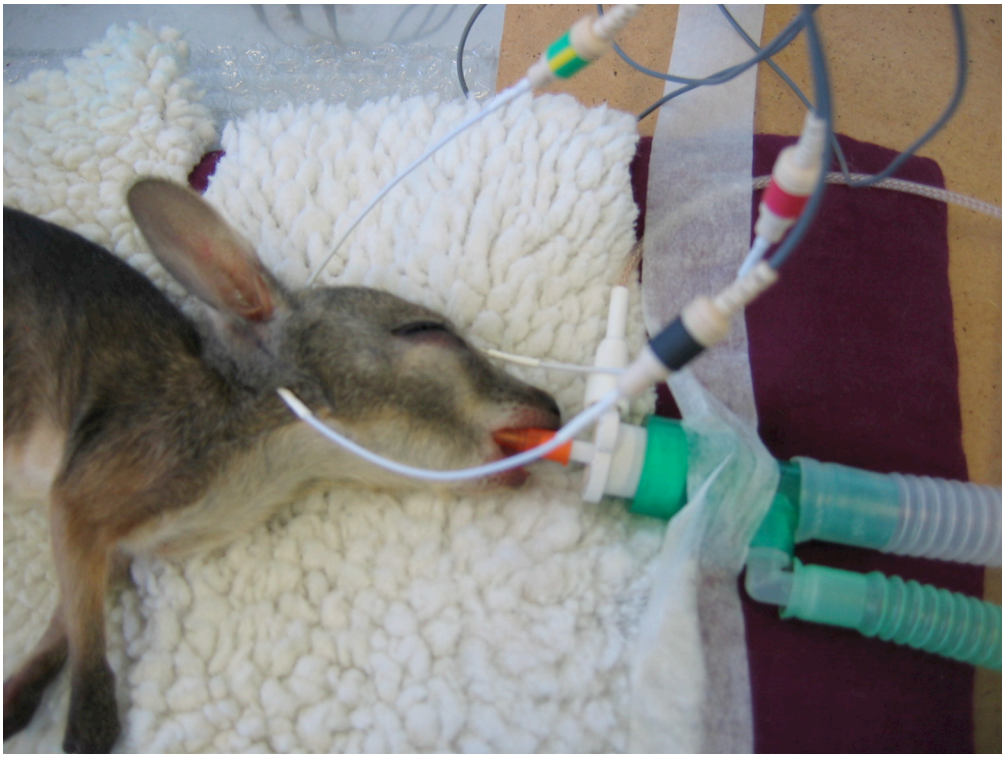
Chapter 2

- Extremely immature at birth -



Parts of the present chapter have been published in the following publication (*see Appendix 2 for a full copy of the proofs of the paper*)

Diesch TJ, Mellor DJ, Johnson CB & Lentle RG. Developmental changes in the electroencephalogram and responses to a noxious stimulus in anaesthetized tammar wallaby joeys (*Macropus eugenii eugenii*). *Laboratory Animals* (in press).

Abstract

The tammar wallaby joey is born extremely immature and most of its neurological maturation occurs *ex-utero* in the maternal pouch. When after birth such animals become capable of conscious perception and therefore able to experience pain, has not previously been investigated. The electroencephalographic (EEG) responses to toe clamping in lightly anaesthetised tammar wallaby joeys were assessed to elucidate whether and when a noxious stimulus can elicit a change in the EEG that would suggest the potential for conscious perception of pain. Parameters examined included frequency spectra between 1-30Hz as well as median (F50) and spectral edge (F95) frequencies and total power (Ptot) of the power spectrum. The EEG of joeys younger than 100-120 days was completely or partly isoelectric to the extent that no EEG response to clamping could be observed. Joeys aged between 140-181 days did not show any significant changes in the parameters investigated, while joeys aged 187-260 days showed a significant decrease in F50 in response to clamping ($p=0.001$). However, multivariate analyses could not distinguish between animals in the two older age groups before or after clamping. The effect of increasing halothane concentration on EEG parameters in the two older groups was also investigated. The EEGs of anaesthetised joeys were compared to a small number of EEGs obtained from joeys not anaesthetised during EEG recordings. The results are discussed in the context of published information on neurological development, sleep-wake EEG differentiation, normal behavioural development and the possible effects of halothane anaesthesia on the EEGs of joeys in the present study. Tentative conclusions were drawn regarding the onset of conscious perception on the basis of such information and the results of the present study were consistent with this. In addition, the absence or presence of eyes open and fur are discussed as markers for estimating when during pouch-life conscious perception and hence the potential for suffering may or may not be possible in joeys whose mothers may be involved in pest control operations or road accidents.

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Electroencephalographic investigations in tammar wallaby joeys (*Macropus eugenii eugenii*) of varying in-pouch ages

2.1 Introduction

In order to ensure that newborn and young animals experience good welfare, it is essential that we know when after birth the capacity to experience negative mental states, such as pain, emerges. This in turn will allow us to undertake actions towards preventing and/or alleviating suffering. There are two prerequisites for suffering. 1) The animal must be sentient, meaning that the animal's nervous system has to be sufficiently developed to relay sensory inputs as electrical impulses from the periphery to the higher centres of the brain (i.e. cerebral cortex), where such impulses can then be interpreted (Mellor & Diesch, 2006, 2007). 2) The animal has to be consciously aware, because in the absence of consciousness there can be no *experience* of pain or any other sensations (Bromm, 1995). Thus, *when*, during development, animals will be able to suffer, depends on the maturation of underlying neurological processes and hence is therefore anticipated to vary depending on the species (Diesch *et al.*, 2008).

The offspring of the tammar wallaby (*Macropus eugenii*), the joey, is born after a short gestation of about 28 days (Tyndale-Biscoe & Janssens, 1988). At birth it emerges from the female reproductive tract and climbs into the maternal pouch, where it permanently remains attached to a teat for about 100 days, and intermittently thereafter, finally leaving the pouch at around 250 days (Tyndale-Biscoe & Janssens, 1988). In comparison to newborns of eutherian mammals, the newborn joey is extremely immature neurologically, so that most neurological maturation occurs *ex-utero* during pouch life (Tyndale-Biscoe & Janssens, 1988). However, some rudimentary neurological function must be present to enable the newborn joey to orient towards the pouch, climb to and enter the pouch and subsequently attach itself to a teat. The newborn marsupial climbs by employing a succession of alternating side-to-side head movements with associated forward movement of the forelimbs (Veitch *et al.*, 2000; Gemmell *et al.*, 2002). It has been suggested that these movements result solely from the action of pattern generators modulating spinal circuitry due to the absence of the influence of higher neurological systems (Pflieger *et al.*, 1996; Ho, 1997; Lentle *et al.*,

2006).

There has been some debate as to how the newborn accomplishes the task of finding its way to the pouch and the teat (Veitch *et al.*, 2000; Gemmell *et al.*, 2002). In the brushtail possum (*Trichosurus vulpecula*), and thus presumably in the tammar wallaby, evidence suggests that the newborn joey does not only find the pouch via a sense of gravity, but that odour and possibly heat emanating from the pouch are also involved (Veitch *et al*., 2000). Metatherians have rudimentary olfactory and vestibular systems at birth (Gemmell & Nelson, 1988, 1989), which, together with observed joey pouch orientation behaviours (Veitch *et al*., 2000), support the involvement of a sense of smell and gravity. Additionally, Merkel cells around the mouth are present at birth in a variety of newborn marsupials (Gemmell *et al*., 1988) and the muzzle of tammar wallaby joeys at birth contains rudimentary vibrissal follicles which are richly innervated (Waite *et al*., 1994). These features may play an important role in initial teat attachment of the joey once it has entered the pouch.

The above capabilities of the newborn joey do not rely on the function of higher brain centres and thus do not require conscious perception. The cerebral cortex of the tammar wallaby joey comprises only two layers of cells at birth and resembles that seen in early human (40 days) and sheep (26 days) embryos (Reynolds *et al.*, 1985). Therefore, it is highly unlikely that metatherian young at birth are capable of conscious perception, as the underlying neurological structures, including the thalamus and cerebral cortex, are not sufficiently developed to support such advanced functions. When after birth this ability develops in metatherians is not known.

It may be possible to estimate the onset of conscious perception in the joey by determining when the structures associated with sensory perception become sufficiently developed and functional.

There is evidence that the cerebral cortex is involved in the experience of pain perception (Jones *et al.*, 1991; Talbot *et al.*, 1991; Jones *et al.*, 1992; Treede *et al.*, 1999). Changes in electroencephalographic (EEG) spectra have been shown to reflect changes in cerebral activity associated with the cognitive perception of pain (Bromm, 1984; Chen *et al.*, 1989). Hence EEG responses to potentially noxious stimulation may

be useful in determining the time at which the apparatus necessary for conscious perception becomes functional.

The minimal anaesthesia model was used for the purposes of the present study, as previous investigations had shown a similarity in the EEG response to potentially painful stimulation in conscious and anaesthetised animals (Ong *et al.*, 1997; Murrell *et al.*, 2003) to that found in conscious humans (Chen *et al.*, 1989) (see Chapter 1). It is a humane model in that it avoids any conscious perception of pain and minimises EEG artefacts created by movement.

The EEG responses of tammar wallabies of different in-pouch ages to toe clamping and changing concentrations of halothane were investigated. Although the minimal anaesthesia model does not demonstrate that animals are capable of conscious perception it does allow inferences to be drawn regarding whether or not the cerebral cortex is capable of responding to systemic stimulation at the different ages investigated. A comparison of baseline EEGs of anaesthetised joeys with the EEGs recorded in a small number of joeys that were not anaesthetised was also undertaken. This was done in order to assess whether developmental changes in the EEG of anaesthetised joeys could also be observed in the non-anaesthetised state.

The results of the study will be discussed in the context of general neurological development, as well as development of sleep-wake EEG patterns and related behaviour, and inferences will be drawn about the onset of conscious perception in these animals. The results will have implications for developmental research in this species and for assessing the potential for suffering in joeys during pest control operations or road accidents.

2.2 Materials and Methods

Animals

Animals were obtained from a colony of feral South Western Mainland tammar wallabies (*Macropus eugenii eugenii*) maintained at Massey University, New Zealand. They were kept in a controlled environment (12:12 light:dark cycle) and fed a pelleted

diet *ad libitum* supplemented with Lucerne hay and fresh fruit with *ad libitum* access to water. Average air temperatures ranged from 15°C in winter to 23°C in summer. Females were mated all year round to ensure a continuous supply of in-pouch joeys. Overall, 29 joeys of varying ages and gender (anaesthetised during EEG recordings n=25, not anaesthetised during EEG recordings, n=4) (Table 2.1) were used in the present study. The Massey University Animal Ethics Committee gave prior approval to this study (Protocols 04/94 and 06/11).

As adult animals were used as breeding stock, no adult EEGs were undertaken although this would have been desirable.

Age estimation

The in-pouch ages of wallaby joeys that were anaesthetised during EEG studies were determined from published data on the relationship between head length (mm) and age (days) (Poole *et al*., 1991). Wallaby joeys that were not anaesthetised during EEG recordings were approximately 137, 155, 189 and 193 days of in-pouch age, as estimated using a weight chart of Massey University animals of known in-pouch ages (unpublished observations).

Table 2.1: General information on the anaesthetised wallaby joeys used in the present study.

Joey	Skull length (mm)	Estimated in-pouch age (days)[#]	Gender	Weight (g)
1	18.69	31[*]	F	5.8
2	37.67	87[*]	M	48.5
3	39.92	94	M	66.5
4	42.86	104	M	73.4
5	43.96	108[*]	M	67.4
6	45.90	114	M	69.5
7	47.46	118	F	82.2
8	49.23	124	F	87.8
9	49.92	127	M	95.3
10	54.35	142	F	121.2
11	55.07	145	M	139.4
12	60.67	164	F	187.3
13	63.22	173	M	258.9
14	63.71	174	M	239.4
15	65.46	181	M	298.6
16	66.81	187	F	423.4
17	67.02	187	M	412.9
18	67.59	189	M	345.6
19	69.00	196	F	412.5
20	69.98	198	M	441.9
21	73.93	215	F	700.0
22	76.67	227	F	665.0
23	78.02	230	M	848.1
24	79.10	238	F	854.0
25	84.42	261	M	1268.4

F = female; M = male

[#] Calculated from skull length (Poole *et al.*, 1991)

[*] Joeys which could not be intubated

Experimental procedure

1) Anaesthetised joeys

Anaesthesia was induced in a chamber using 4-5% halothane (Merial, Parramatta, Australia) in oxygen (100%). Once loss of the righting reflex and absence of general body movements was achieved, joeys were intubated using a guidewire and anaesthesia was maintained with halothane in a flow of oxygen (100%) delivered through a calibrated vaporiser with intermittent positive pressure ventilation. Endtidal halothane was monitored and titrated to 1.0% initially (baseline). Stainless steel needle electrodes (0.3mm diameter; Medelec, Oxford Instruments Medical Systems, UK) were placed subdermally at the midline over the frontal sinus (non-inverting electrode), over the left mastoid process (inverting electrode) and caudal to the occipital process (common electrode).

Recordings were made in a Faraday cage to minimise electrical contamination. Data collection began once a stable endtidal halothane concentration of 1.0% was reached (usually within 5 minutes of electrode placement). A baseline EEG was then recorded for 5 minutes. Following this a supramaximal (as indicated by significant tissue damage) mechanical stimulus was applied using a haemostat clamp (6.5cm long, 3-5mm wide) placed across the fourth phalange of the right hind leg, closed until the first ratchet caught and released after 30 seconds. EEG recordings continued for five minutes after the clamp had been removed. The endtidal halothane concentration was then titrated to 1.2%, and finally to 1.4%, and data were collected for five minutes in each case once these concentrations were stable, which was generally within 5 minutes of adjusting the vaporiser to the higher settings.

Two of the joeys did not receive toe clamping so that EEG data from them related only to endtidal halothane concentrations of 1.0%, 1.2% and 1.4%. In two joeys the order of the experiment was reversed so that recording started with an endtidal halothane concentration of 1.4%, ending with the post-clamping recordings. These exceptions to the present protocol occurred as the original experimental protocol did not involve toe clamping. Similar results were obtained whether or not data from these animals were included, so their data were included in the statistical analyses.

As the youngest joeys were too small for intubation (Table 2.1) the EEG was recorded for 5 minutes after their removal from the induction chamber and placement of electrodes. These joeys did not receive further halothane anaesthesia during this recording period.

All joeys were placed onto a heating blanket (Gaymar, USA) at 40ºC or a heated pad (temperature not recorded) and a small sheep wool blanket was placed over them to prevent excessive heat loss. Rectal temperature was not measured.

The candidate did observe withdrawal responses to clamping in some joeys. However, this was not documented rigorously and hence it is not known whether this behavioural response was equally present in the younger as well as the older groups.

At the conclusion of the experiment joeys were euthanased by intracardiac or intraperitoneal injection of an overdose of pentobarbitone sodium (Pentabarbitone 300, National Veterinary Supplies, Auckland, New Zealand). The euthanased joeys were then weighed, their head length was recorded using digital Vernier callipers (Model 500-322, Mitutoyo UK Ltd, UK) and their tissues were collected for other studies (Lentle *et al.*, 2006; Lentle *et al.*, 2007a; Lentle *et al.*, 2007b).

2) Joeys not anaesthetised during EEG recordings

Anaesthesia was induced in a chamber using 5% sevoflurane (Abbott, Auckland, NZ) in oxygen (100%). Once loss of righting reflex and absence of general body movements was achieved joeys were removed from the chamber and stainless steel needle electrodes (0.3mm diameter; Medelec, Oxford Instruments Medical Systems, UK) were placed as described above. During electrode placement anaesthesia was maintained using sevoflurane in oxygen given via face mask.

Preventing excessive heat loss was achieved as described above. The joeys were then covered with a cardboard box with the electrode wires passing to the exterior recording devices. The box, in excluding light, reduced body movement and hence excessive

artefact in the EEG (unpublished observations). The joeys were not disturbed during recording sessions. The EEG was recorded for approximately 30 minutes after electrode placement. At the end of the recording period joeys were euthanased as previously described and weighed thereafter to estimate their age.

EEG recordings

All EEGs were recorded continuously at a sampling rate of 1kHz using an Apple Macintosh personal computer and Powerlab 4/20 data recording system (Powerlab^{TM} data acquisition system®, AD Instruments Ltd, Bella Vista NSW, Australia) with compatible recording software (Chart 5, Powerlab^{TM} data acquisition system®, AD Instruments Ltd, Bella Vista NSW, Australia). The biological amplifier (World Precision Instruments Inc, Saratosa, Florida, USA) was set at a gain of 1000 with the low-pass filter at 0.1Hz and the high-pass filter at 0.5kHz. Analysis of the EEG was performed off-line following completion of the experiment.

EEG analyses

1) Anaesthetised joeys

EEG traces from joeys younger than about 100 days were nearly completely isoelectric (Table 2.2), as judged by relative EEG power of the EEG power spectrum compared to non-isoelectric EEG traces. Traces from older joeys also showed isoelectric periods interspersed between EEG epochs. Isoelectric periods were of 300msec or longer duration during which the amplitude of the EEG was at least 4-5 times lower than that of the remaining trace. This was assessed visually (see Figure 2.1). The EEG recordings over the first 3 minutes at a stable endtidal halothane concentration of 1% (baseline recording), over the first 3 minutes immediately after toe clamping and over the first 3 minutes at stable endtidal halothane concentrations of 1.2% and 1.4%, were used for examining isoelectric periods. The proportion of time occupied by isoelectric periods (%) and the total number and mean duration of these periods were recorded for all animals for each of these defined periods (Table 2.2).

Spectral analysis was performed using a customised Fast Fourier Transform (FFT)

program (Spectral Analyser, CB Johnson, Massey University, New Zealand, 2006), to calculate the median frequency (F50), spectral edge frequency (F95) and total power (Ptot) for consecutive 1-second segments as well as the power for frequencies of 1-30Hz. Samples of continuous EEG of a duration of 20 seconds each were obtained from the beginning of baseline (1.0% endtidal halothane), during clamping, immediately after clamping and at the beginning of stable endtidal halothane concentrations of 1.2% and 1.4%. Where short isoelectric periods or artefacts were present (<5sec), calculated spectral data were substituted by the preceding value calculated in order to avoid invalid data (i.e. non-stationary data). In five joeys, isoelectric periods were too common to allow this approach. Therefore, five periods of the EEG traces (>3000msec) were selected at equivalent recording periods as those described above, subjected to FFT analysis and the resultant values were combined for each recording period (i.e. baseline, clamping, post-clamping, 1.2% and 1.4% endtidal halothane) for each animal.

Total power and EEG power (log power) of the individual frequency bands have been reported here as arbitrary units. This was done as the set-up of the present study did not allow for the amplification effect of the signal to be taken into consideration. Hence no units could be ascribed. As the same system was used throughout the study and no changes were made to the recording apparatus, all values were recorded on the same scale and can be directly compared with each other without the use of defined units.

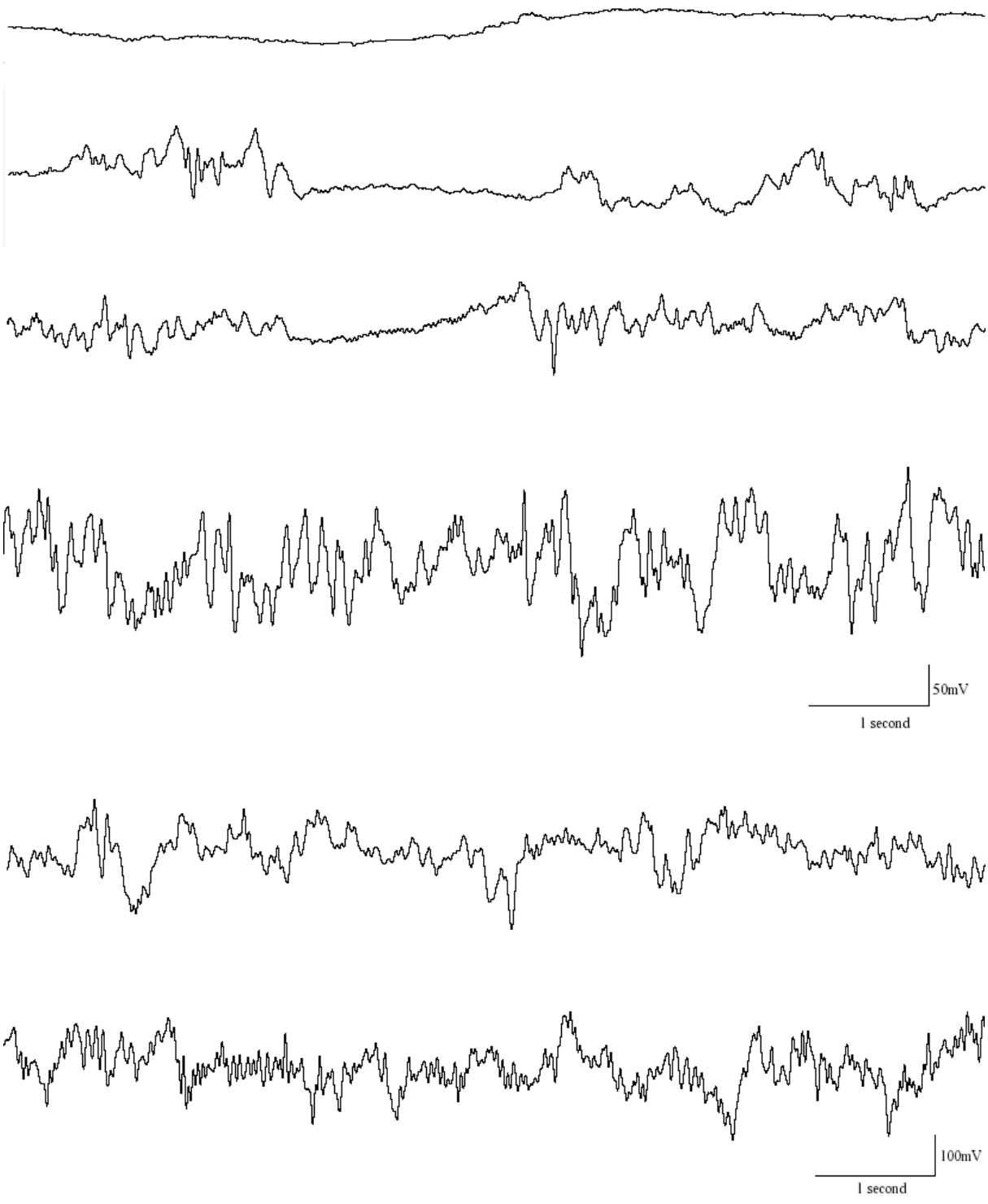

Figure 2.1: EEG traces of joeys approximately 94 days, 124 days, 145 days, 173 days, 198 days and 261 days of in-pouch age (from top to bottom), showing an isoelectric EEG at 94 days and isoelectric epochs for joeys at 124 and 145 days of in-pouch age. Note the different scale for the two oldest joeys – the first scale shown relates to the four traces above it and the second to the two traces above it.

***Table 2.2**: Percentage of time occupied by isoelectric EEG periods (%), average duration of isoelectric periods (msec) and number of isoelectric periods present (N) during 3 minutes of EEG recordings during baseline, post clamping and at endtidal halothane concentrations of 1.2% and 1.4%. Joeys that could not be intubated (n=3) and hence only had a 5-minute EEG record taken are not included in this table, but had an isoelectric EEG throughout the 5 minutes (100%).*

	1.0%			**After clamping**			**1.2%**			**1.4%**		
Age (days)	*%*	*msec*	*N*	*%*	*msec*	*N*	*%*	*msec*	*N*	*%*	*msec*	*N*
94	**99**	35426	5	**99**	35220	5	**100**	180000	1	**100**	89620	2
104	**94**	8033	20	**92**	6284	25	**96**	11906	14	**100**	179279	1
114	**97**	21908	8	**91**	7131	23	**96**	13236	13	**99**	59520	3
118	**78**	3804	37	**78**	3807	37	**77**	5990	23	**94**	15455	11
124	**55**	2010	49	**52**	2078	45	**57**	2314	44	**68**	3140	39
127	**87**	5421	29	**80**	4380	33	**78**	3803	37	**81**	4675	31
142	**5**	657	13	**7**	851	15	**11**	1045	19	**22**	1222	32
145	**13**	1072	22	**11**	1015	20	**16**	909	32	**29**	1408	37
164	**0**	0	0				**2**	377	9	**3**	719	8
173	**0**	0	0	**0**	0	0	**0**	0	0	**1**	350	1
174	**2**	663	6	**2**	616	7	**6**	925	11	**9**	860	19
181	**0**	0	0	**0**	0	0	**1**	595	4	**4**	651	10
187	**0**	0	0				**1**	356	5	**3**	449	10
187	**0**	0	0	**1**	350	1	**1**	440	1	**4**	635	11
189	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0
196	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0
198	**0**	0	0	**0**	0	0	**0**	0	0	**1**	345	2
215	**0**	0	0	**0**	0	0	**1**	665	2	**1**	665	2
227	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0
231	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0
238	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0
261	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0	0

2) Joeys not anaesthetised during EEG recordings

The EEG traces of the four joeys that were not anaesthetised during EEG recordings were assessed for the presence of isoelectric periods (see definition above). Spectral analysis was then performed as above, using the specialised Fast Fourier Transform (FFT) program.

It is commonly known that sevoflurane is a short-acting anaesthetic due to its low solubility (Eger & Johnson, 1987; Strum & Eger, 1987). In addition, due to the size of wallaby joeys compared to adult wallabies we anticipated that recovery would be even faster in the joeys, as small animals have greater ventilation rates and cardiac output (Eger & Johnson, 1987). Although we do not know the sevoflurane recovery times for tammar wallabies, we used EEG traces from 10 minutes after termination of anaesthesia exposure onwards for FFT analysis. Residual anaesthetic concentrations that may have been present in the joeys at this time were not considered to have been sufficiently high to significantly affect spectral data. This is supported by a study where 3-month-old rats were reported to right themselves after about six minutes and to pass a Rotarod test on average after 15 minutes following two hours of sevoflurane anaesthesia at 1.6 MAC (Eger & Johnson, 1987).

The EEG traces of the present study were inspected for movement artefact and five non-contaminated 30-second EEG periods were chosen for FFT analysis from 10 minutes after the EEG recordings had commenced (i.e.10 minutes after anaesthetic exposure had been terminated).

As joeys were covered with a cardboard box to reduce light levels, we were not able to record their behaviour. Therefore, it was not possible to determine whether the animals were aroused or asleep during the recording period. Although movement artefact is present in the EEG traces and would suggest an aroused joey, this was not considered to be a reliable indicator of arousal/sleep state as joeys may have been aroused without causing movement artefact.

To determine the behavioural state of the joeys, EEG traces with apparent low voltage

high frequency waves (LVHF, awake-like) and apparent high voltage low frequency waves (HVLF, sleep-like/quiet awake) were used for the purpose of the present study. The joey aged 155 days did not show HVLF EEG characteristics after recovery and hence only LVHF EEG data are available for this animal. The EEGs of the other three joeys did show both EEG characteristics, but movement artefact during the putative awake-like periods was common preventing meaningful FFT analyses. Thus, the data presented relate to HVLF EEGs of joeys aged 137, 189 and 193 days and awake-like EEG periods of the joey aged 155 days. However, in the absence of direct behavioural observations and the potential to correlate them with EEG traces, small movement artefact might have been present during the selected periods and not interpreted as such.

Statistical analysis

Statistical analysis was performed in SPSS 11.0 for MAC OSX (SPSS Inc., USA) and Prism 5 for MAC OSX (GraphPad Software, Inc., USA). Data were tested for normality using the Shapiro-Wilks statistic (if $n<50$) or the Kolmogorov-Smirnoff statistic (if $n>50$) and normal probability plots. The Levene's test was used to test for homogeneity of the data where these were normally distributed. Data are presented as mean ± standard error of the mean (SEM). Differences were considered significant at $p=0.05$ or less. Degrees of freedom reported (e.g. df 1, 130) represent the number of groups and overall number of data points used in individual statistical tests.

1) Anaesthetised joeys

Grouping

The following groups were established: joeys younger than 130 days (n=9; not included in statistical analyses due to high incidence of isoelectric periods), joeys aged between 140 and 181 days (n=6; five joeys with clamping and one without) and joeys aged between 187 and 260 days (n=10; nine joeys with clamping and one without). The cut-off of 185 days was used to separate groups because, during this time, major physiological changes occur as indicated by the achievement of homeothermic control and changes in milk composition, behaviour and food intake (Setchell, 1974; Green *et al.*, 1983; Tyndale-Biscoe & Janssens, 1988).

Spectral analysis

Median (F50) and spectral edge (F95) frequencies and total power (Ptot) were calculated using EEG traces of 20 seconds duration at the beginning of baseline (1.0% stable endtidal halothane concentration), during clamping and immediately after clamping, as well as at the beginning of 1.2% and 1.4% stable endtidal halothane concentrations.

Spectral data were not normally distributed and non-parametric tests were employed for statistical evaluation. Whether or not spectral parameters were affected by clamping or increases in endtidal halothane concentrations was investigated using the non-parametric Friedman's test (equivalent of a one-sample repeated measures ANOVA) followed by Dunn's post-hoc test. Differences in baseline spectral parameters between the two age groups were investigated using the Mann Whitney test for two unrelated samples.

Subsequently, frequency spectra means (1-30Hz) for all groups before, during and after clamping were graphed and evaluated. The gross differences in the shapes of frequency curves using frequency spectra for each frequency (1-30Hz) were examined by the two-tailed Kolmogorov-Smirnoff test. A similar procedure was used to investigate data obtained during increases in endtidal halothane concentrations.

To further investigate the differences in frequency spectra with age, clamping and change in anaesthesia concentrations, a Principal Component (PC) analysis was performed on log-transformed data using frequency spectra for 1-30Hz and F50, F95 and Ptot. The PC loadings were evaluated and the PC scores were plotted against each other in reduced dimensions to explore the data.

2) Joeys not anaesthetised during EEG recordings

The mean and standard error of the mean for F50, F95 and Ptot were calculated for each of the four in-pouch joeys. Frequency spectra means (1-30Hz), combining the five EEG periods for each joey, were also plotted.

3) Comparison between joeys anaesthetised and not anaesthetised during EEG studies

Three of the non-anaesthetised joeys were compared to anaesthetised joeys of similar ages. They were grouped according to anaesthesia/no anaesthesia and also according to age (younger/older), so that the following four groups emerged:

Older joeys anaesthetised (n=2; 189 and 196 days)
Older joeys not anaesthetised (n=2; 189 and 193 days)
Younger joeys anaesthetised (n=2; 142 and 145 days)
Younger joeys not anaesthetised (n=1; 137 days).

The EEG of the joey aged 155 days was not included in the comparative analysis as there were no anaesthetised joeys of similar age for comparative purposes.

For each animal 20 seconds of EEG data, baseline data in the case of anaesthetised joeys, were used for analyses. Mean and standard error of the mean for F50, F95 and Ptot were calculated, including that for the wallaby aged 155 days, which, however, was not included in the subsequent statistical analysis.

A principal component (PC) analysis was performed on log-transformed data using frequency spectra for 1-30Hz. The PC loadings were evaluated and the PC scores were plotted against each other in reduced dimensions to explore the data.

2.3 Results

General information

Information on gender, estimated age, head length (mm) and weight (g) is presented in Table 2.1.

Anaesthetised joeys

1) Isoelectric periods

The proportion of time occupied by isoelectric EEG periods (%), as well as the number of isoelectric periods and their average durations during 3-minute periods are presented in Table 2.2. In the majority of animals younger than 130 days, 75% or more of the baseline EEG at 1.0% endtidal halothane comprised isoelectric periods. In each joey, this percentage increased with time (i.e. with increase in anaesthetic level). The percentage of time occupied by isoelectric periods in the EEG was markedly lower in animals aged >130 days. The EEG of joeys aged between 140 and 145 days exhibited only 5% to 13% isoelectric periods during baseline observations. Again, this percentage increased with the duration of the experiments in each joey of this age. From 160 days onwards the majority of animals did not exhibit any isoelectric periods during baseline observations, but isoelectric periods tended to become more evident as each experiment progressed. This effect was more pronounced in a number of joeys aged 160-181 days than in those aged 187-200 days, and was absent in the majority of joeys older than 200 days.

The number and duration of individual isoelectric periods were variable. The number tended to increase and subsequently to decrease with increasing in-pouch age, whereas their duration generally decreased with increasing age (Table 2.2). The joey aged 124 days showed the highest number of isoelectric periods at the times it was examined.

The proportion of time occupied by continuous EEG activity during baseline observations, as opposed to isoelectric traces, approached 100% by about 160 days of in-pouch age (Figure 2.2).

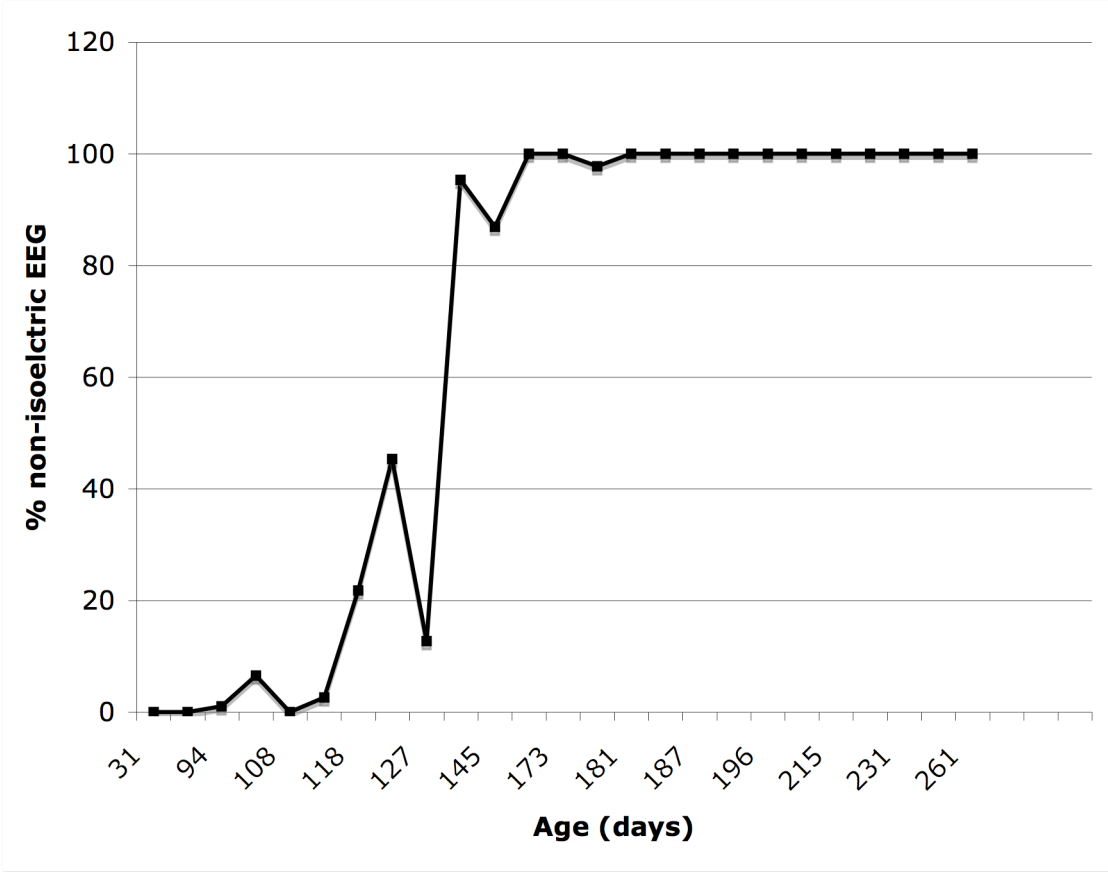

***Figure 2.2**: Changes in the proportion of time occupied by non-isoelectric EEG patterns at different in-pouch ages for baseline (1.0% endtidal halothane) observations in anaesthetised joeys. Each data point represents one animal.*

2) Spectral analysis in response to clamping

Median frequency (F50), spectral edge frequency (F95) and total power (Ptot)

There were no significant differences in F50 at baseline (before clamping) between joeys aged 140-181 days and those aged 187-260 days ($p=0.207$; df 1, 279; $Z=-1.262$; 5.60±0.14Hz before about 185 days, 5.96±0.17Hz afterwards). However, both baseline F95 and Ptot were significantly lower in the younger compared to the older joeys (F95: 23.52±0.21Hz *versus* 24.18±0.13Hz, $p=0.014$, df 1, 279, $Z=-2.455$; Ptot: 47.4±2.4 compared to 89.4±1.8 arbitrary units, $p\leq0.001$, df 1, 279, $Z=-10.780$).

According to the Friedman's test, there were significant overall changes in response to clamping in the older joeys (F50 $p=0.001$), but not in the younger joeys, as changes in F50 and Ptot between treatments only approached significance (F50 $p=0.052$; Ptot $p=0.085$) in the latter joeys (Table 2.3). Median frequency (F50) values of both groups decreased in immediate response to the clamp (before versus during clamping) and then increased again during the after-clamping period. In the older joeys, the post-hoc Dunn's test showed that the significant differences in F50 in response to clamping calculated by the Friedman's test were due to a significant decrease in F50 between before-clamping and during-clamping data and a significant increase in F50 between during-clamping and after-clamping data.

Graphic comparisons

Visual inspection showed that there were differences in the power of frequency spectra between age groups during baseline (before clamping) (Figure 2.3). The two-tailed Kolmogorov-Smirnoff test results showed that EEG power of frequency spectra for all frequencies investigated (1-30Hz) was significantly higher ($p<0.001$) in the older than the younger joeys.

***Table 2.3**: Results of the non-parametric Friedman test, including p-values, Chi-Square statistic, degrees of freedom (df), means and standard error of the mean (SEM) for EEG parameters from anaesthetised joeys before, during and after clamping. Means denoted with the different letters indicate significant differences between means within an age group with significant levels as follows: * p<0.001; + p=0.001-0.01 and ' p=0.01-0.05).*

Age (days)		**F50 (Hz)**			**F95 (Hz)**			**Ptot (arbitrary units)**		
		Before	**During**	**After**	**Before**	**During**	**After**	**Before**	**During**	**After**
140-181	*p-value*	*0.052*			*0.330*			*0.085*		
(n=5)	χ^2	*5.930*			*2.220*			*4.940*		
	df	*2, 99*			*2, 99*			*2,99*		
	Mean	5.60^{a}	4.85^{b*}	5.02	23.52	23.15	23.35	47	56	46
	SEM	0.14	0.18	0.16	0.21	0.25	0.19	2.4	3.5	2.3
187-260	*p-value*	*<0.001*			*0.310*			*0.362*		
(n=9)	χ^2	*14.014*			*2.344*			*2.033*		
	df	*2, 179*			*2, 179*			*2, 179*		
	Mean	5.96^{a*}	5.12^{b}	$6.13^{a'}$	24.18	24.25	24.39	89	92	92
	SEM	0.17	0.17	0.22	0.13	0.15	0.13	1.8	2.7	1.8

F50 = median frequency

F95 = spectral edge frequency

Ptot = total power

χ^2=Chi-Square Friedman statistic

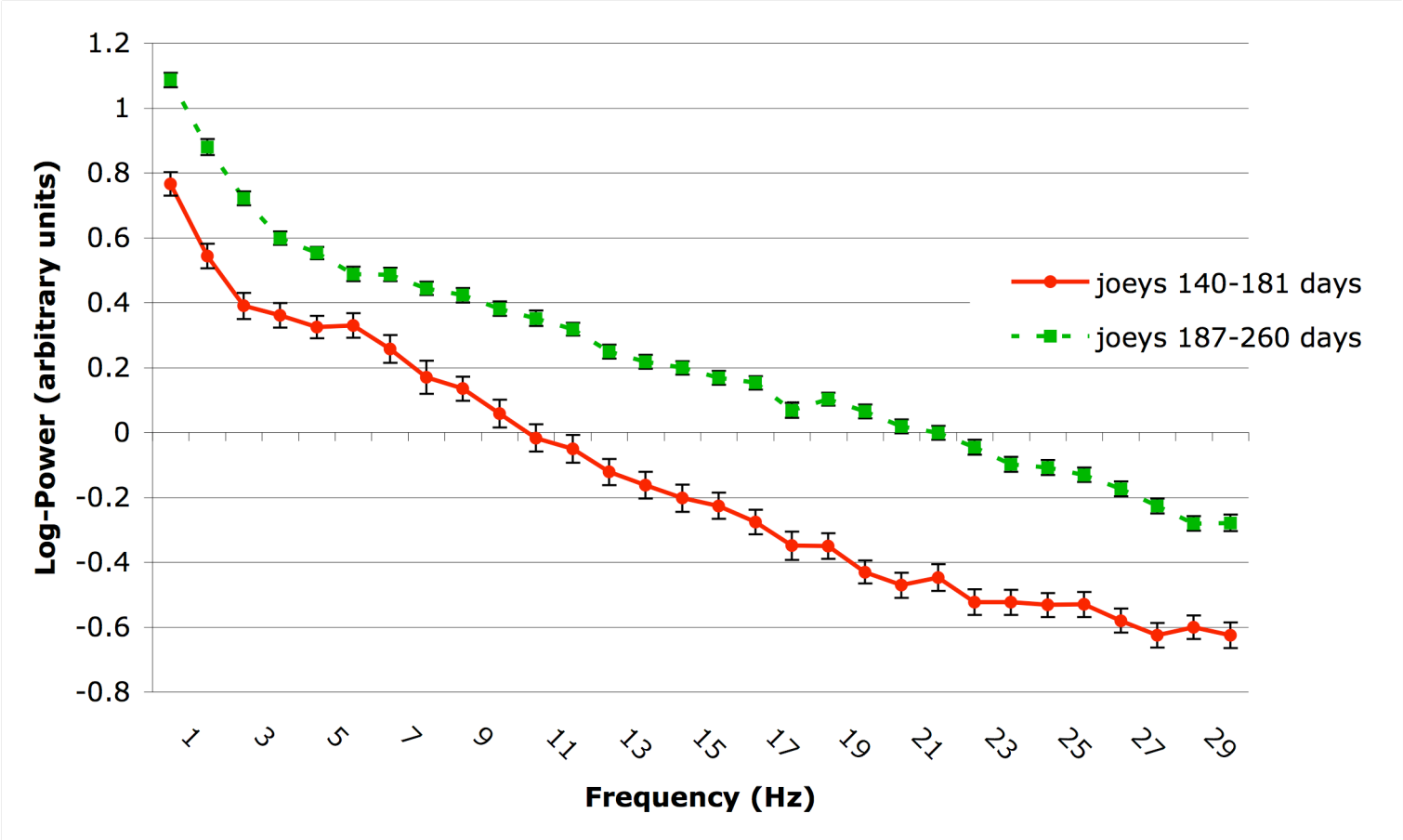

Figure 2.3: Means of log-transformed power in frequencies (1-30Hz) of the EEG power spectrum at baseline (1.0% endtidal halothane concentrations) for the two age groups of anaesthetised joeys. Standard errors of the means are shown as vertical bars.

Direct comparison of frequency spectra means for baseline, clamping and post-clamping showed differences at various frequencies. These differences were visually identified by plotting mean power of log-transformed frequency spectra (before, during and after clamping) against frequency in joeys aged 140-181 days (Figure 2.4) and those aged 187-260 days (Figure 2.5). Further, these were evaluated statistically with the two-tailed Kolmogorov-Smirnoff test using frequency spectra at each frequency for the two age groups, comparing before and during clamping, and during and after clamping as well as before and after clamping spectra. The test showed that there was a significant change in EEG power at 27Hz in response to clamping in the younger joeys (before *versus* after clamping and during *versus* after clamping) and at 28Hz (during versus after clamping). In older joeys, significant changes in frequencies of 9, 11, 12 and 16Hz (before *versus* during clamping), 11, 17, 18, and 26Hz (during *versus* after clamping) and at 18 and 23Hz (before *versus* after clamping) were observed (Table 2.4).

Table 2.4**: **Results (significant p-values) of the two-tailed Kolmogorov-Smirnoff test comparing frequency spectra between treatments (before, during and after clamping) in the two age groups of anaesthetised joeys.

Frequency	**Joeys 140-181 days**			**Joeys 187-260 days**		
(Hz)	*B vs. C*	*C vs. AC*	*B vs. AC*	*B vs. C*	*C vs. AC*	*B vs. AC*
2						
4						
6						
9				0.047		
10						
11				0.000	0.002	
12				0.005		
15						
16				0.019		
17					0.012	
18					0.023	0.009
19						
22						
23						0.021
24						
26					0.034	
27		0.001	0.007			
28		0.026				
29						
30						

B *vs*. C: Before versus during clamping

C vs. AC: During versus after clamping

B *vs*. AC: Before versus after clamping

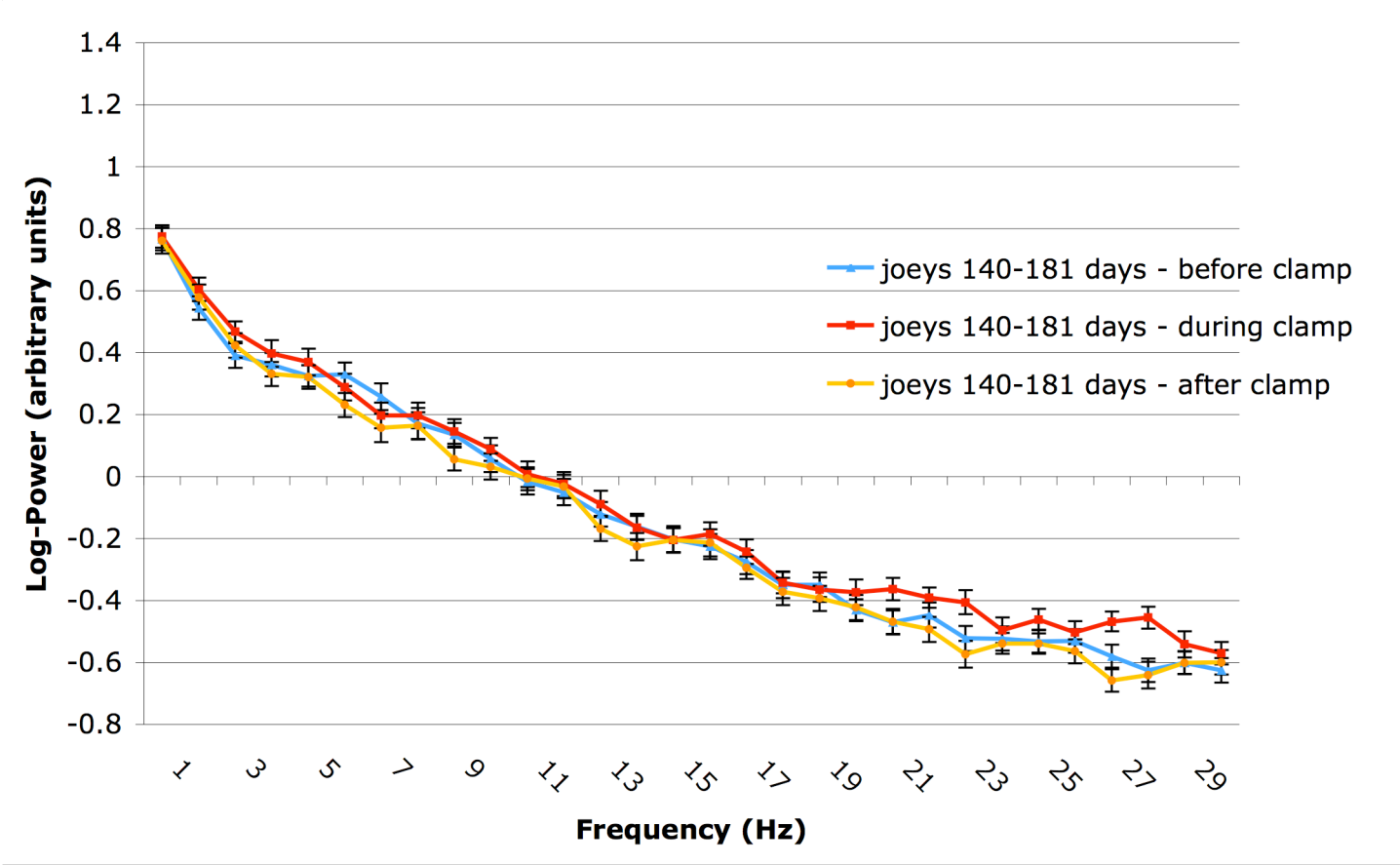

Figure 2.4: Means of log-transformed power in frequencies (1-30Hz) of the EEG power spectrum of anaesthetised joeys aged 140-181 days before, during and after clamping. Standard errors of the means are shown as vertical bars.

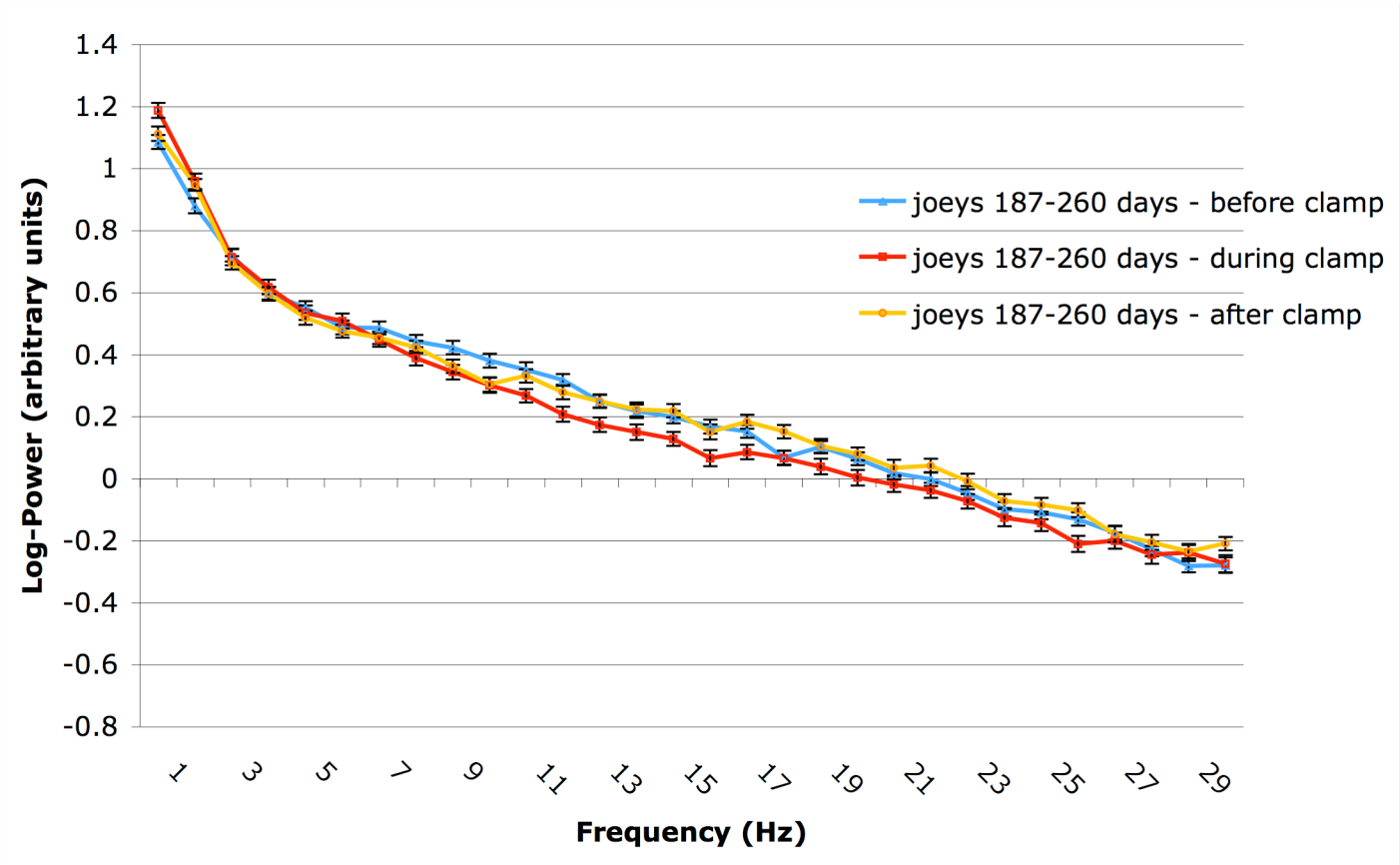

Figure 2.5: Means of log-transformed power in frequencies (1-30Hz) of the EEG power spectrum of anaesthetised joeys aged 187-260 days before, during and after clamping. Standard errors of the means are shown as vertical bars.

Principal Component Analysis (PCA)

Eigenvalues and component scores of the component matrix are presented in Table 2.5. Multivariate analysis of the frequency spectra identified three Principal Components with Eigenvalues >1 that together contributed 52.7% of the overall variation in the data. Component loadings of Principal Component 1 (PC1) were high for all frequencies (1-30Hz) (all loadings >0.5). Hence, this factor could be seen to represent overall power of EEG activity. The loadings of PC2 were high for frequencies of 1-12Hz and those for PC3 were high for frequencies of 1-5Hz, 24Hz and 28-30Hz. Thus, PC2 can be seen to represent frequencies between 1-12Hz and PC3 to represent frequencies of 1-5, 24 and 28-30Hz.

Contrasts were present in both PC2 and PC3, but not in PC1. In PC2 there was a contrast between frequencies of 1-12Hz (positive) and frequencies of 16-30Hz (negative). In PC3 there was a contrast between frequencies of 1-5Hz, 24Hz and 28-30Hz (positive) and frequencies of 7-16Hz (negative).

Figure 2.6 shows the separation of PC scores along the 1st and 2nd Principal Component axes according to age and treatment. Neither of the components showed a clear distinction according to treatment or treatment*age. However, the PC analysis was able to distinguish between the two age groups according to PC1, with the younger joeys scoring low (low EEG power) and the older joeys scoring higher (higher EEG power). PC1 (power of the EEG) contributed 42.7% to the overall variation while PC2 (frequencies 1-12Hz) contributed only 6.1%. Separation according to PC3 (3.9% of overall variation) did not yield any distinctions between ages or treatments.

Table 2.5: Eigenvalues and component scores of the component matrix of all frequencies for Principal Components (PCs) 1 to 3 calculated by Principal Component Analysis for EEG traces before, during and after clamping.

Eigenvalues	**PC 1**	**PC 2**	**PC 3**
Total	12.80	1.83	1.16
Percentage of variance	42.7%	6.1%	3.9%
Frequency	**PC 1**	**PC 2**	**PC 3**
1Hz	0.544	0.320	0.368
2Hz	0.607	0.398	0.303
3Hz	0.552	0.360	0.322
4Hz	0.540	0.417	0.205
5Hz	0.527	0.404	0.171
6Hz	0.545	0.402	-0.009
7Hz	0.561	0.326	-0.209
8Hz	0.586	0.249	-0.265
9Hz	0.580	0.259	-0.238
10Hz	0.567	0.247	-0.196
11Hz	0.627	0.226	-0.258
12Hz	0.642	0.123	-0.336
13Hz	0.647	-0.016	-0.280
14Hz	0.681	-0.085	-0.231
15Hz	0.701	-0.066	-0.182
16Hz	0.667	-0.117	-0.266
17Hz	0.731	-0.164	-0.062
18Hz	0.718	-0.137	0.074
19Hz	0.708	-0.209	0.029
20Hz	0.755	-0.205	0.026
21Hz	0.742	-0.154	0.061
22Hz	0.757	-0.125	0.086
23Hz	0.741	-0.206	0.071
24Hz	0.706	-0.220	0.115
25Hz	0.715	-0.219	0.097
26Hz	0.672	-0.227	0.085
27Hz	0.678	-0.224	0.080
28Hz	0.638	-0.251	0.142
29Hz	0.672	-0.185	0.160
30Hz	0.675	-0.200	0.167

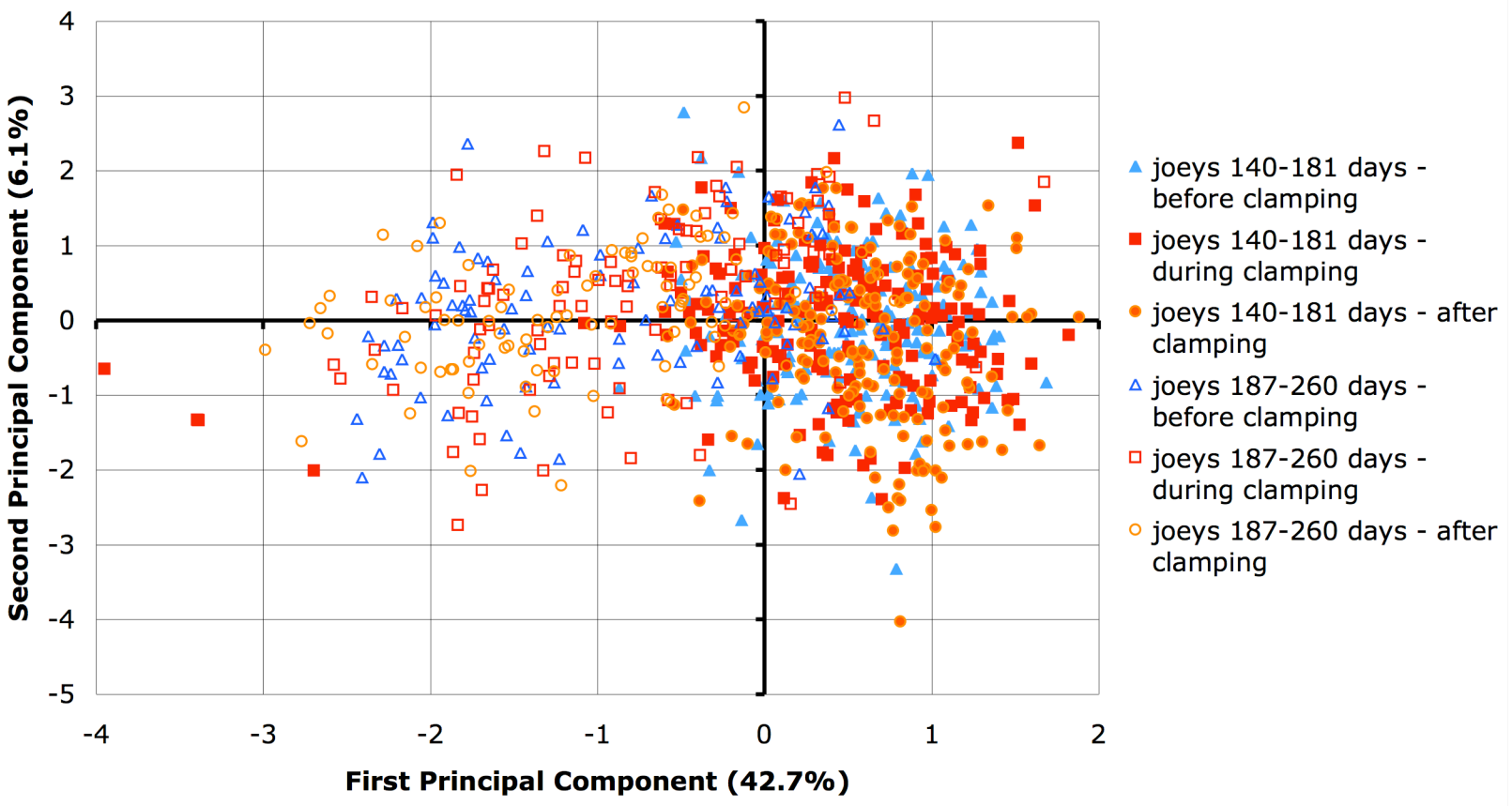

Figure 2.6: Factor scores calculated for frequencies (1-30Hz) by Principal Component Analysis plotted against the 1st and 2nd Principal Component axes, showing separation according to age along the first axis (1st Principal Component) and separation according to frequencies of 1-12Hz along the second axis (2nd Principal Component). Factor scores for both age groups and treatments (before, during and after clamping) are shown and include 20 data points per joey for each treatment.

3) Spectral analysis in response to changing endtidal halothane concentrations

Median frequency (F50), spectral edge frequency (F95) and total power (Ptot)

According to the Friedman's test, there was a significant decrease in both F50 and F95 with changing endtidal halothane concentrations in the older joeys ($p<0.001$ and $p=0.013$; respectively). There was also a significant decrease in F50 with changing endtidal halothane concentrations in the younger joeys ($p=0.001$) (Table 2.6). Thus, F50 for both groups and F95 for older joeys decreased in response to increasing anaesthesia levels. The post-hoc Dunn's test showed that the differences in F50 in the older joeys were due to differences between 1.0% and 1.4%, as well as between 1.2% and 1.4% data. With regard to F95 differences between 1.0% and 1.4% were significant. The significant difference in F50 in the younger joeys was due to significant differences between 1.0% and 1.2% and between 1.0% and 1.4% data.

Graphic comparisons

Direct comparison of frequency spectra means for 1.0%, 1.2% and 1.4% endtidal halothane EEGs showed differences at various frequencies. These differences were visually identified by plotting mean power of log-transformed frequency spectra (before, during and after clamping) against frequency for joeys aged 140-181 days (Figures 2.7) and 187-260 days (Figure 2.8). Further, these were evaluated statistically with the two-tailed Kolmogorov-Smirnoff test using frequency spectra at each frequency for the two age groups, comparing spectra at 1% and 1.2%, 1.0% and 1.4% and 1.2% and 1.4% endtidal halothane concentration. The test showed that there was a significant change in EEG power in response to changes in anaesthetic concentration in the younger joeys at 6, 9 and 23Hz (1% versus 1.2% and 1% versus 1.4%) and in the older joeys at 18Hz (1.0% versus 1.2%), at 1 and 2Hz (1.0% versus 1.4%) and at 1, 21, 22 and 23Hz (1.2% versus 1.4%) (Table 2.7).

***Table 2.6**: Results of the non-parametric Friedman test, including p-values, Chi-Square statistic, degrees of freedom (df), means (M) and standard error of the mean (SEM) for EEG traces at 1.0%, 1.2% and 1.4% endtidal halothane concentrations. Means denoted with different letters indicate significant differences between means within an age group with significant levels as follows: * p<0.001; + p=0.001-0.01 and ' p=0.01-0.05).*

Age (days)		**F50 (Hz)**			**F95 (Hz)**			**Ptot (arbitrary units)**		
		1.0%	**1.2%**	**1.4%**	**1.0%**	**1.2%**	**1.4%**	**1.0%**	**1.2%**	**1.4%**
140-181	*p-value*	*<0.001*			*0.277*			*0.456*		
(n=6)	χ^2	*14.355*			*2.568*			*1.570*		
	df	*2, 106*			*2. 106*			*2, 106*		
	Mean	5.56^{a}	4.82^{b+}	4.81^{b+}	23.52	23.21	23.48	54	54	56
	SEM	0.13	0.17	0.17	0.18	0.19	0.19	2.4	2.3	2.50
187-260	*p-value*	*<0.001*			*0.013*			*0.090*		
(n=10)	χ^2	*16.460*			*8.680*			*4.810*		
	df	*2, 199*			*2, 199*			*2, 199*		
	Mean	5.90^{a*}	5.83^{a}	5.23^{b}	24.08^{c}	24.00	23.44^{c}	89	93	96
	SEM	0.16	0.16	0.16	0.12	0.13	0.14	1.70	1.9	2.0

F50 = median frequency

F95 = spectral edge frequency

Ptot = total power

χ^2= Chi-Square Friedman statistic

***Table 2.7**: Results (p-values) of the two-tailed Kolmogorov-Smirnoff test comparing frequency spectra between treatments (1.0%, 1.2% and 1.4% endtidal halothane) in the two age groups of anaesthetised joeys.*

Frequency	**Joeys 140-181 days**			**Joeys 187-260 days**		
(Hz)	*1.0% vs. 1.2%*	*1.0% vs. 1.4%*	*1.2% vs. 1.4%*	*1.0% vs. 1.2%*	*1.0% vs. 1.4%*	*1.2% vs. 1.4%*
1					0.000	0.003
2					0.030	
6	0.033					
7						
9	0.007					
18				0.009		
21						0.004
22						0.016
23	0.017		0.014			0.022
26						
29			0.047			

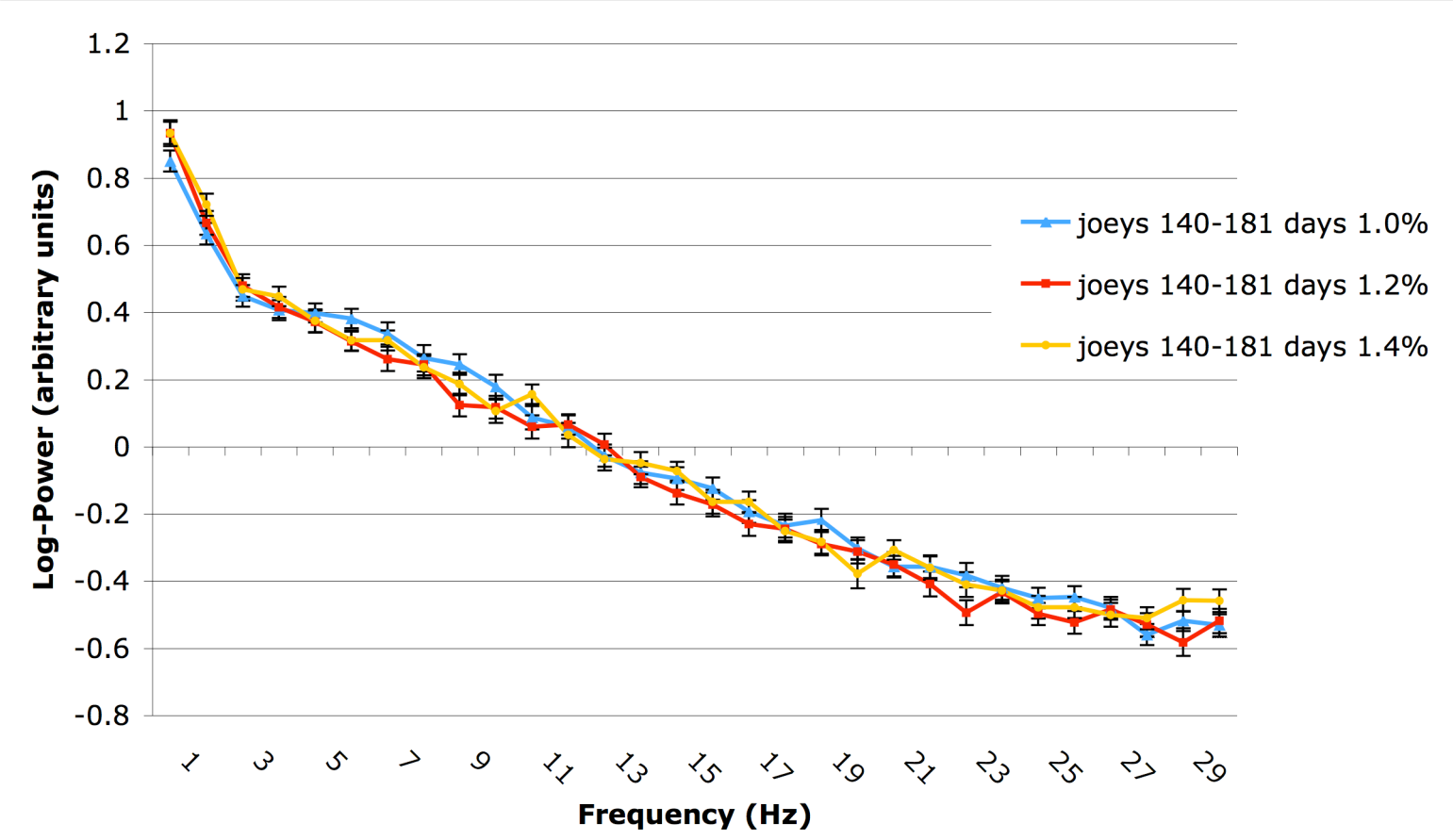

***Figure 2 7**: Means of log-transformed power in frequencies (1-30Hz) of the EEG power spectrum of joeys aged 140-181 days for 1.0%, 1.2% and 1.4% endtidal halothane EEGs. Standard errors of the means are shown as vertical bars.*

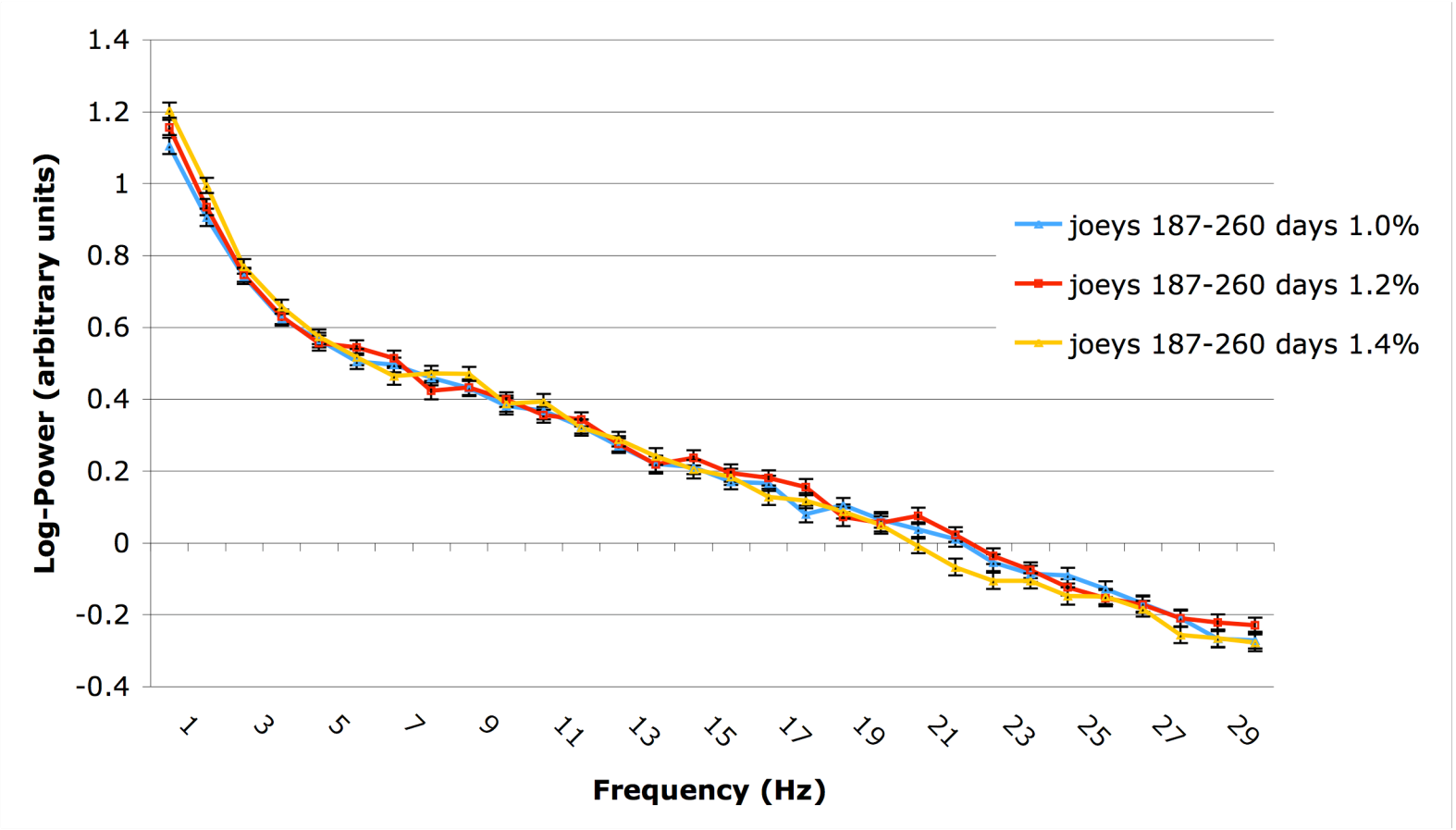

***Figure 2.8**: Means of log-transformed power in frequencies (1-30Hz) of the EEG power spectrum of joeys aged 187-260 days for 1.0%, 1.2% and 1.4% endtidal halothane EEGs. Standard errors of the means are shown as vertical bars.*

Principal Component Analysis (PCA)

Eigenvalues and component scores of the component matrix are presented in Table 2.8. Multivariate analysis of the frequency spectra identified four Principal Components with Eigenvalues >1 that together contributed 50.8% of the overall variation in the data. Component loadings of Principal Component 1 (PC1) were high for all frequencies (1-30Hz) (majority >0.5). Hence, this factor could be seen to represent overall power of EEG activity. The loadings of PC2 were high for frequencies of 1-11Hz, those for PC3 were high for frequencies of 1-4Hz and 24-30Hz and those for PC4 were high for frequencies of 5-8Hz and 27-29Hz. Thus, PC2 can be seen to represent frequencies between 1-12Hz, PC3 to represent frequencies of 1-4 and 24-30Hz and PC4 those of 5-8 and 27-29Hz.

Contrasts were present in all PCs except PC1. In PC2 there was a contrast between frequencies of 1-11Hz (positive) and frequencies of 16-30Hz (negative). In PC3 there was a contrast between frequencies of 1-4Hz and 24-30Hz (positive) and frequencies of 7-16Hz and 19Hz (negative). In PC4 there was a contrast between frequencies 5-8 and 27-29Hz (positive) and frequencies of 1-3, 10-13Hz and 15, 17 and 18Hz (negative).

Figure 2.9 shows the separation of PC scores along the 1st and 2nd Principal Component axes according to age and treatment. Neither of the components showed a clear distinction according to treatment or treatment*age. However, as before, the PC analysis was able to distinguish between the two age groups according to PC1, with the younger joeys scoring low (low EEG power) and the older joeys scoring higher (higher EEG power). PC1 (power of the EEG) contributed 38.3% to the overall variation, while PC2 contributed only 5.5%, PC3 3.6% and PC4 3.4%.

Table 2.8 Eigenvalues and component scores of the component matrix of all frequencies for Principal Components (PCs) 1 to 4 calculated by Principal Component Analysis for EEG traces at 1.0%, 1.2% and 1.4% endtidal halothane concentration.

Eigenvalues	**PC 1**	**PC 2**	**PC 3**	**PC 4**
Total	11.5	1.66	1.07	1.00
Percentage of variance	38.3%	5.5%	3.6%	3.4%
Frequency	**PC 1**	**PC 2**	**PC 3**	**PC 4**
1Hz	0.497	0.356	0.247	-0.37
2Hz	0.53	0.438	0.303	-0.227
3Hz	0.573	0.312	0.284	-0.162
4Hz	0.52	0.383	0.312	-0.071
5Hz	0.53	0.355	0.078	0.229
6Hz	0.505	0.355	-0.054	0.462
7Hz	0.503	0.333	-0.188	0.423
8Hz	0.533	0.283	-0.216	0.242
9Hz	0.595	0.269	-0.289	0.011
10Hz	0.557	0.181	-0.209	-0.284
11Hz	0.617	0.116	-0.19	-0.143
12Hz	0.65	0.081	-0.147	-0.109
13Hz	0.635	0.034	-0.177	-0.109
14Hz	0.626	-0.056	-0.237	-0.094
15Hz	0.656	-0.075	-0.147	-0.104
16Hz	0.666	-0.158	-0.214	-0.015
17Hz	0.676	-0.123	-0.083	-0.106
18Hz	0.692	-0.109	-0.045	-0.130
19Hz	0.698	-0.194	-0.101	-0.049
20Hz	0.671	-0.19	-0.055	-0.022
21Hz	0.713	-0.165	-0.032	-0.077
22Hz	0.695	-0.14	-0.037	0.017
23Hz	0.682	-0.207	0.017	0.029
24Hz	0.689	-0.206	0.108	0.049
25Hz	0.655	-0.22	0.134	0.033
26Hz	0.664	-0.206	0.104	0.048
27Hz	0.633	-0.198	0.171	0.124
28Hz	0.608	-0.223	0.244	0.165
29Hz	0.609	-0.238	0.288	0.214
30Hz	0.586	-0.143	0.268	0.099

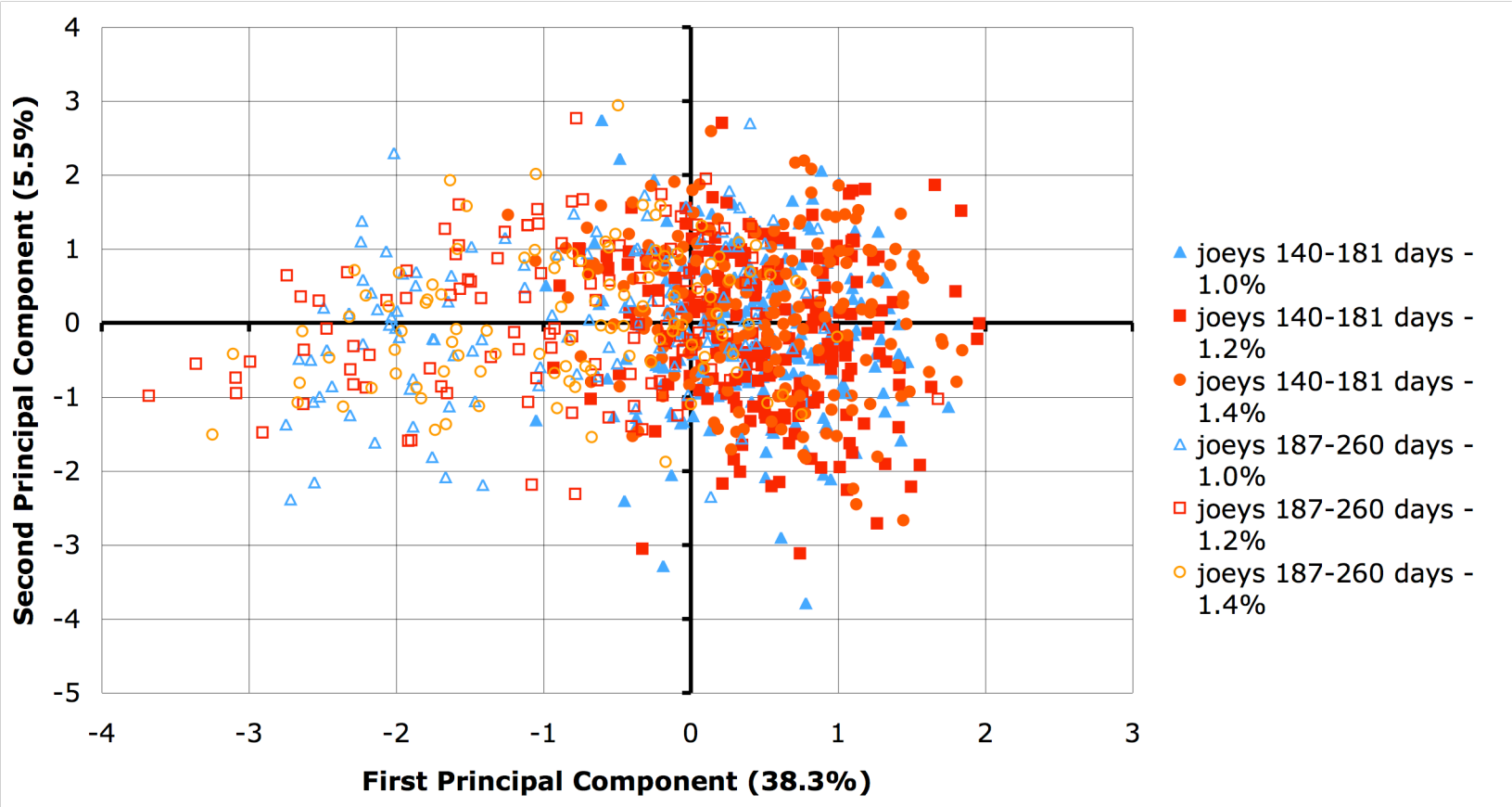

Figure 2.9: Factor scores calculated for frequencies (1-30Hz) by Principal Component Analysis plotted against the 1st and 2nd Principal Component axes, showing separation according to age along PC1 and separation according to frequencies of 1-11Hz along PC2. Factor scores for both age group and treatments (1.0%, 1.2% and 1.4% endtidal halothane) are shown and include 20 data points per joey per each treatment.

Non-anaesthetised joeys

1) Isoelectric periods

Isoelectric periods were only observed in the EEG trace of the joey aged 155 days. However, these were only present during the first 2 minutes of the 21-minute EEG recording.

2) Spectral analysis

Median frequency (F50), spectral edge frequency (F95) and total power (Ptot)

Means and standard error of the means are presented in Table 2.9. Statistical analyses were not performed due to the small number of animals.

***Table 2.9**: Mean and standard error of the mean (SEM) for F50, F95 and Ptot (five EEG periods each of 30 sec duration) for non-anaesthetised joeys (n = 1 for each age).*

Wallaby age		**F50 (Hz)**	**F95 (Hz)**	**Ptot (arbitrary units)**
137 days	*Mean*	4.97	24.74	19.72
	SEM	0.11	0.09	0.33
155 days	*Mean*	6.08	24.67	34.03
	SEM	0.19	0.16	0.86
189 days	*Mean*	4.95	25.44	59.69
	SEM	0.06	0.05	0.38
193 days	*Mean*	3.73	23.80	51.34
	SEM	0.06	0.98	0.50

F50 = median frequency

F95 = spectral edge frequency

Ptot = total power

Graphical Comparison

Visual inspection showed that there were differences in the EEG power of frequency

spectra between the three joeys (Figure 2.10). The power of all frequencies in the joey aged 137 days was lower than that of the other two joeys. The EEG power of the joey aged 189 days was higher than of the joey aged 193 days for the majority of frequencies.

Comparison between joeys anaesthetised and not anaesthetised during EEG studies

Means and standard error of the means for the four groups are presented in Table 2.10. Median frequency and Ptot were higher in both younger and older joeys that were anaesthetised compared to those that were not. The reverse was true for F95 for both younger and older joeys. The differences in F50 and Ptot between anaesthetised and not anaesthetised joeys were greater at the older ages, while that of F95 was greater between anaesthetised and not anaesthetised joeys at the younger ages.

Eigenvalues and component scores of the component matrix are presented in Table 2.11. Multivariate analysis of the frequency spectra identified five Principal Components with Eigenvalues >1 that together contributed 61.4% of the overall variation in the data. Component loadings of Principal Component 1 (PC1) were high for all frequencies (1-30Hz) (majority of loadings >0.5). Hence, this factor could be seen to represent overall power of EEG activity. The loadings of PC2 were high for frequencies of 23-30Hz, those for PC3 were high for frequencies of 1-4, 6, 10-12 and 30Hz, those for PC4 were high for frequencies of 6-8, 11, 12 and 28-30Hz and those for PC5 were high for frequencies of 4-6, 18, 23 and 24Hz.

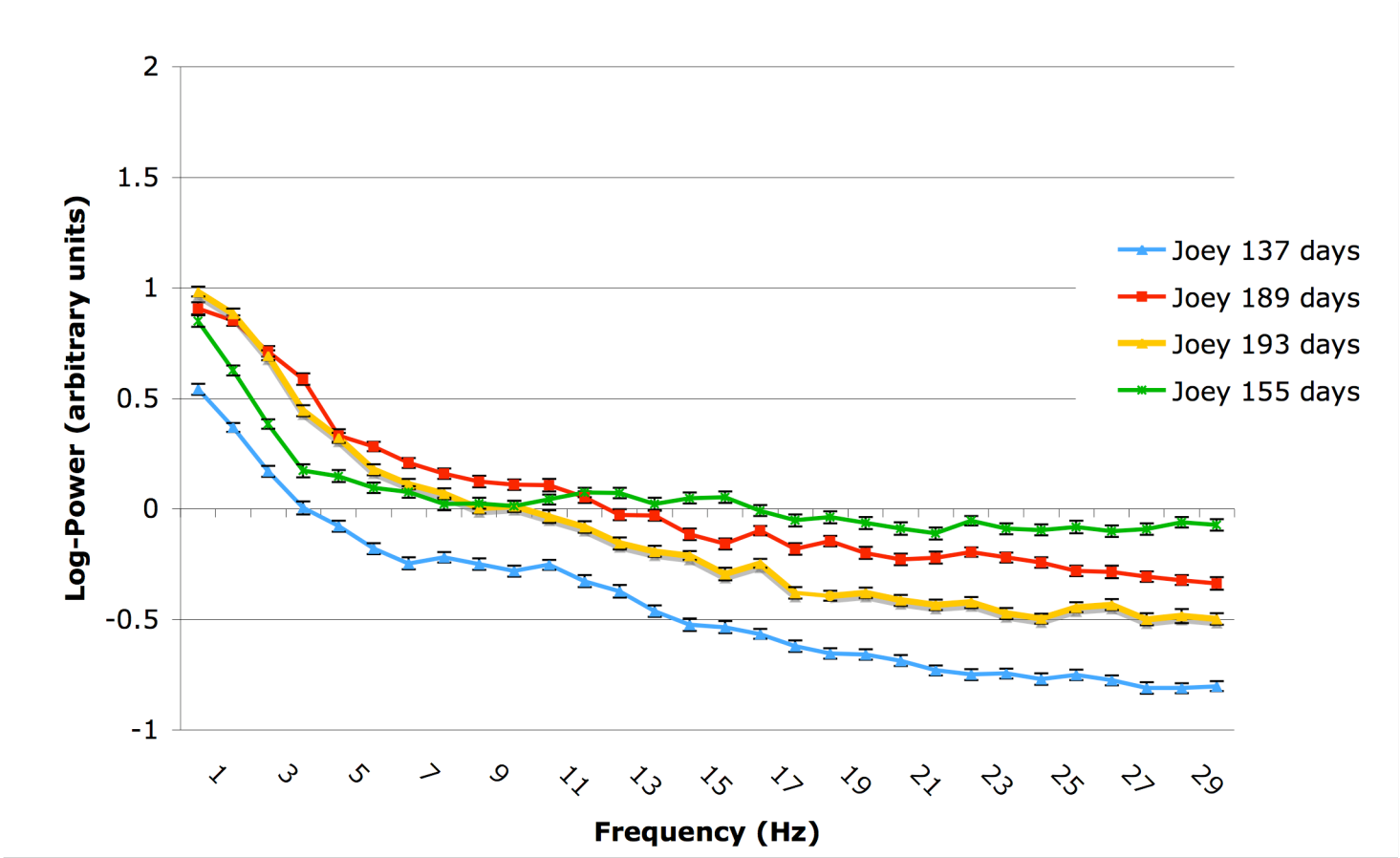

Figure 2.10: Means of log-transformed power in frequencies (1-30Hz) of the EEG power spectrum for the three non-anaesthetised joeys. Standard errors of the means are shown as vertical bars.

Contrasts were present in all PCs apart from PC1. In PC2 there was a contrast between frequencies of 23-30Hz (positive) and 1, 6-10 and 12-15Hz (negative), In PC3 there was a contrast between frequencies of 1-4, 6, 10-12 and 30Hz (positive) and 13, 14, 16-23 and 26Hz (negative), in PC4 there was a contrast between frequencies of 6-8, 11, 12 and 28-30Hz (positive) and 1, 3, 4, 9, 17, 20, 21, 24 and 25Hz (negative) and in PC5 there was a contrast between frequencies of 4-6, 18, 23 and 24Hz (positive) and 1, 8, 12, 13, 20, 21, 26, 28 and 29Hz (negative).

***Table 2.10**: Mean, standard error of the mean (SEM) and number of data points used (n) for F50, F95 and Ptot calculations for younger (137-145 days) and older (189-196 days) joeys that were anaesthetised (baseline – 1.0% endtidal halothane) or not anaesthetised.*

Wallaby age		**F50 (Hz)**	**F95 (Hz)**	**Ptot (arbitrary units)**
Young joeys	*Mean*	4.39	25.02	23.89
Not anaesthetised	*SEM*	0.38	0.36	1.00
(n=1)	*n*	20	20	20
Young joeys	*Mean*	4.98	23.52	27.34
Anaesthetised	*SEM*	0.21	0.39	1.26
(n=2)	*n*	40	40	40
Old joeys	*Mean*	3.92	24.57	53.82
Not anaesthetised	*SEM*	0.28	0.35	1.74
(n=2)	*n*	40	40	40
Old joeys	*Mean*	5.37	24.22	88.18
Anaesthetised	*SEM*	0.33	0.25	3.17
(n=2)	*n*	40	40	40

F50 = median frequency

F95 = spectral edge frequency

Ptot = total power

Table 2.11: Eigenvalues and component scores of the component matrix of all frequencies for Principal Components (PCs) 1 to 5 calculated by Principal Component Analysis for comparison of anaesthetised and non-anaesthetised joeys.

Eigenvalues	**PC 1**	**PC 2**	**PC 3**	**PC 4**	**PC 5**
Total	13.23	2.09	1.32	1.13	1.03
Percentage of variance	44.1%	7.0%	4.4%	3.8%	3.4%
Frequency	**PC 1**	**PC 2**	**PC 3**	**PC 4**	**PC 5**
1Hz	0.559	0.122	0.377	-0.408	-0.214
2Hz	0.422	0.259	0.631	-0.238	0.262
3Hz	0.546	0.312	0.464	-0.079	-0.165
4Hz	0.39	0.472	0.207	0.044	-0.366
5Hz	0.462	0.48	0.035	0.547	0.044
6Hz	0.382	0.497	-0.014	0.025	0.545
7Hz	0.507	0.458	-0.293	0.002	0.256
8Hz	0.57	0.396	-0.241	-0.182	-0.094
9Hz	0.61	0.309	-0.119	-0.116	-0.044
10Hz	0.73	0.176	-0.026	-0.040	0.025
11Hz	0.653	0.069	0.010	-0.165	0.254
12Hz	0.717	0.072	-0.070	-0.231	0.141
13Hz	0.672	-0.045	-0.271	-0.161	-0.289
14Hz	0.752	0.036	-0.217	-0.146	-0.057
15Hz	0.744	0.034	-0.063	-0.17	-0.154
16Hz	0.736	-0.185	-0.175	-0.138	-0.055
17Hz	0.749	0.015	0.016	0.057	-0.185
18Hz	0.753	-0.084	-0.032	0.172	-0.073
19Hz	0.77	-0.099	-0.142	0.075	-0.032
20Hz	0.805	-0.0120	-0.075	0.001	-0.153
21Hz	0.805	-0.092	0.037	0.012	-0.091
22Hz	0.769	-0.095	-0.011	-0.009	-0.022
23Hz	0.796	-0.203	-0.039	0.056	0.049
24Hz	0.736	-0.253	0.126	0.012	0.008
25Hz	0.682	-0.082	-0.043	0.028	0.031
26Hz	0.682	-0.3	-0.058	0.042	0.084
27Hz	0.636	-0.188	0.158	0.159	0.098
28Hz	0.589	-0.404	0.040	0.077	0.234
29Hz	0.685	-0.433	0.205	0.112	0.156
30Hz	0.677	-0.219	0.215	0.073	0.104

Figure 2.11 shows the separation of PC scores along the 1st and 2nd Principal Component axes according to age and whether joeys were anaesthetised or not. The PC analysis was able to distinguish between the two age groups (young and old), with the younger joeys scoring low (low EEG power) and the older joeys scoring higher (higher EEG power) on PC1 (overall EEG power). In addition, the PC analysis was able to distinguish the older joeys according to whether they were anaesthetised or not, also along PC1, where joeys that were not anaesthetised scored lower (lower EEG power) than those that were anaesthetised. The younger joey that was not anaesthetised, and to some extent the older joeys that were not anaesthetised, scored relatively higher on PC2 (frequencies of 23-30Hz) than the joeys that were anaesthetised. Due to the contrast present in this component, joeys that were not anaesthetised showed a higher proportion of frequencies between 23-30Hz present in their EEGs, while those joeys that were anaesthetised showed a tendency to an increase in frequencies of 1, 6-10 and 12-15Hz and lower proportion of frequencies between 23-30Hz. PC1 (power of the EEG) contributed 44.5% to the overall variation, while PC2 (frequencies of 23-30Hz) contributed only 5.3%. Separation according to PCs 3,4 and 5 did not yield any distinctions between ages or whether or not animals had been anaesthetised.

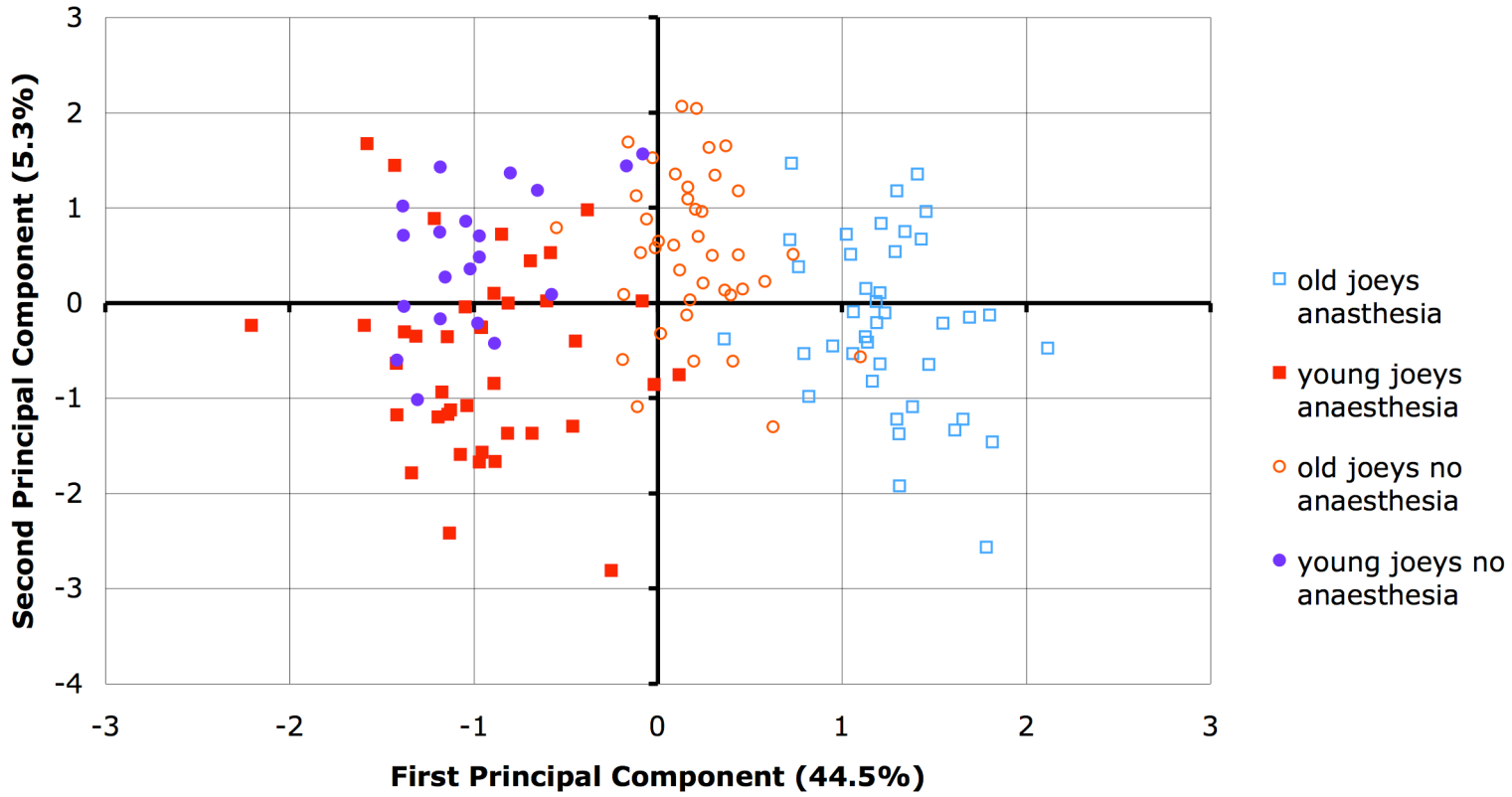

Figure 2.11: Factor scores calculated for frequencies (1-30Hz) by Principal Component Analysis plotted against the 1st and 2nd Principal Component axes for both age group (137-145 days = young; 189-196 = old) and treatments (anaesthesia or no anaesthesia) are shown and include 20 data points per joey per treatment.

2.4 Discussion

The present study aimed to investigate the cerebrocortical (EEG) responses of lightly anaesthetised tammar wallaby joeys of varying ages to toe clamping and the responsiveness of the EEG to different halothane concentrations. As far as the candidate is aware this is the first investigation of this type in any metatherian species. The main findings were as follows. First, there was little or no EEG activity in joeys aged up to about 100 days of in-pouch age, but EEG epochs of sufficient duration for FFT analysis were present by about 140 days. Second, the percentage of time occupied by isoelectric periods decreased, and that occupied by EEG activity increased, with age. Third, during each observation period of each individual joey aged up to about 190 days, the percentage of time occupied by isoelectric EEGs in joeys increased with increasing endtidal halothane. Fourth, EEG power in all frequencies significantly increased with in-pouch age. Fifth, for the parameters investigated there was an age-related progression in EEG responses to clamping, although overall the responses were marginal to moderate. The responses ranged from no effects on EEG parameters in animals between 95-130 days due to the absence of EEG activity through a marginal response in joeys aged 140-181 days to a moderate response in those aged 187-260 days. Sixth, changes in halothane concentrations led to only marginal changes in EEG parameters in the intermediate and older age groups. Seventh, in a small number of joeys there were differences in EEG parameters between anaesthetised and non-anaesthetised joeys according to age.

Postnatal developmental changes of in-pouch tammar wallaby joeys

Before examining the results obtained in the present study on anaesthetised and non-anaesthetised in-pouch joeys, it would be beneficial to describe the normal progression of cerebrocortical development, brain electrical activity and behaviour of tammar wallaby joeys after birth. This knowledge will be essential for an integrated analysis of the development of pain perception *per se,* and conscious perception in general, in the tammar wallaby pouch young. Such an analysis may allow inferences to be drawn regarding the welfare status of joeys involved in pest control operations or during road accidents in Australia and New Zealand.

1) Features of brain development

At birth the developing cerebral cortex of the tammar wallaby consists of two layers of cells and apparently resembles that seen in the 40-day human embryo and 26-day sheep embryo (Reynolds *et al.*, 1985). The cortical plate is not seen throughout the cerebrum until 10-15 days after birth and a subplate zone develops between 20 and 70 days after birth (Reynolds *et al.*, 1985). The layers of the mature cortex are the same as for eutherian mammals - six cortical layers that overlie the white matter (Mayner, 1989). These layers are well established in the tammar wallaby by 197 days, with large pyramidal cells being prominent in layer IV and the subplate zone being no longer distinguishable (Reynolds *et al.*, 1985). Layer IV is a specific cortical target for thalamocortical afferents (Sheng *et al.*, 1991), while neurons of layers V and VI have been shown to project to the thalamus (Marotte & Sheng, 2000). The overall laminar development of somatosensory and visual cortices is similar (Sheng *et al.*, 1991; Waite *et al.*, 1991; Marotte *et al.*, 1997; Pearce *et al.*, 2000; Mark *et al.*, 2002). Layer IV is distinguishable around 72-75 days (Mark *et al.*, 2002), around the time when clusters of afferents in layer VI and cell bodies in layer V and VI can be seen (76 days) (Marotte *et al.*, 1997).

In comparison to the protracted development of the cerebral cortex, the brainstem and upper spinal cord are relatively advanced at birth, enabling the newborn joey to crawl from the birth canal into the pouch and attach itself to a teat (Waite *et al*., 1998). In addition, the facial skin is richly innervated and head turning and sucking reflexes are expressed from birth (Waite *et al.*, 1994).

Thalamic afferents begin to concentrate in layer IV of the visual cortex only after 90 days. Thalamocortical connections between nuclei of the thalamus and the primary visual cortex have been shown to resemble the mature pattern by about 118 days (Sheng *et al.*, 1990; Marotte & Sheng, 2000), while corticothalamic connections resembling those of the adult appear by about 85 days (Sheng *et al.*, 1990). Functional thalamocortical synapses in the visual cortex have been reported to be present from approximately 46 days after birth, as the first synaptic response to electrical stimulation of the optic nerve could be recorded at that age in deep layers of the cortex (Pearce *et al.*, 2000). However, it has not been reported when spontaneous activity is present in these circuits.

Studying the development of auditory function, Hill *et al.* (1998) showed that auditory brainstem responses could first be evoked in animals aged 85 to 94 days by bone conduction. In animals older than 115 days auditory brainstem responses were evoked in all subjects by either bone or air-conducted stimuli (Hill *et al.*, 1998). However, it is suggested that there is continuing development of cochlear sensitivity up to 140 days due to a continuing improvement in thresholds for bone-conducted clicks after the ear canal has opened (125-130 days) (Hill *et al*., 1998). Threshold and latency values for air-conducted click-evoked auditory brainstem responses in pouch young older than 179 postnatal days generally corresponded to adult values (Hill *et al.*, 1998).

In a study by Waite *et al*. (1991), evoked cortical potentials recorded from the primary somatosensory cortex in response to stimulation of the whisker pad of tammar wallaby joeys were first observed at 85 days. However, only the negative N2 wave was discernable, while the negative waves N1 and N3, as well as the positive waves P1-4, were absent (Waite *et al.*, 1991). The adult form of the evoked potential was only apparent at 180 days. In a subsequent study, somatosensory evoked cortical activity in response to electrical stimulation of the infraorbital nerve was recorded in joeys between 55 and 138 days using current source density analysis (Mark *et al.*, 2002). The first synaptic activity within the cortex was observed by 72 days, which is slightly earlier than observed by Waite *et al.* (1991) and could have been due to the different methods employed in the two studies. Mark *et al.* (2002) suggest that the increase in cytochrome oxidase reactivity observed in their study in layer IV from 75 days may represent an increase in metabolism and postsynaptic activity in this layer and that the intensity of cytochrome oxidase reactivity, which becomes gradually clearer in layer VI from 75-90 days, presumably reflects an increase in thalamocortically induced synaptic activity.

2) Development of behaviour in unanaesthetised wallaby joeys

Little information is available on the behavioural development of tammar wallaby joeys, presumably because it initially spends most of its time in the pouch and is thus not easily seen. The joey remains permanently attached to the teat until approximately 100 days after birth (Tyndale-Biscoe & Janssens, 1988) and, as indicated below, major

behavioural development probably occurs only once the joey detaches from the teat and begins to suck intermittently. Opening of the external ear canal occurs between 125-130 days and eye-opening occurs at around 140 days (Hill *et al.*, 1998). In rat pups, opening of the ear canals and eyes, which occurs around 10-12 and 14 days after birth, respectively, is associated with vigorous motor activity and an increased interest in objects (Bolles & Woods, 1964). If we extrapolate to the wallaby joey, increased motor activity and interactions with its environment (i.e. its mother) may emerge from 120 to 140 days of in-pouch age. By about 180 days tammar wallaby joeys put their heads out of their mother's pouch (Tyndale-Biscoe & Janssens, 1988) and begin to nibble grass. At about 190 days the joey will exit the pouch for the first time and it finally leaves the pouch permanently at around 250 days, terminating milk intake by about 300-350 days (Tyndale-Biscoe & Janssens, 1988).

3) Sleep-Wake Cycle Development

The development of rapid eye movement (REM) and non-REM sleep, and associated sleep-wake cycles, has not been investigated for the tammar wallaby or any other species of diprotodont metatherians. However, one study reports on the development of sleep-wake cycles in the polyprotodont Virginia opossum (*Didelphis virginiana*) (Walker & Berger, 1978). Joeys of this species occupy the pouch on average for 70-90 days and then ride on the mothers' backs until weaning at around 100-110 days (McManus, 1974; Krause & Saunders, 1994; Darlington *et al.*, 1999). Prior to 60 days the EEG of opossum joeys was apparently uniform, regardless of behaviourally and physiologically scored states of arousal, and consisted mainly of isoelectric activity. Continuous low-voltage EEG activity appeared from about 60 days, first EEG signs of non-REM sleep occurred around 65 days and at about 75 days a distinction between REM and non-REM sleep states could be made. Fully established sleep-wake cycles would be expected to be present soon after this time as judged by behavioural development of the joeys. Although Walker and Berger (1978) report periods of so-called wakefulness from 48 days onwards (recordings started at 48 days), clear differentiation into sleep-wake states is not expected until after REM-non-REM sleep differentiation which was shown to begin from about 65 days (Walker & Berger, 1978).

The pattern of EEG development described above for the Virginia opossum is also

likely to be seen in tammar wallaby joeys, as it appears to be common to all species observed to date including placental mammals and birds (Mellor & Diesch, 2007).

There are several developmental factors that may be relevant to the onset of REM-non-REM EEG differentiation. In appears that, at least in some animal species born moderately immature, the onset of REM-non-REM differentiation is closely associated with eye-opening. In rats, eye-opening occurs at around 14 days after birth (Bolles & Woods, 1964) and REM-non-REM differentiation begins around 10 to 12 days after birth (Jouvet-Mounier *et al.*, 1970), and this is similar in mice where eye-opening and REM-non-REM differentiation occurs around 12 days after birth (Daszuta & Gambarelli, 1985; Findlater *et al.*, 1993). In humans however, eye opening occurs well before EEG differentiation (Prof Alistair Gunn, personal communication) and this may also be the case for other species born neurologically mature. Even if eye opening is not directly (causally) related to the development of REM-non-REM differentiation, it may nevertheless be useful for estimating when altricial animals may be in the process of sleep state differentiation, as it would suggest that neurological maturation has progressed to a point where sleep state differentiation has already occurred or is about to occur. However, this would have to be further explored.

Second, there may be a functional relationship between the development of endothermic thermoregulation and the development of sleep states. The link between thermoregulation and sleep regulation in addition to circadian and homeostatic processes has been reviewed previously (McGinty & Szymusiak, 2001; Parmeggiani, 2003; Gilbert *et al.*, 2004). The preoptic area (POA) of the hypothalamus has been shown to be involved in promoting sleep by a variety of studies applying various experimental techniques (McGinty & Szymusiak, 2001). For example, lesions of the POA induce insomnia or induce partial sleep loss (Nauta, 1946; Sallanon *et al.*, 1989; John & Kumar, 1998; Schmidt *et al.*, 2000) showing that this area is critical in sleep production. Involvement in thermoregulatory control by the POA has also been observed (McGinty & Szymusiak, 2001). In addition, a link between thermoregulation and sleep regulation via the POA has been demonstrated in a variety of species where local warming of the POA triggered non-REM sleep or slow wave EEG activity (Roberts & Robinson, 1969; Benedek *et al.*, 1982) and local cooling suppressed sleep

(McGinty *et al.*, 1996). Importantly, even in the absence of input from the suprachiasmatic nuclei, which are essential for circadian rhythms of body temperature and sleep-wake activity, sleep-temperature coupling persists (Baker *et al.*, 2005).

Generally, a functional link between development of endothermic thermoregulation and the development of sleep states has not been proven and hence caution has to be exercised when using the development of thermoregulation as a means to estimate the timing for REM-non-REM EEG differentiation. However, even if there were no causal links between the two, using the development of one to predict the development of the other may nevertheless be possible. This is because both will depend on functional development of the POA and necessary interconnections with a variety of brain regions. Hence the development of the two systems would be anticipated to overlap to some extent, allowing cautious estimates to be made regarding the timing of development of one, when the timing of the other is known.

The onset of thermoregulatory capabilities in tammar wallaby joeys occurs between approximately 140 and 149 days (Hulbert, 1988) in conjunction with development of pelage, which also appears around 140 days (Tyndale-Biscoe & Janssens, 1988). In addition, thyroid gland size increases between 140 and 180 days with an associated increase in the amount of labelled iodine (I-131) sequestered by the thyroid gland (Setchell, 1974). During this period - 140-180 days after birth - the ability to elevate oxygen consumption in response to low ambient temperatures also begins to develop and at about 180 days it follows a similar pattern to that found in a fully endothermic vertebrate (Setchell, 1974). The animal possesses a full pelage and is apparently homeothermic by about 180 days (Setchell, 1974; Tyndale-Biscoe & Janssens, 1988). If we accept that there is a link between development of endothermic thermoregulation and the differentiation of sleep states, the observations on development of thermoregulation made above are in agreement with the suggestion that REM-non-REM differentiation may begin around the time of or shortly after eye-opening (i.e. around 140 days after birth).

Present study: Anaesthetised tammar wallaby joeys

The results of the present study will be assessed against the normal developmental

background just described for tammar wallaby joeys.

1) Isoelectric periods

Isoelectric periods were present during baseline observations in joeys up to about 140 days and one joey aged about 174 days. The proportion of time occupied by those periods progressively decreased with in-pouch age (Table 2.2). No published reports have been found of previous studies in which the EEGs of developing tammar wallaby joeys have been studied, so that apparently no information is available on the presence of isoelectric periods during development in these animals. However, as joeys are born with a very immature brain it may be anticipated that the EEG would be initially silent and may mature into the adult EEG patterns via several developmental stages. These may include sporadic spikes of EEG interspersed by isoelectric periods followed by a continuous EEG, as has been reported to occur in eutherian mammals (Ellingson & Rose, 1970) and birds (Mellor & Diesch, 2007). When these phases would normally occur during development of the wallaby is not entirely clear as each joey in the present study was anaesthetised. Accordingly, if this anaesthesia had been disproportionately neurosuppressive in younger joeys (a possibility further discussed below) the present observations might suggest that isoelectric periods persist until older in-pouch ages in anaesthetised joeys than they would in non-anaesthetised joeys. Indeed, in the present joeys that were not anaesthetised during EEG recording, i.e. after anticipated recovery from brief anaesthesia, no isoelectric periods were observed even in the youngest joey aged 137 days.

The increase in the proportion of time occupied by isoelectric periods during each observational period on individual joeys aged up to 187 days, and in one joey at about 215 days, may have been due to several factors. First, this effect may have been brought about by increased suppression of brain electrical activity due to increasing concentrations of halothane anaesthesia. Second, sustained anaesthesia rather than dose of halothane may have led to an increase in isoelectric periods during the course of observations in each joey. Third, anaesthesia-related factors such as hypoxaemia, hypercapnia, cardiovascular depression or altered thermal status may have affected cerebral functioning. Although we did not measure indices associated with these factors, it does not seem likely that hypoxaemia, hypercapnia or hypothermia are responsible.

The joeys received positive pressure ventilation using 100% oxygen that should have prevented hypercapnia and hypoxaemia, and the joeys were maintained on a heated pad and covered with a small blanket to prevent heat loss. While the younger animals may have cooled down during intubation, placing them onto a heat source after intubation is anticipated to have prevented a fall in body temperature to levels where EEG function would have been suppressed sufficiently to produce isoelectric periods. As an additional point, it is not likely that the joeys became severely hypoglycaemic, as this might be expected to induce seizure activity with joeys being kept in a warm environment (Mellor & Cockburn, 1986), not an increase in isoelectric epochs.

As there was an increase in isoelectric periods with increasing halothane concentrations, at least in the youngest joeys, it seems likely that anaesthesia itself or prolonged exposure to anaesthesia was involved in the increasing incidence of isoelectric periods during studies of individual joeys. Although halothane does depress the EEG with regard to the frequency spectrum, it has not been reported to induce EEG burst suppression (Murrell *et al.* 2008) unless presented in very high concentrations (i.e. endtidal halothane of 9%) (Michenfelder & Theye, 1975), and this possibility cannot be ruled out in developing animals without further study. The application of the light anaesthesia model in immature animals is discussed in the General Discussion (Chapter 7)

2) Changes in EEG power with age

The significant increase with age in EEG power at all frequencies (1-30Hz) (Figures 2.3, 2.6 and 2.9) suggests that there is a corresponding widespread increase in electrical activity in those parts of the brain accessed by EEG recording. First, this may be due to progressive maturation of neurological structures involved in the production of the EEG (i.e. increase in neuronal numbers, connectivity and myelination). Additionally, a lesser suppressive impact of halothane anaesthesia in older animals, as suggested by the possible later reduction in or disappearance of isoelectric periods with in-pouch age, may also play a role. Second, the increase in EEG power with age may also be indicative of maturation of subcortical neurological processes and their impact on brain electrical activity. A major neurological system that influences cortical arousal and which could thus bring about an increase in EEG power with age due to its progressive

maturation is the ascending reticular activating system (ARAS). This system consists of a variety of brainstem nuclei and their connections to the cortex via the thalamus, hypothalamus and basal forebrain (Siegel, 2004). There does not seem to be any information available on the development of the components of this system in marsupials.

3) Responsiveness to toe clamping

Joeys younger than 130 days

As EEG activity in the present joeys aged up to 130 days was completely absent or only existed in the form of sporadic spikes, no cerebral response to clamping could be observed. We did not record behavioural responsiveness, such as limb withdrawal, nor responsiveness of autonomic variables such as heart rate or breathing rate in response to clamping. However, even if such responses had been observed, their presence would not have indicated conscious perception of pain as such. First, the joeys were anaesthetised, and second such responses can be elicited via subcortical activation of the autonomic nervous system and motor reflexes in response to sensory information being transmitted via nociceptive pathways (Livingston & Chambers, 2000). Whether it would be possible for noxious stimulation to cause arousal sufficient to induce the onset of EEG activity has, as far as can be ascertained, not been investigated. However, this was not observed here in response to the supramaximal stimulus in anaesthetised joeys.

Joeys aged 140-181 days and 187-260 days

Joeys aged 140-181 days did not show an overall response to clamping as assessed by the spectral parameters F50, F95 and Ptot before, during and after clamping. A significant overall response to clamping was observed in joeys aged 187-260 days in F50.

Previous studies investigating the cerebrocortical responsiveness of adult animals anaesthetised with halothane have observed an increase in F50 and F95, also known as EEG desynchronisation/arousal (Johnson *et al.*, 2005b; Orth *et al.*, 2005; Gibson *et al.*, 2007; Murrell *et al.*, 2007). A decrease in F50, as observed in the present study, is commonly referred to as synchronisation or paradoxical arousal. This has also been

observed in anaesthetised infants and children in response to skin incision (Oshima *et al.*, 1981) and in rat pups in response to tail clamping (Chapter 3). The causes of these differences between adult and young animals and humans apparently remain unclear (Otto, 2008). An additional complication is that in newborn and young lambs, which are neurologically mature at birth, the EEG responded to noxious stimulation with desynchronisation (Johnson *et al.*, 2005a; Johnson *et al.*, 2009). Further studies will be necessary to elucidate the causes of these differences.

The significant changes in F50 presented here were accompanied by significant differences in the EEG power at various frequencies in response to clamping (Table 2.4, Figures 2.4 and 2.5). Multivariate analysis however was not able to distinguish between EEGs before, during and after clamping (Figure 2.6).

There are several possible explanations for the above findings. First, it is likely that the numbers of animals used, and the need for pooling animals of different ages into one group to increase numbers, contributed to the quantitatively small EEG changes in response to clamping. Reconfiguring the analyses by including data from only the youngest (140-170 days) and oldest (older than 220 days) joeys in order to prevent possible contamination by inclusion of data from intermediate joeys however did not affect the results. Hence, the low number of animals used could be a likely cause for the absence of significant changes in response to clamping.

Second, the quantitatively small EEG responses to toe clamping observed could be due to suppressive effects of halothane anaesthesia acting on the central nervous system of the joeys. The time occupied by isoelectric periods was small in those joeys where EEG responsiveness to toe clamping could be investigated, which suggests that suppression by halothane was not pronounced. It may nevertheless be possible that elements of halothane suppression were still sufficient to affect EEG responsiveness to sensory stimulation without causing isoelectric periods.

Third, immaturity of the nervous system may be a reason for the absence of a response in the younger joeys. The response of older joeys was indeed more pronounced than that of younger joeys as judged by significant age-related differences in some parameters in response to clamping. The absence of a significant response in the younger joeys is

consistent with their developmental immaturity, just as the presence of a significant general response in F50 in older joeys is consistent with the more advanced development of their neurological systems (see section below on neurological development in tammar wallabies).

Fourth, the brain regions normally involved in EEG responsiveness to potentially noxious stimulation may be smaller in the wallaby compared to eutherian mammals and hence a sufficiently strong response signal may not have been detected within the EEG after clamping. In order to determine whether this may be so one would have to investigate the situation in a fully mature animal so as to prevent developmental factors from affecting the results. Unfortunately, no data on the responsiveness of the EEG to potentially noxious stimulation are available in adult tammar wallabies.

Alternatively, the fact that metatherians do not have a corpus callosum, but have 3 alternative interhemispheric pathways (Rowe, 1990), may play a role in the results of the present study. In both, polyprotodont (e.g. North American opossum *Didelphis virginiana*) and diprotodont marsupials (e.g. possums, wallabies, kangaroos), two large fibre bundles interconnect the two cerebral hemispheres. There is the hippocampal commissure connecting the hippocampi of the two sides and there is also the anterior commissure, which carries fibres towards or from the olfactory bulbs, amygdala, basal ganglia and cerebral cortex (Johnson Jr., 1977). In addition to these two pathways, diprotodontid marsupials have an additional fibre tract, the fasciculus aberrans. In this tract, fibres from the dorsal regions of the neocortex travel via the internal capsule (rather than just via the external capsule as is the case in polyprotodont metatherians) to meet the anterior commissure, which seems to be a more direct path between the two cortical hemispheres (Johnson Jr., 1977). Whether these structures support pain perception and if so, whether pain perception may be qualitatively different from that experienced in animals with a corpus callosum, are not known.

In the tammar wallaby, the anterior commissure appears at about day 14 of in-pouch age, the fasciculus aberrans by about day 18 and the hippocampal commissure at about day 35 (Ashwell *et al.*, 1996a; Ashwell *et al.*, 1996b; Shang *et al.*, 1997). At about day 150, anterior commissure axon numbers peak and, thereafter until adulthood, approximately 60% of them are lost (Ashwell *et al.*, 1996a; Ashwell *et al.*, 1996b;

Shang *et al.*, 1997). At the time of peak axon numbers, myelination of commissural axons apparently begins and at about day 216 4% of commissural fibres were myelinated compared to around 60% in the adult (Ashwell *et al*., 1996a). Between days 80 and 165 anterior commissural neurons are evident throughout the neocortex, but thereafter the neurons are restricted to localised regions in cortical layers III and V (Shang *et al.*, 1997).

The fact that only a small number of commissural neurons are myelinated by 216 days may suggest that information processing in joeys may be immature until late in pouch life and could thus have affected the EEG responses to clamping in the joeys of the present study.

Sixth, although the joeys were maintained on a heated pad, it is possible that body temperature dropped to a level where EEG responsiveness to external stimulation was reduced. As body temperature was not recorded the success of maintaining normothermia could not be assessed and hence a temperature effect on EEG responsiveness to toe clamping cannot be discounted.

Seventh, wallaby joeys may be protected from potentially painful stimulation by milk-derived opioid receptor ligands or other milk-derived factors until the time of weaning (Teschemacher *et al.*, 1997; Goody & Kitchen, 2001;Teschemacher, 2003). It has been shown in rat pups that the provision of casein-rich milk is a critical component in maintaining μ-receptor-mediated stress-induced analgesia rather than switching to δ-receptor-mediated stress-induced analgesia, which usually occurs at around the time of weaning. Thus milk-derived compounds appear to reach the central nervous system and exert effects on opioid receptor populations. In tammar wallabies, genes for α-casein and β-casein have been shown to be expressed during phase 2 lactation (from birth to 200 days) and a 30% increase in total casein in milk during phase 3 of lactation (200-330 days), mainly due to an increase in β-casein, has also been observed (Nicholas, 1988; Bird *et al.*, 1994). The quantitatively small EEG responses to toe clamping in joeys in the present study could therefore be partly due to milk ingestion and associated release of opioid receptor ligands into the blood stream and thereafter into the central nervous system. The fact that older joeys would be more likely to increase milk intake

with age to accommodate growth and maturation, associated with the observed increase in casein during phase 3 of lactation, supports the present findings that even in the older joeys EEG responses to clamping were moderate. According to this hypothesis, milk-derived opioid receptor ligands could be present well before the neurological development is at a stage where pain perception would be possible, so that one may doubt the presence of such a system on this basis. However, it may well be possible that the presence of these factors plays an essential role for development of opioidergic and other systems in the developing animal before their involvement in pain perception/nociception.

Lastly, it is possible that the age-related differences in the diameter or innervation of the fourth phalange of the hind limb played a role in the differences in responsiveness to clamping observed in the present joeys.

4) Responsiveness to increasing halothane concentrations

The EEGs of joeys aged 140-181 and 187-260 days responded to increasing halothane concentrations. In the older joeys there were only significant changes in spectral parameters (F50 and F95) in response from 1.0% to 1.4%, but not 1.2% endtidal halothane concentration. However, in the younger joeys significant changes in F50 occurred with a rise from 1.0% to 1.2% as well as to 1.4% endtidal halothane concentration. These significant changes in spectral parameters were accompanied by significant differences in the EEG power of various frequencies in response to increasing endtidal halothane concentrations (Table 2.7, Figures 2.7 and 2.8). However, overall, the response to increasing halothane concentrations was small (i.e. only few frequencies were affected; Table 2.7). This is supported by the results of the multivariate analysis (Figure 2.9). As is the case with the data in response to clamping, the small numbers of animals used in the present study may be responsible for the low significance in EEG responsiveness to increasing endtidal halothane concentrations.

As EEG responsiveness to changes in halothane concentrations was tested after exposing joeys to toe clamping, it is also possible that clamping might have interfered causing the relatively small EEG response observed in the present joeys. It would have been better to investigate the effect of halothane concentration on the EEG without the

previous study of EEG responsiveness to clamping. A better design would have been to test the effects of different levels of halothane anaesthesia applied in random order. However, as the number of joeys available for study was restricted and as our primary focus was to investigate EEG responsiveness to a potentially noxious stimulus, we were not able to follow a randomised design.

The differing responses to increasing endtidal halothane concentrations in spectral parameters in the two age groups seem to indicate that the impact of halothane anaesthesia changes over the course of development. The fact that the EEGs of the younger joeys' responded to both 1.2% and 1.4% endtidal halothane concentration suggests that their nervous system was more susceptible to the effects of halothane anaesthesia than was that of the older joeys in which the EEG only responded significantly to 1.4% endtidal halothane concentration.

Comparison between anaesthetised and non-anaesthetised joeys of the present study

A possible contribution of halothane anaesthesia to the presence of isoelectric periods observed in anaesthetised joeys is supported by the absence of isoelectric periods after recovery from anaesthesia in joeys that were not anaesthetised during EEG recordings. However, the small number of joeys available for comparison precludes anything but a tentative suggestion.

The increase in EEG power with age in anaesthetised joeys reported above was not related to the effects of anaesthesia, as this was also observed in joeys not anaesthetised during EEG recordings (Figure 2.10). Rather, increasing EEG power with age seems to represent a maturational phenomenon.

Comparing the EEG spectral data of joeys that were not anaesthetised during EEG recordings with those of age-equivalent anaesthetised joeys it was shown that anaesthesia seemed to affect both younger (137-145 days) and older (189-196 days) joeys, but in different ways, judged by the differential effects of anaesthesia on F50, F95 and Ptot at the different ages (Table 2.10). This is also supported by the results of the PCA, as joeys that were not anaesthetised apparently had a greater proportion of higher frequencies (23-30Hz) and a lower proportion of lower frequencies (1, 6-10 and

12-15Hz) present in their EEG signal compared to anaesthetised joeys. In addition, older joeys that were not anaesthetised had lower overall power compared to those that were anaesthetised.

Overall, it appears that there may be differences in the EEG response to anaesthesia in general, rather than exposure to varying anaesthetic concentrations, between ages. The presence of isoelectric periods in the younger joeys during anaesthesia and their absence in older anaesthetised joeys is consistent with this, although continuing neurological maturation confounds this interpretation. However, as the number of joeys used for comparative purposes was small caution is necessary when interpreting these data. It may well be possible that individual variation rather than variation due to anaesthesia may be involved in the differences observed.

Development of conscious perception

Some of the neurological structures involved in the production of REM and non-REM sleep states are also involved in the production of consciousness; namely the thalamus, the cortex and thalamocortical connections (Evans, 2003). Thus before REM and non-REM sleep states have developed conscious perception seems unlikely. Once REM-non-REM differentiation has begun however, conscious perception may also become possible. Whether such perception may differ qualitatively from that of mature animals is not known.

As mentioned above, no information is available on REM-non-REM sleep differentiation in tammar wallabies. If we accept the argument that eye-opening and thermoregulation coincide with REM-non-REM sleep differentiation (see above), we would expect joeys to become capable of conscious perception no earlier than about 140 days. The fact that joeys put their heads out of the pouch by 180 days and start to nibble grass at that time, which shortly thereafter is followed by the first pouch exist, suggests that joeys are capable of some form of conscious perception by the age of 180 days or so. This is also partly supported by the data of the present study, although the overall EEG responses to toe clamping in anaesthetised joeys were quantitatively small at that age.

Experimental limitations

- The implications of anaesthesia on the outcome of the present study are discussed in more detail in the General Discussion (Chapter 7).

- Age is a major confounding factor as this is associated with developmental changes that may lead to indirect effects of halothane anaesthesia on EEG parameters. This has been discussed in the General Discussion and will hence not be elaborated on here.

- Rectal temperature of the present joeys was not measured and hence it cannot be assured whether or not any of the joeys became hypothermic in response to anaesthesia, which, if they did to a significant degree, might have affected the results of the present study.

- The diameter of the fourth phalange may or may not have affected the results. Nociceptive input may be related to diameter and hence differences in results between joeys of the two age groups may be related to these differences rather than differences in cerebral processing *per se*.

- Due to cranial to caudal development of the nervous system, stimulation of the forelimb may have been a more appropriate stimulus.

- Withdrawal of the hind leg stimulated was not recorded and may have proven useful in the interpretation of the present data. In addition, possible contamination of EEG traces by movement artefact has not been taken into consideration.

Suggestions for future research

- Further EEG studies (including recordings of electro-occulograms and electromyograms) would be necessary to assess the onset of sleep-wake states and their development with age in non-anaesthetised joeys.

- The collection of brains of joeys exposed to noxious stimulation may be

informative should further studies in this area be undertaken. These brains could be assayed for the induction of c-fos activity to assess whether and which brain centres may be activated in response to noxious stimulation in developing joeys and whether there may be changes with age (Bullitt, 1990).

2.5 Conclusions

Although the results of the present study do not inform us directly about whether or not tammar wallaby joeys are able to experience pain, they do suggest that there may be age differences in the ability of the cerebral cortex to respond to potentially noxious stimulation. Overall, the EEG responses to toe clamping and the changes in halothane anaesthesia concentrations were small for all joeys used in the present study. The older joeys (187-260 days) showed a marginally stronger response to clamping in some EEG spectral parameters than did the younger joeys. The extent to which halothane anaesthesia, the number of animals used or other factors might have affected the significance of the observed differences in the present study remains uncertain.

Nevertheless, on the basis of the present results and the analysis of literature on neurological and behavioural development in the tammar wallaby, we make the following cautious suggestions.

- Absence of sustained EEG activity before 100-120 days of in-pouch life in the present anaesthetised joeys, in addition to the published neurological and behavioural evidence for cerebral immaturity, suggest that conscious perception and an associated ability to experience pain in tammar wallaby joeys younger than 100-120 days is doubtful.
- Despite the paucity of EEG information regarding REM-non-REM sleep differentiation we suggest that this does not occur before eye opening and the onset of thermoregulatory development, which begin at around 140 days. As the younger joeys of the present study (140-181 days) showed a marginal response to toe clamping (i.e. changes during clamping), it is possible that animals can experience pain from this point onwards. Judging by the non-significant overall EEG response to clamping (due to lack of responsiveness in the EEG after clamp removal) and the lower EEG power present in younger joeys, it is possible that this experience may be qualitatively different from that of older

and mature animals. However, such an experience may nevertheless cause perceived pain and any associated suffering.

- The behavioural repertoire of joeys referenced in the previous sections and the responses of the older joeys (187-260 days) to potentially noxious stimulation observed in the present study, suggest that joeys older than 180 days are capable of conscious perception and hence are able to experience pain.

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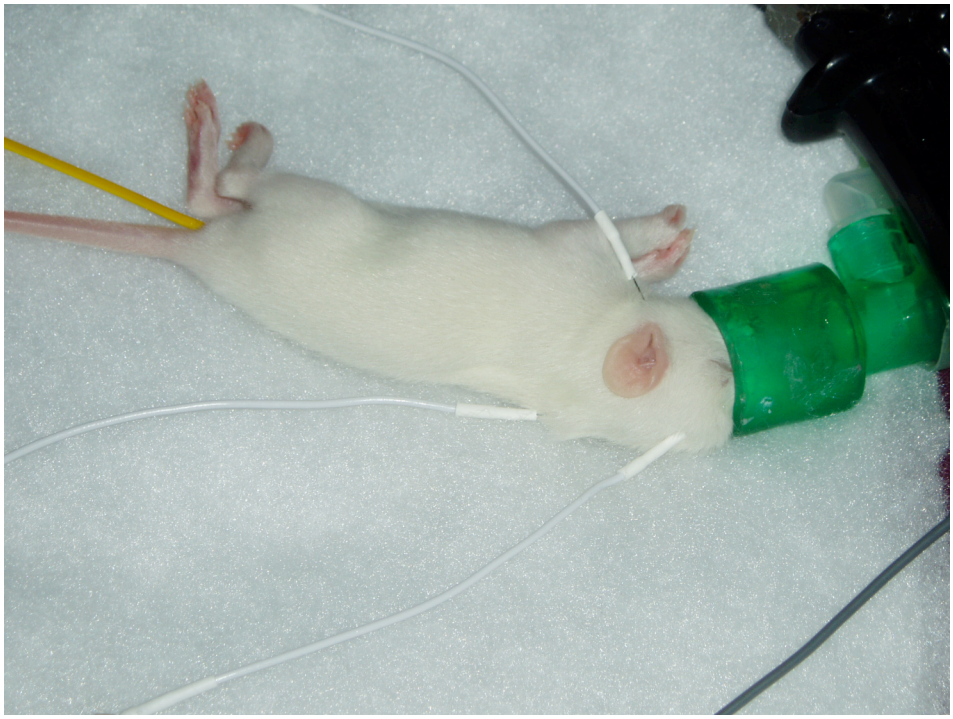
Chapter 3

- Neurologically moderately immature at birth -



Parts of the present chapter have been published in the following publication (*see Appendix 2 for a full copy of the proofs of the paper*)

Diesch TJ, Mellor DJ, Johnson CB & Lentle RG. (2009). Electroencephalographic responses to tail clamping in anaesthetised rat pups. *Laboratory Animals* **43,** 224-231.

Abstract

Whether newborn and young rats experience pain differently from the adult, or whether they experience pain at all, is not presently known. Previous studies have focussed on the development of spinal nociceptive mechanism, which do not inform us about pain perception as such. Electroencephalographic (EEG) responses to tail clamping in lightly anaesthetised young rat pups (5-22 days) were investigated to elucidate when after birth they may become capable of experiencing pain and hence capable of conscious perception. Median frequency (F50) and spectral edge frequency (F95) of the power spectrum over a range of 1-30Hz were determined before and after the application of the noxious stimulus and power spectra were compared by multivariate analysis. There was a gradual increase in EEG power with age. Pups aged 5-7 days had isoelectric traces and hence no EEG response to clamping could be observed. Rats aged 12-14 days showed a significant decrease in F95 ($p=0.002$). In contrast, rats aged 21-22 days showed a clear EEG response to clamping judged by significant reductions in F50 ($p=0.028$) and F95 ($p<0.001$). The results are discussed in the context of published information on neurological development, sleep-wake EEG differentiation, normal behavioural development and the possible effects of halothane anaesthesia on the EEGs of rat pups in the present study. Tentative conclusions were drawn regarding the onset of conscious perception on the basis of such information and the results of the present study were consistent with this. Thus, 1) an absence of a differentiated EEG and neurological evidence suggest that the ability to experience pain, and hence conscious perception, in rat pups younger than 10-12 days is doubtful; 2) the marginal response to tail clamping in 12-14 day-old pups together with the behavioural repertoire of non-anaesthetised rats suggest that pups at this age might be able to experience pain and should be given the benefit of doubt; and 3) the cerebral responses, the observed behaviour and the adult-like sleep-wake EEG patterns in rats aged 18-20 days suggest that rats are normally capable of conscious perception and are hence able to experience pain from 18 days onwards.

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Electroencephalographic responses to tail clamping in anaesthetised rat pups aged 5 to 22 days

3.1 Introduction

It has been shown that nociceptive processes in the newborn and young rat differ from those in the adult. Thresholds to mechanical stimulation of the skin are lower in younger rats and withdrawal reflexes are often inappropriate (Holmberg & Schouenborg, 1996; Fitzgerald & Jennings, 1999; Fitzgerald, 2005), pups show an increased sensitivity to noxious thermal and deep mechanical stimuli (Hu et al., 1997), cutaneous receptive fields of dorsal horn cells are relatively larger and less well organized (Fitzgerald & Jennings, 1999; Fitzgerald, 2005) and evidence suggests that the rat's descending inhibitory pathways are not functionally effective until about 10 to 12 days after birth (Fitzgerald & Beggs, 2001). Underlying these differences are developmental differences in anatomy. For instance, low threshold Aβ-fibres, which in adults are present in laminae III and IV of the dorsal horn of the spinal cord, extend collaterals to superficial neurons in the substantia gelatinosa in neonates and these disappear once C-fibre terminal synaptogenesis in that region is complete (Fitzgerald *et al.*, 1994; Fitzgerald & Jennings, 1999). In addition, these anatomical changes are associated with delayed maturation of interneurons and neurotransmitter systems, together with changing expression of key molecules, receptors, receptor subunits and ion channels during development (Alvares & Fitzgerald, 1999; Narsinghani & Anand, 2000; Pattinson & Fitzgerald, 2004; Fitzgerald, 2005). This information on the development of nociceptive processes at subcortical level does not clarify whether the newborn and young rat might also *experience* pain differently from the adult, nor indeed, whether it experiences pain at all.

There is now evidence that the cerebral cortex is involved in the experience of pain perception (Jones *et al.*, 1991; Talbot *et al.*, 1991; Jones *et al.*, 1992; Treede *et al.*, 1999). Although information on neurological development of higher centres of the brain is available, little is known about the maturation of central nociceptive processes in the brainstem, thalamus and cortex (Narsinghani & Anand, 2000; Fitzgerald & Beggs,

2001), or the development of the conscious perception of pain.

Changes in electroencephalographic (EEG) spectra have been shown to reflect changes in cerebral activity associated with the cognitive perception of pain (Bromm, 1984; Chen et al., 1989). Investigating the development of the EEG of newborn rats may therefore aid our understanding of the onset and development of conscious perception of pain in these animals.

There is disagreement in the literature about when EEG activity can first be detected in the rat. Some researchers have reported weak EEG activity to be present from days 1 to 4 after birth (Yoshii & Tsukiyama, 1951; Tuge *et al.*, 1960; Snead & Stephens, 1983; Tucker *et al*., 2009). The majority of studies suggest that EEG activity is present by 5 to 7 days after birth, being initially characterised by continuous, but irregular, low-voltage slow waves (Ellingson & Rose, 1970). Studies on the development of EEG differentiation (i.e. development of rapid eye movement (REM) and non-REM sleep) show that during the first 10 to 12 days after birth rats show signs of undifferentiated sleep (i.e. sleep-like states of unconsciousness) (Jouvet-Mounier *et al.*, 1970; Gramsbergen, 1976; Mirmiran & Corner, 1982; Titkov *et al.*, 2005). REM-non-REM differentiation begins from about 10 to 12 days after birth and during this period sleep-wake cycles also appear. Whether or not the wakefulness associated with developing sleep-wake cycles can be said to be 'conscious wakefulness' (Evans, 2003) is not apparent from the literature available. Adult-like sleep-wake patterns, which presumably include conscious wakefulness, have been reported to appear from about 18 to 20 days after birth (Jouvet-Mounier *et al.*, 1970; Gramsbergen, 1976; Frank & Heller, 1997). Although these studies do not necessarily provide evidence that the newborn and young rat are able to experience pain, they do provide information on when pain experience is unlikely (i.e. before electrical activity is present in the cortex), when it might be present (i.e. once cortical electrical activity is present and EEG differentiation into REM-non-REM sleep cycles begins to develop) or when we can be confident that it is present (i.e. once the EEG exhibits fully differentiated REM, non-REM and conscious waking cycles).

The aim of this study was to investigate cerebral responses to a noxious stimulus in young rat pups before, during and after EEG differentiation to elucidate when after birth

they may become capable of experiencing pain. As previous studies have shown a similarity in the EEG response to potentially painful stimulation in conscious and anaesthetised animals (Ong *et al.*, 1997; Murrell *et al.*, 2003) and in conscious humans (Chen et al., 1989), the minimal anaesthesia model was used for the purposes of the present study (see Chapter 1). Advantages of this model are that the use of anaesthetised animals is a humane way of investigating nociceptive processes and that contamination of EEG records by movement artefact is minimised.

As animals are anaesthetised, use of this model does not demonstrate whether they do or do not experience pain, but it does allow inferences to be made whether the cerebral cortex is capable of responding to potentially noxious stimulation at the different postnatal ages investigated and by extension, whether conscious perception is possible at the ages investigated. Accordingly, EEG responses to tail clamping were examined in rat pups at 5-7 days, 12-14 days and 21-22 days after birth.

3.2 Materials and Methods

Animals

Sprague Dawley rats were obtained from an outbred colony in the Small Animal Production Unit at Massey University, New Zealand, where they were maintained in a controlled environment (12:12 light/dark cycle with dawn and dusk transitional periods, room temperature 22°C ± 1°C) and fed a commercially available pelleted diet *ad libitum* with *ad libitum* access to water. Female rats were mated and, after birth, 2 to 3 pups from each litter were grouped according to postnatal age as follows: 5-7 day pups [5 days n=2, 6 days n=9, 7 days n=4], 12-14 day pups [12 days n=2, 13 days n=10, 14 days n=2] and 21-22 day pups [21 days n=6, 22 days n=8]. The eyes of 5-7 day pups were closed. Those of 12-14 day pups were closed or open, either partially or fully. Those of the oldest pups (21-22 days) were fully open. Rat pups were weaned at 20 days and fed a pelleted diet *ad libitum* with *ad libitum* access to water. Their gender was not recorded. The study was approved by the Massey University Animal Ethics Committee (Protocol 06/12).

Experimental procedure

The pups were weighed and anaesthesia was then induced in a chamber using 4-5% halothane (Merial, Parramatta, Australia) in oxygen (100 %). A heat lamp (250W) placed above the chamber prevented excessive loss of body heat during induction. After pups lost their righting reflex they were removed from the chamber and were placed onto a heated wheat bag. Rectal temperature was monitored in pups in the two older groups using a temperature probe (Dual Input Digital Thermometer, Dick Smith Electronics, NZ), whereas, for the smaller 5-7 day pups, the temperature between the animal and the wheat bag was recorded rather than rectal temperature. This was done to prevent tissue damage and nociceptive stimulation in the smallest pups, which could have affected the subsequent response to noxious stimulation. Additionally, a soft insulating cover was placed over the pups (Soffban; Shoof International, Cambridge, NZ).

A facemask was fitted and secured by one drop of cyanoacrylate adhesive (Selleys, Auckland, New Zealand) on the side of the face. Anaesthesia was maintained with 2.0% halothane administered by a calibrated dialled vaporizer into a 1.5L/min flow of oxygen. Stainless steel electrodes (0.3mm in diameter; Medelec, Oxford Instruments Medical Systems, UK) were placed in each pup subdermally (12-14 and 21-22 day pups) or, at a shallow angle, through the skull bones (1-2mm deep; 5-7 day pups). A three-electrode montage was used with the non-inverting electrode in the midline over the frontal sinus, the inverting electrode over the left mastoid process and the ground electrode caudal to the occipital process.

Recording in a Faraday cage minimised electrical contamination. The animals were allowed to stabilise for two minutes after electrode placement, following which a baseline electroencephalogram (EEG) was recorded for five minutes. Supramaximal mechanical stimulation was then achieved via a haemostat clamp (6.5cm long, 3-5mm wide) placed across the tail between its mid-point and the tip. It was closed until the first ratchet caught and was held for 10 seconds before release. EEG recording continued for 5 minutes after this stimulus. All pups were then euthanased by injecting an overdose of barbiturate anaesthetic (Pentabarbitone 300, National Veterinary Supplies, Auckland, New Zealand).

Pup behaviour

The incidence of general body movements, audible vocalisations and, in some cases (12-14 and 21-22 day pups) breathing changes, in response to electrode placement and the tail clamp were recorded when obvious changes occurred, but were not systematically evaluated. Although rat pups may have emitted ultrasonic vocalisations, equipment for recording those was not available.

EEG recordings

The EEG was recorded continuously at a sampling rate of 1kHz using an Apple Macintosh personal computer. Isolated biological amplifiers (World Precision Instruments Inc., Florida, USA) were set at a gain of 1000 with a pass band of 0.1Hz to 0.5kHz. Data was digitised using an analogue to digital recording system (Powerlab 4/20, Powerlab^{TM} Data Acquisition System®, AD Instruments Ltd) using compatible recording software (Chart 5, Powerlab^{TM} data acquisition system®, AD Instruments Ltd). Analysis of EEG data was done off-line after completion of each experiment.

ECG analysis

ECG (electrocardiogram) artefact was present in the majority of traces during isoelectric periods of the EEG, which allowed heart rate to be calculated as an index of the rat pups' physiological stability throughout the study (5-7 day pups n=10, 12-14 day pups n=12, 21-22 day pups n=9). A sample (800msec to 6000msec long) was taken approximately every 30 seconds where possible and beats per minute (BPM) were calculated. The average BPM were recorded for the following periods: before baseline (pre-baseline), during baseline and during or after clamping (post-clamping). In addition, BPM were calculated for three consecutive periods before and three consecutive periods during and after clamping (600 to 5000 msec) in order to determine the immediate effect of clamping on heart rate.

EEG analysis

Electroencephalographic traces of 5-7 day pups were nearly completely isoelectric, as judged by relative EEG power of the EEG power spectrum. Traces of older rat pups

also showed isoelectric periods interspersed between EEG epochs (EEG periods of 300msec or longer duration during which the amplitude was at least 4-5 times lower than that of the remaining trace, assessed visually; Figure 3.1).

EEG data were analysed during the last minute before (baseline recording) and the first minute after tail clamping. The percentage of isoelectric periods of 300msec or longer during these times was recorded, as were the number and average duration of isoelectric periods. EEG epochs containing artefact were excluded from analysis.

The traces from 5-7 day pups showed regular electrical excursions amongst isoelectric EEG periods, which may have been either epochs of EEG activity or breathing movement artefacts. If they were movement artefacts, the EEGs of all 5-7 day pups would have been largely isoelectric (100% isoelectric). Nevertheless, as the origin of these periods of electrical excursions remained unclear, a conservative approach was adopted by excluding them from the calculation of the proportion of the time that isoelectric periods were present, so that the percentage was less than 100%. Mean features of 10 of these electrical excursions immediately before and 10 such excursions immediately after clamping were recorded for 5-7 day pups. Parameters included the amplitude of the electrical excursion from its lowest to highest points (mV), its duration (msec) and the interval between periods (msec).

In order to improve precision of EEG spectral analysis only extended EEG epochs were examined before and after clamping in 12-14 and 21-22 day rat pups. These represented the last five epochs of EEG activity before clamping and the first five such epochs after it, which usually occurred within 30 seconds before and after clamping. EEG epochs contaminated with movement artefact were not considered for analysis. The absence of epochs of EEG activity of sufficient duration precluded EEG spectral analysis in 5-7 day rat pups. A power spectrum was created for each epoch using Chart 5 calculating frequency spectra (Powerlab^{TM} data acquisition system®, AD Instruments Ltd; FFT size 1024 samples, Cosine bell, 50% overlap, bin width 0.97/0.98Hz); uneven samples were fitted using zero padding. A 1-30Hz filter was set to remove noise from the data and to exclude any DC component. Median frequency (F50) and spectral edge frequency (F95) were calculated from frequency spectra using standard statistical techniques.
EEG power (log power) of the individual frequency bands has been reported here as

arbitrary units. This was done as the set-up of the present study did not allow for the amplification effect of the signal to be taken into consideration. Hence no units could be ascribed. As the same system was used throughout the study and no changes were made to the recording apparatus, all values were recorded on the same scale and can be directly compared with each other without the use of defined units.

Duration of EEG epochs varied from 0.5-27 seconds (<1sec n=38, 1-2sec n=118, 2-3sec n=51, 3-4sec n=32, 4-5sec n=15, 5-10sec n=12 and 10-27sec n=4). It was assumed that EEG epochs of up to 10 seconds were stationary. The epochs of 10-27 seconds duration (n=4) were tested for stationarity using a specialised FFT (Fast Fourier Transform) program (Spectral Analyser, CB Johnson, Massey University, New Zealand, 2006). The program calculated the median frequency (F50) and spectral edge frequency (F95) for consecutive 1-second segments. The F50 and F95 for the first 5-10 seconds and last 5-10 seconds of each epoch were compared and no statistically significant differences were found over the duration of any such sustained EEG epoch.

Although the ECG contamination will have affected frequencies lower than 30Hz, its effects were apparently minimal with little impact on EEG spectral analysis. This was tested by investigating the overall contribution of the ECG to the power spectrum of the EEG. After pentabarbitone injection, the EEG was abolished, but heart rate could still be recorded for several minutes. This allowed a comparison of the overall contribution of the ECG to the power spectrum in absence of the EEG, which was then extrapolated to the normal EEG.

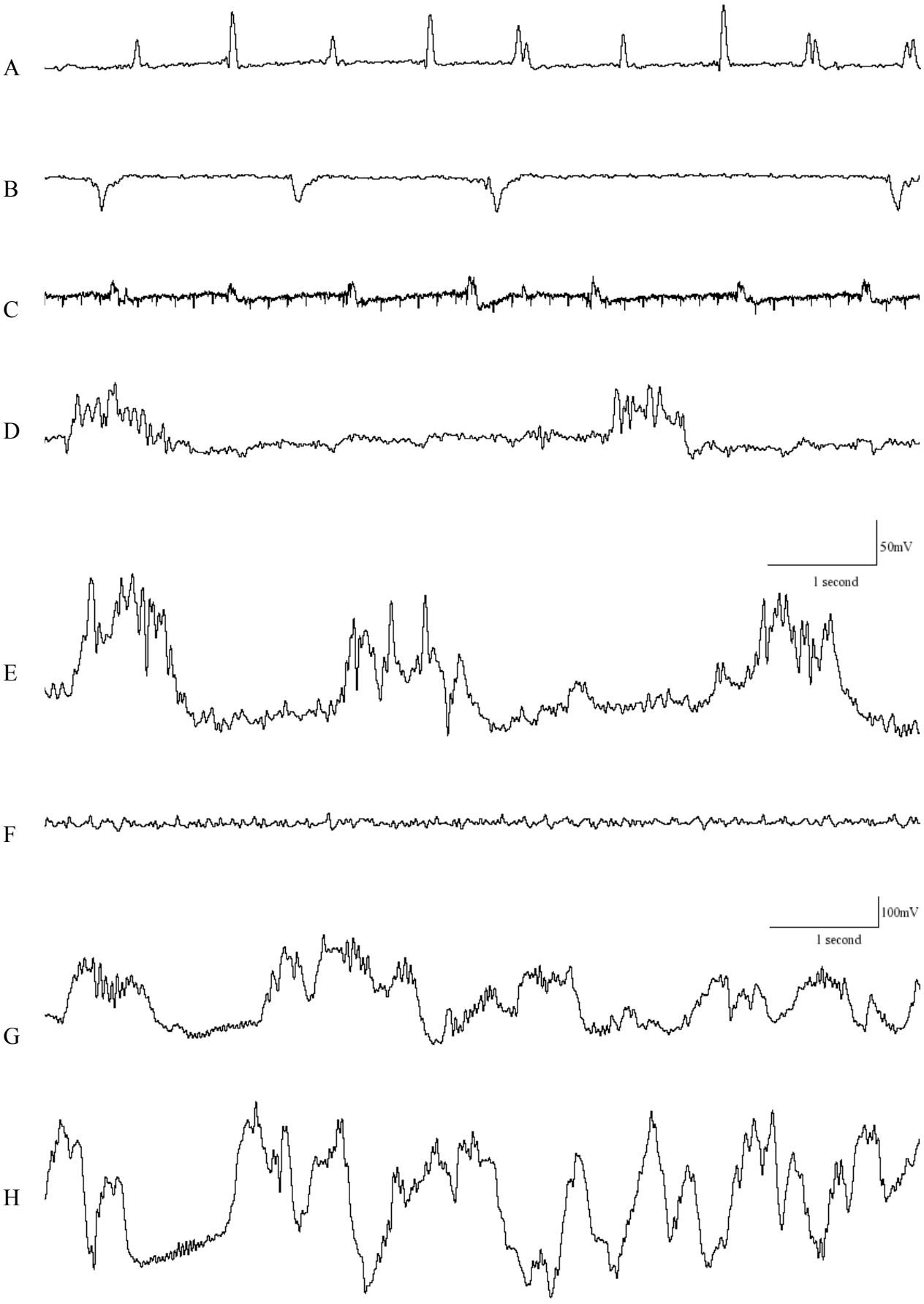

Figure 3.1: EEG traces of 5-7day-old rat pups (A, B and C; trace C showing ECG artefact), of 12-14 day rats (D and E) and of 21-22day-old rats (G and H). Trace F shows the EEG (background electrical noise) after injection of pentabarbitone. Note the differences in scale for traces A-F (50mV) and G-H (100mV).

Statistical Analyses

Statistical analysis was undertaken using SPSS 11.0 for MAC OSX (SPSS Inc., USA). Data are presented as mean ± standard error of the mean. Differences were considered significant at p=0.05 or less. Data were tested for normality using the Shapiro-Wilks test (if n<50) or the Kolmogorov-Smirnoff test with Lilliefors Significance correction (if n>50) and normal probability plots. For data that were not normally distributed a logarithmic transformation was undertaken and used for statistical analysis if this led to a normal distribution of the data. Degrees of freedom reported (e.g. df 1, 130) represent the number of groups and overall number of data points used in individual statistical tests.

1) Heart rate

As overall heart rate data were not normally distributed, the non-parametric Friedman test was used to determine whether heart rate was stable over the course of the experiment. Heart rate data immediately before and after clamping were normally distributed. Due to the small sample size both a paired-sample t-test and a non-parametric Wilcoxon signed rank test were performed to evaluate the significance of any effect of the clamp on heart rate.

2) Isoelectric periods, features of electrical excursions (5-7 day) and EEG epochs (12-14 and 21-22 day)

The EEG data were not normally distributed and using logarithmic and square root transformations did not improve this. Hence, the non-parametric Wilcoxon signed rank test was applied to all parameters to detect significant effects of clamping (i.e. before and after comparisons). Between-group comparisons were conducted using the non-parametric Kruskal Wallis test with Mann-Whitney post-hoc tests.

3) Spectral analysis

F50 and F95 were calculated for each EEG epoch and a mean was then calculated for the five EEG epochs before clamping and the five epochs after clamping for each animal. These mean values were used for statistical analyses. Age differences in F50

and F95 were assessed using the independent-sample t-test after log-transformation of data. The effect of clamping on F50 and F95 was assessed using paired-sample t-tests. For this purpose, F50 data for 21-22 day pups had to be log-transformed to generate a normally distributed data set. Subsequently, standardised frequency spectra sums of log-transformed data (1-30Hz) as well as means of log-transformed frequency spectra (Figure 3.2 and 3.3) for all groups (12-14 day before and after clamping; 21-22 day before and after clamping) were graphed and evaluated. A two-tailed Kolmogorov-Smirnoff test was then run to examine the gross difference in the shapes of frequency curves using frequency spectra for each frequency as well as frequency spectra sums. In order to further investigate these differences statistically, a Principal Component (PC) analysis was done on log-transformed data (frequencies 1-30Hz, 10 EEG periods per animal – 5 before and 5 after clamping). The PC loadings were investigated and the PC scores were plotted against each other in reduced dimensions to explore variation with treatment and with age. The scores were then used in a one-way ANOVA with the Tukey post hoc test to determine statistical differences between the groups.

3.3 Results

General information

Mean body weight and temperature (skin and rectal) are shown in Table 3.1. Mean body weight increased with age. Due to methodological differences body temperature differences were not anticipated to have any relevant biological significance. Due to this and the differences in body temperature measurements, statistical comparison was not undertaken.

Table 3.1: Details of weight and rectal/skin temperature of rat pups of the three ages.

	Weight (g)	Temperature (°C)		
		Pre-baseline	Baseline	Post-clamp
5-7 day pups	**15.49**[a]	**39.9**[ST]	**40.1**[ST]	**40.2**[ST]
	15[b]	15	15	15
	2.65[c]	1.4	1.7	1.9
	0.68[d]	0.37	0.43	0.49
12-14 day pups	**31.33**	**38.1**[RT]	**38.5**[RT]	**38.6**[RT]
	13	13	13	13
	3.17	1.0	1.1	1.1
	0.88	0.28	0.31	0.31
21-22 day pups	**58.14**	**37.9**[RT]	**38.3**[RT]	**38.5**[RT]
	14	14	14	13
	3.19	0.8	0.9	0.97
	0.85	0.21	0.23	0.27

[a]Mean
[b]Number of animals
[c]Standard deviation
[d]Standard error
[ST]Skin temperature
[RT]Rectal temperature

Behaviour

Body movements, recorded as present or absent but not characterised, were observed during electrode placement in 69% of 5-7 day and 12-14 day pups, and in 64% of 21-22 day pups. Likewise, body movements were observed in response to tail clamping in 93, 46 and 50% of 5-7, 12-14 and 21-22 day pups, respectively.

There were audible vocalisations in response to electrode placement and clamping, respectively, in 15% and 69% of animals in 5-7 day pups, but none were observed in the older pups.

Gasping and/or a change in breathing rate (subjectively assessed) occurred at or after clamping in 12-14 and 21-22 day pups.

Heart rate

Means for heart rate data are presented in Table 3.2. Overall, heart rate did not differ significantly during the experiment ($p=0.269$; df 3, 119, Chi-Square=3.929), so that in this respect the pups of all ages appeared stable. Heart rate did not significantly differ in 5-7 day pups before and after clamping ($p=0.069$, df 1, 9, $t=-2.067$). For both 12-14 and 21-22 day pups heart rate was significantly higher after clamping ($p=0.027$, df 1, 11, $t=-2.548$ and $p=0.002$, df 1, 8, $t=-4.492$; respectively).

Table 3.2: Heart rate data (BPM) of rat pups of the three ages during the pre-baseline, baseline and post-clamp phases of observation. Data include details for the entire 5-minute pre-baseline, 5-minute baseline and 5-minute post-clamp periods, as well as data for the period immediately prior to (baseline) and immediately after (post clamp) the clamping, which were used to determine the immediate short-term effect of clamping on heart rate.

	Heart rate (BPM)				
	Pre-baseline	**Baseline**	**Post-clamp**	**Baseline**	**Post-clamp**
	Entire period	Entire period	Entire period	Immediately before clamping	Immediately after clamping
5-7 day pups	**321**[a]	**328**	**325**	**323**	**339**
	9[b]	10	10	10	10
	39.8[c]	38.5	23.4	32.1	26.9
	13.3[d]	12.2	7.4	10.2	8.5
12-14 day pups	**338**	**335**	**334**	**335**	**341**
	12	12	12	12	12
	24.7	18.3	18.3	19.7	20.4
	7.1	5.3	5.3	5.7	5.9
21-22 day pups	**365**	**358**	**383**	**356**	**395**
	10	9	9	9	9
	31.6	33.0	33.5	34.1	39.5
	10.0	10.4	10.6	11.4	13.2

[a]Mean

[b]Number of animals

[c]Standard deviation

[d]Standard error of the mean

Isoelectric periods, characteristics of periods of electrical excursions and EEG epochs

1) 5-7 day pups

The percentage of time occupied by isoelectric EEG periods and the average duration of these periods did not differ significantly before and after clamping in this group (percentage: 78±3 and 75±2% respectively, p=0.363, df 1, 29, Z=-0.909; duration: 1915±728 and 1722±530msec, respectively, p=0.510, df 1, 27, Z=-0.659). However, the

amplitude of electrical excursions was significantly lower (42±5.5 *versus* 63±11.5mV p=0.006, df 1, 27, Z=-2.731) and they were significantly shorter (1428±400 *versus* 1704±490msec, p=0.026, df 1, 27, Z=-2.229) for the 10 excursions observed before compared to after clamping. The interval between electrical excursions was not significantly different before and after clamping for the 10 excursions investigated.

2) 12-14 day pups

The percentage of time occupied by isoelectric EEG periods and the average duration of these periods did not differ significantly before and after clamping in this group (percentage: 55±4 and 51±5%, respectively, p=0.108, df 1, 25, Z=-1.608; duration: 1733±209 and 1715±234msec, respectively, p=0.753, df 1, 25, Z=-0.314). There were also no significant differences in the frequency of isoelectric EEG periods (periods/1min) before and after clamping (21±1.9 *versus* 19.3±1.7 periods/1min, p=0.085, df 1, 25, Z=-1.721) and the durations of epochs of EEG activity before and after clamping (1794±178 and 2002±250msec, respectively, p=0.101, df 1, 25, Z=-1.642).

3) 21-22 day pups

The percentage of time occupied by isoelectric EEG periods did not differ significantly before and after clamping in this group (23±3 and 19±2%, p=0.074, df 1, 27, Z=-1.789). The duration of isoelectric EEG periods was significantly longer before than after clamping (709±40 *versus* 614±30msec, p=0.009, df 1, 27, Z=-2.606). The frequency of isoelectric EEG periods (periods/1min) before and after clamping did not differ significantly (18.6±1.6 and 18.7±1.3 period/1min, respectively, p=0.624, df 1, 27, Z=-0.491) and nor did the duration of epochs of EEG activity before and after clamping (p=0.056; 2391±317 *versus* 3249±675msec, df 1, 27, Z=-1.915).

4) Between-group comparison

The percentage of time occupied by isoelectric EEG periods before clamping was significantly different between the groups (p<0.001, df 2, 41, Chi-Square=33.27). The post-hoc Mann-Whitney tests showed that this difference was highly significant

between all groups (5-7 day pups *versus* 12-14 day pups $p<0.001$, df 1, 27, $Z=-3.801$; 5-7 day pups *versus* 21-22 day pups $p<0.001$, df 1, 28, $Z=-4.583$; and 12-14 day pups *versus* 21-22 day pups $p<0.001$, df 1, 26, $Z=-4.270$), with 5-7 day pups having the highest, 12-14 day pups intermediate and 21-22 day pups the lowest values (78±3, 55±4 and 23±3%, respectively). The duration of isoelectric periods before clamping was significantly different between the three age groups ($p<0.001$, df 2, 40, Chi-Square=23.791). Mann-Whitney post-hoc tests showed that the duration of isoelectric periods before clamping in 5-7 and 12-14 day pups was longer (1915±728 msec and 1733±209 msec, respectively) compared to that of 21-22 day pups (709±40 msec) (5-7 *versus* 21-22 day pups $p<0.001$, df 1, 27, $Z=-3.997$; 12-14 *versus* 21-22 day pups $p<0.001$, df 1, 26, $Z=-4.125$), but did not differ between each other ($p=0.094$, df 1, 26, $Z=-1.698$). The frequency of isoelectric EEG periods (periods/1min) before clamping did not differ significantly between the 12-14 and 21-22 day pups (21±1.9 and 18.6±1.6 period/1min; $p=0.867$, df 1, 26, $Z=-0.195$). Duration of epochs of EEG activity did not differ between age groups (12-14 *versus* 21-22 day pups 1794±178 versus 2391±317 sec; $p=0.061$, df 1, 26, $Z=-1.893$).

Spectral analysis

The absence of epochs of EEG activity in 5-7 day pups precluded EEG spectral analysis and hence only results for 12-14 and 21-22 day pups are presented.

Epochs of EEG activity in 12-14 and 21-22 day pups used in spectral analyses did not have the same durations. Whole epochs of 500msec or longer were used for analysis in order to prevent loss of important data.

1) Median frequency (F50) and spectral edge frequency (F95)

The F50 in 12-14 day pups before and after clamping did not differ significantly (4.87±0.38 *versus* 4.66±0.33Hz; $p=0.648$, df 1, 12, $t=0.468$), but in 21-22 day pups it was significantly reduced in response to clamping (3.35±0.16 *versus* 2.99±0.07Hz; $p=0.028$, df 1, 13, $t=2.471$). The F95 significantly decreased in response to clamping in pups of both ages (12-14 day pups: 19.37±0.49 *versus* 18.57±0.53Hz, $p=0.002$, df 1, 12, $t=4.061$; 21-22 day pups: 15.65±0.80 *versus* 11.61±0.80Hz, $p<0.001$, df 1, 13, $t=5.652$).

Both F50 and F95 before clamping significantly decreased with age between 12-14 and 21-22 days (F50: 4.87±0.38Hz *versus* 3.35±0.16Hz, p=0.029, df 1, 25, t=5.352; F95: 19.37±0.49Hz *versus* 15.65±0.80Hz, p=0.019, df 1, 25, t=6.322).

2) Graphic comparisons

Direct graphical comparison of standardised frequency spectra sums and means of log-transformed data showed differences in the profiles of the curves before and after clamping. These differences were found to be at frequencies of 13-14, 16-17 and 27Hz for 12-14 day pups and at 9, 12-15 and 19-30Hz for 21-22 day pups (Figure 3.2 and 3.3)

Visual analysis revealed that there were differences in all frequencies (1-30Hz) between the two ages (Figure 3.4). This was evaluated statistically in two ways. First, the two-tailed Kolmogorov-Smirnoff test showed significant differences between frequency spectra sums for the EEG of 12-14 and 21-22 day pups before ($p<0.001$, df 1, 59, $Z=2.195$) as well as after ($p=0.007$, df 1, 59, $Z=1.678$) clamping. There were no significant differences in frequency spectra sums within age groups before and after clamping (12-14 day pups, $p=0.952$, df 1, 59, $Z=0.516$; 21-22 day pups, $p=0.236$, df 1, 59, $Z=1.033$). Second, using frequency spectra for the different age groups for each frequency, rather than sums for frequency spectra (as above), the two-tailed Kolmogorov-Smirnoff test showed that there were significant differences in all frequencies between 12-14 and 21-22 day pups ($p<0.001$, df 1, 134, Z ranges from 2.456 to 5.550) (Table 3.3; Figure 3.4). It also showed that there were no significant differences in any of the frequencies before and after clamping in 12-14 day pups ($p=0.063$, df 1, 129, $Z=1.316$). There were significant differences in frequencies at 13-16Hz and 22-30Hz in 21-22 day pups before and after clamping (Table 3.3; Figure 3.3).

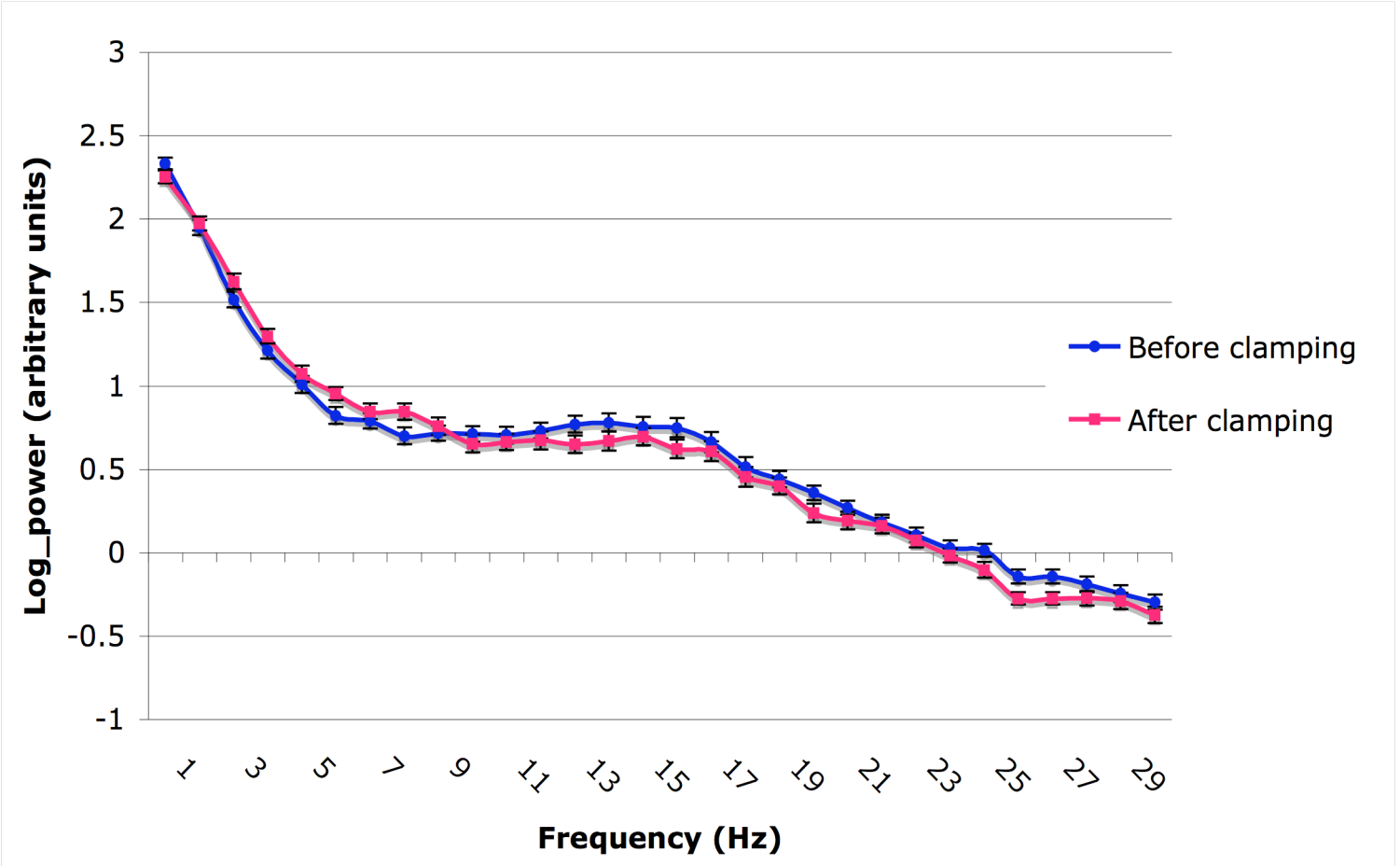

Figure 3.2: Mean power (log) in frequencies (including standard error of the mean bars) of the EEG spectrum of 12-14 day pups before and after clamping for all epochs of EEG activity.

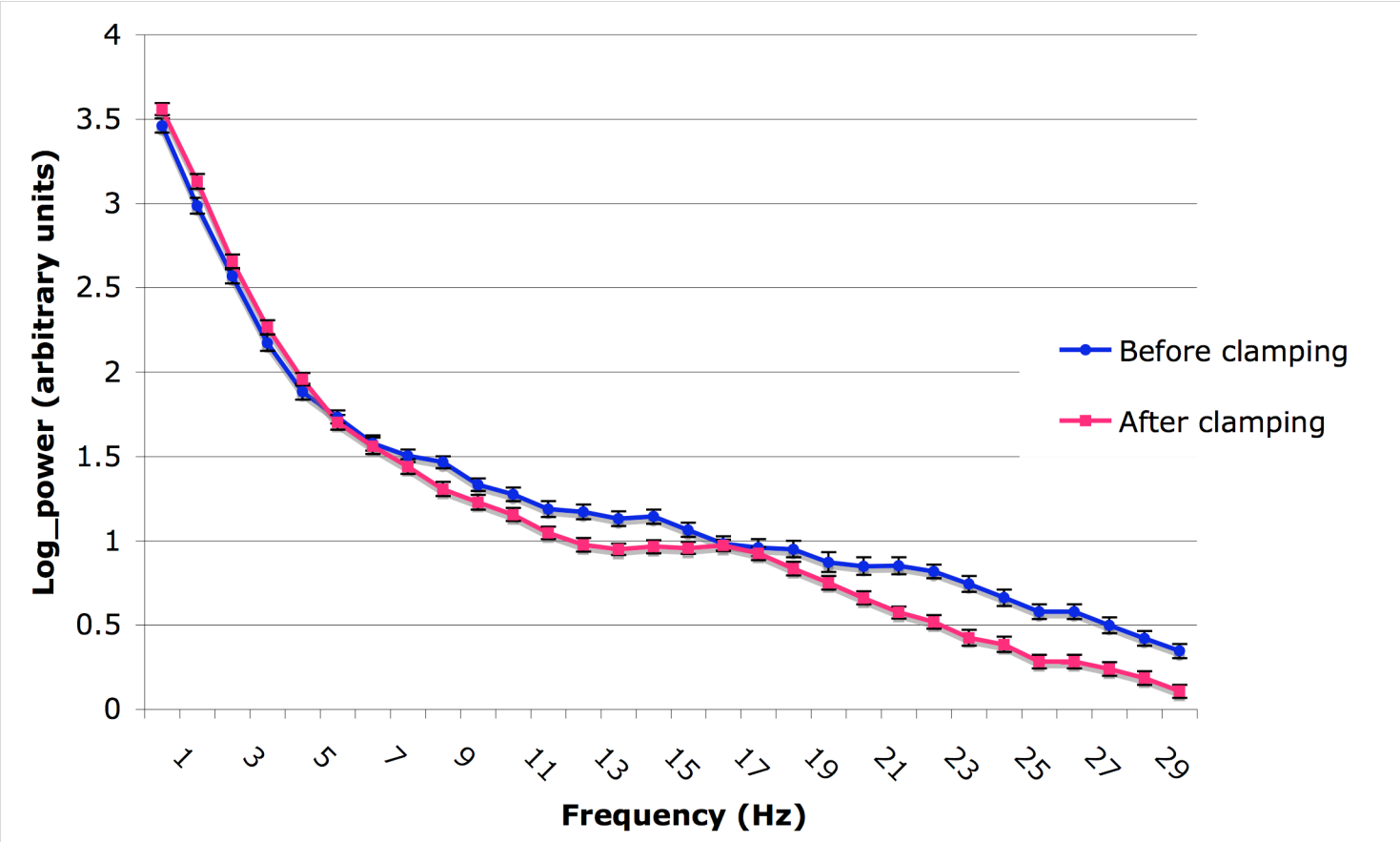

Figure 3.3: Mean power (log) in frequencies (including standard error of the mean bars) of the EEG spectrum of 21-22 day pups before and after clamping for all epochs of EEG activity.

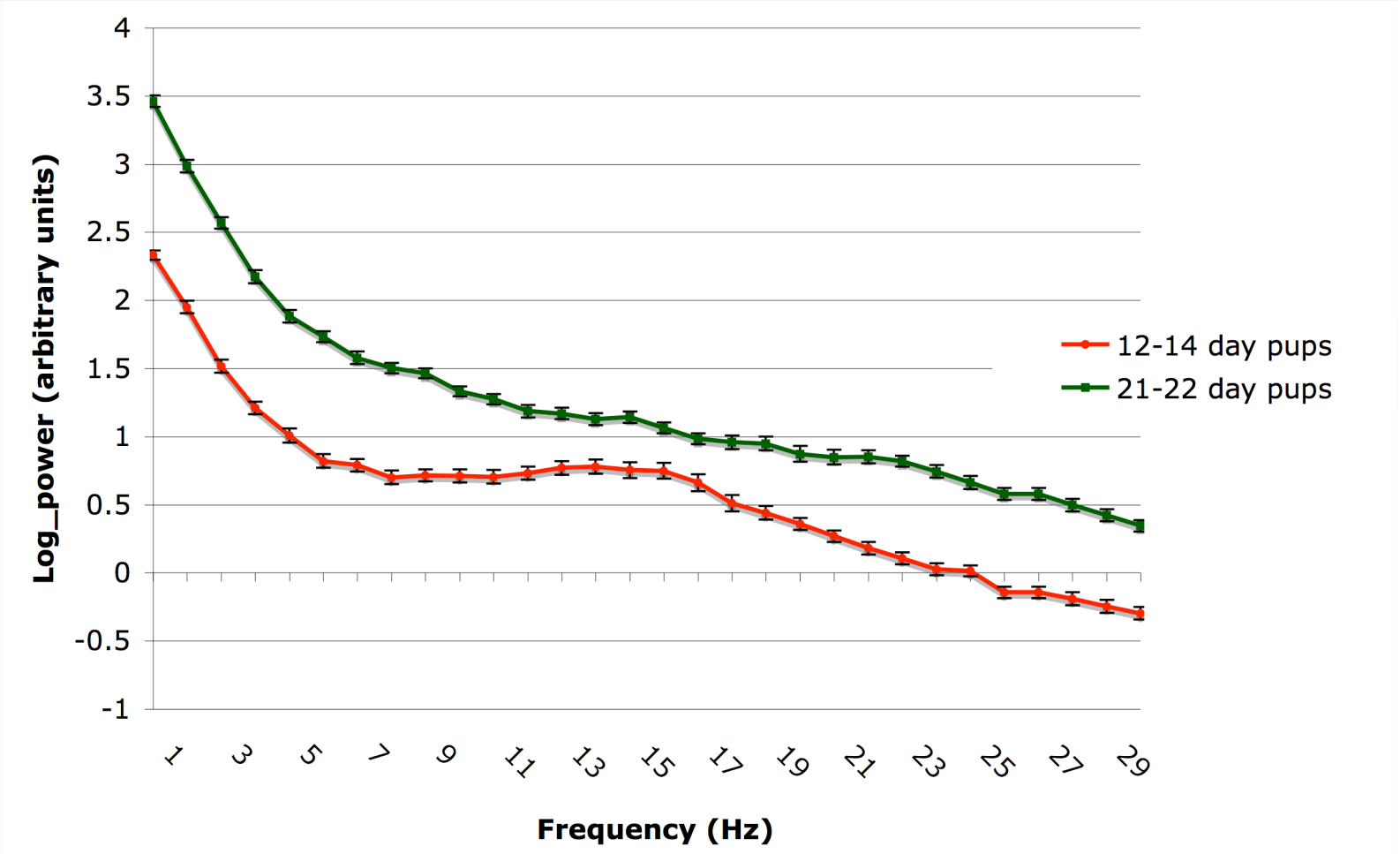

Figure 3.4: Mean power (log) of the EEG spectrum frequencies (including standard error of the mean bars) for all epochs of EEG activity during baseline observations for 12-14 and 21-22 day pups.

3) Principal Components (PC) Analysis

The factor analysis identified five Principal Components (Eigenvalues >1) that contributed 78.5% of the overall variation in the data (Table 3.4). Component loadings showed that Component 1 strongly correlated with all frequencies (1-30Hz) (all loadings ≥0.6). Hence, this factor could be seen as representing overall power and possibly the duration of epochs of EEG activity. Component 2 correlated with frequencies in the range of 14-25Hz, Component 3 with frequencies of 9-17Hz, Component 4 with frequencies of 12-14Hz and of 22-29Hz, and Component 5 with frequencies of 7-10Hz and 19-25Hz. However, these loadings were smaller than 0.4 in the majority of cases.

Contrasts (positive and negative component loadings) were present in Components 2 to 5, but not in Component 1. In Component 2 there was a contrast between frequencies of 1-12Hz (negative) and 14-25Hz (positive). In Component 3 there was a contrast between frequencies 9-17Hz (positive) and 19-30Hz (negative). Component 4 had contrasts between frequencies of 1-7Hz and 16-19Hz (negative) and 12-14Hz and 22-29Hz (positive). In Component 5 there was a contrast between frequencies 7-10, 14 and 19-25Hz (positive) and 2, 12, 16, 17 and 26-30Hz (negative).

Overall, the data obtained by the PC analysis agree with the findings of the two-tailed Kolmogorov-Smirnoff test. It was possible to distinguish between the two age groups and the two treatment groups on the basis of the PC scores along the 1^{st} and 4^{th} axes of variation (Figure 3.5). Component 1 (power and possibly duration of EEG epochs) contributed 57.6% to the overall variation in the data, while Component 4 (frequencies 12-14Hz and 22-29Hz) contributed only 3.6%. Separation according to components 2, 3 and 5 did not yield any distinctions between ages or treatment.

The results of the one-way ANOVA using PC scores showed that there were significant differences in scores between the two ages before clamping in Component 1 (overall power of the EEG) ($p<0.001$, df 3, 266, $F=189.01$), and in scores before and after clamping in Component 1 for 21-22 day pups ($p=0.001$) and in Component 4 (12-14Hz and 22-29Hz) for 12-14 and 21-22 day pups ($p=0.047$ and $p<0.001$, respectively; df 3, 266, $F=25.45$). Thus, 12-14 day pups had significantly lower PC1 factor scores than 21-

22 day pups, indicating that the overall EEG power was lower in the younger group. The separation of data according to PC4 showed that no distinction was possible on the basis of PC score of EEG frequencies of 12-14 day pups before and after clamping, but that this was possible in 21-22 day pups.

Table 3.3: P-values and Kolmogorov-Smirnoff Z-statistics (K-statistic) calculated by the two-tailed Kolmogorov-Smirnoff test comparing power of frequency spectra between 12-14 day pups and 21-22 day pups (baseline observations) before and after clamping.

Frequency (Hz)	**12-14 day *versus* 21-22 day pups**		**12-14 day pups Before *versus* After**		**21-22 day pups Before *versus* After**	
	K-statistic	*p-value*	*K-statistic*	*p-value*	*K-statistic*	*p-value*
1	5.55	<0.001	0.97	0.310	1.01	0.255
2	5.30	<0.001	0.53	0.945	1.10	0.179
3	5.37	<0.001	1.23	0.098	0.76	0.609
4	4.97	<0.001	0.79	0.562	0.76	0.609
5	4.44	<0.001	1.05	0.218	1.27	0.080
6	4.77	<0.001	1.05	0.218	0.76	0.609
7	4.30	<0.001	0.88	0.425	0.59	0.875
8	4.38	<0.001	1.32	0.063	1.18	0.122
9	4.50	<0.001	0.61	0.845	1.35	0.052
10	4.17	<0.001	0.61	0.845	1.18	0.122
11	3.80	<0.001	0.53	0.945	1.35	0.122
12	2.92	<0.001	0.53	0.945	1.27	0.080
13	3.11	<0.001	0.70	0.708	1.61	0.012
14	2.69	<0.001	1.05	0.218	1.86	0.002
15	2.46	<0.001	0.61	0.845	1.52	0.020
16	2.62	<0.001	0.70	0.708	1.52	0.020
17	2.65	<0.001	0.61	0.845	0.59	0.875
18	2.76	<0.001	0.88	0.425	0.68	0.751
19	3.16	<0.001	0.61	0.845	0.76	0.609
20	3.09	<0.001	1.05	0.218	1.01	0.255
21	3.56	<0.001	0.88	0.425	1.35	0.052
22	3.89	<0.001	0.61	0.845	2.20	<0.001
23	4.36	<0.001	0.79	0.562	2.54	<0.001
24	4.34	<0.001	0.88	0.425	2.11	<0.001
25	4.34	<0.001	0.97	0.310	1.70	0.007
26	3.92	<0.001	1.32	0.063	2.45	<0.001
27	4.18	<0.001	1.32	0.063	1.94	<0.001
28	3.96	<0.001	0.88	0.425	2.20	<0.001
29	4.03	<0.001	0.79	0.562	2.20	<0.001
30	4.36	<0.001	1.14	0.148	2.11	<0.001

Table 3.4: Eigenvalues and component scores of all frequencies for Principal Components (PCs) 1 to 5 calculated by Principal Component Analysis.

Eigenvalues	**PC 1**	**PC 2**	**PC 3**	**PC 4**	**PC 5**
Total	17.27	2.13	2.04	1.08	1.03
Percentage of variance	57.6%	7.10%	6.78%	3.60%	3.42%
Frequency	**PC 1**	**PC 2**	**PC 3**	**PC 4**	**PC 5**
1Hz	0.866	-0.171	-0.042	-0.195	-0.096
2Hz	0.828	-0.277	-0.062	-0.222	-0.130
3Hz	0.815	-0.353	-0.054	-0.189	0.018
4Hz	0.835	-0.279	-0.007	-0.218	0.059
5Hz	0.823	-0.290	0.079	-0.189	0.034
6Hz	0.838	-0.265	0.056	-0.175	0.052
7Hz	0.812	-0.272	0.082	-0.107	0.122
8Hz	0.776	-0.377	0.062	-0.019	0.181
9Hz	0.788	-0.266	0.157	0.021	0.148
10Hz	0.788	-0.175	0.225	0.016	0.111
11Hz	0.743	-0.242	0.257	0.089	-0.040
12Hz	0.675	-0.119	0.389	0.240	-0.115
13Hz	0.619	-0.003	0.497	0.370	0.058
14Hz	0.607	0.183	0.517	0.278	0.202
15Hz	0.597	0.350	0.503	0.081	-0.010
16Hz	0.576	0.489	0.404	-0.180	-0.235
17Hz	0.620	0.507	0.277	-0.315	-0.186
18Hz	0.699	0.399	0.061	-0.359	-0.059
19Hz	0.742	0.383	-0.167	-0.224	0.120
20Hz	0.720	0.313	-0.281	-0.025	0.250
21Hz	0.745	0.302	-0.234	0.074	0.301
22Hz	0.775	0.258	-0.282	0.115	0.194
23Hz	0.798	0.181	-0.273	0.114	0.186
24Hz	0.784	0.112	-0.217	0.172	0.221
25Hz	0.763	0.124	-0.273	0.110	0.127
26Hz	0.821	-0.010	-0.274	0.218	-0.302
27Hz	0.821	-0.010	-0.274	0.218	-0.302
28Hz	0.789	-0.0003	-0.257	0.205	-0.345
29Hz	0.801	0.0064	-0.188	0.143	-0.291
30Hz	0.772	0.049	-0.119	0.049	-0.259

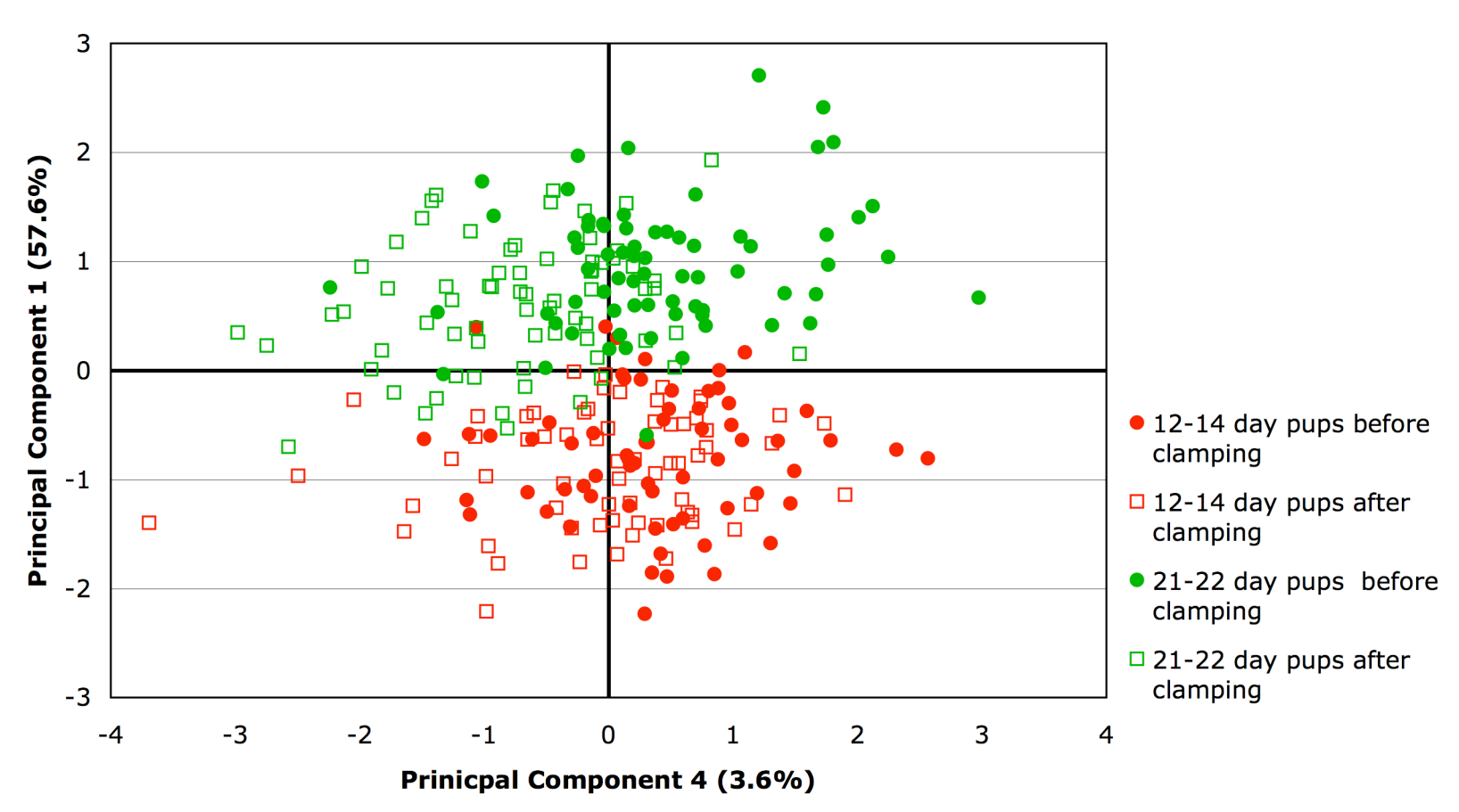

Figure 3.5: Factor scores plotted against the 1st and 4th Principal Component axes showing separation according to age (12-14 day pups versus *21-22 day pups) and tail clamping (12-14 and 21-22 day pups before* versus *after clamping).*

3.4 Discussion

As far the candidate is aware, this is the first investigation of the cerebral responses of young rat pups to potentially noxious stimulation, in particular EEG responses of anaesthetised pups to tail clamping at ages up to 21-22 days after birth. The main findings were as follows. First, there was little or no EEG activity in anaesthetised rat pups at 5-7 days, but EEG epochs were present by 12-14 days. Second, between 12-14 days and 21-22 days the percentage of time occupied by isoelectric periods decreased and that of EEG activity increased in the anaesthetised pups. Third, for the parameters investigated, there was an age-related progression in response to clamping, with no response in 5-7 day pups due to the absence of EEG activity, a marginal EEG response in 12-14 day pups and a significant EEG response in 21-22 day pups. Fourth, behavioural and physiological responses to electrode placement and clamping were evident at all ages.

Postnatal changes in unanaesthetised pups

Before examining the results obtained in the present study on anaesthetised rat pups, it would be beneficial to describe the normal progression of cerebrocortical development, brain electrical activity and behaviour in the rat pup over the first few weeks after birth.

1) Features of brain development

The central nervous system of young rat pups undergoes substantial physiological and anatomical changes during the first few weeks after birth, such that cortical neurons do not acquire their 'mature' characteristics until days 21-30 days (Erecinska et al., 2004). Myelination of cortical axons begins around day 10, continuing up to day 50 (Erecinska et al., 2004), and axonal and dendritic proliferation occurs between 6 and 18 days (Eayrs & Goodhead, 1959; Erecinska *et al.*, 2004). While the sprouting of dendrites is complete by day 12, dendritic branching increases rapidly from days 18 and 24 (Eayrs & Goodhead, 1959; Erecinska *et al.*, 2004) and the number of transmitter-containing synaptic vesicles rises until about day 28 after birth (Erecinska et al., 2004). Action potentials elicited from pyramidal neurons of cortical layer V in an *in vitro* preparation apparently become shorter in duration, larger in amplitude and exhibit a decrease in

input resistance over the first 14 to 21 days after birth, during which there are also increases in cell size and area (McCormick & Prince, 1987). Synaptogenesis in the parietal cortex occurs mainly between days 14 and 21 with few synaptic junctions visible at days 12 and 13 (Aghajanian & Bloom, 1967).

Thalamocortical afferents have been reported to form a dense plexus within layers IV and V below the cortical plate by the day of birth (Catalano et al., 1991) and achieve the adult laminar pattern and density by about 7 days (Metherate & Aramakis, 1999). Somatosensory evoked cerebral potentials elicited by electrical stimulation of peripheral nerves or the thalamus have been observed from 1-5 days after birth (Mares & Faladova, 1975; Verley & Axelrad, 1975) suggesting that thalamocortical connections are functional at that time. However, the features of these potentials differ greatly from those of the adult with regard to their shape, length of latent period and fatigability. Auditory evoked cerebral potentials via acoustic stimulation cannot apparently be elicited before days 10-13 (Iwasa & Potsic, 1982). These have been shown to differ from those of the adult in amplitude, latency and complexity, and apparently change rapidly between 14 and 21 days after birth, maturing over several weeks thereafter (Mourek *et al.*, 1967; Iwasa & Potsic, 1982; Metherate & Aramakis, 1999).

The developmental changes listed above are associated with changes in cerebral function, reflected in the EEG and pup behaviour (see below). A continuous or intermittent EEG consisting of irregular slow waves of low amplitude can be detected in rat pups by days 5-7 (Yoshii & Tsukiyama, 1951; Crain, 1952; Tuge *et al.*, 1960; Deza & Eidelberg, 1967; Jouvet-Mounier *et al.*, 1970; Mares *et al.*, 1979; Snead & Stephens, 1983; Aristakesian, 1997). The EEG is undifferentiated initially and differentiation into sleep-wake patterns occurs between 12 and 18 days after birth, with an adult pattern being attained around 18-20 days (Jouvet-Mounier *et al.*, 1970; Gramsbergen, 1976; Frank & Heller, 1997).

2) Changes in pup behaviour

As would be expected, brain maturation over the first 2 to 4 weeks after birth is associated with marked changes in behavioural patterns (Bolles & Woods, 1964). Initially rat pups show basic social behaviours including huddling with littermates and

sucking from their mother. Pups can crawl, stretch and yawn and show rudimentary forms of grooming. From days 9 to 10 pups start walking and exploring their cage; sniffing and orienting away from the nest and their mother. Self-grooming is now prolonged and carried out in the adult sitting posture. By days 12 to 13 they show vigorous locomotor activity such as running and jumping, and extended exploration of the cage can also be observed. Around day 14 eye-opening occurs and is associated with an increased interest in objects. Allogrooming is the main form of interactive behaviour between pups. Pups aged 17 to 20 days have been observed to be active, with fighting between siblings being prominent. At day 19 pups noticed their observer for the first time and approached with interest. Pups were weaned by removal from the mother and placed into clean cages/containers at day 21; this was accompanied by a reduction of their activity. However, a high level of activity (fighting) was resumed the following day and continued up to 29 days of age. Rats between 30 and 36 days showed less interest in the observer and considerably less resistance to disturbance (such as from littermates). Basic sexual behaviour begins at this age. Thus, rat pup behaviours progress during the first four weeks from mainly self-directed activities (i.e. grooming and sleeping) and mother-directed activities (i.e. sucking), to elaborate exploration of the environment and inter-pup behaviours.

Anaesthetised pups

The results of the present study will be assessed against the developmental background just described for unanaesthetised rat pups.

1) Isoelectric periods

Isoelectric periods were present in the EEG of all rat pups and the proportion of time they occupied decreased progressively with age (5-7 day pups 78%, 12-14 day pups 55% and 21-22 day pups 23%) with a concomitant increase in EEG activity.

Most previous studies of unanaesthetised rat pups have not reported the presence of isoelectric periods in the EEG at the present postnatal ages. We expect that if isoelectric traces had been observed they would have been reported. Thus, the high incidence of isoelectric EEG periods in all of the present pups probably does not accurately reflect

when continuous EEG activity would normally become apparent. This difference may be due to experimental methodology or to improvements in EEG recording equipment and analytical techniques during the intervening 30 to 40 years. In addition, strain differences in the age of onset of continuous EEG activity might exist. Whether such differences could last throughout the developmental period is not apparent. However, it seems unlikely that the high incidence of isoelectric periods in the present pups would be due to rat strain.

It is probable, therefore, that exposure to halothane anaesthesia and/or associated factors such as hypoxaemia, hypercapnia, cardiovascular depression, altered thermal status or even the exposure to vapours from the fixative used to secure the facemask might be involved. Unfortunately the study design does not allow differentiation of direct effects of halothane or indirect effects of other factors on the EEG, despite the stable heart rates and body temperatures of the pups. Nevertheless, it is possible that halothane concentrations were sufficiently high to substantially suppress the electrical activity of the cerebral cortex. Differences between the three age groups could be related to differences in neurological immaturity, body size, water content and other physiological variables such as cerebral blood flow (see also Chapter 6). Although halothane does depress the EEG with regard to the frequency spectrum, it has not been reported to induce EEG burst suppression (Murrell *et al.*, 2008) unless presented in very high concentrations (i.e. endtidal halothane of 9%) (Michenfelder & Theye, 1975) and this possibility cannot be ruled out in developing rat pups without further study.

2) Increased EEG power with age

As shown by the Principal Component analysis there was an overall increase in power with age for all frequencies of the EEG investigated (1-30Hz) (Figure 3.3). Such widespread changes throughout the brain could be a sign of progressive maturation of neurological structures involved in the production of the EEG (i.e. increase in neuronal numbers, connectivity and myelination) as well as progressive maturation of the ascending reticular activating system (ARAS).

The ARAS has been shown to influence cortical arousal (Siegel, 2004), which can be defined as a tonic readiness in cerebral networks bringing neural circuits closer to

threshold rather than bringing about arousal and waking as such (Steriade & McCarley, 1990, 2005). This system consists of a variety of brainstem nuclei and their connections to the cortex via the thalamus, hypothalamus and basal forebrain. The reticular formation of the brainstem utilises glutamate as its primary neurotransmitter, while the pedunculopontine tegmental nucleus (PPT) and the laterodorsal tegmental nucleus (LDT) contain cholinergic cells (producing acetylcholine). Other brainstem nuclei exert serotonergic (raphe nuclei), noradrenergic (locus coeruleus) and dopaminergic (substantia nigra and ventral tegmental area) influences (Jones, 2003). There are two major neural pathways by which cortical arousal is achieved. There is a dorsal pathway via the thalamus and a ventral pathway via the hypothalamus and basal forebrain (Jones, 2003; Siegel, 2004). These pathways have been shown to use the following neurotransmitters and neuromodulators to activate the cortex: glutamate, acetylcholine, histamine, hypocretin (orexin) and nitric oxide (Jones, 2003; Siegel, 2004; Marino & Cudeiro, 2006).

Cholinergic PPT nucleus neurons have been shown to increase in cell area between 12 and 15 days, with peak hypertrophy occurring at around 15 days (Kobayashi *et al.*, 2004). These changes seem to overlap with a period of change in firing properties of PPT neurons (Kobayashi *et al.,* 2004). In addition, cholinergic neurons of the LDT seem to develop their cholinergic properties during the second week after birth and mature functionally thereafter (Ninomiya *et al.*, 2005).

Histamine H1 and H3 receptors have been reported to reach their adult levels by 16 or 23 days after birth, depending on the brain region investigated, although histamine brain concentrations have been reported to be high initially and declined from birth to reach adult levels by day 16 (Ryu *et al.*, 1995).

Orexin-A- and -B-like immunopositive cells and fibres in the lateral hypothalamic area (LHA) were not detected from 0 to 10 days after birth in rats, but were observed after 15 days (Yamamoto *et al.*, 2000). The prepro-orexin mRNA in the LHA also showed a marked increase between 15 and 20 days (Yamamoto *et al.*, 2000). This is also supported by the sharp increase observed in orexin immunoreactive axon density between 16 and 21 days after birth (Steininger et al., 2004).

Other systems of the ARAS are also anticipated to change with regard to their function as the rat matures. However, it is beyond the scope of this chapter to present a detailed review for each of the regions and neurotransmitters involved.

Inhibitory postsynaptic activity in the rat cannot be observed over the first ten days after birth (Sutor & Luhmann, 1995). Initially, activation of $GABA_A$ receptors of immature neurons leads to membrane depolarisation and can potentially lead to excitation (Owens & Kriegstein, 2002). There is a developmental transition of GABAergic neurotransmission over the first 7 to 14 days after birth (Cherubini *et al.*, 1991; Owens & Kriegstein, 2002; Rivera *et al.*, 2005) so that by at least the end of the second week hyperpolarizing inhibitory transmission can be observed. This switch seems to be related to KCC2, a neuronal chloride-extruding K^+/Cl^- co-transporter and related protein and mRNA, which have been reported to peak at 9 days after birth in the hippocampus and to increase between 1 and 15 days after birth in the cortex (Rivera *et al*., 2005).

Taking all these observations into consideration, it appears that the timing of the development of the ARAS and inhibitory activity in the CNS of the rat overlap with the development of REM-non-REM EEG patterns.

3) Responsiveness to tail clamping

5-7 day pups

As EEG activity was mainly absent in 5-7 day pups no cerebral response to tail clamping could be observed. Nevertheless, the animals responded to clamping, as amplitude and duration of electrical excursions in the raw trace increased significantly after clamping, suggesting that pups then took deeper slower breaths. Heart rate also tended to increase. Rat pups of this age showed movement and/or audible vocal responses both to EEG electrode placement and tail clamping. As these rat pups exhibited isoelectric traces, and more importantly were anaesthetised, the presence of such responses is not an indication of pain perception as such, and is much more likely to be due to subcortical activation of the autonomic nervous system and motor and vocal reflexes via nociceptive pathways (Livingston & Chambers, 2000). As EEG responses of older pups were not associated with audible vocalisations or more marked

movement responses, it is likely that the greater behavioural responses observed in 5-7 day pups were due to immature descending inhibition (Narsinghani & Anand, 2000; Fitzgerald & Beggs, 2001; Fitzgerald, 2005).

It is possible that noxious stimulation may cause arousal sufficient to induce an onset of EEG activity. However, this was not observed here in response to the supramaximal stimulus. The increased amplitude observed in ECoG excursions in 5-7 day pups in response to tail clamping is difficult to explain. As mentioned in the materials and methods section, the candidate speculates that these electrical excursions in 5-7 day pups were more likely to be breathing movement artefact than ECoG activity, so that the increase in amplitude in response to tail clamping may represent an increase in autonomic nervous system activity rather than increased arousal sufficient to lead to the onset of EEG activity.

12-14 and 21-22 day pups

Rat pups aged 12-14 and 21-22 days responded to the clamp with a significant increase in heart rate, changes in breathing pattern (as for 5-7 day pups) and movements. Despite the presence of isoelectric periods it was possible to obtain EEG spectra before and immediately after clamping. A qualitatively small response to clamping in 12-14 day pups was observed, as shown by the significant change only in F95 in response to clamping. This is in contrast to 21-22 day pups, which showed clearer cerebral responses to clamping as judged by significant changes both in the F50 and F95, and by the Principal Component analysis, which was able to discriminate between EEG epochs before and after clamping.

In contrast, several non-EEG studies have shown that young rats are in some respects more susceptible to potentially noxious stimulation than older rats (Holmberg & Schouenborg, 1996; Hu *et al.*, 1997; Narsinghani & Anand, 2000; Fitzgerald, 2005). However, these studies have mainly relied on behavioural parameters for pain assessment such as withdrawal reflexes, tail flick responses and paw lifting or licking. Although some reflex responses to noxious stimulation have been shown to correlate well with perceived unpleasantness in humans (Willer, 1977; Willer, 1984) this does not

show that these reflexes are associated with pain experience as such. Reflexes can occur in the unconscious animal, whereas an animal has to be conscious in order to experience pain. Hence, behavioural data may not be helpful in elucidating whether newborn animals experience pain and of what character such pain may be in comparison to the adult animal. However, various studies have shown that the EEG responses to potentially noxious stimulation are demonstrable in anaesthetised animals (Murrell *et al.*, 2003; Johnson *et al.*, 2005a; Johnson *et al.*, 2005b) and that they correspond to EEG responses to painful stimulation seen in conscious animals (Ong *et al.*, 1997).

As already mentioned in Chapter 2, previous studies investigating the cerebrocortical responsiveness of adult animals anaesthetised with halothane have observed an increase in F50 and F95, also known as EEG desynchronisation/arousal (Johnson *et al.*, 2005b; Orth *et al.*, 2005; Gibson *et al.*, 2007; Murrell *et al.*, 2007). A decrease in F50/F95, as observed in the present study, is commonly referred to as synchronisation or paradoxical arousal. This has also been observed in anaesthetised infants and children in response to skin incision (Oshima *et al.*, 1981). The causes of these differences between adult and young animals and humans apparently remain unclear (Otto, 2008). An additional complication is that in newborn and young lambs, which are neurologically mature at birth, the EEG responded to noxious stimulation with desynchronisation (Johnson *et al.*, 2005a; Johnson *et al.*, 2009). Further studies will be necessary to elucidate the causes of these differences.

The differences in cerebral responses to potentially noxious stimulation between rats of different ages could have several physiological causes. First, differences may be of a developmental nature. As detailed above, rat pups exhibit substantial neurological changes during the first 2 to 3 weeks after birth, which could account for the absence of an EEG response to clamping in the 5-7 day pups and the differences in response between the two older age groups, albeit, all observed in anaesthetised pups. Developmental changes in the reticular activating system may play a role in the differing responses between the two older groups. These could include changes in the levels of neurotransmitters or neuromodulators, receptor distribution or subtypes and myelination of cells. In addition, maturational changes in peripheral and central nociceptive pathways may be involved, or even age-related differences in tail diameter and tail innervation.

Second, possible actions of endogenous modulators of neural activity such as opioid receptor ligands and endogenous neuroactive steroids may contribute. It has been shown that opioid systems are functional by 10 days after birth (Kehoe & Blass, 1986; Blass, 1996) and that gustatory and olfactory stimulation through intraoral infusions of milk are capable of engaging central opioidergic systems (Blass & Fitzgerald, 1988). Postabsorptive mechanisms following milk ingestion are another possible means of opioidergic activation, which may occur via hydrolysis of milk-protein derived opioid receptor ligands, but this has not yet been fully demonstrated (Teschemacher *et al.*, 1997; Teschemacher, 2003). A recent study however, has shown that provision of casein-rich milk is a critical component in maintaining μ-receptor-mediated stress-induced analgesia (SIA) rather than switching to δ-receptor-mediated SIA which usually occurs at around the time of weaning (Goody & Kitchen, 2001). This seems to suggest that, at least at this time after birth, milk-derived compounds can reach the central nervous system and exert effects on opioid receptor populations. Overall, it may be possible that exogenous opioid peptides play a role in the central nervous system in developing animals, where such factors can potentially cross the intestinal wall into the circulation and in turn reach the central nervous system by crossing the blood brain barrier, if this should be sufficiently permeable. Therefore, such exogenous factors might also exert analgesic properties during the period of milk ingestion in rat pups. The reduced cerebral response observed in the younger animals in the present study could therefore be partly due to previous milk ingestion and associated release of opioid receptor ligands into the blood stream and central nervous system. The fact that 21-22 day pups were weaned just prior to the time of the experiment (one day earlier) is consistent with this idea. However, it may be argued that the functionality of the opioid modulation in pups from about 10 days after birth, and hence the potential for opioid modulation of pain perception, appears to be at odds with the suggestion presented later in this chapter, that conscious perception does not appear to be likely at this age (see below).

Alternatively, the differing responses of the 12-14 day and 21-22 day pups could be due to actions of neuroactive steroids. The neuroactive steroids allopregnanolone (5α-pregnane-3α-ol-20-one) and pregnanolone (5β-pregnane-3α-ol-20-one), both

progesterone metabolites, have potent anaesthetic, analgesic and sedative properties (Majewska, 1992; Paul & Purdy, 1992). They exert their effects via the $GABA_A$ receptor by enhancing GABA binding, bringing about increased inhibition in the central nervous system (Majewska, 1992; Paul & Purdy, 1992; Belelli & Lambert, 2005). Interestingly, allopregnanolone has been reported to be mildly elevated in the rat pup cerebral cortex at 10-14 days after birth (Grobin & Morrow, 2001), which is consistent with allopregnanolone involvement in the different EEG responses observed here. However, Grobin and Morrow (2001) found that $GABA_A$ receptor-mediated Cl^- influx was depressed in cortical tissue from 12 day-old rat pups, suggesting that these receptors were less sensitive, which they suggested could be ascribed to changes in subunit composition or cell loss due to apoptosis. Nevertheless, it is possible that elevated levels of allopregnanolone at this time may make up for the receptors' low sensitivity thereby still exerting some degree of neuroinhibition. Further research would be necessary to elucidate this.

Third, as already discussed, the age-related differences in the cerebral response to tail clamping could be due to differential effects of halothane anaesthesia acting on the central nervous system of rats at different stages of neurobiological development.

Naturally, changes in the responses to clamping may be due to a combination of the above factors rather than a single one.

Development of conscious perception

It has been suggested by Evans (2003) that, as sleep is a form of unconsciousness, the mechanisms that underlie it are major contributors to the physiological bases for consciousness and unconsciousness (see Chapter 1).

Researchers have reported active sleep and quiet sleep periods in rats over the first 1-2 weeks after birth, which are interspersed by so-called periods of wakefulness. These states are identified using behaviours usually seen in EEG-differentiated sleep-wake cycles (Frank & Heller, 2003), for example, myoclonic twitching against a background of muscle atonia (sleep) and behaviours such as locomotion and stretching against a background of high muscle tone (wakefulness) (Blumberg et al., 2005; Karlsson et al.,

2005). It has been demonstrated that neural mechanisms that govern these behavioural states in rat pups at one week of age are similar to those governing sleep-wake cycles in adult rats (Karlsson et al., 2005; Seelke et al., 2005). However, the EEG of rats during the first 10-12 days after birth does not exhibit REM-non-REM differentiation and consists of a pattern that seems to represent undifferentiated sleep (Titkov et al., 2005). According to this evidence it is seems unlikely that these animals would be capable of conscious perception and hence capable of *experiencing* pain. Although sensory information reaches higher brain centres at this age (see section on evoked potentials), it may well be possible that integration and interpretation of such information does not yet occur due to immaturity of the nervous system.

REM and non-REM sleep and wake patterns differentiate between days 12 and 18 and this is associated with major changes in neurophysiology as outlined above. However, it is not known when, during this period, wakefulness may be conscious as opposed to unconscious (i.e. when rat pups begin to perceive consciously) (Zeman, 2001; Evans, 2003). Judging by the presence of basic exploratory behaviour and interactions with littermates via allogrooming, in addition to the above-mentioned evidence for sleep-wake cycle development, basic conscious perception may be present from as early as 12-14 days. At this time the pups can leave the nest faster than the mother can retrieve them (Bolles & Woods, 1964) and hence, by then, they may need to be capable of some level of perception in order to return to the nest and survive. Whether such perception has to be conscious in order for the pups to return is not clear. A simultaneous ability to consciously experience pain may enhance the ability of the pups to avoid harm and may thereby aid survival once maternal protection is no longer assured. In addition, rat pups aged 15-16 days begin to display differences between REM sleep and waking behaviours, which are apparently accompanied by the emergence of two neuronal populations of which one is REM sleep specific and the other mainly wake specific (Corner, 1990). These data suggest that conscious waking behaviour may become more pronounced around day 15 or 16. This is supported by rat pup behaviour at this age. Pups have been described as being very active at this age, exploring their living space and being interested in and manipulating objects (Bolles &Woods, 1964).

By days 18-20, adult-like sleep-wake patterns are present, which suggests that animals at this age and older are likely to be capable of conscious perception. This may explain

why Bolles and Woods (1964) found that rats only started noticing their observers and interacting with them from about day 19 onwards.

Finally, conscious perception may develop gradually, not as an on-off phenomenon, so that once the capability for it is present, levels of conscious perception may increase as brain function matures. Overall though, we do not know enough about the physiological bases of conscious perception to do more than speculate about this at present.

1) Comparison with fetuses that are neurologically mature at birth

In fetuses of animal species that bear precocial young, such as sheep, REM-non-REM EEG differentiation occurs before birth (Mellor *et al*., 2005) and prior EEG activity shows a similar pattern of development to that of the postnatal rat pup. Initially, pre-cortical and cortical structures remain electrically silent, and this is followed by irregular electrical spikes and short epochs of sustained EEG activity. Continuous undifferentiated EEG activity then develops and subsequently differentiates into the two discrete states resembling REM and non-REM sleep. These sleep-like states occupy 95% of the fetal EEG in late pregnancy (Mellor *et al*., 2005). Although the other 5% have previously been interpreted as periods of wakefulness, recent evaluations suggest that they represent sleep state transitions instead (Mellor *et al*., 2005). Therefore, sleep-wake cycles, and thus conscious perception, do not appear to occur before birth, only becoming evident for the first time after birth in sheep (Mellor & Diesch, 2006).

It follows from the above that equivalent stages of EEG differentiation occur prenatally in sheep and postnatally in rats, and that conscious or unconscious wakefulness becomes apparent in rat pups at or soon after REM-non-REM differentiation, but not in fetal lambs. This could be related to the different physiological environments of the brain in postnatal rat pups and prenatal lambs. There is evidence that the lamb is maintained in sleep-like states throughout pregnancy, and even throughout labour, by a variety of suppressive factors that inhibit neural activity, including adenosine, neuroactive steroids, prostaglandin D_2, a possible placental inhibitor, warmth and cushioned tactile stimulation of the uterine environment (Mellor et al, 2005). The onset of conscious perception occurs after birth and is associated with the withdrawal of neuroinhibitors and a concomitant increase in neuroactivators and novel sensory

stimulation (Mellor & Diesch, 2006). In contrast, rat pups are not exposed to a plethora of neurosuppressors at the time of EEG differentiation, like those seen in the sheep fetus. Thus, it is not surprising that wakefulness can be observed during this time. Although the neuroactive steroid allopregnanolone is slightly elevated around 10 to 14 days (Grobin & Morrow, 2001) and might exert some form of neurosuppression, its concentrations are apparently not sufficient to abolish wakefulness.

Experimental limitations

- Age is a major confounding factor as this is associated with developmental changes which may have impacted on the effect of anaesthesia on the different age groups. This has been discussed in the general discussion in Chapter 7 and will hence not be elaborated on here.

- A supplementary study to assess the impact of a range of halothane concentrations on pup EEGs at different ages in the absence of clamping would have been helpful to determine the extent of cortical suppression by halothane in immature rats.

- As animals were too small for intubation within the present laboratory set-up, end tidal halothane partial pressure could not be monitored and normalisation of anaesthetic depth was not possible. However, even if intubation had been possible, equal halothane end tidal partial pressures would not necessarily have indicated that anaesthetic depth was equal between the pups of the different ages, as MAC has previously been shown to change during development (Gregory et al., 1969; Lerman et al., 1983; Lerman et al., 1986; Orliaguet et al., 2001). Although depth of anaesthesia was not assessed during the study, the fact that movement in response to noxious stimulation was present in 46 to 93% of animals (depending on age) suggests that a light plane of anaesthesia was maintained. It is possible however that animals became hypoxic during the experiment as pups were not intubated and hence not ventilated. Hypoxaemia could have led to an isoelectric EEG.

- Tail diameter may or may not have affected the results. Nociceptive input may

be related to tail diameter and hence differences in results between 12-14 day and 21-22 day pups may be related to these differences rather than differences in cerebral processing *per se*. However, it is possible that a smaller tail diameter being stimulated with the same haemostat clamp would be exposed to a greater proportionate area of contact possibly leading to greater nociceptive input than stimulation of a tail with a larger diameter. Thus, in these terms, responses to clamping might have been expected to be greater in the 12-14 day pups than the 21-22 day pups. In addition, it has to be acknowledged that tail diameter differences between the rat pups of the different ages may not be sufficiently great to lead to significantly different nociceptive input.

- Due to cranial to caudal development of the nervous system, stimulation of the forelimb may have been a more appropriate stimulus.

- The fact that electrode placement differed between age groups could also have impacted on the results of the present study. However, as the youngest rat pups showed an isoelectric EEG, it is anticipated that recordings using subdermal electrodes rather than intracranial electrodes would not have provided different results. In addition, the major results of the present study, namely the differences in response between 12-14 day and 21-22 day pups, would not have been affected by these differences, as electrode placement was the same for these two age groups.

Suggestions for improvements and future research

- A pilot study could have been undertaken to measure the EEG in non-anaesthetised rat pups. Comparisons between these EEGs and those of anaesthetised rat pups could subsequently be used to evaluate effects of halothane on the EEG. However, EEG recordings in non-anaesthetised animals can also be problematic, as is discussed in Chapter 4.

- Whether EEG excursions in the youngest pups were related to breathing movements could have been investigated in a subset of rat pups by administering a neuromuscular block and ventilating the rats, if adequate tools

for intubation could be sourced and intubation were successful.

- The collection of brains of rat pups exposed to noxious stimulation may be informative should further studies in this area be undertaken. These brains could have been assayed for the induction of c-fos activity to assess whether and which brain centres are activated in response to noxious stimulation in developing rat pups (Bullitt, 1990).

3.5 Conclusions

Although the results of the present study do not inform us directly about whether or not rat pups are able to experience pain, they do suggest that there may be differences in the amount and character or nature of the pain experienced by newborn rats of different ages. This could be explained by developmental differences and/or the presence of endogenous factors that may have the capacity to affect neural activity directly or have antinociceptive properties. However, the extent to which exposure to halothane anaesthesia might have affected the results of the present study remains uncertain. Nevertheless, on the basis of the present results and the related literature traversed here, it is possible to make the following suggestions.

1) Absence of a differentiated EEG, together with the neurological and behavioural evidence for cerebral immaturity, strongly suggest that conscious perception and an associated ability to experience pain in rat pups younger than 10-12 days is doubtful.

2) Although neurological development is on-going between 10 and 18 days after birth and REM-non-REM sleep cycles are still differentiating, the marginal response to tail clamping in 12-14 day rat pups, together with the behavioural repertoire when not anaesthetised (Bolles and Wood, 1964), suggest that pups might be capable of experiencing pain from this age onwards. Although this experience may be qualitatively different from that of mature animals, it may nevertheless cause negative mental states or suffering. On the basis of the precautionary principle, therefore, pain relief should be provided when significantly invasive procedures are conducted on rat pups of this age.

3) The adult-like sleep-wake EEG patterns present in rat pups aged from days 18-20, pup behaviour at that age and the cerebral responses observed in the present study suggest that animals older than 18 days are normally capable of conscious perception and hence are able to experience pain.

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- Neurologically mature at birth –

Lambs

Chapter 4

Abstract

Sheep are a precocial species, in which by definition most neurological development occurs before birth. However, conscious perception appears to be precluded due to the operation *in utero* of a range of potent neuroinhibitory mechanisms that maintain states of unconsciousness in the mature fetus throughout the last half of pregnancy. Hence, the evidence is that conscious perception usually does not occur until some time after birth. However, when after birth consciousness, and thus conscious perception, may occur for the first time is, as far as can be ascertained, not known. Behavioural arousal is observed in the normal healthy newborn very soon after the onset of rhythmic breathing and is associated with behaviour that is suggestive of conscious perception, such as interactions with the dam and, once the lamb is able to stand successfully, seeking the teat. However, it is possible that full consciousness is *not* necessary for such behaviours to occur. Increased arousal may not necessarily include conscious perception, as it is possible to be awake but unconscious.

The present study measured EEG activity and behaviour in lambs 3 to 30 minutes, 1 to 4 hours and 1 to 2 days after birth. This was done to determine whether changes occurred in the EEG over the first few minutes and days after birth in an animal species born neurologically mature. Such changes, if present, may indicate neurological maturation beyond birth associated with exposure to a new environment or the potential for modulation of consciousness perception for minutes or hours after birth. Over the first 30 minutes after birth lambs were in an aroused state and non-REM sleep was apparently not common. Lambs aged 1 to 4 hours and 1 to 2 days however showed an increasing proportion of non-REM sleep and a concomitant decrease in arousal. In addition, there was an increase in EEG total power with time after birth indicating waning of modulation of conscious perception and/or neurological maturation. Although it cannot be ascertained whether arousal observed in the present lambs is associated with consciousness or not, it is suggested that the onset of conscious perception is a gradual process and that therefore full conscious perception is probably not apparent before 5 minutes after birth and that it may take up to several hours for it to become fully established. This however does not necessarily indicate that suffering would not be present in these animals and the benefit of doubt should be given if harmful procedures are to be undertaken.

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Ontogeny of the electroencephalogram (EEG) and associated behaviours of lambs over the first 2 days after birth

4.1 Introduction

As already mentioned in previous chapters, sentience and consciousness are prerequisites for suffering, meaning that an animal, which is neither sentient nor conscious, is not capable of experiencing negative mental states, such as pain. Therefore, when during ontogeny animals develop the capacity to suffer will depend on when they become sentient and consciously aware, i.e. when they have the capacity to consciously perceive sensations. This, in turn, depends on when during ontogeny underlying neurological processes develop and become functional.

Sheep are a precocial species, in which by definition most neurological development occurs before birth. During late gestation the sensory systems of the fetal lamb, including audition, proprioception, gustation, olfaction and cutaneous senses, are well developed and functional (Bradley & Mistretta, 1975). In addition, differentiation of REM-non-REM[1] sleep-like EEG patterns and behaviour occur well before birth from about 115 days of gestation (Clewlow *et al.*, 1983; Szeto & Hinman, 1985). Arriving at this neurophysiological stage prenatally indicates, as outlined above (Chapters 1, 2 and 3) and elsewhere (Mellor & Diesch, 2006), that fetal lambs develop the capacity for sentience before birth. However, conscious perception appears to be precluded due to the operation *in utero* of a range of potent neuroinhibitory mechanisms that maintain states of unconsciousness in the mature fetus throughout the last half of pregnancy (Mellor *et al.*, 2005). Hence, the evidence suggests that conscious perception usually does not occur until some time after birth (Mellor *et al.*, 2005; Mellor & Diesch, 2006) (see Chapter 1). However, when after birth consciousness, and thus conscious perception, may occur for the first time is, as far as can be ascertained, not known.

The rupture of the umbilical cord at birth is associated with the loss of the placental supply of oxygen and an associated increase in carbon dioxide tensions in the newborn,

[1] Rapid eye movement and non-rapid eye movement sleep

all of which apparently play a role in the onset of breathing at birth (Mellor & Diesch, 2006). While the loss of the placenta also means that placental supply of the potent neuroinhibitor adenosine will be lost, hypoxaemia, occurring as a result of any delay in the onset of breathing, will lead to a transient increase in adenosine concentrations until breathing has finally commenced. Thus, before the onset of breathing, oxygenation of the brain will not be sufficient to support conscious processes and adenosine concentrations will be increased, thereby exerting neuroinhibitory effects. This is supported by absence of behavioural signs of consciousness immediately after birth when the newborn lamb lies immobile on the ground (Photograph 1) or when it first lifts and/or shakes its head before the onset of rhythmic breathing (Mellor *et al.*, 2009). Even after rhythmic breathing has been established and once the lamb rights itself to sternal recumbency with its head up (Photograph 1), full consciousness seems unlikely at this point (Mellor *et al.*, 2009), until arterial oxygen tensions rise above and adenosine concentrations fall sufficiently below the threshold for awareness (Mellor & Gregory, 2003). Arterial oxygen tension threshold levels for awareness in mature postnatal animals appear to be around 28mmHg (Brierly *et al.*, 1980; West *et al.*, 1984; Hattingh *et al.*, 1986), which in the newborn lamb is reached within about the first 15 minutes after birth (Mellor & Pearson, 1977; Berger *et al.*, 1990). However, more recent studies in human infants showed that oxygen saturation reaches 90% within the first 5 minutes after birth (Kamil *et al.*, 2006; Mariani *et al.*, 2007; Altuncu *et al.*, 2008). In addition, adenosine has a very short half-life so that the onset of breathing would lead to a rapid decrease in its circulating and central concentrations (Moser *et al.*, 1989), thereby lifting any major neuroinhibition exerted via adenosine. Thus, conscious perception may be possible as soon as several minutes after birth. However, the presence of other residual neuroinhibitory factors may modulate this.

Arousal is observed in the normal healthy newborn very soon after the onset of rhythmic breathing and is associated with behaviour that is suggestive of conscious perception, such as interactions with the dam and, once the lamb is able to stand successfully, seeking the teat. However, it is possible that full consciousness is *not* necessary for such behaviours to occur. Increased arousal may not necessarily include conscious perception, as it is possible to be awake but unconscious (Zeman, 2001; Mellor *et al.*, 2005).

Photograph 1: Twin lambs soon after birth with the first-born twin in sternal recumbency holding its head up and the second-born twin still lying immobile on the group. (With permission by Mark Oliver, Auckland University, Auckland, New Zealand)

In support of this, animals that are born extremely immature, such as marsupial pouch young (Chapter 2), and apparently even those born moderately immature, such as rat pups (Chapter 3), have the capacity to orient towards and attach to the nipple in the absence of higher brain structures (marsupial joeys) or the interconnections that are necessary for conscious perception (e.g. rat pups). Thus, consciousness is apparently not necessary for such behaviours suggesting that they are predominantly reflex in character in such newborns. In lambs, teat seeking and sucking are, at least in part, mediated by a variety of reflex mechanisms. Initially the lamb orientates itself towards the mother

helped by auditory and visual cues and begins to explore her body (Nowak, 2006). Tactile stimulation of the face apparently promotes oral exploration. In addition, heat emanating from the udder and olfactory cues from the inguinal region of the ewe are also important factors in guiding the lamb to locate the teat (Nowak, 2006). However, the presence of reflex mechanisms during teat seeking and bonding does not preclude consciousness. Just because teat seeking occurs in the newborn marsupial pouch young and young rat pups apparently without consciousness does not necessarily imply that consciousness is absent in neurologically mature newborns such as lambs.

As noted above, REM-non-REM differentiation has been used previously (Chapters 2 and 3) to indicate the earliest stage at which brain mechanisms may have matured sufficiently to support conscious perception in animals born neurologically immature. This is because neural functions required for REM and non-REM sleep are linked to brain mechanisms that also support conscious perception (Evans, 2003). However, this approach will not aid in answering the question of when, after birth, neurologically mature newborns may attain the capacity for conscious perception for the first time, as REM-non-REM differentiation is already complete by the time of birth in these animals.

The electroencephalogram (EEG) may nevertheless provide useful information for estimating the time at which consciousness may first appear after birth. However, it appears that such EEG data are not presently available.

The aim of the present study was to measure EEG activity and behaviour in lambs as soon as possible after birth for up to 30 minutes. For comparisons, EEGs were also recorded in lambs after they had their first intake of colostrum. In addition, the EEG one to two days after birth was recorded in several lambs. This was done to determine whether changes occurred in the EEG of an animal species born neurologically mature over the first few minutes and days after birth. Such changes, if present, may indicate neurological development beyond birth associated with exposure to a new environment or the potential for modulation of consciousness perception for minutes or even days after birth.

4.2 Materials and Methods

The research was carried out on Massey University Sheep Farms in the Manawatu region of New Zealand, and was approved by the Massey University Animal Ethics Committee (Protocol 06/40), Palmerston North, New Zealand.

Animals

Romney ewes (n=75) were mated in autumn (April) to lamb over a 3-week period in spring (September). Sheep were maintained on pasture throughout pregnancy and thereafter.

Ewes close to lambing were inspected for signs of labour at regular intervals (approximately every 15 minutes) for 20 hours a day. Signs included pawing at the ground, repeatedly standing up and lying down, lying with one hind leg stretched out with or without lifting the head while displaying abdominal straining, presence of a fluid filled bag protruding from the vulva or broken membranes with fluid leaking from the vulva, and the presence of the lamb's head and/or front hooves just inside, or visibly outside the vulva.

Once ewes were close to birth they were calmly herded into a semi-enclosed barn, where they were offered *ad libitum* access to water and where they were kept for several hours after giving birth to ensure bonding with their lambs. Once it was ensured that bonding had occurred and lambs were sucking successfully the animals were returned to pasture.

Experimental procedure

The time when lambs were completely free of the birth canal was recorded as the time of birth. The lambs were then brought to the recording area, where they were held gently but firmly by an assistant for the entire observation period (up to 30 minutes after birth). Although it was attempted to keep stimulation to a minimum this was not always feasible.

Electrodes for electroencephalographic (EEG) recordings were placed and EEG

recording commenced immediately. A video camera was used to record the behaviour of the lambs. The EEG was recorded until 30 minutes after birth. If lambs remained relatively immobile, 30 minutes of EEG were recorded. However, if lambs struggle or otherwise became active before this time, EEG recording was terminated early. This was done as the EEG traces of such animals were contaminated with movement artefacts and hence could not be used for analyses.

After the initial recordings were concluded, rectal temperature of the lamb was measured using a digital thermometer (Becton Dickinson Ltd., Auckland, New Zealand). The lamb was then weighed and subsequently a 5ml blood sample was taken from the jugular vein into a sodium heparin vacutainer (Becton Dickinson Ltd., Auckland, New Zealand). Blood samples were put on ice immediately or processed within several minutes after taking them (see below). Lamb gender was noted and the animal was then returned to its dam.

Blood samples were mixed gently and a drop of blood was removed from the heparinised tube to measure blood glucose concentrations using a glucometer (Accu-Chek Advantage System; Roche Diagnostics, Auckland, New Zealand). Packed cell volume (PCV) was measured in two capillary tubes using a purpose-built centrifuge and specialised PCV reader. The remaining blood was centrifuged for 15 minutes at 3,000rpm and the plasma transferred to 1.5 ml Eppendorff tubes. Plasma samples were frozen at -20°C to await further analysis.

In case of twins or triplets, the first-born lamb was usually used for experimental purposes. However, if recordings were already underway using a lamb of another dam, the twin or second triplet were also used. All lambs were removed from the dam and were returned simultaneously after the first EEG recording. Lambs were rubbed together to allow spread of the aroma of amniotic fluid and to facilitate bonding of the mother with all of her lambs. All lambs, except one which was subsequently hand reared, were successfully bonded to their dams by the end of the experiment.

Lambs were aided in sucking where necessary. Once the lambs had a suck and had become sleepy another 5 to 15 minutes of EEG were recorded (i.e. between 1 and 4 hours after birth) and, when otherwise possible (i.e. when there were no newborn lamb

EEGs being recorded). A second record was not obtained from all lambs that also had a birth record taken. In five lambs, ten minutes of EEG were recorded at about one day (n=2) or about two days (n=3) after birth. These lambs did not have a prior birth record taken.

EEG recordings

In order for electrodes to be placed quickly after birth (i.e. to improve ease of electrode placement), amniotic fluid was removed from the head of the lamb using a towel or head wool was cut short at the sites of electrode placement with an electric shaver. Stainless steel electrodes (Medelec, Oxford Instruments Medical Systems, UK) were placed at the midline over the frontal sinus (non-inverting electrode), over the left mastoid process (inverting electrode) and caudal to the occipital process (common electrode) and were secured with cyanoacrylate adhesive (Selleys, Auckland, New Zealand) onto the wool of the lamb.

The electroencephalogram (EEG) was recorded continuously at a sampling rate of 1kHz using an Apple Macintosh personal computer and Powerlab 4/20 data recording system (Powerlab^{TM} data acquisition system®, AD Instruments Ltd, Bella Vista NSW, Australia) using compatible recording software (Chart 5, Powerlab^{TM} data acquisition system®, AD Instruments Ltd, Bella Vista NSW, Australia). Isolated biological amplifiers (World Precision Instruments Inc., Florida, USA) were set at a gain of 1000 with a low-pass filter of 0.1Hz and a high-pass filter of 0.5kHz. Analysis of EEG data was done off-line after completion of the experiment.

EEG analysis

Large segments of EEG records were not useable for analysis due to movement artefact. Useable EEG segments of EEG records were obtained from 16 lambs up to 30 minutes, from 12 lambs 1 to 4 hours and from 5 lambs 1 to 2 days after birth.

For all artefact-free EEG epochs longer than 3 seconds in duration Fast Fourier Transform (FFT) analysis was performed. The EEGs between 3 and 30 minutes after birth were categorised visually, where possible, according to whether low voltage high

frequency (LVHF), high voltage low frequency (HVLF) or an intermediate (INT) EEG was present (Figure 4.1). HVLF was not very common. The numbers of useable EEG data points per period per EEG state were calculated and are presented as a percentage of total number of data points per period in Table 4.3, Section 4.3. In addition, the state each lamb was in was identified with useable EEG segments as soon as possible after birth (~ 3 minutes) and at about 5, 10, 15, 20, 25 and 30 minutes after birth. If artefact was present at that prescribed time point the state of the nearest useable EEG segment thereafter was used. The number of lambs whose EEGs showed the characteristics of LVHF, INT and HVLF states at the time points above are presented in Table 4.4, Section 4.3.

For lambs 1 to 4 hrs and 1 to 2 days after birth, EEG traces were again inspected for EEG epochs without artefact and subjected to FFT analysis. Few periods of artefact were present in lambs 1 to 2 days. In lambs of both age ranges the non-artefactual data were categorised according to the presence of LVHF, INT or HVLF EEG segments.

Spectral analysis was done using a specialised FFT program (Spectral Analyser, CB Johnson, Massey University, New Zealand, 2006). The program calculated the median frequency (F50), spectral edge frequency (F95) and total power (Ptot) for consecutive 1-second epochs as well as the power for frequencies of 1 to 30Hz.

In order to account for the relative contribution of each frequency (1 to 30Hz) to total power, the power of the spectrum within specified frequency ranges was calculated as a percentage of total power, and has been referred to in the following sections as ‘relative power’. Thus, the relative power was calculated for the frequency ranges of 1-3Hz, 4-6Hz, 7-9Hz, 10-12Hz, 13-15Hz, 16-18Hz, 19-21Hz, 22-24Hz, 25-27Hz and 28-30Hz. The purpose was to reduce the size of the data set and to facilitate presentation.

In the other experimental chapters of this thesis (Chapters 2, 3 and 6) relative power was not calculated and unadjusted values were used for statistical analyses. This was done, as comparisons were made between pre-and post stimulating events within individual animals rather than between different age groups represented by different animals. As the present study was aimed at investigating age differences in EEG parameters, relative power for each frequency range was calculated and used for

statistical analyses, instead of the initial EEG power output, to help ensure that any effects on EEG power from the recording set-up or other external sources would have minimal effects on the data to be compared.

Lamb behaviour analysis

The behaviour of lambs between 3 and 30 minutes after birth (n=24) and between 1 and 4 hrs after birth (n=14) was investigated. The behavioural analysis was not restricted to those lambs from which EEG data were analysed. Video records were not available for lambs 1 to 2 days after birth. For lambs 1 to 4 hours after birth a 5-minute-period of the video was selected for analysis, usually starting around 4 to 5 minutes after recording started unless the recording duration was too short for this approach.

After watching several of the videos an ethogram (i.e. catalogue of behaviours) was created for the purpose of this study. It included behaviours that were related to the onset of breathing and those that appeared to be associated with increased or decreased arousal.

Behaviours where frequency of occurrence was measured

a) Behaviours associated with the onset of breathing

Sneezing

Head shaking

b) Behaviours associated with increased arousal

Head movements

Vocalisations

Whole body movements (struggle, swimming type of movement involving lunging forward)

Sucking attempts

Behavioural states (where the proportion of time displayed was measured)

Eyes open/closed - closed suggesting decreased arousal

Head up/resting - resting suggesting decreased arousal

Continuous focal sampling of video records was done using JWatcher V1.0 Behaviour Analysis Software for Macintosh operating systems (http://galliform.psy.mq.edu.au/jwatcher/).

Plasma sample analysis

Plasma was analysed for lactate concentrations by an enzyme reaction technique using a commercial kit (Lactate kit #11822837; Roche Diagnostics Ltd., Auckland, New Zealand). During this procedure L-lactate is oxidized to pyruvate by the enzyme lactate oxidase. The hydrogen peroxide produced by this reaction is then used in an enzymatic reaction to generate a coloured dye. The intensity of the colour formed is proportional to the L-lactate concentration. Plasma lactate concentrations were determined in duplicate for each sample and the two values were then averaged.

Statistical analysis

Statistical analysis was performed in SPSS 11.0 for MAC OSX (SPSS Inc., Chicago ILL, USA) and Prism 4.0 for MAC OSX (GraphPad Software Inc., San Diego CA, USA). Data are presented as means ± standard error of the mean (SEM). Differences were considered significant at $p<0.05$.

1) General information

The birth data gathered, including rectal temperature, weight, PCV, and plasma glucose and plasma lactate concentrations, were statistically assessed by comparing values of these variables between lambs that were 1) assisted at birth and those that were not, 2) between female and male lambs, and 3) between single lambs and multiples.

The Shapiro-Wilks test and normal probability plots were used to assess normality of the data. Levene's test was used to assess the variances of the data where those were normally distributed. For parameters that were normally distributed and had equal variances (homogeneity of variances) two-tailed independent sample t-tests were used to assess differences between the groups. For those parameters where this was not the case, logarithmic or square root transformations were done. If these were successful and

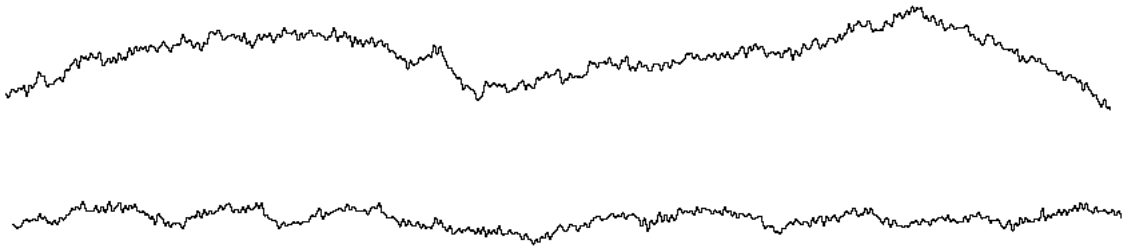
produced normally distributed data with homogenous variances the two-tailed independent sample t-test was again used, while for those where normality and homogeneity could not be achieved the non-parametric Mann-Whitney test was used to assess differences between the groups.

2) EEG

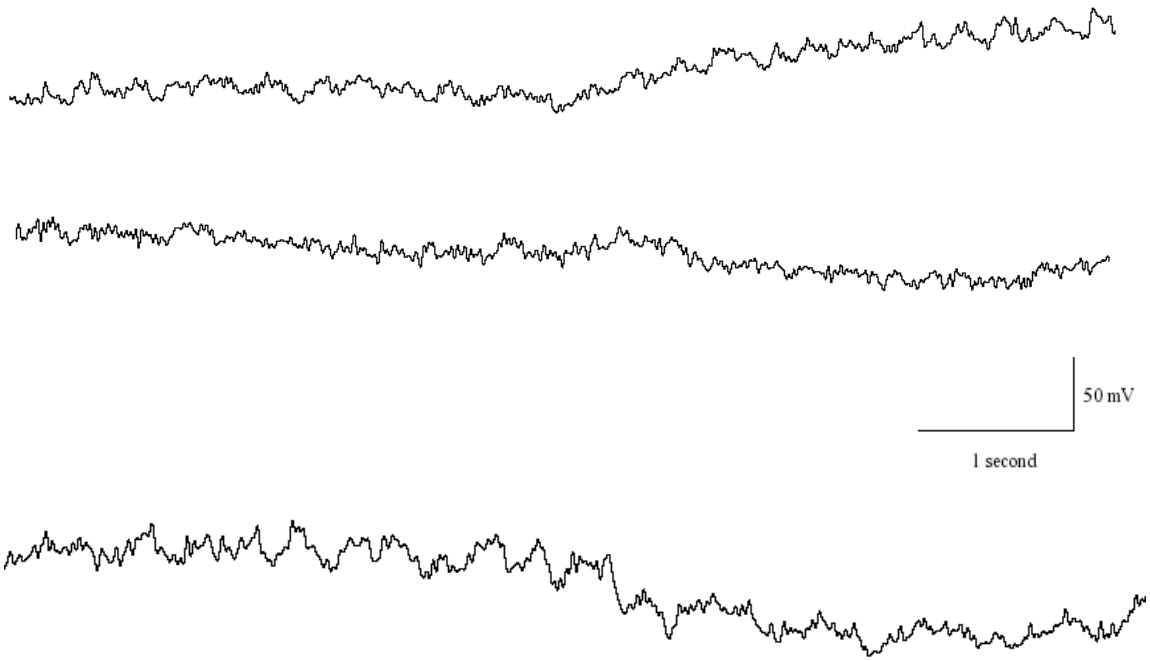
The mean value for each EEG parameter was calculated for each animal for each state and each period of observation (time after birth). This mean value was then used for further statistical analysis.

Before statistical analyses were done, data were tested for normal distribution and homogeneity of variance. This was done using the Kolmogorov-Smirnoff test with Lillifors significance corrections ($n>50$), Shapiro Wilks test ($n<50$) and normality plots as well as the Levene's test, respectively. Where necessary, transformations were done to create a normally distributed data set with homogenous variance. For F50, F95 and Ptot a logarithmic transformation was undertaken for this purpose. As relative EEG power for the ten frequency bands was presented as percentage and therefore did not follow a normal distribution, these parameters also underwent transformations. Here, the proportion of each data point (data point divided by 100) was calculated followed by an arcsine (inverse sine) square root transformation (Martin and Bateson, 1998). Transformed data were again tested for normality and homogeneity by the tests mentioned above.

Low Voltage High Frequency EEG (LVHF)



Intermediate EEG (INT)



High Voltage Low Frequency EEG (HVLF)

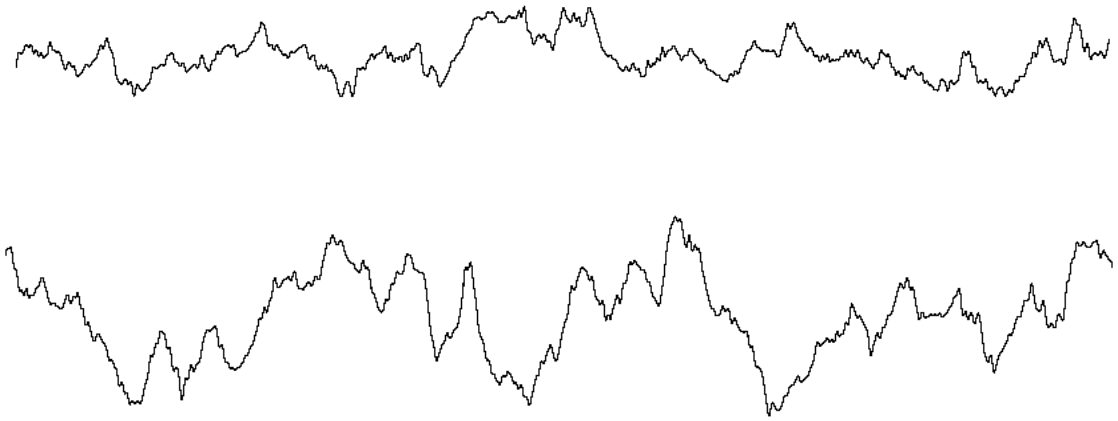


Figure 4.1: EEG traces of newborn (3 to 30 minutes) and young (1 to 4 hours) lambs showing the differences between LVHF, HVLF and INT EEGs

Comparisons within age groups

Changes in EEG parameters over the first 3 to 30 min after birth

The birth EEG data (3 to 30 minutes after birth) were divided into six periods equivalent to 3 to 5, 5 to 10, 10 to 15, 15 to 20, 20 to 25 and 25 to 30 minutes after birth as well as two periods equivalent to 3 to 15min and 15 to 30 min after birth.

As only few animals (n=4) exhibited HVLF EEGs for short periods of the time, this state was not included in the statistical analyses and the comparison between the age groups over the first 30 minutes after birth was restricted to LVHF and INT EEGs.

There was an uneven number of lambs contributing data to the six EEG periods immediately after birth, and within those an uneven number of lambs contributing to the three EEG states, so that the number of data points per period and per state varied greatly (see Table 4.4, Section 4.3).

Six periods

For both, LVHF and INT EEGs, one-way analyses of variance (ANOVA) with Tukey post-hoc tests were performed on transformed spectral data and a non-parametric Kruskal Wallis test with post-hoc Dunn's tests was done for non-transformed data. Due to the uneven distribution of data over the six periods and the two states, there were too many missing values to perform a repeated measure ANOVA or the non-parametric equivalent, the Friedman's test, which may be a more adequate test for differences in the lambs over time.

In addition, a multivariate Principal Component (PC) Analysis was used to further elucidate the changes in EEG power that may have occurred over the first 30 minutes after birth. This was done separately for LVHF and INT EEG data. Data calculated for the ten frequency bands were transformed first by adjusting them to a proportion followed by an arcsine square root transformation for each value. The PC loadings were evaluated and the PC scores were plotted against each other in reduced dimensions to explore the data.

Two periods

For both, LVHF and INT EEGs, independent sample t-tests were performed for transformed data, while the non-parametric Mann-Whitney test was performed for non-transformed data. In addition, for animals for which data were available for both periods, the related samples t-test or the non-parametric two-related samples Wilcoxon test was performed.

Differences between EEG states in lambs 3 to 30 minutes, 1 to 4 hours and 1 to 2 days after birth

Differences in spectral parameters (F50, F95 and Ptot) and the ten frequency bands between EEG states per age group were investigated. Mean data were used assessing the differences between LVHF and INT in lambs 3 to 30 minutes after birth, between LVHF, INT and HVLF in lambs 1 to 4 hours and between INT and HVLF in lambs 1 to 2 days. Where two states were compared independent sample t-tests were performed on transformed data, while non-parametric Mann-Whitney tests were performed for non-transformed data. For 1 to 4hr lambs, one-way ANOVA with Tukey post-hoc test was done for transformed data and the non-parametric Kruskal-Wallis test with post-hoc Dunn's test for non-transformed data.

Comparisons between age groups

The EEG data within each of the three EEG states (LVHF, INT, HVLF) were compared between the three age groups (3 to 30 minutes, 1 to 4 hours and 1 to 2 days after birth) to determine whether changes occurred with time. As there were no significant differences over the first 3 to 30 minutes in any of the EEG parameters investigated (see results section), data of lambs of this age were not subdivided into any groups as was done in the pervious section.

Where two ages were compared (LVHF and HVLF) independent sample t-tests were performed on transformed data, while non-parametric Mann-Whitney tests were performed for non-transformed data. For age comparisons of INT EEG data, one-way ANOVA with Tukey post-hoc tests were done for transformed data and the non-parametric Kruskal-Wallis test with post-hoc Dunn's test was performed for non-transformed data.

3) Behaviour

Intra-observer reliability was assessed in a subset of lambs (n=24). This was done by re-scoring the behaviours of these lambs on a separate occasion (4 months after the initial scoring had been done) and comparing the original scores with the new scores for each behaviour/state using Spearman rank correlation (Martin & Bateson, 1998). A non-parametric test was used, as the data were not normally distributed.

Behavioural data were transformed into proportions of overall period length, while time spent with eyes open/closed and head up/resting were transformed into percentages of overall period length. This was done to take into consideration the duration of each period investigated (up to 300,000msec). First, non-parametric Spearman rank correlations between behaviours and states were undertaken to assess how the overall data related to each other. Spearman rank correlation coefficients (r) were used to judge the association between two sets of behaviours rather than the p-value obtained by these calculations. This was done, as small correlations (weak associations) can be significantly different if the sample size is large enough (Martin & Bateson, 1998). Hence, $r < 0.4$ indicates a low correlation, $r = 0.4\text{-}0.7$ a moderate correlation and $r > 0.7$ a high correlation (Martin & Bateson, 1998).

The scores for the behaviours/states recorded were then compared between ages. This was done using two age groups for lambs between 3 and 30 minutes after birth (i.e. 3 to 15 minutes plus 15 to 30 minutes) and data for lambs 1 to 4 hours. As data for all variables were not normally distributed, the non-parametric Friedman's test with the post-hoc Dunn's test were used to assess whether there were significant changes in any of the behaviours with age.

4) Correlation of EEG and behaviour data

Due to financial constraints, software to link the timing of video recordings with that of EEG recordings was not available for the present study. This would not be a problem if usable EEG segments of several minutes in duration were available so that overlap between EEG and behavioural recordings could be guaranteed. However, useable EEG

in the present study were usually of short duration (seconds rather than minutes) so that matching behaviour with EEG would have been inaccurate, as the linking could have been out by several seconds. Hence, it was not possible to assess behavioural changes associated with changes in EEG states, although this would have been desirable.

4.3 Results

General information

Overall, data from 40 lambs were collected. However, the EEGs of only 16 lambs 3 to 30 minutes after birth, 12 lambs 1 to 4 hours after birth, and 5 lambs 1 to 2 days after birth were usable for spectral analysis due to the high incidence of movement artefact in the EEG traces. In addition, one lamb had to be excluded from analyses due to a birth injury. The general information on the lambs which had their EEG data and/or behaviour assessed is presented in Table 4.1 below. Birth data for the 1 to 2 day-old lambs were not available.

Half of the ewes (n=12/24) were assisted during labour (i.e. lamb position adjusted or lamb pulled) due to lambs being too large for natural delivery (mainly singles) or due to malpresentation of the lambs (i.e. one leg and head only, tail only or tail and one leg).

Table 4.1: General information for lambs used in data analysis. PCV=packed cell volume; B=behaviour; 1st= EEG 3 to 30min after birth, 2nd=EEG 1 to 4hrs after birth.

Lamb #	Analysis	Litter size	Gender	Birth rank	Assistance Yes/No	Weight (Kg)	Rectal Temp (°C)	PCV (%)	Lactate (mmol/L)	Glucose (mmol/L)
2	1st EEG + B	Single	Male	1	Y	6.5	40.3			
6	1st EEG + B	Single	Male	1	Y	5.0	39.6	49	3.28	1.3
7	1st EEG + B	Twin	Male	1	Y	5.0	40.0	39	4.68	<0.6
9	B	Single	Male	1	N	6.8	40.1	43	4.82	1.1
12	2nd EEG +B	Single	Male	1	N	5.8	40.3	48.5	3.34	<0.6
16	B	Single	Female	1	Y	5.2	40.5	46	5.58	1.4
17	B	Single	Female	1	Y	5.8	40.6	53.5	3.32	1.1
18	B	Single	Female	1	N	5.6	40.4	46.5	3.71	2.2
21	2nd EEG + B	Twin	Female	2	Y	5.0	40.2	45.5	2.42	<0.6
22	1st + 2nd + B	Single	Male	1	N	6.4	39.5	46	2.13	0.6
23	1st + 2nd + B	Single	Male	1	N	6.2	39.4	45	4.11	<0.6
24	1st + 2nd + B	Twin	Male	1	N	4.4	40.8	52.5	2.65	<0.6
25	1st + 2nd + B	Single	Male	1	N	5.4	40.2	46.5	4.11	4.2
26	1st EEG + B	Twin	Female	2	Y	4.2	39.8	49	8.8	3.6
27	1st + 2nd + B	Triplet	Female	2	Y	4.4	39.1	35.5	6.22	5
28	1st EEG + B	Twin	Female	2	Y	3.2	40.9	49.5	4.15	0.6
29	1st + 2nd + B	Single	Male	1	Y	6.6	40.6	51	3.85	2.2
30	2nd EEG + B	Single	Male	1	Y	7.0	40.6	40	5.6	<0.6
32	1st + 2nd + B	Single	Female	1	N	5.2	40.6	49	3.7	1.1
34	1st EEG	Triplet	Male	3	Y	5.0	40.6	44	4.55	1.2
35	1st + 2nd + B	Twin	Male	1	N	5.5	40.1	42	3.18	<0.6
36	1st + 2nd + B	Twin	Male	2	N	5.0	39.8	45	3.76	0.8
37	1st EEG + B	Twin	Male	1	N	5.0	40.8	44	4.85	0.8
38	B	Twin	Female	2	N	5.0	40.5	45.5	6.96	1.8

General data comparison between groups

Means and SEMs for the three comparison groups are presented in Table 4.2. There were no significant differences in rectal temperature between lambs that were assisted and those that were not ($p=0.902$, df 22, $t=0.125$), between female and male lambs ($p=0.490$, df 23, $Z=-0.690$), and those that were single lambs and multiples ($p=0.588$, df 22, $t=0.595$).

There were no significant differences in weight between lambs that were assisted and those that were not ($p=0.366$, df 22, $t=-0.924$). However, females tended to be lighter than males although this difference was not significant ($p=0.057$, df 23, $Z=-1.903$). Single lambs were significantly heavier than multiples ($p<0.001$, df 23, $Z=-3.786$).

There were no significant differences in PCV between lambs that were assisted and those that were not ($p=0.791$, df 21, $t=-0.269$), between female and male lambs ($p=0.497$, df 21, $t=0.691$) and those that were single lambs and multiples ($p=0.455$, df 21, $t=0.762$).

Plasma lactate concentrations did not significantly differ between lambs that were assisted and those that were not ($p=0.203$, df 21, $t=1.313$). There were also no significant differences between female and male lambs ($p=0.176$, df 21, $t=1.401$), and those that were single lambs and multiples ($p=0.104$, df 21, $t=-1.697$).

Plasma glucose concentrations were also not significantly different between lambs that were assisted and those that were not ($p=0.464$, df 14, $t=0.753$), between female and male lambs ($p=0.380$, df 14, $t=0.907$), and those that were single lambs and multiples ($p=0.629$, df 14, $t=-0.494$).

Table 4.2: Means and SEMs of lamb birth parameters comparing lambs that were assisted with those that were not, female with male lambs, and single lambs with multiples. PCV = packed cell volume

Parameters	**Assistance**		**Gender**		**Number of lambs born**	
	Yes	***No***	***Female***	***Male***	***Single***	***Multiple***
Weight (kg)	5.2^{Mean}	5.5	4.8	5.7	5.7	4.5
	0.3^{SE}	0.2	0.3	0.2	0.2	0.3
	12^{N}	12	9	15	17	7
Rectal temperature (°C)	40.2	40.2	40.3	40.2	40.3	40.1
	0.2	0.1	0.2	0.1	0.1	0.2
	12	12	9	15	17	7
PCV (%)	46	46	47	45	46	45
	1.7	0.8	1.6	1.0	1.0	1.8
	11	12	9	14	16	7
Plasma glucose concentration	2.05	1.58	2.10	1.53	1.60	2.17
(mmol/L)	0.53	0.42	0.53	0.42	0.33	0.77
	8	8	8	8	10	6
Plasma lactate concentration	4.77	3.94	4.98	3.96	3.93	5.27
(mmol/L)	0.53	0.36	0.68	0.29	0.25	0.82
	11	12	9	14	16	7

Summary of EEG results

Due to the large amount of data presented in the following sections of this chapter, a summary of the major results for EEG data is presented here, followed by an in-depth account of the results.

1) EEG differences between states

- In lambs 3 to 30min after birth, there were no significant differences between the features of their LVHF and INT EEGs.
- In lambs 1 to 4hrs, F50 was significantly higher in INT EEGs than HVLF EEGs. In addition, significant differences in the frequency ranges of 1-4Hz, 4-6Hz, 10-12Hz, 13-15Hz and 19-21Hz were observed. However, there were again no differences between LVHF and INT EEG states.
- In lambs 1 to 2 days, F50 was higher in INT than HVLF EEGs. In addition, significant differences in the relative EEG power of the frequency bands of 1-3Hz, 16-18Hz and 19-21Hz were observed between states.

2) EEG changes with time after birth

- There was a decrease in the presence of movement artefact in EEG traces with time after birth
- There was a decrease in the proportion of LVHF EEG with age, such that LVHF EEG was not observed in lambs 1 to 2 days after birth
- HVLF EEG was absent between 3 and 15 minutes after birth and its incidence was low between 15 and 30 minutes after birth
- There was an increase in the proportion of HVLF EEG with time after birth
- There were no significant differences in EEG parameters (F50, F95, Ptot) in INT EEGs over the first 30 minutes after birth
- When comparing all age groups, Ptot increased with time after birth in INT and HVLF EEGs. In addition, relative EEG power of the frequency bands of 16-18Hz, 22-24Hz, 25-27Hz and 28-30Hz decreased with time after birth in INT EEGs.

Detailed report of EEG results

1) The first 30 minutes after birth

As only few animals (n=4) exhibited HVLF EEG traces, which overall did not contribute much to the overall EEG recordings during the first 3 to 30 minutes (Tables 4.3 and 4.4), HVLF EEG data were not included in the statistical analyses. When measured as a percentage contribution of the three states to the overall useable EEG, the LVHF state was more prominent than the INT state (Table 4.3). However, this may have been confounded by the inability to obtain useable EEG records when animals were in an aroused state and exhibited a high degree of movement. This is represented as the proportion of artefact present for each 5-minute period over the first 5 to 30 minutes after birth (Table 4.3).

Table 4.3: Percentage contribution of the three EEG states (LVHF = low voltage high frequency, INT = intermediate and HVLF = high voltage low frequency) to the total number of seconds available for EEG analysis from 16 lambs for each time period investigated over the first 30 minutes after birth and percentage contribution of the three EEG states as well as artefact to the total number of seconds recorded for the 16 lambs.

EEG state	3-5min	5-10min	10-15min	15-20min	20-25min	25-30min
LVHF	58.1%	63.9%	41.6%	22.0%	52.7%	51.4%
INT	41.9%	36.1%	58.4%	54.9%	44.0%	46.5%
HVLF	0%	0%	0%	23.1%	3.3%	2.1%
LVHF	*	4.9%	6.8%	2.8%	10.6%	5.7%
INT	*	2.8%	9.6%	7.0%	8.8%	5.2%
HVLF	*	0%	0%	3.0%	0.7%	0.2%
Artefact	*	92.3%	83.6%	87.2%	79.9%	88.8%

* 3-5-minute data was not included as time periods varied in duration

Table 4.4: Number of lambs whose EEGs showed the characteristics of LVHF, INT and HVLF states or whose EEG could not be used at the selected time points after birth due to movement artefact, assessed by scan sampling at the stipulated time.

	LVHF	**INT**	**HVLF**	**Artefact**
Time after birth		***Total number of lambs n = 16***		
3-5 min	5	3	0	8
5 min	5	8	0	3
10 min	7	8	0	1
15 min	5	8	0	3
20 min	4	9	0*	3
25 min	7	6	0*	3
30 min	8	5	0*	3

*Zero because of scan sampling – see Table 4.5

State comparison

The means and SEMs of all spectral parameters for LVHF and INT EEGs are presented in Table 4.5. As there were no significant differences between the two EEG states (Table 4.6) the following statistical analyses only take one of the states, namely INT EEGs, into consideration. This was done, as the age comparisons (see further below) focussed on INT EEGs due to the absence of LVHF EEGs in 1-2-day-old lambs.

Table 4.5: Means and SEMs for F50, F95 and Ptot and the ten frequency bands for the LVHF and INT EEGs of the first 30 minutes after birth. N=number of animals

	LVHF	**INT**
	N=13	N=15
F50 9Hz)	**6.49**	**6.80**
	0.42	0.45
F95 (Hz)	**26.70**	**26.68**
	0.20	0.22
Ptot (arbitrary units)	**21.4**	**21.8**
	2.11	1.98
1-3Hz (%)	**37.7**	**35.5**
	1.17	1.67
4-6Hz (%)	**11.7**	**12.7**
	0.38	0.57
7-9Hz (%)	**8.3**	**8.6**
	0.21	0.17
10-12Hz (%)	**7.3**	**7.4**
	0.15	0.14
13-15Hz (%)	**6.7**	**6.9**
	0.12	0.17
16-18Hz (%)	**6.3**	**6.6**
	0.22	0.33
19-21Hz (%)	**5.8**	**6.1**
	0.21	0.30
22-24Hz(%)	**5.6**	**5.7**
	0.29	0.23
25-27Hz (%)	**5.3**	**5.4**
	0.28	0.30
28-30Hz (%)	**5.4**	**5.3**
	0.33	0.33

Table 4.6: Results of the independent sample t-tests and Mann-Whitney tests comparing spectral parameters between LVHF and INT EEGs for lambs 3 to 30 minutes after birth. * Z-statistic

	3 to 30 minutes after birth statistics
F50 (Hz)	$0.695^{\text{p-value}}$ $-0.397^{\text{statistic}}$
F95 (Hz)	0.908 -0.115*
Ptot (arbitrary units)	0.897 -0.130
1-3Hz (%)	0.299 1.059
4-6Hz (%)	0.259 -1.129*
7-9Hz (%)	0.076 -1.774*
10-12Hz (%)	0.633 -0.483
13-15Hz (%)	0.189 -1.313*
16-18Hz (%)	0.629 -0.484*
19-21Hz (%)	0.507 -0.672
22-24Hz (%)	0.655 -0.452
25-27Hz (%)	0.959 -0.052
28-30Hz (%)	0.773 -0.291

Age comparison - Spectral parameters (F50, F95 and Ptot)

When comparing the six age groups, there were no significant changes in F50, F95 or Ptot in INT EEGs with age (Table 4.7). The same was the case for comparisons with only two age groups (3 to 15 minutes plus 15 to 30 minutes). In addition, when performing related-samples tests on the same data, there were again no significant differences between the two ages. The means and SEMs are presented in Table 4.8.

Table 4.7: Results of the independent sample t-tests, one-way ANOVAs, Mann-Whitney tests and related samples Wilcoxon signed rank tests comparing spectral parameters in INT EEGs between the different age groups between 3 and 30 minutes after birth.

Six age groups		**F50**	**F95**	**Ptot**
Independent samples	p-value	0.642	0.720	0.865
	F-statistic	0.678	0.573	0.373
Two age groups				
Independent samples	p-value	0.886	0.661	0.569
	t-statistic/Z-statistic*	-0.145	-0.438*	-0.578
Related samples	p-value	0.888	0.508	0.087
	t-statistic/Z-statistic*	-0.145	-0.663*	-1.923

Table 4.8: Mean and standard error of the mean (SEM) for F50, F95 and Ptot of EEGs recorded in 16 lambs aged up to 30 minutes after birth using mean data for individual lambs. Values for the INT EEG state are presented. N=number of animals

	Minutes after birth					
	~3 to 5	**5 to 10**	**10 to 15**	**15 to 20**	**20 to 25**	**25 to 30**
INT	N=4	N=12	N=11	N=10	N=9	N=9
F50	**8.06**$^{\textbf{Mean}}$	**6.31**	**7.17**	**7.58**	**6.45**	**7.31**
	1.85^{SEM}	0.40	0.49	0.76	0.54	0.78
F95	**26.77**	**26.57**	**26.93**	**26.72**	**26.39**	**27.02**
	0.73	0.25	0.24	0.36	0.29	0.22
Ptot	**25.2**	**21.9**	**22.3**	**25.2**	**21.6**	**21.7**
	4.73	2.81	1.97	2.67	2.72	2.54

Age comparison - Frequency band data (1-30Hz)

There were no significant changes in relative EEG power in any of the ten frequency bands in INT EEGs with time after birth over the first 30 minutes after birth (Table 4.9). This was the case no matter if the six or the two age groups were used for comparison. In addition, the related-sample comparisons between two age groups did not yield any significant differences in any of the frequency bands either. The means and SEMs are presented in Tables 4.10.

Table 4.9: Results of the independent sample t-tests, one-way ANOVAs, Mann-Whitney tests and related samples Wilcoxon signed rank tests comparing relative power in the ten frequency ranges in INT EEGs between the different age groups between 3 and 30 minutes after birth. *non-parametric test statistic

INT			
Age groups	**Six**	**Two**	
	Independent	**Independent**	**Related**
Frequency range	**(df 5, 49)**	**(df 1, 23)**	**(df 1, 9)**
1-3Hz	0.737	0.995	0.961
	0.550	-0.006	0.051
4-6Hz	0.890	0.827	0.957
	0.334	-0.219*	-0.055
7-9Hz	0.816	0.139	0.218
	0.443	-1.478*	-1.323
10-12Hz	0.209	0.269	0.417
	1.492	1.132	-0.851
13-15Hz	0.313	0.194	0.224
	1.222	-1.339	-1.305
16-18Hz	0.223	0.622	0.717
	1.450	-0.493*	0.375
19-21Hz	0.510	0.801	0.971
	0.857	0.255	-0.037
22-24Hz	0.640	0.852	0.519
	0.680	0.189	0.671
25-27Hz	0.497	0.933	0.670
	0.887	0.085	0.440
28-30Hz	0.739	0.870	0.448
	0.548	-0.164*	0.794

Table 4.10: Means and SEMs of relative EEG power (%) of the ten frequency bands for INT EEGs for lambs 3 to 30 minutes after birth. N=number of animals

Frequency bands	**3-5min**	**5-10min**	**10-15min**	**15-20min**	**20-25min**	**25-20min**
	N=4	N=12	N=11	N=10	N=9	N=9
1-3Hz	**32.5**$^{\textbf{Mean}}$	**37.0**	**34.6**	**34.2**	**36.8**	**34.2**
	4.84^{SEM}	1.83	1.70	2.18	1.67	2.34
4-6Hz	**11.7**	**12.4**	**12.6**	**11.9**	**12.7**	**12.2**
	1.03	0.68	0.53	0.54	0.65	0.33
7-9Hz	**8.7**	**8.5**	**8.4**	**8.6**	**8.9**	**8.7**
	0.37	0.29	0.27	0.21	0.07	0.21
10-12Hz	**8.2**	**7.3**	**7.6**	**7.6**	**7.5**	**7.7**
	0.25	0.18	0.15	0.20	0.20	0.27
13-15Hz	**7.1**	**6.6**	**7.2**	**7.12**	**7.0**	**7.2**
	0.40	0.23	0.16	0.26	0.11	0.29
16-18Hz	**7.5**	**6.8**	**6.4**	**6.6**	**6.0**	**6.2**
	0.76	0.40	0.18	0.33	0.26	0.37
19-21Hz	**6.7**	**5.8**	**6.1**	**6.4**	**5.7**	**6.3**
	0.76	0.35	0.27	0.36	0.29	0.35
22-24Hz	**6.9**	**5.8**	**5.9**	**6.0**	**5.4**	**6.1**
	1.42	0.24	0.31	0.42	0.38	0.41
25-27Hz	**5.2**	**5.0**	**5.8**	**6.0**	**5.1**	**5.7**
	0.72	0.24	0.40	0.53	0.42	0.41
28-30Hz	**5.7**	**4.9**	**5.6**	**5.6**	**4.9**	**5.8**
	1.47	0.40	0.37	0.55	0.40	0.51

Multivariate statistical analysis

Principal Component Analysis Eigenvalues and component scores of the component matrix for INT EEGs over the first 30 minutes after birth are presented in Table 4.11. Two Principal Components with Eigenvalues >1 contributed 55.6% to the overall variation in the data.

Component loadings of Principal Component 1 (PC1) were high for 13-15Hz, 16-18Hz, 19-21Hz, 22-24Hz, 25-27Hz and 28-30Hz and for 4-6Hz and 7-9Hz in PC2. PC1 therefore represents frequencies between 16-30Hz, while PC2 represents frequencies between 4-9Hz. Contrasts were present in PC1 between frequency bands 1-3Hz (negative) and 13-15Hz, 16-18Hz, 19-21Hz, 22-24Hz, 25-27Hz and 28-30Hz (positive), and in PC2 between frequency bands of 4-6Hz and 7-9Hz (positive) and 28-30Hz (negative).

Figure 4.2 shows the separation of PC scores along the 1st and 2nd Principal Component axes according to age group for INT EEGs. Neither of the two components showed a clear distinction between the ages, as is supported by the results other statistical results.

Table 4.11: Eigenvalues and Principal Component score calculated by PCA for INT EEGs of the first 30 minutes after birth.

Eigenvalues	**INT**	
	PC1	**PC2**
Total	4.08	1.48
Percentage	40.8%	14.8%
Frequency ranges	**INT** **PC1**	**PC2**
1-3Hz	-0.919	-0.290
4-6Hz	-0.209	0.508
7-9Hz	0.198	0.633
10-12Hz	0.431	0.487
13-15Hz	0.524	0.329
16-18Hz	0.691	0.120
19-21Hz	0.774	-0.024
22-24Hz	0.763	-0.192
25-27Hz	0.746	-0.360
28-30Hz	0.694	-0.458

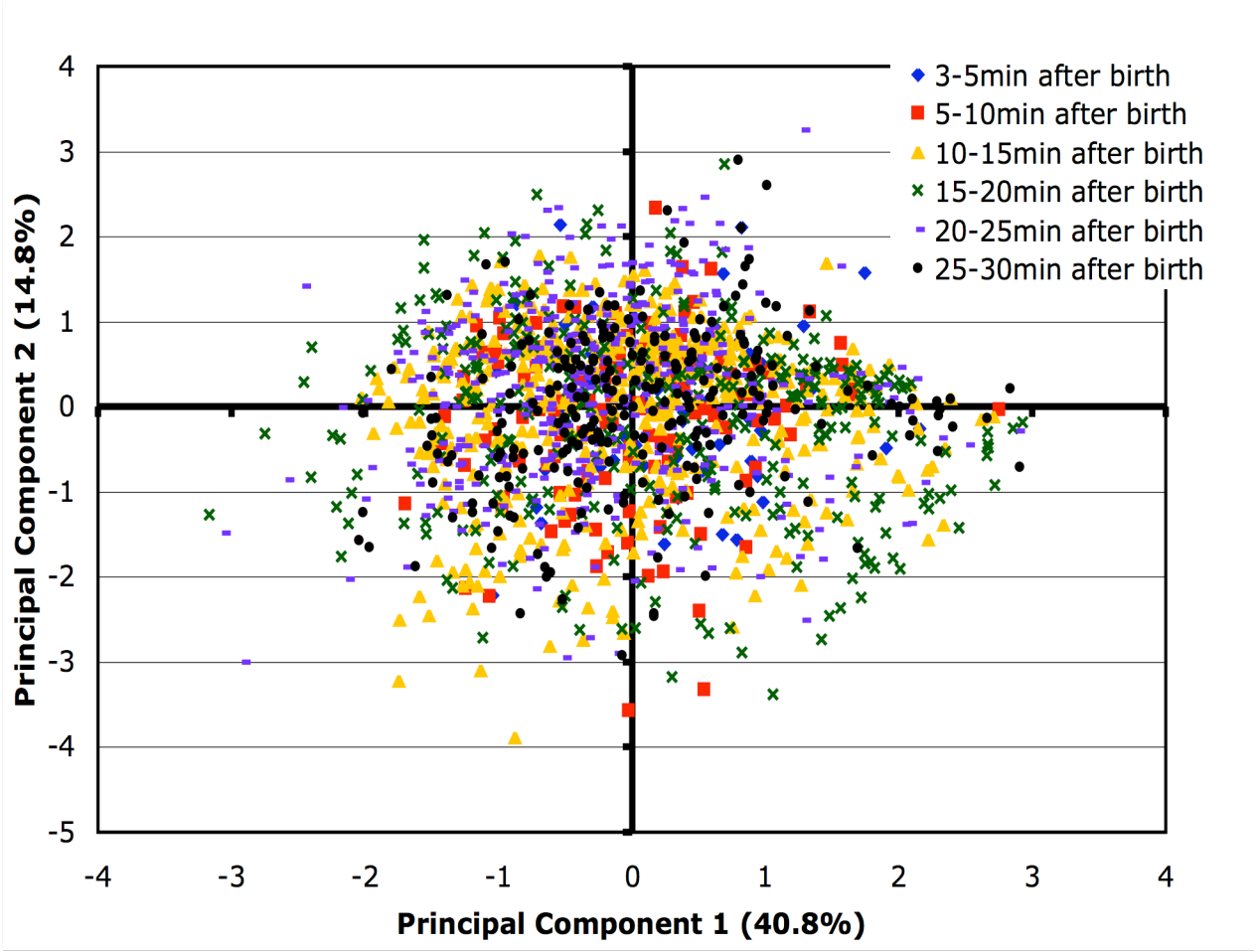


Figure 4.2: Principal Component factor scores plotted against the 1st and 2nd Principal Component axes according to age group for INT EEGs of lambs over the first 30 minutes after birth.

2) 1 to 4hrs

The means and SEMs for spectral data for LVHF, INT and HVLF EEGs are presented in Table 4.12.

There were significant differences between the three states in F50 and the frequency bands of 1-3Hz, 4-6Hz, 10-12Hz, 13-15Hz, 16-18Hz and 19-21Hz. Results for statistical tests are presented in Table 4.13 below. Although there were significant differences in F50 between the three states, the Tukey post-hoc test was not able to differentiate between them. However, using another post-hoc test (LSD), it was shown that F50 was significantly higher in LVHF and INT EEGs than HVLF EEGs (Table 4.12).

For the frequency band of 1-3Hz, relative EEG power was significantly lower in INT EEGs than HVLF EEGS, while for frequency band 10-12Hz relative EEG power was significantly higher in INT than HVLF EEGs. In the frequency band of 4-6Hz relative EEG power was significantly lower in LVHF than in HVLF EEGs. For frequency bands of 13-15Hz relative EEG power was higher in INT compared to HVLF EEGs, while for frequency bands of 16-18Hz and 19-21Hz LVHF and INT were higher in relative EEG power than HVLF EEGs. There were no significant differences between LVHF and INT EEG states.

Table 4.12: Means and SEMs for LVHF, INT and HVLF for lambs 1 to 4 hours after birth. N=number of animals

Spectral parameters	**LVHF**	**INT**	**HVLF**
	N=11	**N=13**	**N=11**
F50 (Hz)	**5.95**	**6.00**	**4.48**
	0.51	0.58	0.32
F95 (Hz)	**26.06**	**25.94**	**25.17**
	0.46	0.40	0.36
Ptot (arbitrary units)	**22.8**	**23.8**	**27.9**
	3.02	2.34	2.51
1-3Hz (%)	**39.2**	**38.3**	**44.3**
	1.89	1.90	1.19
4-6Hz (%)	**11.9**	**12.9**	**13.6**
	0.48	0.27	0.31
7-9Hz (%)	**8.4**	**8.8**	**8.2**
	0.32	0.09	0.19
10-12Hz (%)	**7.4**	**7.6**	**6.9**
	0.22	0.13	0.19
13-15Hz (%)	**6.6**	**6.7**	**5.9**
	0.13	0.21	0.20
16-18Hz (%)	**6.1**	**6.0**	**5.1**
	0.21	0.21	0.20
19-21Hz (%)	**5.7**	**5.5**	**4.6**
	0.35	0.28	0.20
22-24Hz (%)	**5.2**	**5.1**	**4.2**
	0.37	0.38	0.25
25-27Hz (%)	**5.0**	**4.6**	**3.8**
	0.54	0.41	0.32
28-30Hz (%)	**4.6**	**4.5**	**3.6**
	0.56	0.49	0.38

Table 4.13: Results of the one-way ANOVA (F statistic) and non-parametric Kruskal Wallis test (Z-statistic) comparing LVHF, INT and HVLF EEGs in lambs 1 to 4 hours after birth. *Z-statistic

Spectral parameters	**1 to 4 hours after birth (df 2,32)**
F50 (Hz)	**0.049**[p-value]
	3.324[statistic]
F95 (Hz)	0.244
	2.821*
Ptot (arbitrary units)	0.219
	1.594
1-3Hz (%)	**0.044**
	3.439
4-6Hz (%)	**0.009**
	5.469
7-9Hz (%)	0.065
	5.463*
10-12Hz (%)	**0.017**
	4.628
13-15Hz (%)	**0.009**
	9.314*
16-18Hz (%)	**0.007**
	9.960*
19-21Hz (%)	**0.011**
	9.085*
22-24Hz (%)	0.078
	2.760
25-27Hz (%)	0.174
	1.850
28-30Hz (%)	0.381
	0.994

3) 1 to 2 days

The means and SEMs for spectral data for INT and HVLF EEGs are presented in Table 4.14 and results for statistical tests are presented in Table 4.15.

F50 was significantly higher in INT than HVLF EEGs and the relative EEG power of the frequency band of 1-3Hz was significantly lower in INT than HVLF EEGs. In addition, the relative EEG power of the frequency bands of 16-18Hz and 19-21Hz was significantly higher in INT than HVLF EEGs.

Table 4.14: Means and SEMs for F50, F95 and Ptot and the ten frequency bands for the INT and HVLF. N=number of animals

Frequency bands	**INT**	**HVLF**
	N=5	**N= 5**
F50 (Hz)	**5.07**	**4.15**
	0.29	0.24
F95 (Hz)	**25.43**	**24.54**
	0.45	0.38
Ptot (arbitrary units)	**35.0**	**39.6**
	4.59	4.00
1-3Hz (%)	**41.2**	**45.0**
	1.38	0.83
4-6Hz (%)	**13.2**	**13.9**
	0.59	0.65
7-9Hz (%)	**9.0**	**8.7**
	0.16	0.31
10-12Hz (%)	**7.5**	**7.0**
	0.15	0.30
13-15Hz (%)	**6.7**	**6.0**
	0.06	0.28
16-18Hz (%)	**5.7**	**5.1**
	0.08	0.20
19-21Hz (%)	**5.0**	**4.4**
	0.15	0.13
22-24Hz (%)	**4.4**	**3.8**
	0.24	0.16
25-27Hz (%)	**3.9**	**3.3**
	0.36	0.29
28-30Hz (%)	**3.6**	**2.9**
	0.46	0.41

Table 4.15: Results of the independent sample t-test (t-statistic) and non-parametric Mann-Whitney test (Z-statistic) comparing INT and HVLF EEGS in lambs 1 to 2 days after birth. *Z-statistic

	Individual means
Spectral parameters	**(df 1, 8)**
F50 (Hz)	**0.039**
	-2.460
F95 (Hz)	0.171
	-1.503
Ptot (arbitrary units)	0.464
	0.769
1-3Hz (%)	**0.044**
	2.394
4-6Hz (%)	0.450
	0.795
7-9Hz (%)	0.386
	-0.917
10-12Hz (%)	0.167
	-1.521
13-15Hz (%)	0.076
	3.153
16-18Hz (%)	**0.047**
	3.938
19-21Hz (%)	**0.023**
	-2.794
22-24Hz (%)	0.078
	-2.020
25-27Hz (%)	0.192
	-1.424
28-30Hz (%)	0.296
	-1.117

4) Comparison between the three age groups

There was a decrease in the incidence of LVHF EEGs with age so that this state was not detected in 1 to 2 day-old lambs, while there was an increase in the incidence of HVLF EEG during EEG recording periods with age (Table 4.16). In addition, there was a gradual decline in movement artefact with age. Due to the small number of lambs exhibiting HVLF EEG at 3 to 30 minutes after birth and the small number of data points available for HVLF EEG per lamb during this period, HVLF EEG spectral data for this age group were not included in the comparative analysis. In addition, as there were no significant differences in any of the EEG parameters between LVHF and INT EEGs in lambs at 3 to 30 minutes after birth as well as 1 to 4 hours after birth, statistical analyses were undertaken for only one of those states. As LVHF EEGs were not present in lambs 1 to 2 days after birth INT EEGs were thus used for this purpose.

Table 4.16: Percentage contribution of the three EEG states to EEG data used for statistical analysis comparing the three age groups and to overall EEG data available (note that HVLF EEG was not used for statistical analysis in lambs 3 to 30 minutes after birth and no statistical analysis was undertaken to determine the changes in EEG movement artefact with age).

Time after birth	*LVHF*	*INT*	*HVLF*	*Artefact*
3 to 30 minutes	48.4%*	51.6%	0%	
	45.9%+	48.7%	5.4%	
	6.7%^	7.1%	0.8%	85.4%
1 to 4 hours	12.2%+	30.6%	57.2%	
	6.6%^	16.7%	31.1%	45.6%
1 to 2 days	0%+	34.4%	65.6%	
	0%^	26.0%	49.6%	24.4%

* Calculated from data used for statistical analyses

\+ Excludes contribution of movement artefact

^ Includes contribution of movement artefact

Spectral parameters (F50, F95 and Ptot)

The results of the statistical tests comparing spectral parameters between the three age groups are presented in Table 4.17, while the means and SEMs for F50, F95 and Ptot for the three age groups at the three EEG states are presented in Table 4.18.

INT EEGs showed an increase in Ptot (p=0.027, df 2,30, F=4.095), which was significant between lambs 3 to 30 minutes and 1 to 2 days after birth (p=0.021) and approached significance between 1 to 4 hours and 1 to 2 days after birth (p=0.080). For INT EEGs at 3 to 30 minutes and 1 to 4 hours after birth, a related-samples comparison was possible. This analysis showed that there were no significant differences between the two age groups in any of the spectral parameters.

For HVLF EEGs, there was a significant increase in Ptot between 1 to 4 hours and 1 to 2 days (p=0.023, df 1,14, t =-2.548).

Table 4.17: Results of the one-way ANOVAs and independent t-tests and the non-parametric Mann-Whitney and Kruskal-Wallis tests comparing F50, F95 and Ptot between EEGs recorded from newborn lambs up to 30 minutes, 1 to 4 hours and 1 to 2 days after birth. *Z-statistic for individual means, ^Chi-Square statistic for individual means, df degrees of freedom

	INT **(df 2, 30)**	**HVLF** **(df 1, 14)**
F50	0.161 p-value	0.612 p-value
	1.939 F-statistic	0.518 t-statistic
F95	0.069	0.315
	5.335^	1.042
Ptot	**0.027**	**0.023**
	4.095	-2.548

Table 4.18: Means and SEMs for F50, F95 and Ptot of EEGs recorded in lambs up to 30 minutes after birth, 1 to 4 hours after birth and 1 to 2 days after birth for INT and HVLF EEGs. N=number of animals

	First 30 minutes	1 to 4 hrs	1 to 2 days
INT	**N=15**	**N=13**	**N=5**
F50	**6.80**	**6.01**	**5.07**
	0.45	0.58	0.29
F95	**26.68**	**25.94**	**25.43**
	0.22	0.40	0.45
Ptot	**21.8**	**23.8**	**35.0**
	1.98	2.34	4.59
HVLF	**N=4**	**N=11**	**N=5**
F50	**3.77**	**4.48**	**4.15**
	0.16	0.32	0.24
F95	**24.92**	**25.17**	**24.54**
	0.31	0.36	0.38
Ptot	**25.9**	**27.9**	**39.6**
	4.07	2.51	4.00

Frequency band data (1-30Hz)

The results of the statistical tests comparing relative EEG power of the ten frequency bands between the three age groups are presented in Table 4.19 and means and SEMs are presented in Table 4.20.

There were no significant differences in any of the frequency bands between the three age groups in HVLF EEGs. There were also no significant changes in INT EEGs with time after birth. For INT EEGs of 3 to 30min-lambs and 1 to 4hr-lambs, a related-sample comparison was possible. This analysis showed that the relative EEG power of the frequency ranges of 16-18Hz (3 to 30min: 7.0±0.51; 1 to 4hrs: 6.0±0.23), 22-24Hz (3 to 30min: 5.9±0.35; 1 to 4hrs: 5.1±0.40), 25-27Hz (3 to 30min: 5.5±0.48; 1 to 4hrs: 4.7±0.51) and 28-30Hz (3 to 30min: 5.4±0.53; 1 to 4hrs: 4.6±0.59) decreased significantly with time after birth.

Table 4.19: Results of the one-way ANOVAs and independent t-tests and the non-parametric Mann-Whitney and Kruskal-Wallis tests comparing the relative power of the ten frequency bands between EEGs recorded from newborn lambs up to 30 minutes, 1 to 4 hours and 1 to 2 days after birth. *Z-statistic for individual means, ^Chi-Square statistic for individual means, df degrees of freedom

	INT	**HVLF**
	(df 2, 30)	**(df 1, 14)**
1-3Hz	0.200[p-value]	0.722[p-value]
	1.700[F-statistic]	-0.362[t-statistic]
4-6Hz	0.330	0.655
	2.218^	-0.457
7-9Hz	0.294	0.181
	1.276	-1.408
10-12Hz	0.507	0.769
	0.695	-0.299
13-15Hz	0.797	0.797
	0.228	-0.262
16-18Hz	0.065	0.924
	5.452^	-0.098
19-21Hz	0.097	0.715
	2.529	-0.373
22-24Hz	0.050	0.371
	3.320	0.924
25-27Hz	0.066	0.352
	2.971	0.962
28-30Hz	0.094	0.324
	2.561	1.021

Table 4.20: Means and SEMs for the ten EEG frequency bands recorded in lambs up to 30 minutes after birth, 1 to 4 hours after birth and 1 to 2 days after birth for the INT and HVLF EEGs. N=number of animals

	INT			**HVLF**	
	3-30min N=15	**1-4hours N=13**	**1-2 days N=5**	**1-4hours N=11**	**1-2days N=5**
1-3Hz	35.62	39.21	42.02	44.88	45.63
	0.25	0.22	0.25	0.17	0.21
4-6Hz	12.60	12.58	12.54	13.48	14.46
	0.10	0.08	0.10	0.07	0.11
7-9Hz	8.70	8.89	8.85	8.14	8.46
	0.06	0.04	0.06	0.04	0.06
10-12Hz	7.54	7.73	7.48	6.81	6.71
	0.05	0.04	0.06	0.03	0.04
13-15Hz	7.02	6.82	6.66	5.87	5.68
	0.05	0.04	0.05	0.03	0.04
16-18Hz	6.23	6.04	5.73	5.06	4.87
	0.05	0.04	0.05	0.03	0.04
19-21Hz	5.88	5.44	4.95	4.54	4.29
	0.05	0.04	0.05	0.03	0.03
22-24Hz	5.75	4.88	4.34	4.08	3.71
	0.05	0.04	0.05	0.03	0.03
25-27Hz	5.39	4.35	3.85	3.71	3.25
	0.05	0.05	0.06	0.03	0.03
28-30Hz	5.30	4.08	3.57	3.46	2.95
	0.06	0.06	0.06	0.03	0.03

EEG recording of non-breathing lamb

The EEG of one lamb that did not breathe after birth, and subsequently died, was recorded from ~ 2 minutes after birth although breathing was not successful by the time EEG recording had commenced. A segment of this lamb's trace, and, for comparison, a LVHF and INT trace of another lamb which did breathe successfully during the same period after birth, are presented in Figure 4.3. It can be seen that the trace was isoelectric, as only background noise could be detected. Thus, this indicates that isoelectric traces were not observed in any of the lambs of the present study breathing successfully.

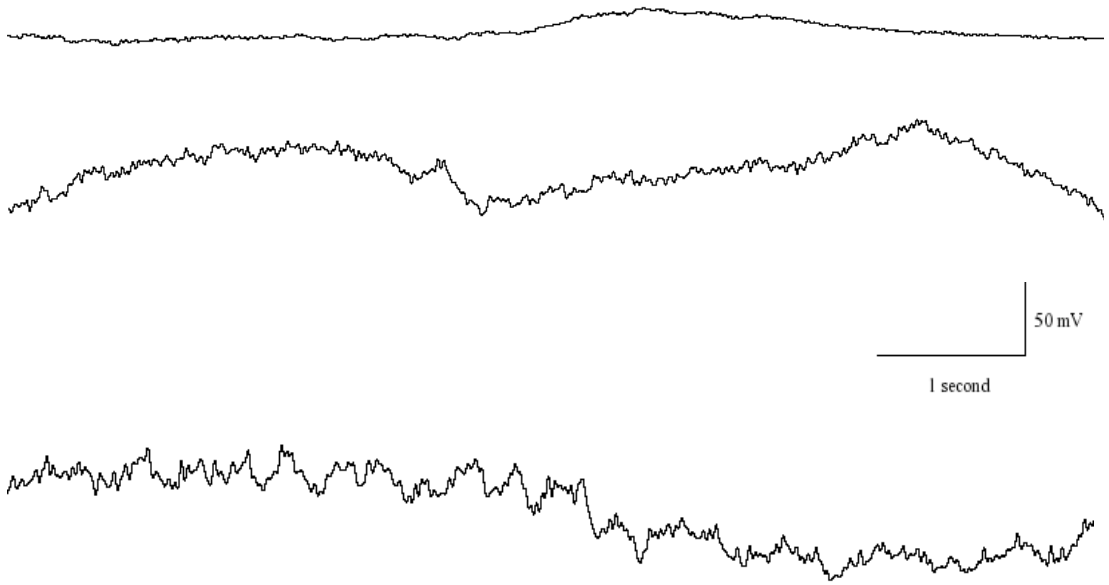


Figure 4.3: Top trace: Isoelectric EEG trace of a non-breathing lamb 3 minutes after commencing EEG recordings (i.e. ~ 5:00 min after birth). Middle trace: LVHF EEG of lamb breathing successfully during the same period. Bottom trace: INT EEG of the same successfully breathing lamb.

Behavioural data

1) Intra-observer reliability

The Spearman rank scores for intra-observer reliability for each behaviour and state are presented in Table 4.21. There is apparently no designated Spearman rank score above which all measures are acceptable and below which none are (Martin & Bateson, 1998). Martin and Bateson (1998) give the guideline of r=0.7 for an important category that is difficult to measure, although for categories of behaviour where measurement is straightforward, reliability should be better than 0.7. Hence, the results of the present study are within these boundaries as all reliability scores were above 0.7.

Table 4.21: Results of the Spearman rank correlation test for assessing intra-observer reliability.

Behaviours	**Spearman rank score (r)**
Sneezing	0.871
Head shake	0.995
Head movement	0.926
Body movement	0.963
Vocalising	0.985
Sucking/teat seeking	0.756
States	
Eyes closed	0.914
Head resting	0.876

2) Assessing categorisation of behaviours of arousal

The main results of the Spearman rank correlations are presented in Table 4.22. As expected there was a strong negative correlation between the states of eyes open and closed and those of head up and head resting. In addition, moderate correlations were observed between eyes open and head resting and eyes closed and head up, thus showing that lambs resting their heads tended to have their eyes closed while those with their head up tended to have their eyes open. In addition, vocalisations, whole body

movements as well as sucking attempts showed moderate to strong positive correlations with eyes open and head up, supporting the categorisation of these variables into "arousal" behaviours.

Table 4.22: Spearman rank correlations (r) and p-values of behaviours and states linked to arousal and number of data points (n). Only those correlations with coefficients >0.500 are presented (moderate and strong correlations).

	Eyes open	Head up	Whole body movements
Eyes closed	-0.865[r]	-0.518	
	<0.001[p]	<0.001	
	34[n]	36	
Head resting	-0.655	-0.998	
	<0.001	<0.001	
	36	36	
Vocalise	0.510	0.705	0.556
	<0.001	<0.001	<0.001
	47	36	57
Whole body movement	0.610	0.704	
	<0.001	<0.001	
	47	36	
Sucking attempts	0.566	0.610	
	<0.001	<0.001	
	47	36	

3) Changes in behaviour with time after birth

The results of the non-parametric Friedman tests are presented in Table 4.23 and the means and SEMs for behavioural states and other behavioural data are presented in Figures 4.4 and 4.5, respectively.

The time spent with head up or head resting did not change with time after birth. Lambs

tended to spend less time with their eyes open between 1 and 4hrs after birth than at the younger ages (p=0.085), although this was not supported by complementary changes in the time spent with eyes closed.

With regard to behaviours associated with arousal, there was a significant decrease in the number of whole body movements and sucking attempts with time after birth. This is in support of the tendency for lambs to spend less time with eyes open at 1 to 4 hours. As would be expected, behaviours that were associated with the onset of breathing, i.e. head shake and sneeze, decreased significantly between 3 to 15 minutes and 1 to 4 hours after birth.

Table 4.23: Results of the non-parametric Friedman test showing p-values, Chi-square statistic and degrees of freedom (number of groups and lambs per group) for behaviours and states comparing lambs 3 to 15 minutes, 15 to 30 minutes and 1 to 4 hours after birth.

Behaviours/states	**P-value**	**Chi-square**	**Degrees of Freedom**
Eyes open	0.085	5.083	3, 6
Eyes closed	0.398	1.853	3, 8
Head up	0.522	1.600	3, 4
Head resting	0.252	2.696	3, 5
Whole body movements	**0.007**	10.09	3, 11
Vocalisations	0.717	0.667	3, 11
Sucking attempts	**0.002**	12.38	3, 11
Head movements	0.174	3.500	3, 11
Sneeze	**<0.001**	20.13	3, 11
Head shake	**0.002**	12.84	3, 11

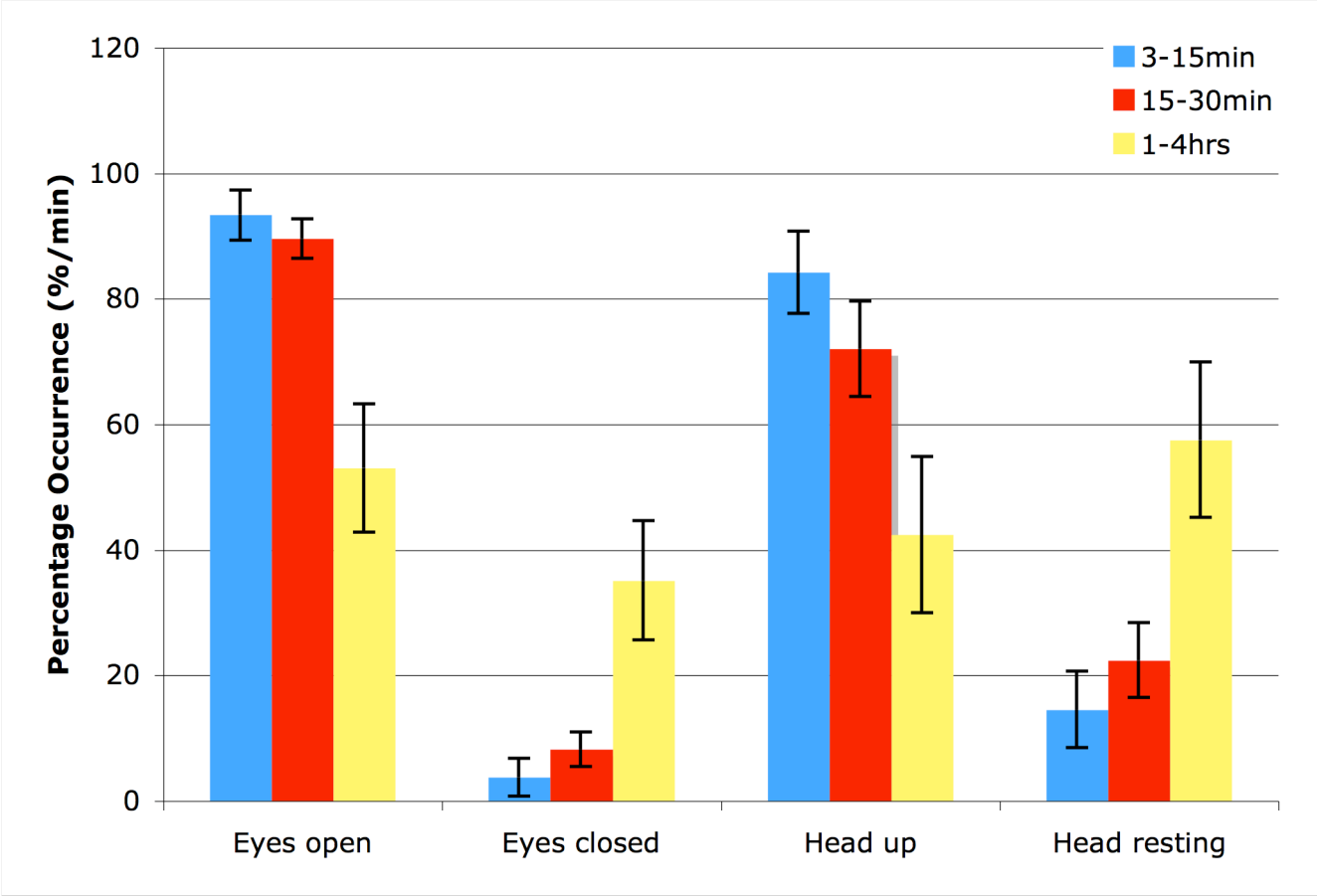


Figure 4.4: Means and SEMs for occurrence of behavioural states (eyes open or eyes closed and head up or head resting) per minute of behavioural observations in lambs at 3 to 15 minutes, 15 to 30 minutes and 1 to 4 hours. There were no significant changes with time.

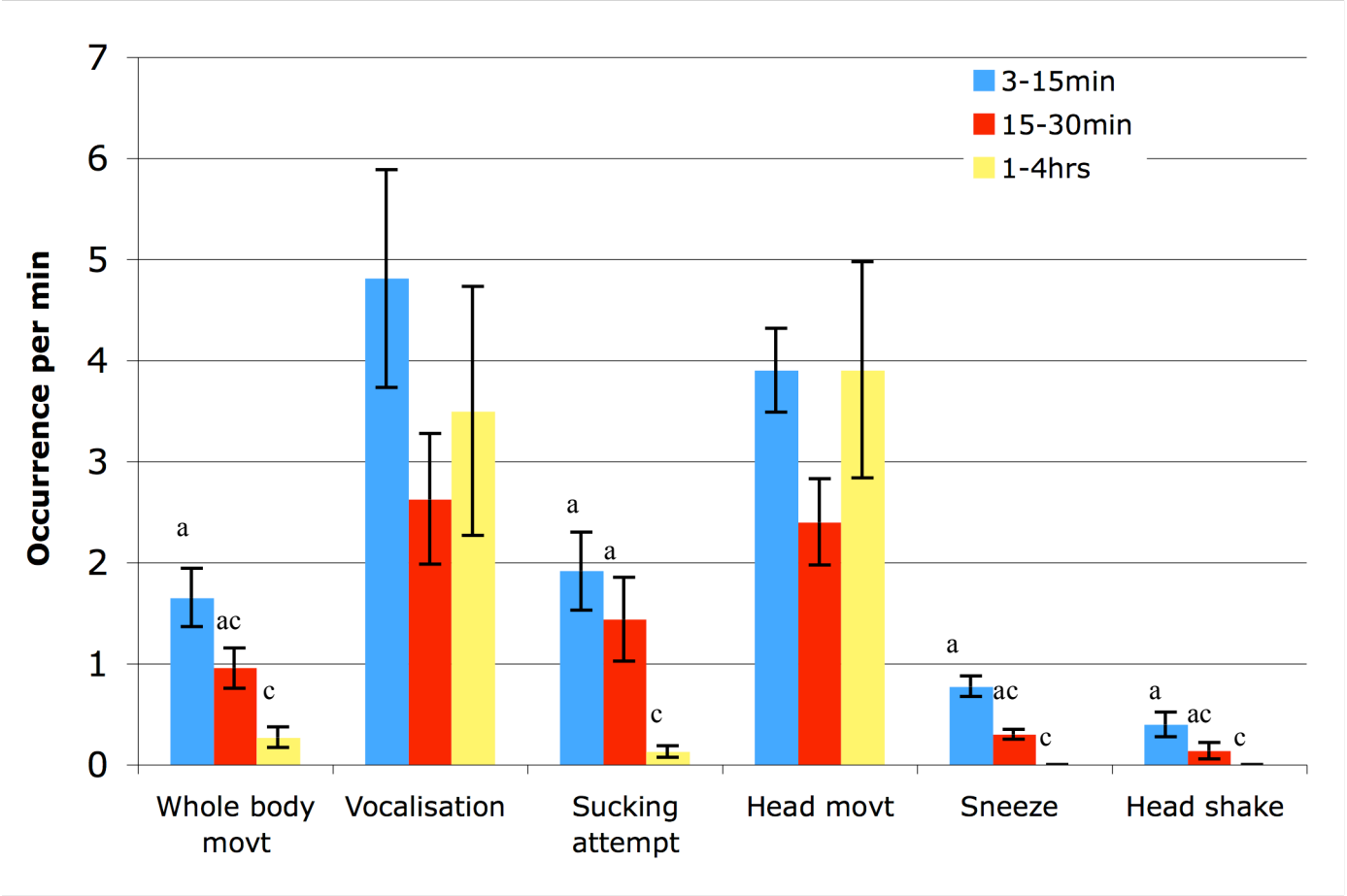


Figure 4.5: Means and SEMs for behaviours per minute of behavioural observations in lambs aged 3 to 15 minutes, 15 to 30 minutes and 1 to 4 hours. Different letters indicate significant differences $p<0.05$.

4.4 Discussion

As far as the candidate is aware, there are no previous studies that have investigated the changes in EEG parameters with age from as close to birth as presented here. The main findings of the present study were as follows. First, lambs were fairly uniform physiologically and appeared healthy with regard to rectal temperature, PCV and plasma lactate and glucose concentrations, although singles were, as expected, heavier than multiples. Second, a high degree of arousal was observed during the first 30 minutes after birth, during which HVLF EEGs were absent or present for only short periods. Third, the incidence of HVLF EEGs increased with time after birth. Fourth, arousal-related behaviours and movement artefact in EEG traces decreased with time after birth supporting a high degree of arousal soon after birth and a subsequent increase in HVLF EEGs. Fifth, EEG parameters did not change over the first 30 minutes after birth. Sixth, changes in EEG parameters between the three age groups were observed, including an increase in Ptot and associated changes in frequency bands with time after birth.

Some key physiological changes around the time of birth

Shortly before, during and immediately after birth the lamb undergoes a variety of physiological changes, which enable it to adapt to extrauterine life. There is an extensive body of literature (original research and review articles) pertaining to these physiological changes (e.g. Blanco *et al.*, 1984; Smeaton & Simpson-Morgan, 1985; Mellor & Cockburn, 1986; Blanco *et al.*, 1988; Martin *et al.*, 1988; Mellor, 1988; Heymann, 1989; Kumar & Hanson, 1989; McMillen & Nowak, 1989; Power, 1989; Rudolph, 1989; Blanco, 1991; Randall, 1992; Smolich *et al.*, 1992; Xu, 1996; Breen *et al.*, 1997; Calder *et al.*, 1997; Sangild *et al.*, 2000; Piccione *et al.*, 2006; Turner *et al.*, 2008). To review these data fully is beyond the scope of this thesis, however a very brief summary of some major physiological changes/adaptations in response to the exposure to the extrauterine environment is presented below.

Physiological adaptations relevant in the present context are those affecting the oxygen status of the fetus and newborn, including maturation of the lungs, the changes necessary to become an air breathing organism and the progression to fully effective

ventilation after the onset of breathing (e.g. Dawes, 1968; Blanco *et al.*, 1984). Allied with these pulmonary changes are cardiovascular responses to the loss of the placental circulation, including a rise in blood pressure and systemic vascular resistance, a drop in pulmonary resistance and an increase in pulmonary blood flow (Askin, 2001), as well as the closure of the foramen ovale, the ductus arteriosus and the ductus venosus. Oxygen consumption increases rapidly after birth, in particular due to the need of the newborn animal to execute respiratory work and to maintain thermogenesis (Birk *et al.*, 1989; Symonds *et al.*, 1995). Between the event of birth and the first intake of energy-rich colostrum the newborn is entirely dependent on stored energy reserves accumulated before birth to fuel heat production (Mellor & Cockburn, 1986). The ability for gluconeogenesis in the liver is very low in the fetal lamb and only develops after birth (Warnes *et al.*, 1977b, a) associated with the appearance of rate-limiting enzymes (Girard, 1986).

Subsequent to these initial changes, postnatal colostrum intake and the maturation of the gastrointestinal tract are of significance, as is discussed in the sections below. The GI tract has been shown to grow during the neonatal period (e.g. increase its linear dimensions, weight and protein content) and this is likely be related to the exposure to colostrum (Widdowson, 1984). The reader is referred to the reviews by Xu (1996) and Sangild *et al*. (2000) for a more detailed account on postnatal GI tract development and adaptation.

EEG development before birth and changes immediately prior to and during birth

Pre-cortical and cortical structures of the fetal lamb's brain are initially electrically silent and sporadic spikes and short periods of sustained EEG activity do not appear before about half-way through gestation. Continuous undifferentiated EEG apparently develops from about 90 days and the EEG differentiates into REM-non-REM sleep-like EEG patterns from about 110-120 days of gestation (Ellingson & Rose, 1970; Clewlow *et al.*, 1983; Szeto & Hinman, 1985). By late pregnancy, REM-non-REM sleep-like EEG patterns occupy 95% of fetal EEG activity present during each day (Szeto, 1992; Mellor *et al.*, 2005). Conscious perception appears to be precluded at this time due to the operation *in utero* of a range of potent neuroinhibitory mechanisms (Mellor *et al.*, 2005).

Fetal states of unconsciousness appear to persist during labour and may even become deeper as labour progresses. Thus, as labour approaches, fetal ECoG activity indicates a predominance of high voltage slow wave patterns observed during non-REM sleep-like states (Berger *et al.*, 1986; Shinozuka & Nathanielsz, 1998), and fetal motor systems remain largely quiescent (Berger *et al.*, 1986; Hasan & Rigaux, 1991). As these changes were observed 4 to 5 hours prior to the spontaneous onset of labour, a time when coordinated uterine contractions are not yet very strong, they were evidently independent of hypoxaemia associated with labour contractions (Shinozuka & Nathanielsz, 1998). Nevertheless, hypoxaemia during labour is likely to affect the EEG and behaviour of the fetus; indeed if hypoxaemia becomes protracted isoelectric EEGs can be observed (Mallard *et al.*, 1992; Watson *et al.*, 2002; Hunter *et al.*, 2003a; Hunter *et al.*, 2003b).

EEG recordings in macaque monkeys before, during and after birth showed that after the initial gasp in the newborn monkey there was an increase in the frequency and amplitude of the EEG compared to the fetal pattern (Esquivel de Gallardo *et al.*, 1964). In a study of human infants a gradual increase in EEG power after clamping of the umbilical cord and the first breath was observed, and the EEG at 5 minutes after birth was indistinguishable from that of a newborn baby several hours old (Rosen & Satran, 1965). In contrast, subsequent EEG recordings in human infants before, during and after labour using specialised suction electrodes did not show any differences between EEGs before and after birth (Rosen & Scibetta, 1970). However, these studies, and others like them, were undertaken about four decades ago when quantitative and statistical methods of EEG analysis were not available and EEG analysis still relied mainly on visual examination of EEG traces. Thus, care has to be taken in the interpretation of such results. More recent investigations on the electroencephalographic changes around the time of birth are apparently not available.

EEG changes after birth observed in the present study

1) Differences in EEG parameters between EEG states

There are three states of vigilance that are distinguished from each other by a set of

physiological signs, including EEG (electroencephalogram) rhythms, muscular tone (electromyogram (EMG) recordings) and eye movements (electrooculogram (EOG) recordings) (Steriade & McCarley, 2005). Wakefulness is characterised by behavioural arousal, an EEG consisting of low-voltage high-frequency (LVHF) waves and voluntary eye-movements. Rapid eye movement (REM) sleep has EEG characteristics indistinguishable from the waking EEG, however, in contrast to the waking state muscular atonia is present and involuntary eye movements occur which are accompanied by spiky PGO (pontogeniculooccipital) potentials (Steriade & McCarley, 2005). In contrast, non-REM sleep, also called slow-wave sleep, is characterised by high-voltage low-frequency (HVLF) waves in the EEG.

The induction and maintenance of wakefulness, REM sleep and non-REM sleep have been attributed to the interactions between multiple wake and sleep-promoting systems widely distributed within the brain from the medulla to the cerebral cortex, and these have been listed in Chapter 1.

The fact that there were no significant differences between LVHF and INT EEGs is interesting. It is not clear, whether this is due to a genuine similarity between the two states (hence suggesting assessment of the EEG was not sensitive enough to classify it into the two states accurately) or whether animal numbers were too small to detect differences between the two states. An alternative is that LVHF EEGs may be representative of an immature form of quiet arousal EEGs of which INT is the maturer version, so that as time after birth progressed the incidence of LVHF EEGs decreased while that of INT EEGs increased. If this were the case, then differences between the two states may have been too small to be significant, in particular with the small numbers of animals available.

LVHF/INT EEGs observed in the present study could be associated with conscious or unconscious arousal (quiet arousal). Indeed movement artefact was very high during the first 30 minutes after birth indicating that lambs were in a state of active arousal for most of this observation period. Due to the high incidence of movement artefact (Table 4.24), indicating the absence of muscle atonia, it is not likely that INT EEGs represented REM sleep EEGs. However, this possibility cannot be excluded for the other two age groups observed here, as supplementary data, such as time-linked

behavioural observations or eye movement data, were not available. Yet, the present lambs did exhibit a general reduction in physical activity between birth and two days of age.

HVLF EEGs observed in the present study are most likely indicative of the presence of non-REM sleep, as a high voltage low frequency EEG trace is a characteristic that is not present in other naturally occurring states of vigilance.

It has to be acknowledged that the incidence of vigilance states considered here may have been over or under-represented, because useable EEG records were only available for short intervals while the EEGs did not exhibit movement artefact, and because the lambs were held during the recordings, which may itself have impacted on their state at the time (see discussion on limitations and drawbacks below).

2) Changes in EEG state and behaviour with age

Notwithstanding the exact identity of the EEG states reported on here, there was a high incidence of wakefulness/arousal (quiet and active) present during the first 30 minutes after birth, while non-REM sleep was apparently absent initially or present for only short periods of time. This is supported by the high incidence of arousal-type behaviours and movement artefact in the EEGs over the first 30 minutes after birth as well as the absence/low incidence of HVLF EEGs over this period (Table 4.24). Over time, the incidence of non-REM sleep increased substantially, which is again supported by the increase in the incidence of HVLF EEGs with age and the increase in behaviours associated with resting/sleeping in lambs aged 1 to 4 hours after birth. Moreover, these changes coincided with a decrease in the incidence of EEG movement artefact and apparent behavioural arousal (Table 4.24).

Table 4.24: Percentage contribution of the three EEG states to EEG data in the three age groups (with ^ and without + consideration of movement artefact). The data presented here are identical to those in Table 4.16 in the results sections of this chapter. The data have been relocated here for convenience.

Time after birth	*LVHF*	*INT*	*HVLF*	*Artefact*
3 to 30 minutes	45.9%+	48.7%	5.4%	
	6.7%^	7.1%	0.8%	85.4%
1 to 4 hours	12.2%+	30.6%	57.2%	
	6.6%^	16.7%	31.1%	45.6%
1 to 2 days	0%+	34.4%	65.6%	
	0%^	26.0%	49.6%	24.4%

High incidence of arousal during the first 30 minutes after birth

Behavioural arousal may be essential for the newly born lamb to interact with its mother and to attempt to suck, thereby ensuring its survival. Colostrum intake before gut closure is essential for the newborn lamb to attain passive immunity, as antibodies are not transferred via the placenta (Mellor & Stafford, 2004), as is the case in some other mammals (Baintner, 2007). Also, colostrum intake is essential to provide energy substrates for the continuous maintenance of heat production by the newborn and young lamb (Mellor & Cockburn, 1986). In addition, sucking is essential for the development of neonatal preference for the dam. If sucking/intake of colostrum is prevented for several hours after birth the subsequent ability of the dam to identify and favour its lamb is impaired (Nowak *et al.*, 1997; Goursaud & Nowak, 1999; Mellor & Stafford, 2004; Nowak *et al.*, 2007). Therefore, the absence of sleep for a short time after birth appears to be beneficial.

As discussed in Chapter 1, several factors that act soon after birth appear to be involved

in the arousal observed in newborn lambs. First, the stimulation associated with the birth process, including strong tactile stimulation, hypoxia and exposure to the cold extrauterine environment (Svensson, 1987; Lagercrantz, 1996; Tang *et al.*, 2000), leads to a surge in plasma noradrenaline concentrations and appears to activate noradrenergic fibres of the locus coeruleus. The locus coeruleus forms part of the ascending reticular activating system (ARAS) (Jones, 2003) and extends noradrenergic neurons throughout the brain including the cerebral cortex, brain stem and spinal cord (Moore & Bloom, 1979; Lagercrantz, 1996; Berridge & Waterhouse, 2003; Jones, 2003). It has major roles in stimulating and maintaining arousal and state-dependent cognitive processes (Svensson, 1987; Berridge & Waterhouse, 2003; Jones, 2003). In addition, neurons of the locus coeruleus project to the olfactory bulb (Shipley *et al.*, 1985) so that activation of this nucleus may facilitate teat-seeking and bonding in the newborn animal (Winberg, 2005). Second, elevated oestradiol concentrations soon after birth are likely involved in the arousal observed in the newborn animal and the initial absence of sleep. 17β-oestradiol is a neuroactive steroid that exerts rapid excitatory effects in a variety of brain areas (Wong *et al.*, 1996; McEwen & Alves, 1999; Woolley, 1999; McEwen, 2002). Among other mechanisms, this seems to be partly due to the induction of short-term facilitation of glutaminergic synaptic transmission (Wong *et al.*, 1996). Third, the neuropeptide orexin, which has previously been shown to be involved in arousal and feeding by exerting central excitatory effects (Kilduff & Peyron, 2000; Willie *et al.*, 2001), may play a role in promoting arousal, hunger and hence feeding in the newborn lamb. Orexigenic fibres form a widespread neuronal network. The fact that the locus coeruleus apparently receives the densest innervation of these orexigenic fibres is consistent with a role of orexin in producing arousal and inhibiting sleep (Bourgin *et al.*, 2000). A recent study has shown that orexin fibres are present in fetal lambs immediately prior to birth and that orexin can be found in the plasma of these fetuses (Dickinson *et al.*, 2008). Unfortunately, this study did not report the values for newborn lambs within several hours of or over the first few days after birth, but only for lambs 15 days old, at which age orexigenic fibre density was greater than that observed in the fetus. The involvement of orexin in the onset of feeding as well as the maintenance of an aroused state soon after birth needs to be investigated further.

Therefore, although concentrations of residual neuroinhibitors such as allopregnanolone are likely elevated at this time (Chapter 5), arousal and its associated interactions with

the dam would be safeguarded by a variety of arousing stimuli overcoming the suppressive effects of these neuroinhibitors. Whether arousal/wakefulness observed during the first half hour or so after birth is associated with conscious wakefulness cannot be judged by the results of the present study and further research would be necessary to assess this. However, as mentioned in the introduction to this chapter, behavioural processes occurring at this time, including teat-seeking, do not appear to rely on conscious processes, as they are also observed in newborn mammals where conscious perception is not yet established. One may ask what types of behaviours could be accepted as indicative of conscious arousal. This of course is difficult to ascertain. It could be argued that finding the teat via learning after several failed attempts is such an indicator. However, even learning can occur on a subconscious level (see Chapter 1 for discussion on fetal learning).

Gradual increase in non-REM sleep and decrease in arousal with age

A high incidence of sleep observed during the first 2 to 3 days after birth (Nowak, 2006) may reflect a high need for memory consolidation in the newborn animal. Various studies have supported the theory that both REM and non-REM sleep play a role in the consolidation of memories and synaptic plasticity associated with memory processes (for reviews see: Benington & Frank, 2003; Walker, 2008). As the newborn animal is exposed to a very different environment than is the fetus, learning about this environment will be an important part of postnatal survival. The initial high incidence of sleep in the newborn may thus allow the animal to lay down the memories necessary to accomplish this. Naturally, there may also be other functions for the increased incidence of sleep observed in lambs for the first few days after birth.

Such a decrease in arousal over the first few days after birth may seem contrary to waning neuroinhibition by residual neuroinhibitors such as allopregnanolone (see Chapter 5). However, these may not be the only factors impacting on the state of consciousness in the newborn lamb.

The present increase in HVLF EEGs with age, and hence presumably an increase in the incidence of non-REM sleep, may be associated with successful colostrum intake, the gradual waning of CNS activating factors, continued presence/production of

neuroinhibitory hormones in the face of waning activating factors (at least for the first day after birth) the adaptation to the extrauterine environment, as well as the establishment of sleep-wake cycles.

Intake of an energy-rich diet has been shown to lead to increased drowsiness and sleep (Danguir, 1987). Newborn lambs over the first 2 to 3 days after birth appear to spend more time resting and sleeping after they have sucked (Nowak, 2006). In satiated animals, the EEG shows a marked increase in HVLF activity (Hockman, 1964) while starvation apparently suppresses sleep (Jacobs & McGinty, 1971). Several physiological mechanisms, which may or may not be interrelated, appear to be involved in the phenomenon. First, changes in metabolic rate in response to food intake play a likely role (Nicolaidis, 2006). Second, gastric stimulation has been shown to induce EEG activity characteristics of sleep (Kukorelli & Juhász, 1977), which seems to be associated with activity in the vagus nerve. Vagal stimulation has also been shown to cause EEG synchronisation (Chase *et al.*, 1967) and the non-REM-promoting effects of increased food intake are apparently inhibited by vagotomy (Hansen & Krueger, 1997). Third, stimulation of the gastrointestinal tract by intraluminal nutrients or mechanical distension in addition to exposure to cholecystokinin (CCK) alters vagal afferent activity (Schwartz *et al.*, 1993). Hence, release of gastrointestinal humoral factors, such as CCK, have also been shown to induce sleep (Mansbach & Lorenz, 1983; Kapás *et al.*, 1991). Fourth, feeding has a direct effect on the immune function of the gut and energy-rich foods have been shown to increase mRNA of the cytokine interleukin-1β (IL-1β) in the liver and hypothalamus of rats (Hansen *et al.*, 1998). Cytokines are known for their role in inflammation, triggering the acute phase response in which somnolence plays an important part (Krueger *et al.*, 1998). It is not certain how IL-1β may bring about its central somnogenic effects, as it is a relatively large and hydrophilic molecule which would not be expected to cross the blood brain barrier (BBB) easily. However, it has been suggested that it may signal the central nervous system (CNS) via vagal afferents (Krueger *et al.*, 1998). Indeed, IL-1β has been shown to increase vagal afferent activity (Niijima, 1996; Hansen *et al.*, 1998).

As already stated above, newborn ruminants, such as lambs, calves and kids, must ingest colostrum soon after birth in order to thrive (Mellor & Stafford, 2004). In these

species, colostrum ingestion not only ensures that increased energy needs can be met, but its intake is also vital to establish protection against infectious agents, as no passive transfer of immunoglobulins occurs via the placenta before birth (Besser & Gay, 1994; Mellor & Stafford, 2004). As colostrum is an energy-rich source of food (Mellor & Cockburn, 1986), it can be assumed that the above-mentioned means of stimulating postprandial sleep will also be present in the newborn herbivore. Thus, ingestion of colostrum and the resultant mechanical stimulation of the gastrointestinal tract, release of CCK and increase in metabolic rate would be expected to increase sleep propensity. In addition, immune factors (cytokines) shown to be involved in this phenomenon appear to be present in colostrum. Colostrum of calves contains IL-1β, which is absorbed into the circulation upon colostrum intake by the calf (Goto *et al.*, 1997; Yamanaka *et al.*, 2003). In addition, other cytokines present in colostrum, such as tumour necrosis factor α (TNF-α) (Yamanaka *et al.*, 2003), also appear to be involved in stimulating sleep (Krueger *et al.*, 1998).

Oral and gastrointestinal stimuli related to sucking have also been shown to lead to activation of opioidergic systems in the newborn (Blass & Fitzgerald, 1988; Blass, 1996) and opioid receptor ligands derived from hydrolysis of milk proteins, such as caseins, may exert analgesic or sedative properties in newborn and young animals during the period of colostrum/milk intake (Teschemacher *et al.*, 1997; Teschemacher, 2003). This has already been discussed in Chapter 1. Opioids not only play a role in analgesia, but they also signal a state of reward, well-being and 'drive-reduction" (Shayit *et al.*, 2003), so that opioidergic activation may well play a role in the drowsiness occurring after food intake.

Although the majority of lambs aged 1 to 4 hours after birth were less active than those at 3 to 30 minutes after birth, there were some whose activity levels were relatively high. The reason for these lambs being more active could be that the amount of colostrum they had taken in was small initially and hence they may have been hungry again during the 1-to-4hr observation period.

It is not clear whether the reduced arousal in the present 1-to-4hr lambs is representative of what occurs in undisturbed lambs at that age, as the present lambs were handled at birth as well as at the 1-to-4hr-observation and this could have affected their behaviour.

As mentioned above, the degree of arousal at this time may be related to previous intake of colostrum.

3) Changes in EEG parameters with age

Comparison of the three age groups

Changes in EEG parameters were observed when comparing the lambs of the three age groups (i.e. birth to 30 min, 1 to 4 hours and 1 to 2 days after birth). In a recent study investigating EEG maturation in goat kids between 15 and 75 days after birth, an increase in the relative power of the frequency band of 12 to 30Hz and a decrease in the relative power of the frequency bands of 4 to 8Hz was also observed (Bergamasco *et al.*, 2006). Indeed, a significant increase in EEG power was present in anaesthetised lambs between 4 to 24 hours and 7 to 11 days of age reported in Chapter 6. However, as these lambs were anaesthetised, differences between age groups could have been affected by age-related differential effects of halothane. The results of the present study are at odds with the findings by Bergamasco *et al*. (2006) as there was a decrease in the relative power of the frequencies between 16 and 30Hz. The difference in age may be the reason for this discrepancy. However, the candidate was not able to locate any other reports in which the development of the EEG has been investigated in terms of power spectral EEG parameters within sleep-wake states from birth in order to verify this.

It is surprising that there was an increase in total power in INT EEGs with time after birth, while the power of frequency bands between 16 and 30Hz decreased with time after birth in INT EEGs and that this was not accompanied by a significant change in relative EEG power in the lower frequencies.

The changes with time after birth observed in the EEG parameters in the present study could have been effected by waning neuroinhibition and/or gradual neurological maturation, increasing animal size and increasing extrauterine experience with respect to differences observed between lambs of the different ages.

Waning neuroinhibition

As already mentioned in Chapter 1, the fetus is exposed to a variety of neuroinhibitory

substances. Previous studies have shown that brain and plasma concentrations of the progesterone metabolite allopregnanolone, a potent neuroinhibitor, are still somewhat elevated 3 days after birth. Thus, it is possible that the changes in EEG parameters observed in the present study with age, are related to the gradual waning of neuroinhibition that may be present in the newborn lamb for some time after birth. The presence of the neuroinhibitor allopregnanolone and its precursors pregnenolone and progesterone over the first 9 days after birth in lambs has been investigated in Chapter 5.

In addition, the composition of mammary gland secretions change continuously over the first few days during the transition from colostrum to mature milk (Nowak, 2006). For example, protein concentrations are highest in colostrum and gradually decline (Nowak, 2006). Although the major proportion of this change is due to a decline in immunoglobulins (Nowak, 2006), it is possible there will also be a concomitant decline in opioidergic precursor concentrations, as colostrum is gradually replaced by milk as a food source.

Continuing neurological maturation

In contrast to the fetus, the postnatal lamb is exposed to a variety of novel environmental stimuli after birth. In light of these environmental differences and the need for the postnatal animal to learn about its environment and how to interact with it, neurological maturation will continue after birth. Such activity-dependent maturation may include the formation of new neural circuitry, which in turn may lead to an increase in EEG power as observed in the present study. For example, it has been suggested that changes in EEG parameters with age in goat kids could be associated with the maturation of integration and interconnections of different brain area feedback loops after birth (Bergamasco *et al.*, 2006).

Whether such maturation would already occur within hours after birth, hence explaining the differences in EEG parameters between lambs aged 3 to 30 minutes and those aged 1 to 4 hours, is not known as far as the candidate is aware. The initial differences observed may be due to waning neuroinhibitory influences or to other immediate physiological responses to birth, and later changes in EEG parameters may be related to

maturational phenomena. Further studies would be necessary to elucidate this.

Absence of EEG changes during the first 30 minutes

There were no significant differences in any of the EEG parameters within the first 30 minutes after birth. However, there was a large proportion of artefact present in EEGs of lambs at this age (Table 4.29) and it is possible that changes over the first 30 minutes after birth could have been present in the EEGs if movement artefact had not prevented FFT analysis.

It is also possible that over the first 30 minutes after birth EEG changes do occur, but that these are very gradual and hence would initially be too small to be registered in the parameters assessed here.

Drawbacks of the study design and other considerations

1) EEG recording in awake animals

The EEG of aroused/alert animals has been successfully recorded using subcutaneous/cutaneous electrodes in a variety of species such as horses, dogs and pigs (Redding, 1964; Redding & Colwell, 1964; Rose *et al.*, 1972; Knecht *et al.*, 1980; Purohit *et al.*, 1981). One precondition for recording meaningful EEG data of alert animals is that the animal is calm and relaxed, showing only minimal movement. If this is not the case movement artefact is introduced into the trace. On the other hand, if an EEG can be obtained from an alert animal without movement artefact, this may mean that the animal may not be in a fully alert state. In humans, vigilance levels fluctuate and boredom, fatigue or monotony may increase the presence of periods of light drowsiness, thereby leading to changes in the EEG including a shift away from alpha-waves (8-13Hz), the so-called "alpha dropout" (Niedermeyer, 1999). Therefore, EEG parameters measured will not be representative of the EEG of an awake and normally behaving alert human. In addition, this may lead to an over-representation of the drowsy or sleep-like EEGs compared to what would have been observed in an animal that would not have been interfered with. In addition, terminating EEG recording sessions early due to lambs struggling would have biased the data towards EEG collection from less active lambs. This has to be taken into consideration when drawing conclusions

from such data.

2) State determination

As mentioned above, sleep-wake states can be distinguished by a set of physiological signs, including EEG (electroencephalogram) rhythms, muscular tone and eye movements (Steriade & McCarley, 2005). In the present study, only EEG recordings were undertaken and hence it was not possible to determine with confidence the presence of the three vigilance states (waking and REM and non-REM sleep) or the presence of intermediate states such as drowsiness. This however would have been of great interest, as there do not appear to be any studies investigating sleep-wake cycle development in lambs from the time of birth.

Behaviour recordings were done in order to assist with sleep-wake state determination. However, it was not possible to link the video records with the corresponding EEG records in a way that would have allowed second-by-second analysis, which would have been necessary due to the short durations of useable EEG traces. The inability to link the two recordings has subsequently led to the purchase of improved equipment for future studies.

3) Data analysis

Overall, the number of lambs from which EEG data were available per age per period and per state was small. Hence, statistical analysis was performed using means for each lamb, but also using all data points available for each lamb as separate data point. This approach has led to some discrepancies in the results, so that significant differences present in the analysis using individual data points were not always present in the analysis using overall means for each lamb. This made interpretation of the data somewhat difficult and hence the candidate has referred to changes as “trends” rather than actual changes.

4) General short comings

It has to be acknowledged that the lambs of the present study were not exposed to a

normal birth environment and this may have affected the results. First, lambs were removed from the dam immediately at birth and held by a technician for the entire 30-minute recording period, so that environmental stimulation differed from that a newborn lamb would normally be exposed to at birth. Second, although lambs were reunited with their dams after the first recording in order for them to bond and suck, they were removed again for the second recording and vocalisations from the ewe could be heard in the recording room. Third, ewes and lambs were kept in a series of pens close to the recording room and were exposed to humans on a regular basis. This may have affected initial bonding and colostrum intake, although mismothering only occurred in one ewe-lamb pair. Fourth, the way lambs were held differed between technicians and may have had an impact on the state of the lamb.

How could the study now be improved?

One way of improving the outcomes of the present study would be to use implanted telemetric electrodes. These could be surgically implanted in the fetus during late gestation, including ECoG electrodes as well as those for recording EMG and EOG activity. This would allow recordings to be made immediately prior to, during and immediately after birth, including the time when breathing may not yet have commenced. In addition, if lambs remain healthy, animals could be followed up for several days in order to investigate the development of EEG states around the time of birth and beyond.

4.5 Conclusions

While the lamb is evidently in a state of heightened arousal soon after birth, this is not necessarily indicative of conscious perception, as arousal can be mediated by lower brain centres without involving consciousness (see Introduction of the present chapter). Thus, it cannot be ascertained from the results of the present study whether the arousal observed in the present lambs was associated with conscious perception. However, full conscious perception appears unlikely for at least the first 5 minutes after birth until oxygen tensions are sufficiently high and adenosine concentrations are sufficiently reduced to support conscious perception (see introduction to this chapter). Even after these have been achieved, full conscious perception may not be present for several more

minutes or even hours. Neuroinhibitory factors, such as progesterone metabolites, which may still be elevated for some time after birth, may play a role here. In addition, the apparently high proportion of sleep observed in lambs over the first few days after birth (Nowak, 2006) suggests that the action of neuroinhibitory factors may be present at the time thereby reducing the amount of time animals spend in a state during which conscious perception is possible. However, further studies are necessary to substantiate these claims, as the data of the present study is not conclusive here.

Overall, the onset of conscious perception in newborn lambs would not be expected to be immediate, like switching on a light, but a more gradual process possibly lasting up to several hours. Thus, conscious perception may initially be qualitatatively different from that of young and adult sheep. However, this may not preclude animals at this stage from suffering in response to noxious stimulation and they should be given the benefit of doubt when invasive procedures are being considered.

4.6 References

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Chapter 5

Abstract

There is evidence suggesting that conscious perception might initially be different in the newborn lamb compared to that of older animals despite their relative neurological maturity at birth. A certain degree of sedation and/or analgesia during the first few days after birth may well play an important role in the survival of the newborn lamb, enabling it to engage in teat seeking and bonding with its dam rather than being distracted by birth injuries or other environmental factors. A variety of factors could be involved in the modulation of conscious perception, such as residual concentrations of neuroinhibitory substances usually present in the fetal lamb before birth. One of these substances is the progesterone metabolite allopregnanolone, which is present in high concentrations in brain and plasma of the fetal lamb and has previously been shown to exert sedative, anaesthetic and analgesic effects via the $GABA_A$ receptor. In order to assess whether neuroactive steroids are present to modulate conscious perception or pain perception for the first few days after birth in animals born neurologically mature, brain and/or plasma concentrations of allopregnanolone and its precursors pregnenolone and progesterone were measured in newborn lambs for the first 9 days after birth. Although brain concentrations of allopregnanolone were low and did not change over the first 9 days after birth, plasma allopregnanolone concentrations remained elevated for the first 12 hours after birth, suggesting that if modulation of conscious perception or pain perception by residual concentrations of allopregnanolone were possible, it would be highest over the first 12 hours after birth. However, $GABA_A$ receptor changes associated with the withdrawal of progesterone metabolites at birth may affect neuroactive steroid sensitivity of the receptor. This and other factors that may affect neuroactive steroid action are discussed. Overall, the interactions between neuroactive steroids and the $GABA_A$ receptor are very complex and have to be taken into consideration when assessing the ability of such steroids to modulated conscious perception.

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Plasma and brain steroid concentrations in newborn lambs between birth and 9 days of age: Potential for modulation of conscious perception

5.1 Introduction

There is evidence that conscious perception might initially be different in neonatal lambs compared to older animals despite their relative neurological maturity at birth. First, as shown in the previous chapter, changes in the composition of electroencephalographic (EEG) spectra seem to occur during the first 1 to 2 days after birth. In addition, a recent study by Johnson *et al.* (2009) investigated the cerebrocortical response of lightly anaesthetised lambs to rubber ring castration over the first 6 weeks after birth. They showed that there were significant changes in responsiveness of the cortex to castration between birth and 5 weeks, especially over the first 10 days after birth, during which the response increased markedly from very low levels during the first 1 to 3 days after birth. The authors suggested that this indicates a change in the degree to which noxious stimuli would be perceived during that early postnatal period.

Some degree of sedation or analgesia during the first few days after birth may well play a part in the survival of the newborn lamb. During the birth process there is potential for trauma and injury to occur and this can sometimes be severe (Mellor & Stafford, 2004). If the newborn lamb at birth were capable of experiencing pain to the extent that would normally be expected to accompany equivalent tissue damage at older ages, this may impede teat-seeking activity, mother-young bonding and colostrum intake thereby jeopardising lamb survival. Modulation of such pain by factors that exert sedative, hypnotic or analgesic effects could therefore be beneficial in this respect. Such sedation may also lead to the inactivity of lambs in response to threatening stimuli which is observed during first few days after birth, which in turn may facilitate grooming and the establishment of the mother-young bond (Mellor *et al.*, 2009).

Naturally, there are a variety of factors, which alone or in combination, may contribute to the changes in EEG spectra observed with age (previous Chapter) and to the reduced

EEG responsiveness of lambs to noxious stimulation over the first few days after birth (Johnson *et al.*, 2009). For example, interactions between the lamb and its mother, including oral and gastrointestinal stimuli related to sucking, may be involved, which may lead to activation of opioidergic systems in the newborn animal, thereby providing comfort and wellbeing and being important in filial attachment (Shayit *et al.*, 2003; Val-Laillet *et al.*, 2004; Nowak *et al*., 2007). Such activation of opioidergic systems may also provide the newborn with a certain degree of analgesia. In addition, opioid receptor ligands derived from hydrolysis of milk proteins, such as caseins, may exert analgesic or sedative properties in newborn and young animals during the period of milk intake (Teschemacher *et al.*, 1997; Teschemacher, 2003). This may especially be so during the first 1-2 days after birth while the intestinal wall is still sufficiently permeable for such factors to cross into the circulation (i.e. before gut closure has occurred; Mellor & Stafford, 2004), and from there into the nervous system should the blood brain barrier (BBB) be permeable to such factors at the time.

Finally, neuroinhibitory agents involved in maintaining unconscious sleep-like states prior to birth, such as the potent neuroactive steroid allopregnanolone, may still be present in sufficiently high concentrations during the first few days after birth to exert their neuroinhibitory effects despite the presence, initially, of activating substances such as have been described in Chapter 1 (Mellor & Diesch, 2006). Naturally one could argue that the action of such sedative factors may impair the animal's ability to engage in feeding processes due to the reduction in the level of consciousness. However, both teat seeking and ingestion of colostrum/milk are not necessarily activities that require full consciousness (see Introduction Chapter 4). In addition, as mentioned in Chapter 1, the presence of a variety of activating factors during the first day or so after birth may, to some extent, counteract the effect of residual neuroinhibitory factors, thereby allowing the newborn to interact with its environment, while still obtaining benefits from possible analgesic/hypnotic properties of residual production of inhibitory agents during the immediate postnatal period.

Allopregnanolone exerts its neuroinhibitory effects by enhancing neuronal inhibition via the $GABA_A$ receptor (Puia *et al.*, 1990; Paul & Purdy, 1992; Purdy & Paul, 1999). However, not only steroid concentrations in plasma and brain, but also receptor subunit composition, density, local distribution and other factors may have an impact on the

effects of neuroactive steroids.

Thus, in order to understand more about the possible modulatory action of neuroinhibitory steroids, such as allopregnanolone, on conscious perception after birth, it is imperative to gain insight into the operation of the $GABA_A$ receptor, how interactions of neuroactive steroids with this receptor bring about its effects and how these may change during ontogeny. These topics are reviewed below. It has to be acknowledged that this area of research is very complex and cannot be discussed here in full. Rather, salient points have been made, and for further details the reader is referred to several recent reviews, which have proven essential for the analysis below (see Lambert *et al*., 2003; Belelli *et al*., 2006; Akk *et al*., 2007; Herd *et al*., 2007).

The $GABA_A$ receptor is a member of the ligand-gated ion channel superfamily and is a heteropentamer constructed of five distinct subunits from combinations of α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π and θ subunits (Lambert *et al.*, 1996; Lambert *et al.*, 2001; Akk *et al.*, 2007). Subunit composition of the $GABA_A$ receptor has been shown to influence its physiological and pharmacological properties. In addition, different combinations of subunits appear to have a distinct distribution throughout the CNS (Lambert *et al.*, 1996; Lambert *et al.*, 2001). GABA also acts on the $GABA_A$ receptor in two distinct modes, both of which impact on neuronal information processing (Belelli *et al*., 2006). One is termed the 'phasic mode', which appears to involve the activation of synaptically clustered receptors by quantally released GABA, while the second, termed 'tonic mode', is mediated by receptors that are located extra-synaptically (Belelli *et al.*, 2006). Synaptic and extra-synaptic $GABA_A$ receptors also exhibit distinct structural and biophysical properties (Belelli *et al*., 2006). Thus, the 'phasic' response is usually mediated by postsynaptic receptors containing a $\gamma 2$ subunit in combination with an α and a β subunit (Belelli *et al.*, 2006; Herd *et al.*, 2007), while the 'tonic' form of inhibition is mediated by receptors located exclusively outside of the synapse which contain a δ subunit in combination with an $\alpha 4$ or $\alpha 6$ subunit and a β subunit (Belelli *et al.*, 2006; Herd *et al.*, 2007).

Neuroactive steroids are derived from peripheral or central sources and are present in the brain in sufficient concentrations to modulate the function of the $GABA_A$ receptor

(Paul & Purdy, 1992; Mellon, 1994; Herd *et al.*, 2007). They bind to a specific site at the receptor which is different from the benzodiazepine recognition site (Pinna *et al.*, 2000). Although neuroactive steroids have been shown to universally prolong the decay of inhibitory postsynaptic currents (IPSCs), the concentrations required to produce this effect vary greatly across different neurons (Herd *et al.*, 2007). Thus, neurosteroid sensitivity of synaptic $GABA_A$ receptors varies greatly between brain regions (Jussofie, 1993) and even across neurons within the same area (Harney *et al.*, 2003). Using recombinant receptors, various studies investigated the relation between neurosteroid sensitivity of $GABA_A$ receptors and their subunit composition (Lambert *et al.*, 2001; Lambert *et al.*, 2003). These studies showed that incorporating subunits that predominantly exist synaptically (i.e. α, β and γ) only had a subtle influence on GABA-modulatory properties of neurosteroids (Lambert *et al.*, 2003; Herd *et al.*, 2007). In contrast, it has been found that extrasynaptic isoforms (containing either α1, 4 or 6, β and δ subunits) seem to be more sensitive to neuroactive steroids than those receptors containing the γ subunit (Belelli *et al.*, 2002).

More recent evidence suggests that subunit composition is not the only determinant of neurosteroid sensitivity of $GABA_A$ receptors (Belelli *et al.*, 2002; Belelli *et al.*, 2006). For example, no obvious associations between allopregnanolone sensitivity thresholds with mIPSCs decay times and subunit composition could be observed (Belelli *et al.*, 2006).

Phosphorylation has been suggested to play a role in the specificity of neurosteroid action (Fáncsik *et al.*, 2000; Pinna *et al.*, 2000; Brussaard & Koksma, 2003; Belelli *et al.*, 2006). Inhibition of protein kinase C (PKC) appears to reduce neurosteroid sensitivity of synaptic $GABA_A$ receptors in CA1 neurones (Harney *et al.*, 2003). However, by which mechanism phosphorylation exerts its effects is not presently known (Belelli *et al.*, 2006). In addition, expression patterns and activity of enzymes involved in neurosteroid synthesis and degradation will play an important part in neuronal inhibition mediated by $GABA_A$ receptors (Smith, 2002; Belelli *et al.*, 2006). Steroidogenic enzyme expression, and thus local neurosteroid production and concentrations, are brain region specific, regulated in a sex-specific manner and change during certain life events such as pregnancy (Pinna *et al.*, 2000; Belelli *et al.*, 2006;

Herd *et al.*, 2007).

Developmental changes in allopregnanolone concentrations have been reported (Grobin & Morrow, 2001; Nguyen *et al.*, 2003a). In rats, these were associated with changes in $GABA_A$ receptor chloride ion influx (Grobin & Morrow, 2001) and alterations in $GABA_A$ receptor subunit expression (Grobin & Morrow, 2000), which in turn appear to be correlated to changes in GABA-mediated IPSCs during postnatal development (Hollrigel & Soltesz, 1997; Mtchedlishvili *et al.*, 2003).

The effects of neuroactive steroids on $GABA_A$ receptor function have also been observed in maturely born species such as guinea pigs and sheep. In guinea pigs allopregnanolone modulation of $GABA_A$ receptors in the immature cerebral cortex was possible and changes in allopregnanolone action were related to changes in $GABA_A$ receptor benzodiazepine pharmacology (Bailey *et al.*, 1999). In sheep fetuses, functional $GABA_A$ receptors are present from at least 85 days of gestation (Crossley *et al.*, 2000). Region-specific changes in binding characteristics with age occurred in hypothalamic and cortical tissues, and allopregnanolone had the ability to modify binding characteristics in late gestation fetuses (Crossley *et al.*, 2000; Crossley *et al.*, 2003).

Aim of study

Concentrations of allopregnanolone and its precursors are elevated during mid-to-late gestation in both plasma and different brain regions in fetal sheep (Nguyen *et al.*, 2003a). However, although the plasma and brain concentrations of these steroids are apparently lower than late-pregnancy values by 3 days after birth and have dropped markedly by 21 days (Petratos *et al.*, 2000; Nguyen *et al.*, 2003a), to date no sequential postnatal studies have been undertaken from the day of birth in lambs. As already mentioned, allopregnanolone has potent sedative, anaesthetic and analgesic effects (Majewska, 1992; Paul & Purdy, 1992; Lancel *et al.*, 1996; Damianisch *et al.*, 2001; Rupprecht, 2003). Whether allopregnanolone or its precursors (see Figure 5.1 for details on enzymatic pathways) would be present during the first few days after birth to bring about modulation of conscious perception is not known.

Therefore, the aim of this study was to measure the concentrations of the neuroactive steroid allopregnanolone and its precursors progesterone and pregnenolone over the first 9 days after birth in plasma and/or brain tissue of newborn lambs. This was done to evaluate whether this neuroactive steroid was present in plasma and brain tissue for the first few days after birth and thus, whether it would have the potential to bring about modulation of conscious perception in animals born neurologically mature.

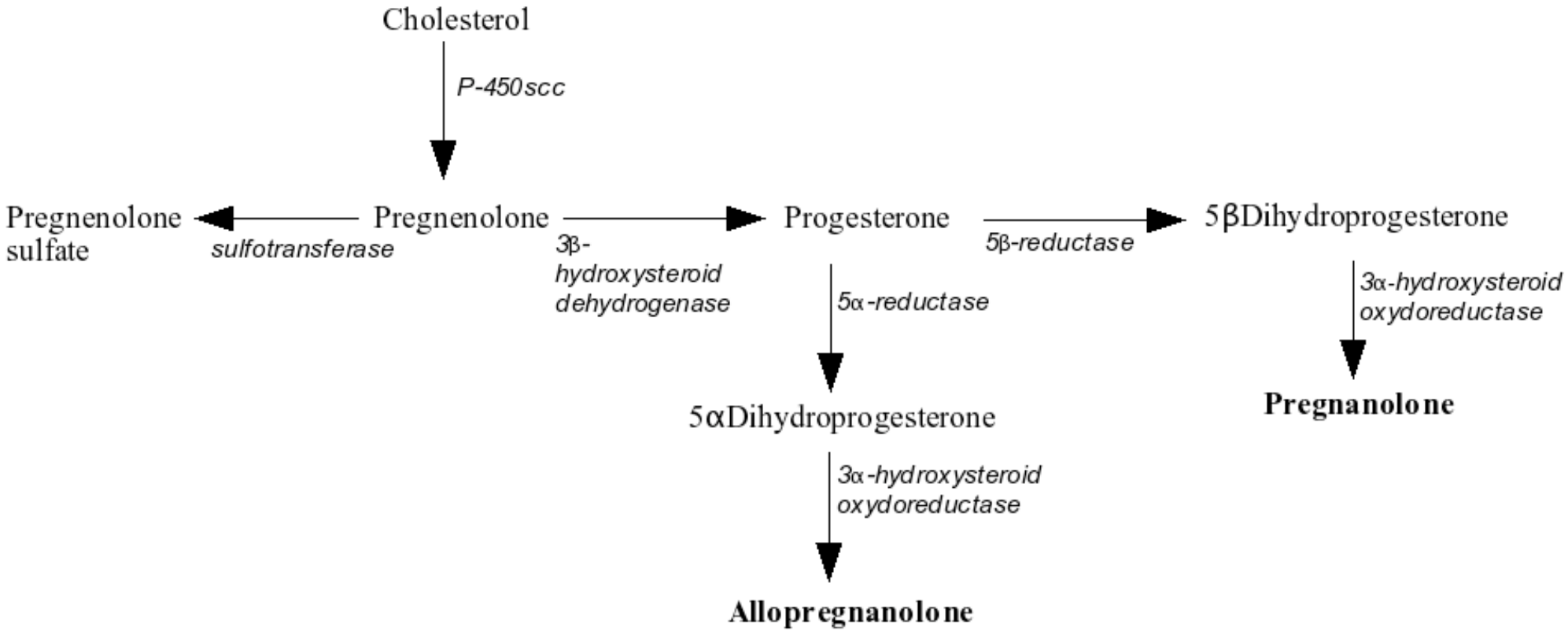

Figure 5.1: Neurosteroid biosynthesis and metabolism showing the precursors for allopregnanolone and pregnanolone and the associated enzymes (adapted from Birzniece et al. (2006)).

5.2 Materials and Methods

Animals

Lambs (Romney/Finn/Texel X) were aged between 4 and 12hrs (n=5), ~24hrs (n=5), ~36hrs (n=5), ~72hrs (n=5), ~7 days (n=5) and ~9 days (n=5) after birth. Lambs were separated from their mothers in the morning and transported to Massey University, where they were maintained in a covered pen. As the mothers did not accompany the lambs they did not have access to food (colostrum/milk) before euthanasia. The study was approved by the Massey University Animal Ethics Committee (Protocol No. 04/93).

Sample collection

Each animal had a blood sample taken via the jugular vein into a 10ml sodium-heparin vacutainer (Becton Dickinson Ltd., Auckland, New Zealand). Blood was put on ice and was centrifuged for 15min at 3,000rpm. Plasma was pipetted into 1.5ml Eppendorff tubes and frozen at -80ºC until analysed.

Lambs were euthanased by overdose of pentabarbitone sodium (400mg/kg; National Veterinary Supplies, Auckland, New Zealand) administered into the jugular vein. The animals were then weighed and the brain was dissected from the skull, was hemisected and each hemisphere was further cut into sections, which were immediately placed onto labelled foil on dry ice. Once frozen the slices were placed into labelled bags. Bags were maintained on dry ice until the end of the dissections and were then frozen at -80ºC at the end of the day. Samples were then transported to Monash University, Melbourne, Australia, where they were maintained at -30ºC until further analysed.

Sample preparation

Brain samples of a variety of brain regions were selected to get a general overview of neurosteroid concentrations after birth. Brain areas examined included the spinal cord (base of skull), medulla, pons, cerebellum, basal ganglia, hippocampus and cerebral cortex. The cerebral hemisphere in which the areas of cortex, hippocampus and basal ganglia could best be discerned was used for analyses. For the hippocampus, both

cerebral hemispheres were used if possible to allow collection of sufficient amounts of tissue for analyses. However, this was not always successful and for some animals hippocampal sections were not available for all assays. The way the tissue was cut and frozen sometimes prevented identification of the hippocampus.

Steroid extractions

1) Allopregnanolone in brain tissue and plasma

Allopregnanolone was extracted from brain tissue and plasma by modification of a method used by Barbaccia *et al.* (1992), as described by Yawno *et al.* (2007). Briefly, 100mg of frozen tissue (±10mg) was weighed out on dry ice and homogenised in ice-cold 50% methanol containing 1% acetic acid. The homogenates were then centrifuged at 3,000rpm at 4°C for 30min (Beckman Coulter Allegra X-15R, Beckman Instruments Inc, Irvine, CA, USA) and the supernatants collected. Ice-cold 50% methanol containing 1% acetic acid was then again added and the homogenate centrifuged at 4,000rpm at 4°C for 15min. This step was then repeated. The supernatants were combined and stored overnight at 4°C. The pellets of brain tissue were frozen at -20°C for further analysis.

Allopregnanolone was extracted from brain homogenates by applying the combined supernatants to Sep-Pak C-18 cartridges (Waters Corp, Milford, MA, USA), which were previously primed with 2.5ml 100% methanol, followed by 2.5ml 50% methanol and finally with 2.5ml 50% methanol + 1% acetic acid. Plasma samples were diluted with ice-cold 50% methanol containing 1% acetic acid (100µl plasma in 900µl solvent) before applying them to the cartridges. After applying the samples to the cartridges they were washed with 2.5ml 50% methanol + 1% acetic acid followed by 2.5ml 50% methanol. The steroids were then eluted by applying 2.5ml 100% methanol and the collected fractions were dried under N_2 at 37°C. Once dried, the steroids were resuspended in 1ml assay buffer (0.1M phosphate buffer at pH 7.4-7.6).

Two additional samples of brain and plasma were extracted in parallel with each extraction run to estimate extraction efficiency. For this purpose, the samples were spiked with tritium-labelled allopregnanolone (25µCi of Pregnan-3α-ol-20one, 5α-

[9,11,12-3H(N)] in 1ml 100% ethanol; PerkinElmer Life and Analytical Science, Boston MA, USA) to give a reading of about 5,000 cpm.

2) Progesterone in brain tissue

The re-suspended sample collected during the allopregnanolone extraction (see above) was also used for progesterone assays. Thus, no separate extraction for progesterone was done. However, two additional samples of brain tissue were extracted in parallel with each extraction run above to estimate extraction efficiency for progesterone. For this purpose, the samples were spiked with tritium-labelled progesterone (250μCi of [1,2,6,7-3H(N)] progesterone in 250ml 100% ethanol; PerkinElmer Life and Analytical Science, Boston MA, USA) to give a reading of about 10,000cpm.

3) Pregnenolone and pregnenolone sulphate in plasma

Pregnenolone

An aliquot of 200μl of each plasma sample was pipetted into a pyrex screw top glass tube and 4ml of diethyl ether were added to each sample. After mixing the samples for 10min using a vortexer followed by a wait of 2min, the tubes were then immersed, one at a time, into an ethanol bath (ethanol with dry ice) to freeze the aqueous layer containing undissolved pregnenolone sulphate. The solvent was pipetted into glass tubes and was then evaporated to dryness under N_2 at 37°C. The diethyl ether extraction was then repeated. Once completed, dried pregnenolone was resuspended in 400μl assay buffer (0.1M phosphate buffer, pH 7.0) and stored at -20°C until further analysis.

Four additional plasma samples were extracted in parallel to estimate extraction efficiency. For this purpose, the samples were spiked with tritium-labelled steroids. Two samples were spiked with pregnenolone tracer (250μCi of Pregnenolone [7-^{3}H(N)]; PerkinElmer Life and Analytical Science, Boston MA, USA) and two samples with pregnenolone sulphate tracer (tritium labelled pregnenolone was used to synthesise pregnanolone sulphate tracer (McKay *et al.*, 1987) to give a reading of about 5,000cpm.

Pregnenolone sulphate

The aqueous layer of the pregnenolone extraction was thawed after the second diethyl ether extraction and 400µl of saturated NaCl were added to each tube followed by 50µl of concentrated HCl and 4ml ethyl acetate. After mixing the samples using a vortexer for 10min with an intermittent break of 2min the tubes were placed into a waterbath with an inbuilt shaker where they were incubated at 37°C over night. The next morning the samples were mixed using a vortexer for 10min with a break of 2min before being centrifuged at 1200rpm at 4°C for 15min. The aqueous layer was then again frozen in an ethanol bath and the solvent pipetted into glass tubes to be evaporated to dryness under N2 at 37°C. The ethyl acetate extraction was then repeated two more times. Dried pregnenolone sulphate was finally resuspended in 400µl assay buffer (0.1M phosphate buffer, pH 7.0) and stored at -20°C until further analysis.

Neurosteroid radioimmunoassays

1) Allopregnanolone in brain tissue and plasma

Allopregnanolone concentrations in brain tissue and plasma were measured in duplicate by a previously validated specific radioimmunoassay (RIA). Allopregnanolone standard (200µg/ml in 100% ethanol; Steraloids Inc., Newport RI, USA) was used to prepare a standard curve with values ranging from 25pg/tube to 8000pg/tube. A polyclonal antiserum raised against allopregnanolone carboxylmethyl ether coupled with BSA was purchased from Dr R.H. Purdy (Department of Psychiatry, Veterans Administration Hospital, San Diego, CA, USA); it had previously been characterised by Bernardi *et al.* (1998) (Table 5.1).

Table 5.1: Allopregnanolone antiserum cross reactivity with closely related steroids as characterised by Bernardi et al. (1998).

Steroid	Cross reactivity (%)
3α-hydroxy-5α-pregnan-20-one	100.00
3α-hydroxy-5β-pregnan-20-one	6.50
Pregn-4-ene-3,20-dione	0.70
5α-pregnane-3,20-dione	0.10
5β-pregnane-3,20-dione	0.10
20β-hydroxy-5α-pregnan-3-one	0.10
5α-pregnane-3α,20α-diol	0.10
3β-hydroxy-5α-pregnan-20-one	0.05
5β-pregnane-3α,20α-diol	0.03
3β-hydroxy-5β-pregnan-20-one	0.01
5α-pregnane-3β,20α-diol	0.01

Antiserum (250µl, working dilution 1/150) and tracer (250µl, ~ 8,000cpm) were added to standards (250µl) and the extracted samples (200µl) were mixed gently and incubated over night. The following day, bound and free fractions were separated by the addition of 200µl of cold charcoal solution (5mg activated charcoal, 0.5mg dextran T70 and 1mg bovine-γ-globulin for each ml of solution, made up with MilliQ water). After centrifuging at 2500rpm at 4°C for 10min, 500µl of the supernatant were aliquoted into a scintillation vial. Scintillation fluid (4ml; EcoSint A, National Diagnostics, Hessle Hull, UK) was then added to all tubes and radioactivity (β) was determined for each sample after vortexing. Radioactivity (β) was determined for each sample by placing samples into a multipurpose scintillation counter (Beckman Coulter LS6000TA/LS6500; Beckman Instruments Inc., Irvine, CA, USA). Values for all tubes were converted from cpm to pg/tube using a specialised computer program (Prince Henry's Hospital Institute of Medical Research Radioimmunoassay System, Monash University, Melbourne, Australia).

Recovery of allopregnanolone was 87.3% from plasma (n=1) and 79.8±4.4% (Stdev) for brain samples (n=5). For the final calculations these recoveries were taken into consideration to correct for extraction losses. The limit of detection (sensitivity) for plasma allopregnanolone was 1.69nmol/L (n=1) and that for brain allopregnanolone was 3.03±0.63pmol/g (Stdev) (n=5). The inter-assay and intra-assay coefficients of variation were 9% and 19%, respectively.

It was unfortunate that the antiserum used for the allopregnanolone assays ran out after the first set of assays, before any re-runs were carried out. New antisera from the same supplier could not be procured, as production had been stopped. Hence, a new antiserum was bought from a different source (AS04 041; Agrisera, Vännäs, Sweden). This meant that any re-runs for allopregnanolone had to be undertaken with the new antiserum instead of the old antiserum. Comparisons between the two antisera regarding binding efficiency showed that they were similar in that respect. When re-assaying the samples using the new antiserum, the values that read below the detection limit in the first assays (i.e. using old antiserum) also read low in the re-runs. The data of the first assays using the old antiserum were therefore used for statistical analyses so that all values were derived from RIAs using the same antiserum.

2) Progesterone in brain tissue

Tissue progesterone concentrations were measured in duplicate by a previously validated specific radioimmunoassay (RIA). Progesterone standard was prepared as a stock solution (4mM) in absolute ethanol, which was further diluted with ethanol to make up working dilutions of 10μM, 200nM and 10nM. A standard curve with values ranging from 0.1pmol/tube to 10.0pmol/tube was prepared using the above working dilutions, evaporating them to dryness in air at 37°C and reconstituting in 150μl of assay buffer (0.1M phosphate buffer, pH 7.4-7.6). Progesterone antibody raised in sheep against progesterone-11-α-BSA (progesterone antiserum S23; 14-9-81; lyophilised 25-9-84) was generously provided by Dr J. Malecki (Bairnsdale, Victoria, Australia). Cross reactivities with other steroids are presented in Table 5.2.

As the samples for progesterone analysis were the same as those extracted for

allopregnanolone analysis, the buffer used for re-suspension was different from that normally used for progesterone RIAs (AP buffer pH 7.4-7.6 while progesterone buffer pH 7.0). However, a test RIA was undertaken to assess the impact of the different buffers on RIA results for samples re-suspended in allopregnanolone buffer. As antiserum binding was improved when using the allopregnanolone buffer and as this was the buffer samples were re-suspended in after the extraction procedures, allopregnanolone buffer was used for progesterone RIAs.

Antiserum (100µl; working dilution 1/66.67) and tracer (50µl, ~ 10,000cpm) were added to standards (150µl) and extracted samples (150µl), which were then mixed gently and incubated over night. The following day bound and free fractions were separated by the addition of 50µl bovine-γ-globulin (20mg/ml assay buffer) and 800µl 30% PEG (polyethlene glycol).

Table 5.2: Progesterone antiserum cross reactivity with closely related steroids (Rice et al., 1986; Billiards, 2003).

Steroid	Cross reactivity (%)
Progesterone	100.00
11α-hydroxy progesterone	43.50
5α-pregnane,3α-ol,20-one	15.90
5β-pregnane,3α-ol,20-one	10.00
Corticosterone	1.05
17α-hydroxy-progesterone	0.70
20α-hydroxy-pregnane,3-one	0.30
11-deoxycortisol	<1.00
5α-pregnane,3β-ol,20-one	<1.00
5α-pregnane,3α,17α-diol-20-one	<1.00
5β-pregnane,3α,17α,20α-triol-20-one	<1.00
5β-pregnane,3α,17α,20α-triol	<1.00
Dehydroepiandrosterone	<0.40
Cortisol	<0.20

After centrifuging at 3200rpm at 4°C for 15min the supernatant was aspirated and the pellet resuspended with 50µl absolute ethanol. Once vortexed, 1.5ml of scintillation fluid (EcoSint A, National Diagnostics, Hessle Hull, UK) was added to all tubes and they were again vortexed and then sonicated for 30min. Radioactivity (β) was determined for each sample and values for all tubes were converted from cpm to pmol/tube using a specialised computer program (Prince Henry's Hospital Institute of Medical Research Radioimmunoassay System, Monash University, Melbourne, Australia).

Recovery of progesterone from brain tissue samples was 82.6±1.8% (Stdev) (n=5). For the final calculations these recoveries were taken into consideration to correct for extraction losses. The limit of detection (sensitivity) for progesterone was 6.41±2.67 pmol/g (Stdev) (n=3). The inter-assay and intra-assay coefficients of variation were 11% and 9%, respectively.

3) Progesterone in plasma – extracted assay

Plasma progesterone concentrations were measured in duplicate by a previously validated extracted radioimmunoassay (RIA). Progesterone standard was prepared as a stock solution (4mM) in absolute ethanol, which was further diluted with ethanol to make up working dilutions of 10µM, 200nM and 10nM. A standard curve with values ranging from 0.1pmol/tube to 10.0pmol/tube was prepared using the above working dilutions, evaporating them to dryness in air at 37°C and reconstituting them with 100µl CMP (charcoal maternal plasma; a steroid-free plasma sample). Extraction of progesterone was achieved by adding 100µl of distilled water (dH2O) and 2ml of hexane to all tubes. The tubes were then vortexed thoroughly and placed individually into an ethanol bath (ethanol and dry ice) to freeze the aqueous layer. The solvent was then decanted into glass tubes and evaporated to dryness under air at 37°C. Samples were then resuspended in 50µl assay buffer (0.1M phosphate buffer, pH 7.0).

Progesterone antibody, raised in sheep against progesterone-11-α-BSA (progesterone antiserum S23; 14-9-81; lyophilised 25-9-84), was generously provided by Dr J. Malecki (Bairnsdale, Victoria, Australia) (see Table 5.2 for cross reactivities).

Antiserum (100µl; working dilution 1/80) and tracer (100µl, ~10,000cpm) were added to standards (100µl) and samples (100µl), which were then mixed gently and incubated over night. The following day bound and free fractions were separated by the addition of 50µl bovine-γ-globulin (20mg/ml assay buffer) and 800µl 30% PEG (polyethlene glycol). After centrifuging at 3200rpm at 4°C for 15min the supernatant was aspirated and the pellet resuspended with 50µl absolute ethanol. Once all tubes had been vortexed, 1.5ml of scintillation fluid (EcoSint A, National Diagnostics, Hessle Hull, UK) was added to all tubes and they were vortexed and then sonicated for 30min. Radioactivity (β) was determined for each sample and values for all tubes were converted to from cpm to pmol/tube using a specialised computer program (Prince Henry's Hospital Institute of Medical Research Radioimmunoassay System, Monash University, Melbourne, Australia). The limit of detection (sensitivity) for progesterone was 0.86nmol/L.

4) Pregnenolone and pregnenolone sulphate in plasma

Plasma pregnenolone, and dissociated pregnenolone from pregnenolone sulphate, were measured in duplicate by a previously validated specific radioimmunoassay (RIA). Pregnenolone standard was prepared as a stock solution (4mM) in absolute ethanol, which was further diluted with ethanol to make up working dilutions of 10µM, 200nM and 10nM. A standard curve with values ranging from 0.01pmol/tube to 10.0pmol/tube was prepared using the above working dilutions, evaporating them to dryness in air at 37°C and reconstituting in 50µl assay buffer (0.1M phosphate buffer, pH 7.0). A commercial pregnenolone antibody was purchased from MP Biomedicals Australasia (Seven Hills NSW, Australia). Cross reactivities with other steroids are presented in Table 5.3.

Antiserum (100µl; 1/150 working dilution) and tracer (100µl, ~5,000cpm) were added to the extracted samples, which were then mixed gently and incubated over night. The

following day bound and free fractions were separated by the addition of 400µl of cold charcoal solution (5mg activated charcoal, 0.5mg dextran T70 and 1mg bovine-γ-globulin for each ml of solution, made up with MilliQ water). After centrifuging at 2000rpm at 4°C for 20min the supernatant was decanted into assay tubes. Scintillation fluid (2ml; EcoSint A, National Diagnostics, Hessle Hull, England, UK) was then added to all tubes and radioactivity (β) was determined for each sample after vortexing. Values for all tubes were converted from cpm to pmol/tube using a specialised computer program (Prince Henry's Hospital Institute of Medical Research Radioimmunoassay System, Monash University, Melbourne, Australia).

Recovery of pregnenolone was 50±11.3% (n=2) and 33±17.0% for pregnenolone sulphate (n=2). For the final calculations these recoveries were taken into consideration to correct for extraction losses. The limit of detection (sensitivity) for pregnenolone was 1.92nmol/L (n=1).

Table 5.3: Pregnenolone antiserum cross-reactivity with closely related steroids (Billiards, 2003).

Steroid	Cross reactivity (%)
Pregnenolone	100.00
Pregnenolone-sulfate	100.00
Progesterone	3.10
5α-dihydroprogesterone	0.85
Desoxycorticosterone	0.03
17α-hydroxypregnenolone	0.02
Cholesterol	<0.025
17α-hydroxyprogesterone	<0.025
20α-dihydroprogesterone	<0.025
Cortisol	<0.025
Dihydroepiandrosterone	<0.025
11-desoxycortisol	<0.025
Corticosterone	<0.025
Androsterone	<0.025
5α-dihydrotestosterone	<0.025
Ethiocholanolone	<0.025
Estradiol-17α	<0.025
Estradiol-17β	<0.025
Estriol	<0.025
Estrone	<0.025
20β-dihydroprogesterone	<0.025
Testosterone	<0.025
Aldosterone	<0.025
Androstenedione	<0.025

Lowry protein assay

The Lowry protein assay was undertaken to determine the amount of protein present in each tissue sample. This was done in order to determine the concentrations of allopregnanolone and progesterone per mg of protein rather than per unit wet weight to take into consideration any changes in tissue water content with age.

The pellets obtained during the homogenisation process during allopregnanolone/progesterone extractions were used for this purpose. First, pellets were resuspended with 1ml Reagent A&S (20µl of reagent S to each ml of reagent A; Bio-Rad Laboratories, Hercules, CA, USA). Samples were then vortexed and where necessary placed into a water bath at 60°C for 5min to ensure that the pellet dissolved. Samples were then diluted (1:4 dilution) by adding 30µl of Reagent A&S to 10µl of resuspended sample. A protein standard curve using Bovine Serum Albumin (BSA) was prepared ranging in concentrations from 0.5mg/ml to 3.5mg/ml.

Water blanks (230µl water), reagent blanks (5µl water), standards (5µl) and samples (5µl) were then added to a clean and non-scratched microplate and an additional 25µl of Reagent A&S and 200µl Reagent B (Bio-Rad Laboratories, Hercules, CA, USA) were added to all wells except water blanks. The plate was allowed to stand at room temperature for 15min and was then read by a Spectra Max Plus^{384} connected to a personal computer with Soft Max Pro Plus software (Molecular Devices, Menlo Park, CA, USA). The absorbance level was set at a wavelength of 650 to 750nm.

Calculation of final values

1) Allopregnanolone

Values obtained by RIA (pg/tube) had to be further processed to calculate the concentrations present in the sample.

- The output from the β-counter (pg/tube) was multiplied by a factor of 1.25, as 200µl of resuspended sample were used in an assay with 250µl of each standard.
- The resultant value was then multiplied by a factor of 1 (results by counter given in pg/ml, resuspension volume was 1ml).

- This value was then adjusted for recovery assessed during the extraction procedure.
- The amount of tissue or plasma used was taken into consideration by dividing the above value by the amount used for extraction to get a final value in pg/mg or pg/μl.
- The molecular weight of allopregnanolone (318.5g/mol) was then used to calculate pmol/mg or pmol/μl of allopregnanolone present in the sample.
- Last, pmol/g for tissue and pmol/ml for plasma were calculated by multiplying the value by a factor of 1000.

2) Progesterone

Values obtained by RIA (pmol/tube) had to be further processed to calculate the concentrations present in the sample.

- The output from β-counter (pmol/tube) was multiplied by a factor of 6.67 (1ml/150μl) to give pmol/ml, as 150μl of 1ml resuspended sample was used for the assay (output from counter was in pmol/tube, each tube containing 150μl sample and standards).
- This value was then adjusted for recovery assessed during the extraction procedure.
- The amount of tissue was taken into consideration by dividing the above value by the amount used for extraction to get a final value of pmol/mg.
- Last, pmol/g were calculated by multiplying the value by a factor of 1000.

3) Pregnenolone and pregnenolone sulphate

Values obtained by RIA (pmol/tube) had to be further processed to calculate the concentrations present in the sample.

- The output from β-counter (pmol/tube) was multiplied by a factor of 8 to give pmol/ml, as 50μl of 400μl resuspended sample was used for the assay (counter output in pmol/tube and added 40μl per tube of sample and standards).
- This value was then adjusted for recovery assessed during the extraction

procedure.

- The amount of tissue was taken into consideration by dividing the above value by the amount used for extraction to get a final value of pmol/mg.
- Last, pmol/g were calculated by multiplying the value by a factor of 1000.

4) Allopregnanolone and progesterone per mg of protein

Values obtained by the Lowry Protein assay had to be further processed to determine the amount of allopregnanolone per mg of protein present in the samples. For this purpose the following calculations were done:

- First, the output from the plate reader (protein concentration in mg/ml) was multiplied by a factor of 4. This was done as the sample to be analysed for protein concentration was diluted (1:4 dilution) after the addition of 1ml of reagent A/S to the pellet.
- The protein content per g wet weight (mg/g of tissue) was then calculated by adjusting the resultant value to the amount of tissue used for each sample (~100mg), then calculating the value per mg and multiplying by a factor of 1000.
- The concentration of allopregnanolone and progesterone present per mg of protein (pmol per mg of protein) was calculated by dividing their concentrations (pmol/g wet weight) by the protein concentration present in the tissue sample (mg/g wet weight).

Comparison with previously published data

Assuming that proportional changes in plasma allopregnanolone concentrations between 3-day-old lambs and other age groups were similar between the present lambs and those observed by Nguyen *et al*. (2003a), previously published values were used as a guide to extrapolate between the present plasma allopregnanolone concentrations of 3-day-old lambs and those for late-gestation fetuses at 132-134 days and 144 days of gestation, and postnatal lambs at 19-26 days of age. Likewise, allopregnanolone concentrations measured in fetuses ~130 days of gestation by Yawno *et al.* (2007) were

used to calculate approximate concentrations for 3-day-old lambs. This was done, as the extraction and assay methods used by Yawno *et al*. (2007) were the same as those used in the present study, while those used by Nguyen *et al*. (2003a) were not, thus allowing a check for methodology differences between studies.

Statistical Analyses

SPSS 11.0 for MAC OSX (SPSS Inc., Chicago ILL, USA) and Prism 5.0 for MAC OSX (GraphPad Software Inc., San Diego CA, USA) were used for all statistical analyses. Mean and standard error of the mean (SEM) were calculated for all parameters. Due to small sample sizes per group ($n \leq 5$) only non-parametric statistical tests were employed. Differences were considered significant at $p<0.05$. Degrees of freedom reported (e.g. df 1, 130) represent the number of groups and overall number of data points used in individual statistical tests. Although the data were part of a time series (12hrs to 9 days after birth) repeated measures statistics were not used, as the data were collected from different animals at each age.

1) Allopregnanolone and Progesterone

Plasma and brain

Comparison of allopregnanolone and progesterone concentrations between ages was only undertaken for those age groups for which data of three or more animals were available for each hormone (i.e. for which concentrations of each hormone were above the detection limit). Hence, mean and SEMs presented in graphs only represent those groups included in statistical analyses.

The non-parametric Kruskal-Wallis test for multiple comparisons and the Mann-Whitney test for paired comparisons were used. Post-hoc Wilcoxon paired sample tests were performed following the Kruskal-Wallis test to determine the ages between which significant differences could be detected.

Statistical analysis was not possible for plasma progesterone data, as these were below the detection limit for all lambs.

Protein calculations

Protein concentrations between the six age groups in general and within the same tissue were compared using the Kruskal-Wallis test. In tissues where significant differences were observed in protein concentrations with age, allopregnanolone and progesterone concentrations per mg of protein were assessed and a Kruskal-Wallis test with post-hoc Dunn's test was employed to assess hormone concentration changes with age. This was done to determine whether changes in protein concentrations with age significantly affected hormone concentrations at those ages.

2) Pregnenolone and pregnenolone sulphate

The non-parametric Kruskal-Wallis test with post-hoc Wilcoxon paired sample tests were performed to determine whether plasma pregnenolone and pregnenolone sulfate concentrations changed significantly over the first 9 days after birth.

5.3 Results

General information

The gender and average weight of lambs per gender and per age group are presented in Table 5.4. One lamb (Lamb 1) weighed below 3kg at euthanasia (2.8kg).

Table 5.4: General information for lambs aged between 12hrs or less and 9 days after birth (n = 5 per group).

Age (days)	Gender (number of animals N)		Mean weight (kg)	Mean weight (kg) Combined genders
0.5 or less	*Female*	N = 3	3.33 ± 0.4	3.54 ± 0.3
	Male	N = 2	3.85 ± 0.8	
1	*Female*	N = 2	4.75 ± 0.3	4.78 ± 0.1
	Male	N = 3	4.80 ± 0.2	
1.5	*Female*	N = 1	5.80	4.56 ± 0.4
	Male	N = 4	4.25 ± 0.3	
3	*Female*	N = 3	5.07 ± 0.3	5.28 ± 0.6
	Male	N = 2	5.60 ± 1.8	
7	*Female*	N = 3	6.60 ± 0.5	6.36 ± 0.3
	Male	N = 2	6.00 ± 0.2	
9	*Female*	N = 4	6.20 ± 0.7	6.50 ± 0.6
	Male	N = 1	7.70	

Allopregnanolone

1) Plasma

Allopregnanolone concentrations (mean±SEM) in lambs aged between 12hrs or less and 9 days after birth are presented in Figure 5.2. None of the samples read below the detection limit.

In addition, mean allopregnanolone concentrations for lamb fetuses approximately 130 days of gestation assessed in an umbilical cord occlusion study by Yawno *et al*. (2007) are presented as the mean of three mean control values for lambs before various treatments (n=5 per group) (Yawno *et al.*, 2007).

The postnatal plasma concentrations of allopregnanolone were significantly different between age groups as assessed by the non-parametric Kruskal-Wallis test (p=0.007, df 5, 29, Chi- Square 15.89). Allopregnanolone concentrations of lambs aged up to 12hrs were significantly higher than those of older lambs (p=0.043, Z=-2.023 for all comparisons; Figure 5.2). Overall, there was a gradual decline in allopregnanolone concentrations with age with values from 1.5 days showing little subsequent change.

Comparison with previously published data

Using proportional changes in plasma allopregnanolone concentrations reported by Nguyen *et al.* (2003a) as a guide for calculation, extrapolating from 3-day-old lambs to fetal lamb values showed that one would anticipate allopregnanolone concentrations at ~130-135 and ~144 days of gestation to be around 58nmol/L. Likewise, extrapolating from 3-day-old lambs to those 19 to 26 days old, one would anticipate allopregnanolone concentrations of 15.5nmol/L for the present lambs. Mean values of control lambs ~130 days of gestation observed by Yawno *et al*. (2007) were 54nmol/L and estimated values for 3-day-old lambs and 19-26-day-old lambs were 31.2nmol/L and 14.2nmol/L, respectively. The values estimated for 3-day-old lambs agree with the ones of the present study.

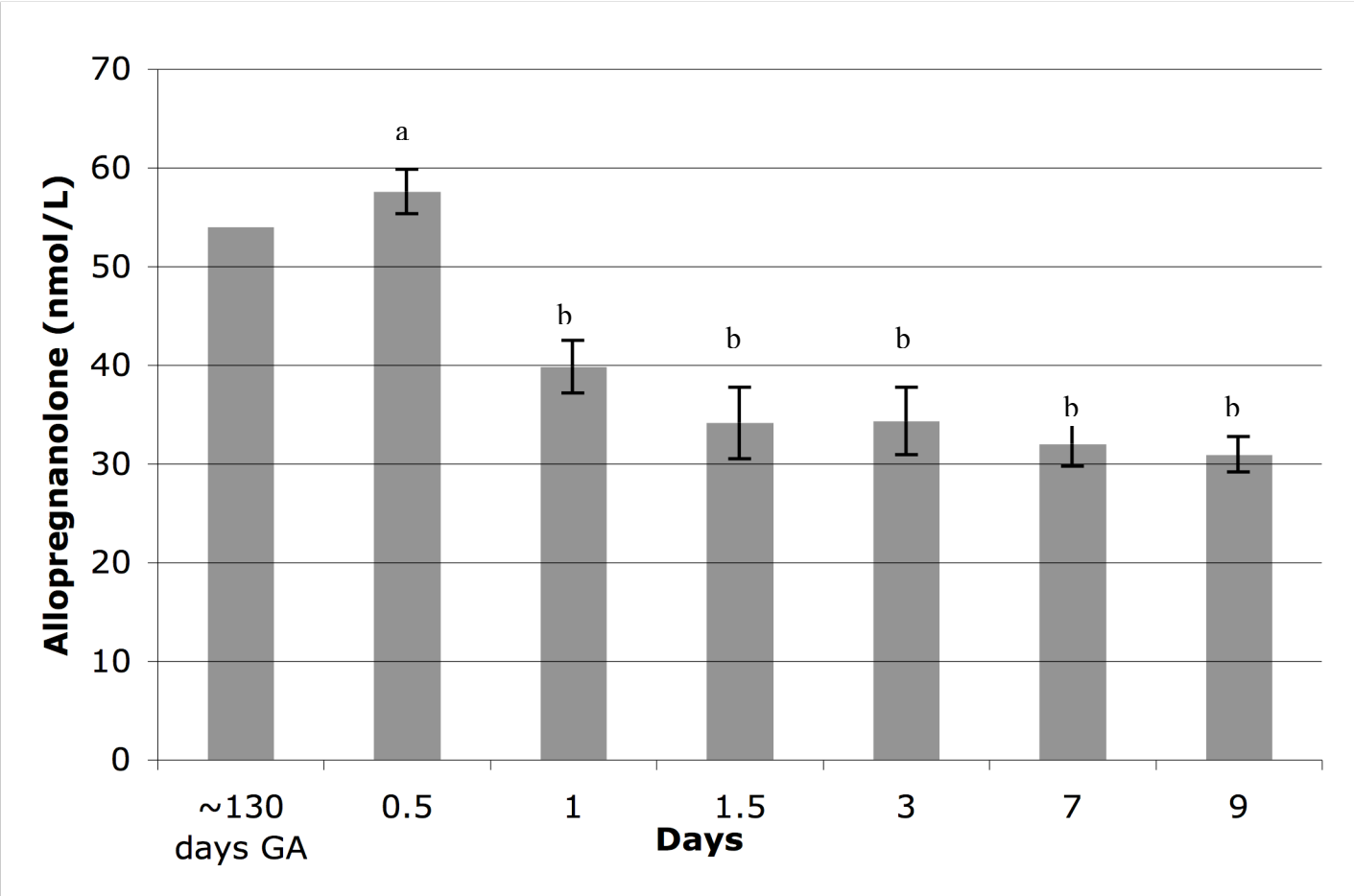

Figure 5.2: Mean and SEM of plasma allopregnanolone concentrations of fetal lambs ~130 days gestational age (Yawno et al., 2007) and the present newborn lambs aged between 12hrs or less and 9 days after birth (n=5 per age group). The fetal data have been included with permission by Tamara Yawno. Values that do not share the same letter are significantly different from each other (p<0.05).

2) Brain tissue

Brain tissue allopregnanolone data fell into two groups, lambs with allopregnanolone concentrations above the detection limit and lambs with allopregnanolone concentrations below the detection limit. The numbers of lambs with values below the detection limit at each age for each brain area are presented in Table 5.5. As undetectable concentrations are not necessarily zero, animals with values below the detection limit have not been included as zero values in the statistical analysis.

The mean and SEM for allopregnanolone concentrations (for wet weight) of all brain regions examined are presented in Table 5.6. The graphs presented in this section display results for those groups used in statistical analysis only (i.e. age groups which had three or more animals with values in the detectable range).

Table 5.5: Number of lambs with allopregnanolone concentrations below the detection limit for each age group and each brain region.

Age (days)	Spinal Cord	Medulla	Pons	Cerebellum	Basal Ganglia	Hippocampus	Cerebral Cortex
0.5 or less (n=5)	0	0	0	0	0	2	1
1 (n=5)	5	0	5	0	3	5	4
1.5 (n=5)	3	0	5	0	3	5	2
3 (n=5)	2	0	2	0	5	3	3
7 (n=5)	2	0	3	0	2	5	5
9 (n=4)	4	0	4	0	4	3	4

Spinal Cord

There were no significant changes in allopregnanolone concentrations in the spinal cord between the ages of up to 12hrs and 3 and 7 days (p=0.110, df 2, 10, Chi-Square 4.412) (Figure 5.3), although numerically the mean values decreased.

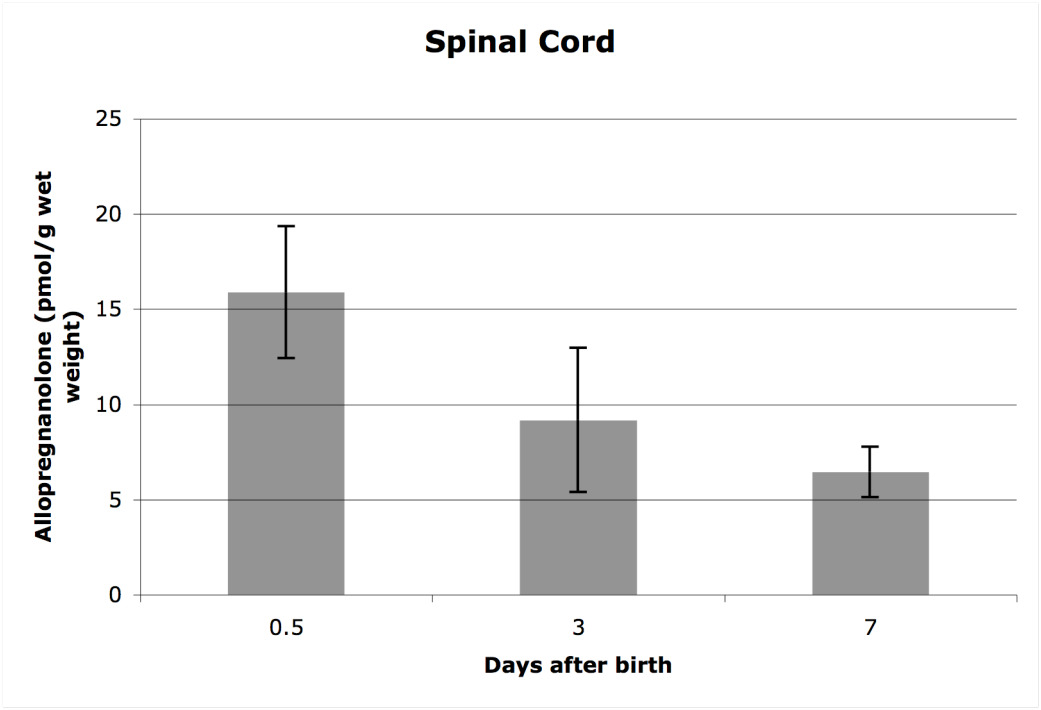

Figure 5.3: Mean and SEM of allopregnanolone concentrations (pmol/g wet weight) of the spinal cord in lambs aged up to 12hrs (n=5), 3 (n=3) and 7 (n=3) days after birth. Number of lambs with values below the detection limit were 0, 5, 3, 2, 2 and 4 for lambs aged 12hrs or less, 1, 1.5, 3, 7 and 9 days, respectively.

Medulla

Allopregnanolone concentrations in the medulla changed significantly with age (p=0.046, df, 5, Chi-Square 11.299). There was a significant decrease in allopregnanolone concentrations between lambs aged up to 12hrs and those aged 1.5 and 3 days (p=0.043, Z=-2.023) and a significant increase in lambs between 1.5 and 7 days (p=-0.043, Z=-2.023) (see Figure 5.4).

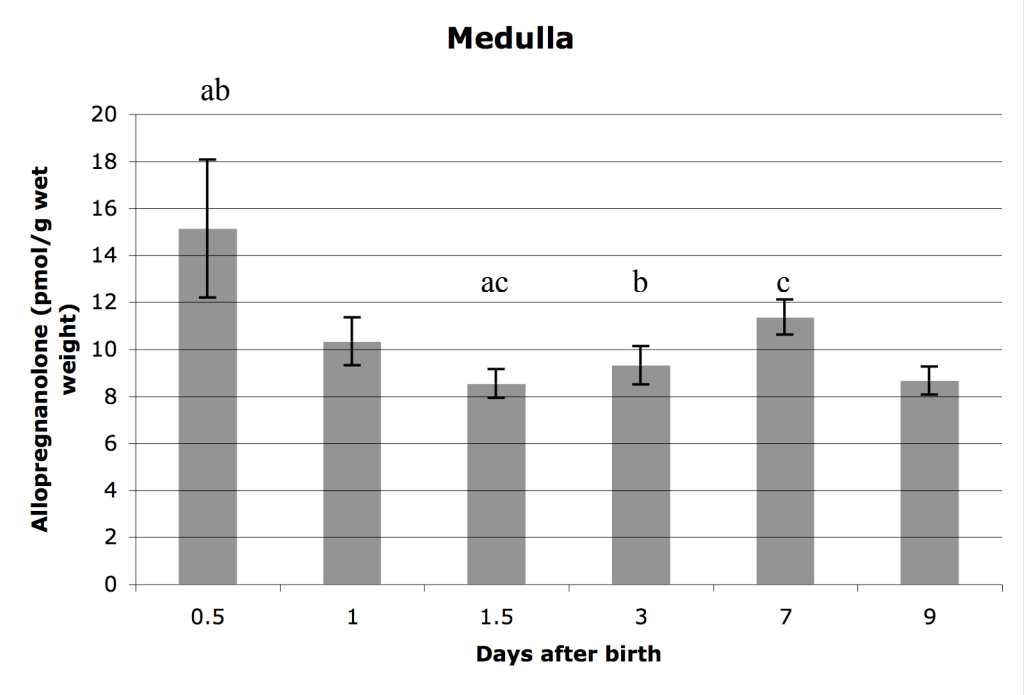

Figure 5.4: Mean and SEM of allopregnanolone concentrations (pmol/g wet weight) in the medulla of lambs aged between 12hrs or less and 9 days after birth (n=5 for all but 9-day lambs where n=4). Values that do share the same letter are significantly different from each other, $p<0.05$). There were no values below the detection limit.

Table 5.6: Means and SEM allopregnanolone concentrations (pmol/g wet weight) and number of lambs per age group in the brain regions investigated (n=5 for each age group but 9-day-old lambs where n=4). Only values of lambs with allopregnanolone concentrations above the detection limit are presented.

Days	*Spinal Cord*	*Medulla*	*Pons*	*Cerebellum*	*Basal Ganglia*	*Hippocampus*	*Cerebral Cortex*
0.5 or less	15.90[Mean] 3.47[SEM] 5[N]	15.13 2.94 5	15.52 3.33 5	10.65 1.74 5	10.50 1.16 5	9.61 1.52 3	7.64 0.73 4
1		10.34 1.02 5		10.56 0.43 5	6.56 0.45 2		17.48 1
1.5	16.30 7.33 2	8.54 0.61 5		12.00 1.61 5	5.74 0.42 2		7.42 1.48 3
3	9.19 3.78 3	9.32 0.81 5	8.19 2.85 3	13.97 1.89 5		8.84 0.27 2	7.02 0.62 2
7	6.45 1.31 3	11.37 0.75 5	6.71 1.56 2	11.08 1.63 5	9.05 1.42 3		
9		8.67 0.59 4		9.56 1.42 4		7.331 1	

Pons

There were no significant changes in allopregnanolone concentrations in the pons of lambs aged up to 12hrs compared to those 3 days after birth (p=0.101, df 7, Z=-1.640) (Figure 5.5), the values in the former lambs were numerically higher.

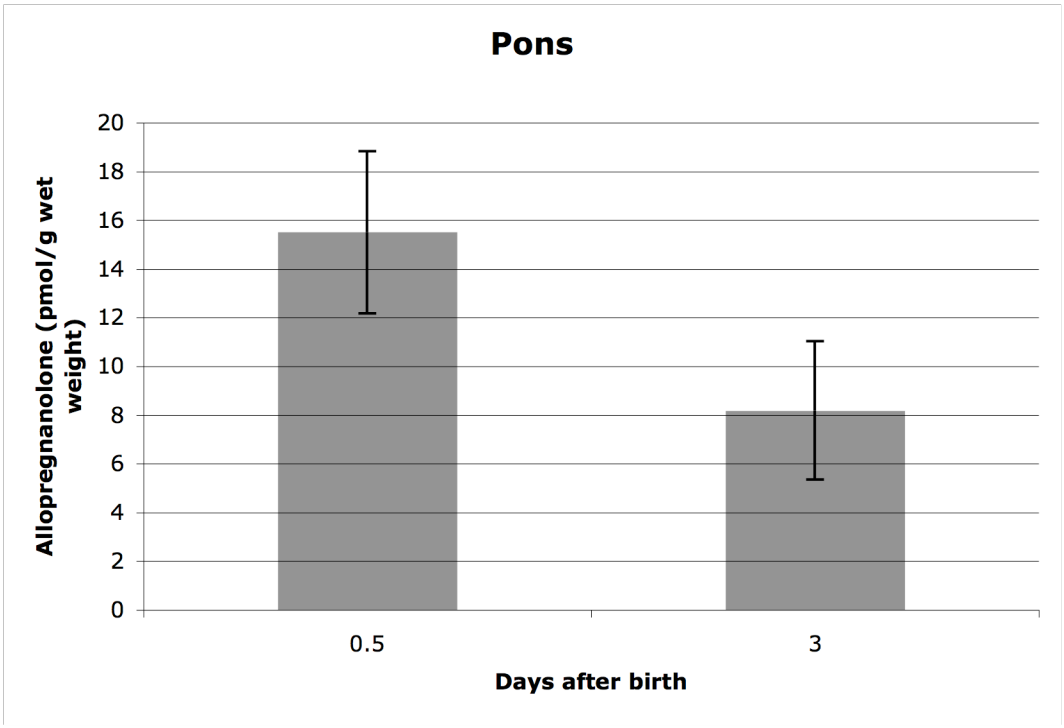

Figure 5.5: Mean and SEM of allopregnanolone concentrations (pmol/g wet weight) of the pons in lambs aged up to 12hrs (n=5) and 3 days (n=3) after birth. Number of lambs with values below the detection limit were 0, 5, 5, 2, 3 and 4 for lambs aged 12hrs or less, 1, 1.5, 3, 7 and 9 days, respectively.

Cerebellum

Allopregnanolone concentrations in the cerebellum did not significantly change between lambs aged up to 12hrs to those aged 9 days after birth (p=0.504, df, 5, Chi-Square 4.320; Figure 5.6).

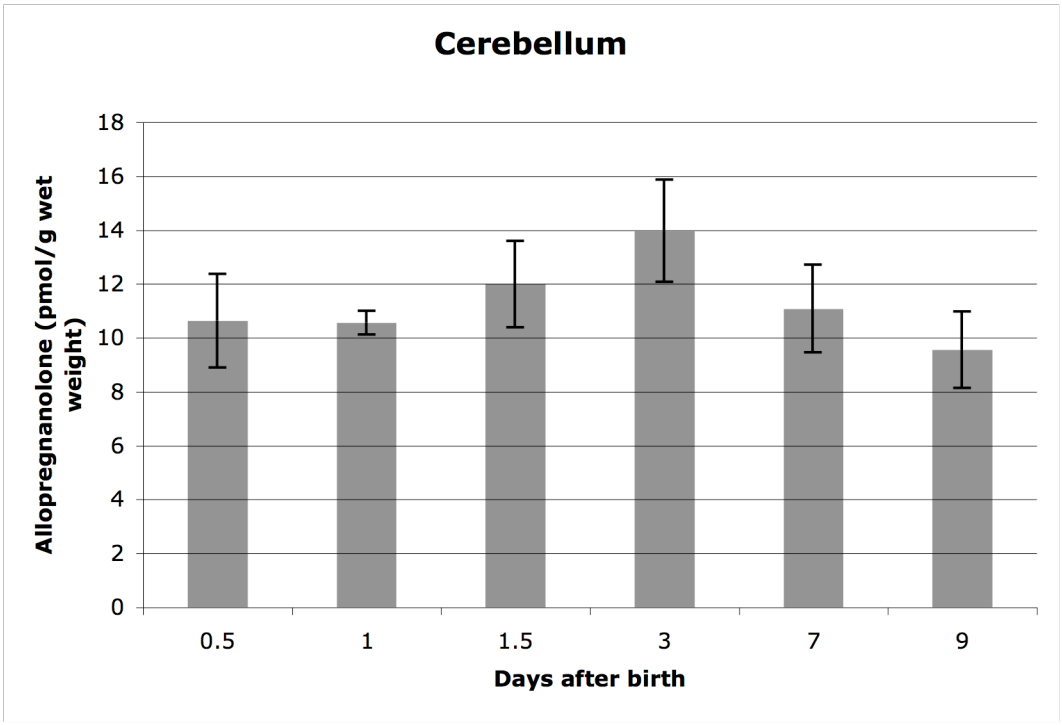

Figure 5.6: Mean and SEM of allopregnanolone concentrations (pmol/g wet weight) of the cerebellum of lambs aged up to 12hrs to 9 days after birth (n=5 for all but 9-day-old lambs where n=4). There were no values below the detection limit.

Basal Ganglia

There were no significant changes in allopregnanolone concentrations in the basal ganglia between the ages of up to 12hrs and 7 days (p=0.655, df 7, Z=-0.447; Figure 5.7).

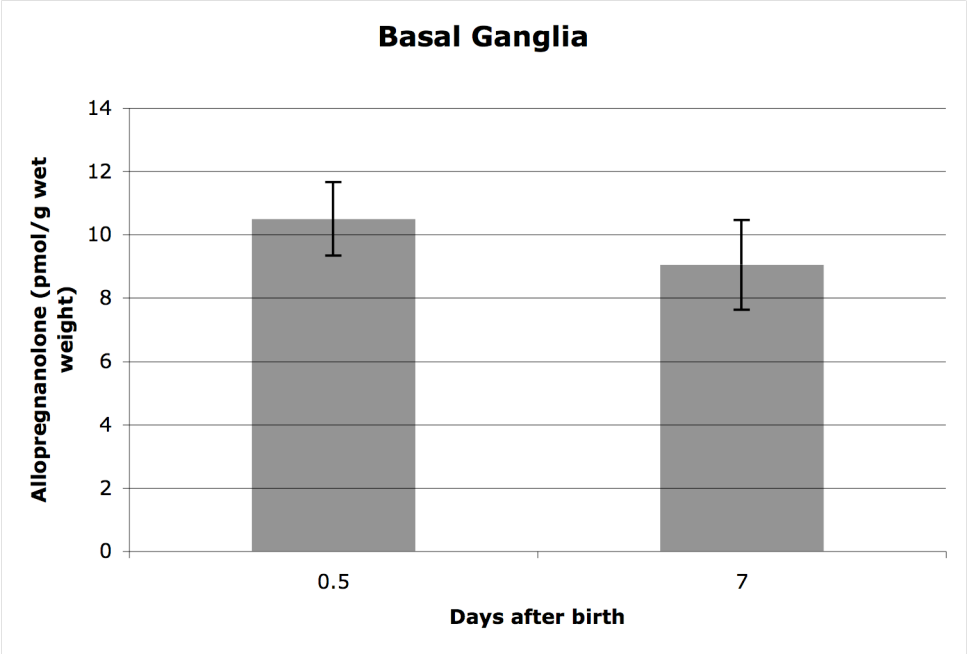

Figure 5.7: Mean and SEM of allopregnanolone concentrations (pmol/g wet weight) of the basal ganglia in lambs aged up to 12hrs (n=5) and 7 days (n=3) after birth. Number of lambs with values below the detection limit were 0, 3, 3, 5, 2 and 4 for lambs aged 12hrs or less, 1, 1.5, 3, 7 and 9 days, respectively.

Hippocampus

The concentrations of allopregnanolone in the hippocampus were below the detection limit for the majority of lambs of all ages (Table 5.5). The data available for hippocampal allopregnanolone concentrations were thus not sufficient for statistical analysis and hence no comparison between ages was possible.

Cerebral Cortex

There were no significant changes in allopregnanolone concentrations in the cerebral cortex between the ages of up to 12hrs and 1.5 days ($p=0.157$, df 4, $Z=-1.414$; Figure 5.8).

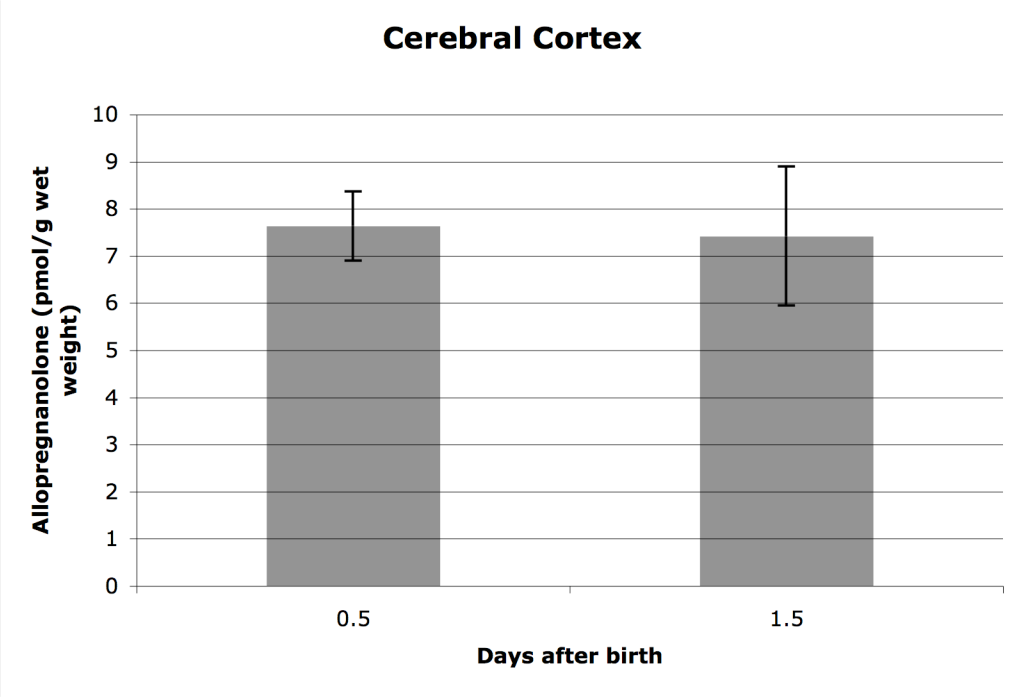

Figure 5.8: Mean and SEM of allopregnanolone concentrations (pmol/g wet weight) of the cerebral cortex in lambs aged up to 12hrs (n=4) and 1.5 days (n=3) after birth. Number of lambs with values below the detection limit were 1, 4, 2, 3, 5 and 4 for lambs aged 12hrs or less, 1, 1.5, 3, 7 and 9 days, respectively.

Progesterone

1) Plasma

Plasma progesterone concentrations were below the detection limit for all lambs.

2) Brain tissue

Brain tissue progesterone data also fell into two groups, those lambs with progesterone concentrations below the detection limit and those with values above the detection limit. The numbers of lambs with values below the detection limit at each age for each brain area are presented in Table 5.7. As undetectable concentrations are not necessarily zero, animals with values below the detection limit have not been included as zero values in the statistical analysis.

The means and SEM for progesterone concentrations (for wet weight) of all brain regions examined are presented in Table 5.8. The graphs presented in this sections display results for those age groups used in statistical analysis only (i.e. age groups

which had three or more animals with values in the detectable range).

Table 5.7: Number of lambs with progesterone concentrations below the detection limit for each age group and each brain region.

Age (days)	Spinal Cord	Medulla	Pons	Cerebellum	Basal Ganglia	Hippocampus	Cerebral Cortex
0.5 or less (n=5)	4	2	3	0	0	1	0
1 (n=5)	5	0	5	0	3	1	1
1.5 (n=5)	3	0	5	0	5	5	1
3 (n=5)	4	0	4	0	5	2	2
7 (n=5)	5	0	5	0	1	3	3
9 (n=5	4	1	4	0	3	4	0

Spinal Cord and Pons

The concentrations of progesterone in the spinal cord and the pons were below the detection limit for the majority of lambs of all ages (Table 5.7). The data available for spinal cord and pons progesterone concentrations were therefore not sufficient for valid statistical analysis and hence no comparison between ages was possible.

Table 5.8: Means and SEM progesterone concentrations (pmol/g wet weight) and number of lambs per age group in the brain regions investigated (n=5 for each age group apart from 9-day-old lambs where n=4). Only values of lambs with progesterone concentrations above the detection limit are presented.

Days	*Spinal Cord*	*Medulla*	*Pons*	*Cerebellum*	*Basal Ganglia*	*Hippocampus*	*Cerebral Cortex*
0.5 or less	63.49 1	62.62^{Mean} 9.71^{SEM} 3^{N}	57.23 2.47 2	46.50 2.64 5	24.40 2.91 5	22.48 2.51 4	19.41 1.13 5
1		53.08 5.03 5		34.91 2.69 5	26.76 3.74 2	18.11 2.93 4	18.29 1.52 4
1.5	16.30 7.33 2	46.63 5.21 5		38.79 5.95 5			16.44 1.05 4
3	50.12 1	56.33 5.01 5	31.84 1	38.22 2.19 5		20.78 6.60 3	15.33 0.67 3
7		51.41 4.09 5		40.73 6.05 5	16.04 2.24 4	17.70 0.29 2	15.83 2.33 2
9		48.32 3.13 4		46.41 5.18 4	16.07 1		21.64 2.44 4

Medulla

The progesterone concentrations in the medulla did not change significantly between lambs aged 12hrs or less and 9 days of age (p=0.647, df 5, 26, Chi-Square 3.343). The means and SEM for progesterone concentrations in the medulla at the different ages are presented in Figure 5.9.

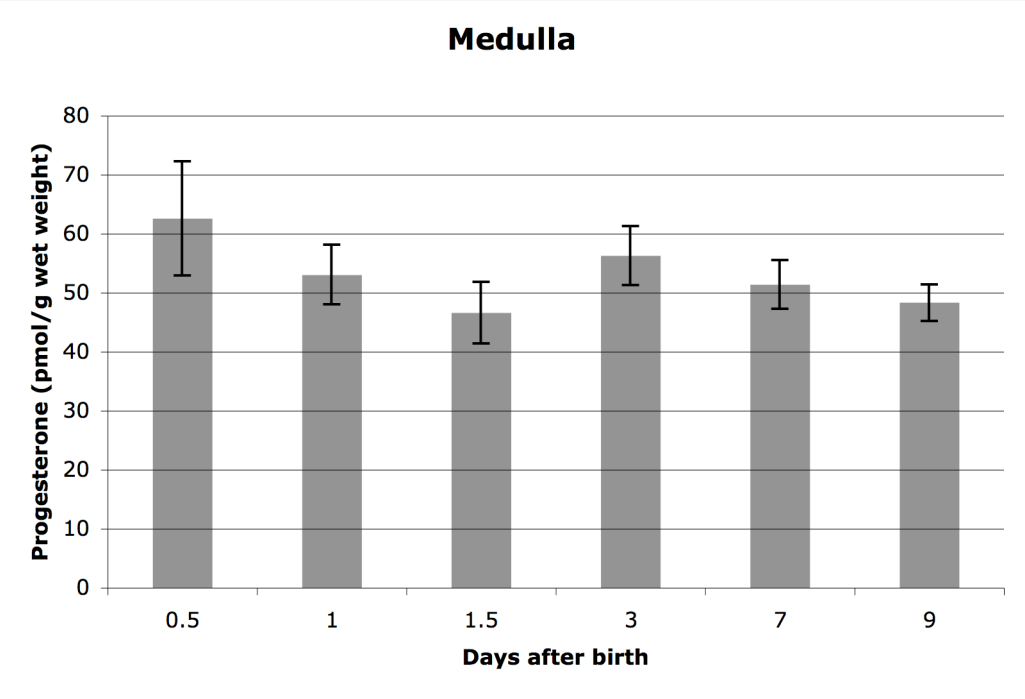

Figure 5.9: Mean and SEM of progesterone concentrations (pmol/g wet weight) of the medulla of lambs aged up to 12hrs to 9 days after birth (n=5 for all but lambs up to 12hrs and 9 days of age where n=3 and 4, respectively). Number of lambs with values below the detection limit were 2, 0, 0, 0, 0 and 1 for lambs aged 12hrs or less, 1, 1.5, 3, 7 and 9 days, respectively.

Cerebellum

The progesterone concentrations in the cerebellum did not change significantly between lambs age 12hrs or less and 9 days of age (p=0.281, df 5, 28, Chi-Square 6.268). The means and SEM for progesterone concentrations in the cerebellum at the different ages are presented in Figure 5.10.

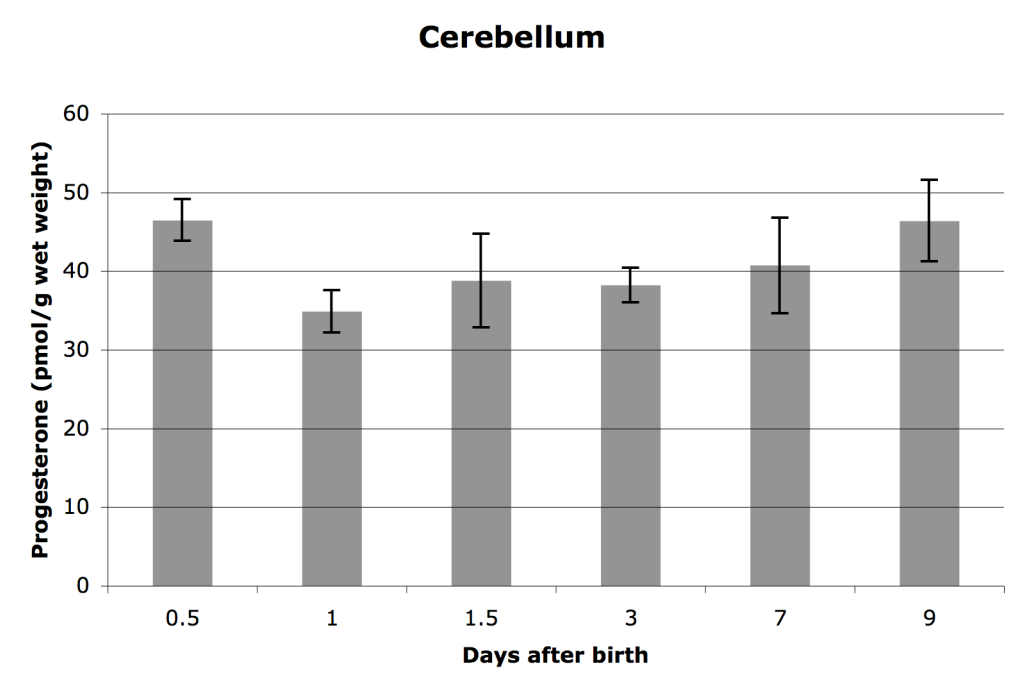

Figure 5.10: Mean and SEM of progesterone concentrations (pmol/g wet weight) of the cerebellum of lambs aged up to 12hrs to 9 days after birth (n=5 for all but 9-day-old lambs where n=4). There were no values below the detection limit.

Basal Ganglia

There were no significant changes in progesterone concentrations in the basal ganglia between the ages of 12hrs or less and 7 days (p=0.050, df 8, Z=-1.960; Figure 5.11).

Hippocampus

There were no significant changes in progesterone concentrations in the hippocampus between the ages of 12hrs, 1 day and 3 days (p=0.523, df 2, 10, Chi-Square 1.295; Figure 5.12).

Cerebral Cortex

There were no significant changes in progesterone concentrations in the cerebral cortex between the ages of 12hrs or less and 9 days (p=0.112, df 4, 19, Chi-Square 7.504; Figure 5.13).

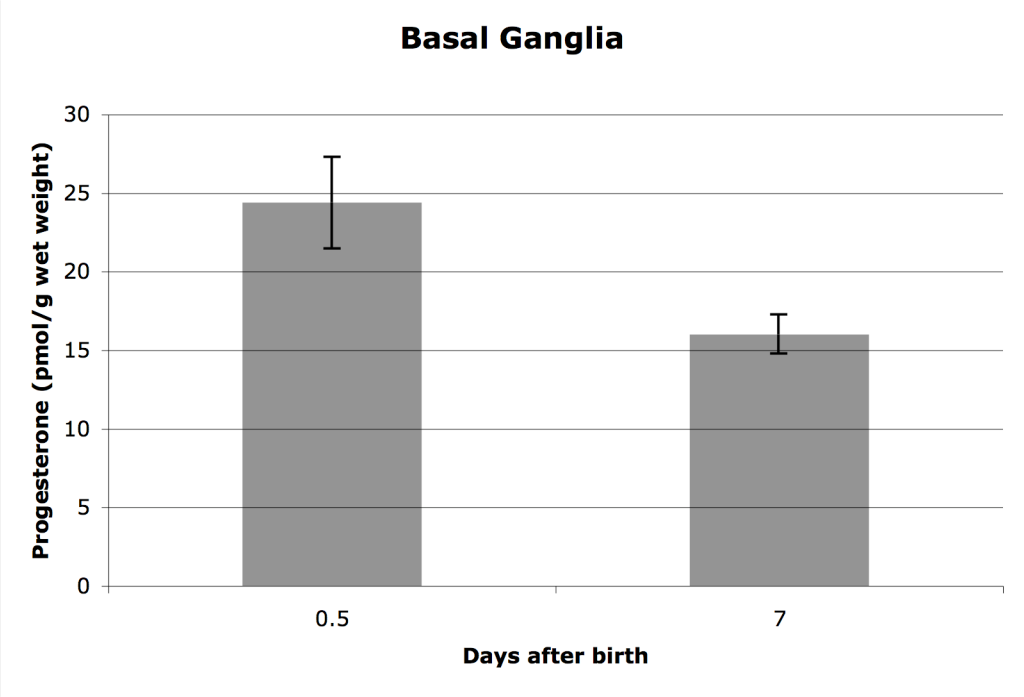

Figure 5.11: Mean and SEM of progesterone concentrations (pmol/g wet weight) of the basal ganglia in lambs aged up to 12hrs (n=5) and 7 days (n=4) after birth. Number of lambs with values below the detection limit were 0, 3, 5, 5, 1 and 3 for lambs aged 12hrs or less, 1, 1.5, 3, 7 and 9 days, respectively.

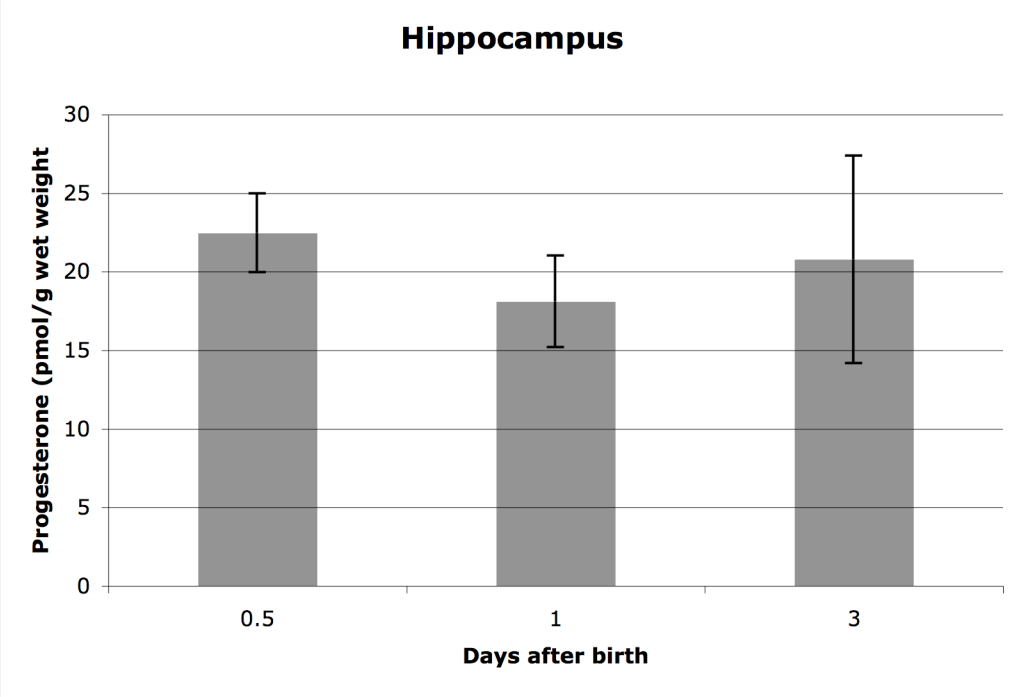

Figure 5.12: Mean and SEM of progesterone concentrations (pmol/g wet weight) of the hippocampus in lambs aged up to 12hrs (n=4), 1 day (n=4) and 3 days (n=3) after birth. Number of lambs with values below the detection limit were 1, 1, 5, 2, 3 and 4 for lambs aged 12hrs or less, 1, 1.5, 3, 7 and 9 days, respectively.

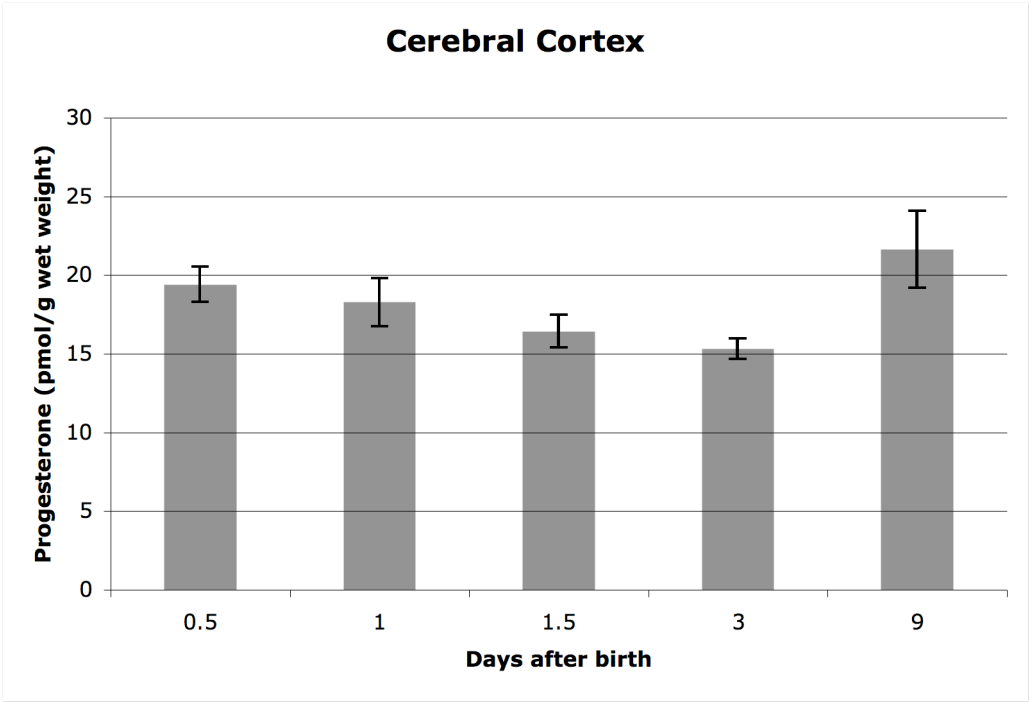

Figure 5.13: Mean and SEM of progesterone concentrations (pmol/g wet weight) of the cerebral cortex in lambs aged up to 12hrs, 1, 1.5, 3 and 9 days after birth (n=5, 4, 4, 3 respectively). Number of lambs with values below the detection limit were 0, 1, 1, 2, 3 and 0 for lambs aged 12hrs or less, 1, 1.5, 3, 7 and 9 days, respectively.

Protein calculations

The protein concentrations per g of wet weight per age group are presented in Table 5.9. There were no significant changes in protein concentrations between age groups when tissue values were pooled for each age group (p=0.117, df 5, 195, Chi-Square 8.814).

There were no significant changes in protein concentrations with age in the spinal cord (p=0.503, df 5, 28, Chi-Square 4.328), pons (p=0.342, df 5,28, Chi-Square 5.647), hippocampus (p=0.208, df 5,22, Chi-Square 7.169), cortex (p=0.963, df 5, 28, Chi-Square 0.994), basal ganglia (p=0.399, df 5, 28, Chi-Square 5.143) and cerebellum (p=0.074, df 5, 28, Chi-Square 10.042).

However, there were significant changes with age in protein concentrations in the

medulla (p=0.006, df 5, 28, Chi-Square 16.277). There were no significant changes with age in the medullar allopregnanolone and progesterone concentrations per mg of protein (p=0.122, df 5, 28, Chi-Square 8.687 and p=0.287, df 5, 28, Chi-Square 6.397; respectively). With regard to allopregnanolone, however, these results are in contrast to those calculated for allopregnanolone concentrations per g of wet weight, where a significant decrease in allopregnanolone concentration was observed between 12 hours and 1.5 days (see previous section on allopregnanolone concentration in brain tissue).

The protein concentrations per g of wet weight, allopregnanolone concentrations per mg of protein and progesterone concentrations per mg of protein for those samples where the steroid concentrations were above the detection limit are presented according to age group and brain region in Tables 5.10 and 5.11.

Table 5.9: General information for protein concentrations (mg/g wet weight) per age group. N= number of tissue samples per age

Protein [ ]	*Mean*	*Median*	*SEM*	*N*
0.5 days	64.69	64.35	1.54	34
1 days	61.25	62.64	1.69	34
1.5 days	63.98	63.63	0.91	34
3 days	63.35	62.65	1.18	35
7 days	62.79	62.77	1.61	33
9 days	59.56	61.32	1.57	27

Table 5.10: Means and SEMs for protein concentrations (g wet weight) and allopregnanolone (AP) concentrations (pmol) per mg of protein for each brain region in each age group for those samples where the AP concentrations were above the detection limit. N=number of animals

	Up to 0.5 days		**1 day**		**1.5 days**		**3 days**		**7 days**		**9 days**	
Brain region	**Protein**	**AP**	**Protein**	**AP**	**Protein**	**AP**	**Protein**	**AP**	**Protein**	**AP**	**Protein**	**AP**
Spinal Cord	58.18^{Mean}	0.27			61.88	0.26	62.01	0.15	65.09	0.10		
	2.95^{SEM}	0.06			0.54	0.12	2.19	0.06	2.28	0.02		
	5^{N}	5			2	2	3	3	3	3		
Medulla	63.93	0.24	50.00	0.21	61.56	0.14	55.37	0.17	61.77	0.19	53.57	0.17
	0.64	0.05	0.91	0.02	1.72	0.01	1.00	0.01	2.48	0.02	6.80	0.02
	5	5	5	5	5	5	5	5	5	5	4	4
Pons	65.75	0.24					65.30	0.13	64.80	0.11		
	1.83	0.05					3.73	0.05	1.60	0.03		
	5	5					3	3	2	2		
Cerebellum	62.26	0.17	62.75	0.17	64.47	0.19	60.48	0.23	54.11	0.20	56.34	0.17
	1.34	0.03	1.34	0.01	3.11	0.02	4.86	0.01	1.96	0.03	2.13	0.02
	5	5	5	5	5	5	5	5	5	5	4	4
Hippocampus	69.03	0.14					63.06	0.14			56.16	0.13
	1.94	0.02					0.41	0.01				
	3	3					2	2			1	1
Basal	75.21	0.14	64.43	0.10	67.70	0.09			53.87	0.19		
Ganglia	4.60	0.02	11.83	0.01	1.93	0.01			8.42	0.06		
	5	5	2	2	2	2			3	3		
Cortex	57.86	0.15	61.76	0.28	62.79	0.12	65.38	0.11				
	8.56	0.04			0.99	0.03	3.94	0.02				
	4	4	1	1	3	3	2	2				

Table 5.11: Means and SEMs of protein concentrations (g wet weight) and progesterone (PROG) concentrations (pmol) per mg of protein for each brain region in each age group for those samples where the PROG concentrations were above the detection limit. N=number of lambs

	Up to 0.5 days		**1 day**		**1.5 days**		**3 days**		**7 days**		**9 days**	
Brain region	**Protein**	**PROG**	**Protein**	**PROG**	**Protein**	**PROG**	**Protein**	**PROG**	**Protein**	**PROG**	**Protein**	**PROG**
Spinal Cord	59.31 1	1.07 1					61.25 1	0.82 1				
Medulla	64.60^{Mean} 0.90^{SEM} 3^{N}	0.97 0.16 3	50.00 0.91 5	1.07 0.11 5	61.56 1.72 5	0.76 0.08 5	55.37 1.00 5	1.01 0.08 5	61.77 2.48 5	0.84 0.08 5	53.57 6.80 4	0.96 0.15 4
Pons	65.73 1.39 2	0.87 0.06 2					58.34 1	0.55 1				
Cerebellum	62.26 1.34 5	0.75 0.05 5	62.75 1.40 5	0.56 0.05 5	64.47 3.11 5	0.63 0.12 5	60.48 4.86 5	0.64 0.05 5	54.11 1.96 5	0.75 0.11 5	56.34 2.13 4	0.84 0.11 4
Hippocampus	68.57 1.44 4	0.33 0.04 4	67.25 6.39 4	0.28 0.05 4			62.88 0.30 3	0.33 0.10 3	73.34 2.17 2	0.24 0.003 2		
Basal Ganglia	75.20 4.60 5	0.32 0.03 5	75.53 1.32 2	0.35 0.04 2					58.58 7.59 4	0.29 0.04 4	63.01 1	0.27 1
Cortex	59.73 6.89 5	0.34 0.03 5	67.26 1.91 4	0.27 0.02 4	63.33 1.33 5	0.26 0.02 4	63.23 3.12 3	0.24 0.02 3	57.14 10.74 2	0.28 0.01 2	62.93 1.63 4	0.34 0.04 4

Pregnenolone and pregnenolone sulphate in plasma

Plasma pregnenolone and pregnenolone sulfate concentrations (mean±SEM) for lambs aged between 12hrs or less and 9 days after birth are presented in Figure 5.14. All values were above the detection limit.

The results of the Kruskal-Wallis test showed that the plasma concentrations of both pregnenolone and pregnenolone sulphate differed significantly between age groups ($p<0.001$, df 5, Chi-Square 24.374 and $p=0.010$, df 5, Chi-Square 15.06; respectively). According to post-hoc Dunn's tests, pregnenolone concentrations were significantly higher in lambs aged 12hrs or less than in lambs aged 3 days and 9 days ($p<0.05$), and those of 1.5-day-old lambs were significantly higher than those of 9-day-old lambs ($p<0.05$). For pregnenolone sulphate the Wilcoxon test showed that significant differences across ages were due to significant differences between lambs aged 1 day and 7 days ($p=0.043$; $Z=-2.023$) and between lambs aged 1.5 days and those aged 7 and 9 days ($p=0.043$; $Z=-2.023$) (see Figure 5.14).

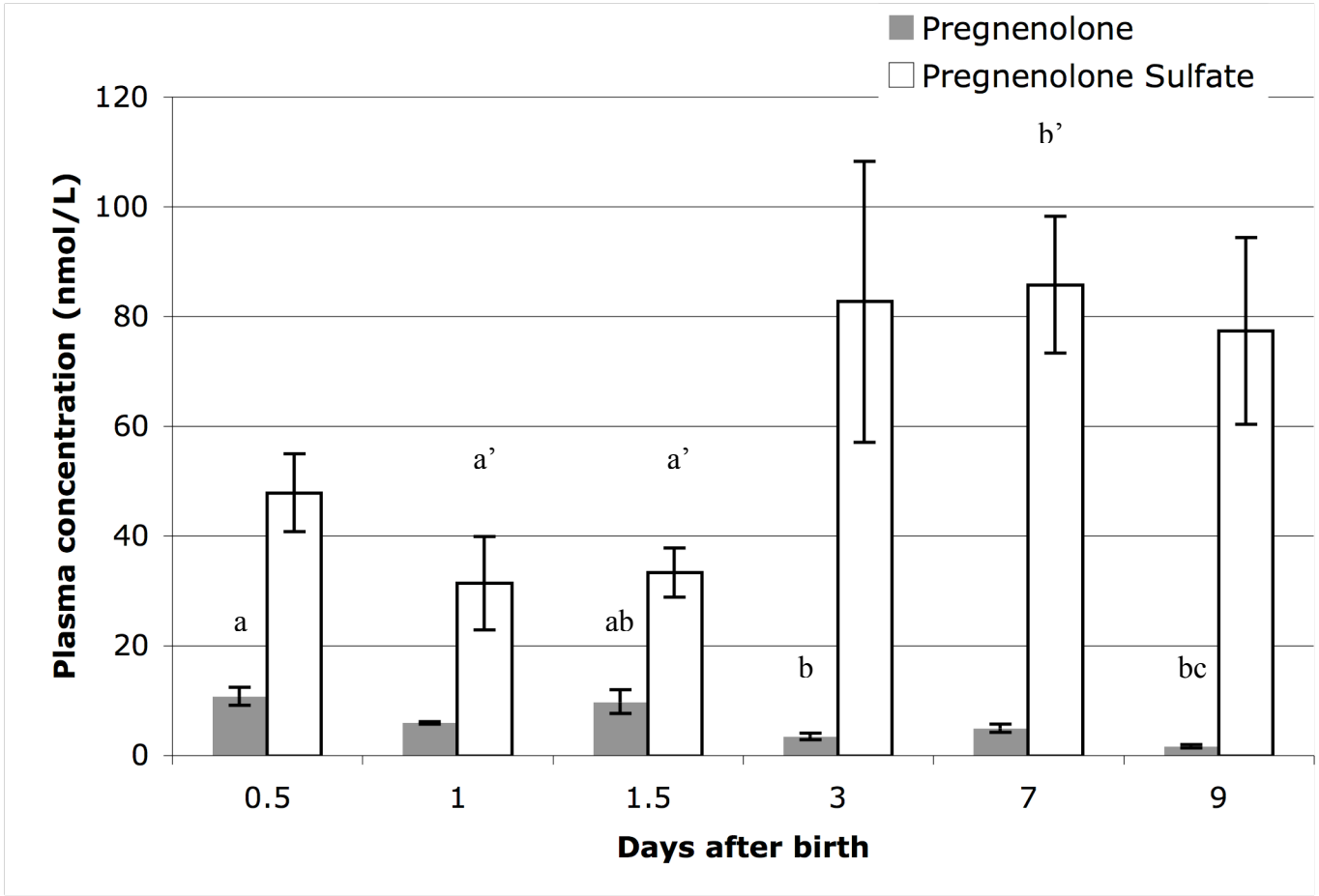

Figure 5.14: Mean and SEM of plasma pregnenolone and pregnenolone sulfate concentrations of newborn lambs aged between 12hrs or less and 9 days after birth (n=5 for all ages but at 12hrs or less n=4). Values that do not share the same letter are significantly different from each other, $p<0.05$).

5.4 Discussion

A sequential analysis was undertaken to determine the concentrations of allopregnanolone and its precursors progesterone and pregnenolone from birth to 9 days after birth in lambs. This was done to assess the potential of allopregnanolone to modulate conscious perception for the first few days after birth in precocial newborn animals. The main findings of the present study are as follows. First, plasma allopregnanolone concentrations were elevated in animals aged 12 hours or less after birth. Second, allopregnanolone concentrations in brain tissues were relatively low compared to published fetal brain levels and there were no differences with age in any brain region. Third, plasma progesterone concentrations were below the detection limit, while brain progesterone concentrations were relatively high compared with those of allopregnanolone. Fourth, plasma pregnenolone concentrations decreased from 3 days onwards, while those for pregnenolone sulphate tended to increase from that age. However, this was not significant for all age groups.

So far allopregnanolone has been addressed prior to progesterone and pregnenolone, as allopregnanolone concentrations have been the main focus of the present study. However, for sake of clarity, the following discussion will begin by addressing pregnenolone and progesterone as precursors of allopregnanolone and will then move onto allopregnanolone (see Figure 1 for enzymatic pathways).

Plasma pregnenolone and pregnenolone sulphate concentrations of newborn lambs

The synthesis of pregnenolone from cholesterol via P450 side chain cleavage enzyme (P450scc) is central to steroid hormone synthesis, as this is the rate-limiting step in steroidogenesis (Compagnone & Mellon, 2000). Cholesterol in the fetus appears to be derived from maternal/placental sources as well as the fetal liver (Woollett, 2008).

As *in vitro* studies have shown, pregnenolone is not only a precursor for the production of pregnane steroids, but in its sulphated form it modulates $GABA_A$ receptor activity in an antagonistic fashion (Paul & Purdy, 1992). However, the excitatory effects of pregnenolone sulphate are not selective for $GABA_A$ receptors, but may also involve the inhibition of glycine receptors (Falkenstein *et al.*, 2000), while enhancement of

glutamate-mediated excitation via NMDA receptors has also been observed (Paul & Purdy, 1992; Compagnone & Mellon, 2000; Falkenstein *et al.*, 2000). In addition, sulphated steroids appear to have the ability to modulate neurotransmitter release presynaptically via sigma (σ) receptors (Gibbs *et al.*, 2006). However, there is considerable controversy over the concentrations of sulphated steroids present in brain tissue, as different analytical techniques have provided vastly different results (Gibbs *et al.*, 2006; Schumacher *et al.*, 2008). Thus, whether sulphated steroids are present in sufficient concentrations in the brain to exert the effects observed in *in vitro* studies, appears, as of yet, unconfirmed.

1) Pregnenolone

Plasma pregnenolone concentrations at three days after birth in the present study were lower than those reported by Nguyen *et al*. (2003a) for lambs of the same age. This however may be due to the different protocols used for extraction and assay techniques for pregnenolone.

Plasma pregnenolone concentrations of the present study tended to decrease from 3 days after birth onwards. However, concentrations for lambs of the three younger groups were not all significantly higher than those for the older three age groups. The most likely reason for such a decline in plasma pregnenolone concentrations is pregnenolone metabolism into other steroid hormones or pregnenolone sulphate. The latter is consistent with the gradual increase in pregnenolone sulphate concentrations with age observed here at the same time as the gradual decline of pregnenolone. Previous studies agree with this, as pregnenolone concentrations for brain and plasma have been shown to decline markedly after birth followed by a further decline between 3 days and 3 weeks (Nguyen *et al.*, 2003a). As plasma pregnenolone concentrations are greatly reduced after fetal adrenalectomy and hypophysectomy (Nguyen *et al.*, 2004b), it seems likely that it is mainly the loss of placental precursors at birth which leads to the associated reduction of pregnenolone concentrations in brain and plasma of newborn lambs, rather than loss of placental supply of pregnenolone *per se*. The reasoning is as follows. The fetal adrenals express P450scc, levels of which increase in late gestation and remain stable at birth and thereafter at least for the first 20 days (Nguyen *et al.*, 2003a). Hence, residual precursors for pregnenolone synthesis present soon after birth

could be metabolised to pregnenolone. With time, however, as concentrations of precursors decline through metabolism and excretion, pregnenolone concentrations would also be expected to decline, as occurred in the present study.

Whether pregnenolone concentrations observed after birth in lambs of the present study would be sufficiently high to bring about neuroinhibitory effects, either via conversion to allopregnanolone peripherally or via entry into the brain and subsequent conversion to allopregnanolone, would need to be investigated by further studies.

2) Pregnenolone sulphate

A recent report has warned of the possible problems with measuring sulphated steroids in plasma and brain via indirect methods, as has been employed in the present study (i.e. measuring unconjugated pregnenolone) (Schumacher *et al.*, 2008). It appears that the fraction presumed to contain pregnenolone sulphate may also contain non-polar components from which pregnenolone can be generated by the application of solvents during the extraction procedure (Schumacher *et al.*, 2008). Thus, the plasma concentrations of pregnenolone sulphate obtained in the present study may be an overestimation of the actual values present. However, pregnenolone sulphate may nevertheless be present in sufficient concentrations to have neuroexcitatory effects.

The passage of sulphated steroids across the blood brain barrier (BBB) is apparently restricted and slow (Hirst *et al.*, 2008). As a decrease in BBB permeability in sheep has been observed between 60% of gestation and maturity (Stonestreet *et al.*, 1996), pregnenolone sulphate present in plasma of newborn sheep would not be anticipated to play a major activating role in the central nervous system. However, it appears that sulphated steroids may nevertheless be able to cross the BBB. A study in late-gestation fetal sheep administered oestradiol-sulphate intravenously showed an increase in HPA-activity, abundance of FOS in the cerebellum and increased uptake of estrone sulphate into various brain regions, which were associated with an increase of oestradiol sulphate in plasma (Wood *et al.*, 2003). Crossing of the BBB by sulphoconjugated steroids may occur with the help of transporters, such as organic anion transporting proteins (OATPs) (Wood *et al.*, 2003; Schumacher *et al.*, 2008). Whether sulphated steroids would be able to cross in sufficient concentrations to exert activating effects would have to be further

investigated.

The presence of sufficiently high concentrations of pregnenolone sulphate in the brain and plasma of lambs around the time of birth may thus, in addition to other factors such as noradrenaline and oestradiol (Chapter 1), play an important role in promoting arousal in the newborn lamb, thereby facilitating its interactions with its mother including sucking. However, further studies would be necessary to determine the concentrations of pregnenolone sulphate in brain tissues of newborn lambs and to assess its ability to modulate GABAergic signalling in the newborn animal.

3) Progesterone in plasma and brain

Progesterone, via the action of 3β-hydroxysteroid dehydrogenase, is a product of pregnenolone metabolism. In the pregnant sheep, the placenta is the major organ of progesterone production (Miller, 1998). Progesterone concentrations are high during the last half of pregnancy as the placenta produces large quantities of this hormone at this time (Bassett *et al.*, 1969; Mellor & Gregory, 2003). However, during the last 3-5 days before birth the production of progesterone by the placenta declines as placental neurosteroidogenesis increasingly switches from progesterone to oestrogen production (Thorburn & Challis, 1979; Mellor & Gregory, 2003). These changes are also reflected in the progesterone concentrations of the fetus, where high concentrations in fetal plasma and brain then decline steadily towards birth (Nguyen *et al.*, 2003a), suggesting that progesterone itself and precursors for progesterone synthesis are supplied by the placenta. However, several organs may be involved in this steroidogenic pathway. This is suggested by the marked reduction of progesterone concentrations in fetal plasma in response to hypophysectomy or adrenalectomy, which seems to indicate that precursors are metabolised to progesterone mainly by the fetal adrenal gland and that progesterone supply by the placenta is not the main source of circulating progesterone in the fetal plasma (Nguyen *et al.*, 2004b).

Once the birth process has been completed, plasma progesterone concentrations in the dam measure below 1.6nmol/L (Mellor *et al.*, 1987). It is expected that, due to loss of the placenta, this decline would also be mirrored in the circulation of the newborn animal.

In the present study, plasma progesterone concentrations were below the detection limit for lambs of all ages. This is in contrast to a previous study in which lambs had plasma progesterone concentrations of approximately 15nmol/L and 5nmol/L at 3days and 19-26days post partum, respectively (Nguyen *et al.*, 2003a). Interestingly, brain progesterone concentrations in the present study were relatively higher than those detected by Nguyen *et al*. (2003a). The differences between the present study and that by Nguyen *et al*. (2003a) could be due to the fact that the extraction as well as the assay procedures varied between the two studies. In addition, the long storage period of plasma samples of the present study (2 years) might have led to a gradual decrease in progesterone levels to below the detection limit at the time of analyses. This however would not explain the higher levels of progesterone obtained in the present brain samples. A variation in the protocol, whereby the buffer originally suggested for use was replaced by the buffer also used for brain extractions and assays (i.e. allopregnanolone assay buffer) might have been responsible. However, as results for RIA assays of brain extracts were not reading below the detection limit, buffer substitution may not have been the problem.

In the present study, plasma progesterone concentrations were below detection levels while allopregnanolone concentrations were still somewhat elevated after birth. This seems at odds with the fact that progesterone is a precursor for allopregnanolone production and that levels of progesterone and allopregnanolone have previously been observed to be related to each other (Paul & Purdy, 1992). However, although plasma progesterone concentrations *per se* were low, it is possible that 5α and 5β–reduced progesterone metabolites, which are precurors for allopregnanolone and pregnanolone synthesis, respectively, were still present in sufficiently high concentrations after the loss of the placenta to sustain allopregnanolone synthesis for the first half day after birth. Unfortunately, no studies have reported on the concentrations of these precursors in the plasma of newborn lambs.

In contrast, brain progesterone concentrations of the present lambs were higher than allopregnanolone concentrations. This could be indicative of a reduction in the metabolism of progesterone to allopregnanolone in neural tissue after birth, thereby leading to a short-term accumulation of progesterone in brain tissue and the relatively

low concentrations of allopregnanolone observed. However, enzymes involved in metabolism of progesterone to allopregnanolone (5α-reductase) are expressed at normal levels after birth (Nguyen *et al*., 2003a) and hence the higher concentrations of progesterone are not easily explained.

The progesterone concentrations in brain tissue were relatively stable over the 9 days after birth as judged by the absence of statistically significant differences between age groups. This is difficult to explain, as one would have expected levels to drop progressively due to local metabolism of progesterone, especially since brain tissue expression of 5α-reductase has been shown to remain stable around birth (Nguyen *et al*., 2003a).

Allopregnanolone in plasma and brain

Progesterone is metabolised to two prominent pregnane steroids, 5α- and 5β-dihydroprogesterone, via the actions of 5α- and 5β-reductase (Figure 5.1). In their turn, these progestagens are converted to allopregnanolone and pregnanolone (not to be confused with pregnenolone, see Figure 5.1), respectively, by the action of 3α-hydroxysteroid oxidoreductase.

In addition to pregnenolone and progesterone, marked reductions in plasma concentrations of allopregnanolone in fetal lambs in response to hypophysectomy or adrenalectomy seem to indicate that allopregnanolone is mainly sourced from metabolism in the fetal adrenal gland and that allopregnanolone supply by the placenta is not the main source of circulating allopregnanolone in the fetal plasma (Nguyen *et al.*, 2004b). Also, it appears that the high levels of allopregnanolone in the fetal brain are maintained by local brain mechanisms and are regulated independently of fetal adrenal gland activity and do not rely on fetal plasma allopregnanolone concentrations (Nguyen *et al.*, 2003b; Nguyen *et al.*, 2004a; Nguyen *et al.*, 2004b; Yawno *et al.*, 2007; Hirst *et al.*, 2008).

Allopregnanolone concentrations in brain tissue of lambs of the present study were relatively low when compared to fetal levels (Nguyen *et al.*, 2003a; Yawno *et al.*, 2007). Brain allopregnanolone concentrations did not change with age after birth,

which, in addition to the generally low concentrations, does not support the notion of residual brain allopregnanolone concentrations being involved in the modulation of conscious perception. However, the elevated plasma allopregnanolone concentrations after birth would suggest that modulation of conscious perception by residual concentrations of allopregnanolone, if present, would occur over the first 12 hours after birth.

Steroid hormones, and their metabolites, are highly lipophilic substances and can therefore readily cross the BBB (Falkenstein *et al.*, 2000). Hence, the possibility of allopregnanolone being able to exert neuroinhibitory effects despite low brain concentrations of allopregnanolone *per se* cannot be ruled out. This is supported by the fact that systemically administered allopregnanolone/pregnanolone have proven GABAergic neuroinhibitory effects (Schulz *et al.*, 1996; Nicol *et al.*, 1999). It is therefore possible that the plasma concentrations of allopregnanolone present in lambs up to 12hrs of age have the potential to modulate conscious perception.

The plasma concentrations of pregnanolone associated with general anaesthesia in humans, when allowing for plasma protein binding of 99%, was reported to be 94nM (Sewell & Sear, 2002, 2004). However, plasma pregnanolone concentrations were not measured in the present study. If we assume that allopregnanolone and pregnanolone exert their actions at similar concentrations, we may nevertheless be able to use this information to assess whether allopregnanolone concentrations in the present lambs would have been sufficiently high to modulate conscious perception. The plasma pregnanolone concentrations reported above are nearly twice that of plasma allopregnanolone concentrations observed in lambs aged up to 12 hours after birth in the present study. It therefore seems likely that, if values twice that of allopregnanolone presented here are capable to produce general anaesthesia, concentrations observed in the present lambs were sufficiently high to affect conscious perception to some degree although not to the point of inducing anaesthesia. Such depressive effects on consciousness however, do not apparently interfere with the lambs' ability to seek the teat, to suck and to interact with the dam.

Plasma allopregnanolone concentrations of newborn lambs aged 12 hours or less were similar to those observed for saline treated lambs 12 and 15 days after birth in a study

investigating the effect of endotoxin on allopregnanolone concentrations (Billiards *et al.*, 2002) and were also similar to plasma allopregnanolone concentrations of 3-day-old lambs of another study (Nguyen *et al.*, 2003a). However, the brain concentrations of allopregnanolone observed were different from the results of the present study. Taking the medulla and cerebellum as an example, allopregnanolone concentrations of lambs of all ages were lower than those reported for saline-treated lambs at 20 days (Billiards *et al.*, 2002) and those reported for 3-day-old lambs (Nguyen *et al.*, 2003a).

These differences may well be due to variations in extraction and assay techniques. In addition, differences in how lambs were handled could have led to an increase in baseline allopregnanolone concentrations, as stress has previously been shown to increase allopregnanolone concentrations in plasma and brain (Purdy *et al.*, 1991; Barbaccia *et al.*, 1996). Finally, relatively low concentrations of allopregnanolone in both brain and plasma in the present study may be due to the time delay between taking the samples and analysis, which was approximately one year (allopregnanolone analyses were done a year before progesterone analyses). However, when extrapolating values of plasma allopregnanolone concentrations of fetal lambs at around 130 days of gestation (Yawno *et al*., 2007), values were similar to those of the present lambs. As Yawno *et al*. (2007) employed the same extraction and assay procedures as used in the present study, this seems to suggest that differences between the present study and those by Billiards *et al.* (2000) and Nguyen *et al.* (2003a) were due to differences in extraction and assay techniques.

It is possible that local areas of the brain regions differ in allopregnanolone concentrations. Such localised elevations of allopregnanolone may well have the potential to exert neuroinhibitory effects leading to modulation of conscious perception, if present in areas involved in the production of the latter, such as the reticular activating system. As only a small amount of each brain area (100mg) was used for analysis, there is potential for such areas to go undetected and such a possibility cannot be discounted. That this could be the case is also supported by the fact that allopregnanolone concentrations of the cerebellum in the present study were readable while those of newborn lambs in the study by Nguyen *et al.* (2003a) were not, even though values were consistently lower in the present study for other brain regions as well as plasma than those reported by Nguyen *et al*. (2003a).

$GABA_A$ receptor development and modulation

As previously discussed in the introduction of this chapter, progesterone metabolites exert their neuroinhibitory effects by acting via $GABA_A$ receptors. Changes in receptor-related properties around the time of birth may affect GABAergic signalling and its modulation by neuroactive steroids. Although this area of research is very complex, an attempt has been made to elucidate the possible changes in GABAergic signalling around the time of birth and how this could affect the potential for neuroactive steroids to modulate conscious perception.

1) Development

In neonatal rats, major changes in GABAergic inhibition in the neocortex take place over the first few weeks after birth (Luhmann & Prince, 1991) and are associated with receptor subunit changes. There is an increase in mRNA for the α1 subunit in the cerebral cortex, the hippocampus and the cerebellum during the second postnatal week and a steady increase in the expression of the γ2L subunit in these regions between 6 and 29 days after birth (Roberts & Kellogg, 2000; Vicini *et al.*, 2001; Yu *et al.*, 2006). Changes in the clustering of α subunits with increasing postnatal age have also been observed (Hutcheon *et al.*, 2004). In addition to subunit composition of the receptor, it is likely that other factors play a role in the changes in GABAergic inhibition observed during postnatal development in the rat, including maturation of the neuronal chloride ion homeostasis (Luhmann & Prince, 1991; Rivera *et al.*, 2005; Tyzio *et al.*, 2008) and possibly maturation of phosphorylation events at the receptor.

However, while such changes are taking place postnatally in rats, this is not anticipated to occur in sheep, as neuronal growth/differentiation is complete and GABAergic neuroinhibitory actions have been shown to exist well before birth (Gluckman, 1982; Johnston & Gluckman, 1983; Nicol *et al.*, 1999; Crossley *et al.*, 2000; Crossley *et al.*, 2003). Therefore, one would expect neuroactive steroids to exert similar neuroinhibitory effects in the newborn animal to those observed previously in the fetus (Nicol *et al.*, 1998; Nicol *et al.*, 1999). Nevertheless, as is discussed in the next section, changes in GABAergic signalling may occur in precocial species around the time of birth despite

their relative neurological maturity.

2) Modulation by neurosteroids

Although from a developmental point of view changes in GABAergic inhibition are not expected in newborn lambs after birth, it is possible that other factors may lead to momentary or even long-term changes in GABAergic signalling around the time of birth. This in turn could have implications for the potential of residual concentrations of allopregnanolone to modulate conscious perception at that time.

Recent studies have shown that acute or long-term exposure to and withdrawal from neurosteroids following prolonged exposure can lead to alterations in subunit expression of $GABA_A$ receptors, especially for those subunits expressed in extra-synaptic receptors, the α4 and δ subunits (Smith *et al.*, 1998a; Smith *et al.*, 1998b; Biggio *et al.*, 2003; Griffiths & Lovick, 2005; Shen *et al.*, 2005; Herd *et al.*, 2007; Maguire & Mody, 2007). A good example for long-term exposure and withdrawal from neuroactive steroids is pregnancy. Endogenous plasma and brain levels of allopregnanolone are elevated during pregnancy in the maternal animal, followed by a steep decline around the time of parturition (Concas *et al.*, 1998; Concas *et al.*, 1999). In rats, the prolonged exposure to these neuroactive steroids during the course of pregnancy apparently leads to a progressive increase in receptor density, which is associated with a decrease in chloride channel function, while delivery was associated with a decrease in receptor density and greater sensitivity of the chloride ion channel (Concas *et al.*, 1998; Concas *et al.*, 1999). In addition, the same authors observed that expression of γ2L subunit mRNA in rat brain was decreased during pregnancy and returned to control values 2 days after delivery. A decrease in the efficacy of GABA and its allosteric modulators at the $GABA_A$ receptors in response to long-term treatment with neuroactive steroids has also been observed by Yu *et al.* (1996). However, other studies have observed subunit changes in response to exposure and withdrawal of neuroactive steroids, which differ from those observed for pregnant rats at delivery by Concas *et al.* (1999). Thus, studies by Smith *et al.* (1998a), Smith *et al.* (1998b), Grobin & Morrow (2000) and Griffiths & Lovick (2005) have shown changes in the α4 subunit as well as the β1 and δ subunits. Discrepancies between these studies and those undertaken by Concas and colleagues could be related to differences in experimental

systems used, as the complex changes in hormones which occur during pregnancy cannot simply be replicated by the administration of a single hormone (Concas *et al*., 1998). Nevertheless, all these studies have shown that $GABA_A$ receptor plasticity is related to changes of neuroactive steroid concentrations.

The fetus, just like the dam, is exposed to high concentrations of neuroactive steroids during pregnancy and the abrupt decline observed around the time of birth seems to fit the description of 'withdrawal'. No studies have apparently been undertaken to investigate whether changes in GABA signalling in response to long-term neurosteroid exposure occur in the fetus. If similar changes were to occur in the fetus as were observed in the dam during pregnancy, this would imply that during intrauterine life, the long-term exposure to high concentrations of neuroactive steroids, such as allopregnanolone, could potentially lead to a decrease in the efficacy of GABA and its allosteric modulators at the $GABA_A$ receptors. However, the increased density of $GABA_A$ receptors in addition to the high concentrations of allopregnanolone present at that time might be able offset some of these effects, thereby helping to maintain the overall state of neuroinhibition suggested to be present in the fetus (see Chapter 1). Indeed, an increase in GABA receptor density with increasing age (and hence with increasing allopregnanolone concentrations) has previously been observed in fetal sheep (Crossley *et al.*, 2000). In turn, the reduction in the concentrations of allopregnanolone and its precursors around the time of birth (Nguyen *et al.,* 2003a) may lead to a reduction in receptor density as well as an increase in the efficacy of GABA and allosteric modulators at the $GABA_A$ receptors, as can be observed in the dam. If this were so, the residual concentrations of allopregnanolone present soon after birth may have the potential to exert neuroinhibitory effects due to increased efficacy of neuroactive steroids at the receptor despite reduced receptor densities and a reduction in neuroactive steroid concentrations.

In the dam, a reduction in neurosteroid sensitivity may be essential to bring about hormonal changes necessary for delivery and lactation. Thus, in adult rats a reduction of neuroactive steroid sensitivity of the $GABA_A$ receptor was observed in the supraoptic nucleus (SON) around parturition, associated with increasing concentrations of oxytocin (Douglas *et al.*, 2000; Koksma *et al.*, 2003). In this particular brain area, in addition to a reduction of allopregnanolone present at $GABA_A$ receptors, the increased levels of

oxytocin present at the time are suggested to bring about neuroactive steroid insensitivity by increasing the level of postsynaptic phosphorylation (Koksma *et al.*, 2003) and in turn leading to further disinhibition of oxytocin neurones. Whether such mechanisms may also play a role in the survival and adaptation in the newborn animal is, as far as can be ascertained, not yet known.

An *in vitro* study undertaken in fetal and neonatal rat cell-attached hippocampal slices showed that the excitatory actions of GABA usually observed in cells during the perinatal period in rats (Owens & Kriegstein, 2002) were switched to inhibitory mode around 1 to 2 hours prior to delivery (Tyzio *et al.*, 2006) and that these changes were associated with a reduction in intracellular chloride ion concentration likely brought about by increased oxytocin concentrations prior to delivery (Tyzio *et al.*, 2006). It has been postulated by Tyzio *et al*. (2006) that such a switch in GABA action to neuroinhibition may help the fetus to adapt to the possible adverse effects, such as hypoxaemia, during delivery. However, as mentioned above, GABAergic signalling is already inhibitory in fetal lambs. It therefore appears more likely that oxytocin, if present in the lamb fetus around the time of birth, would bring about a reduction in neurosteroid sensitivity of $GABA_A$ receptors as is observed in the maternal animal. But what could be the benefit of such a change in neuroinhibition at a time, when such inhibition is vital to protect the lamb from birth-related insults, such as hypoxaemia?

Oxytocin release has been shown to increase in response to stressors in adult mammals (Nishioka *et al.*, 1998; Ebner *et al.*, 2000). Birth is a very stressful process and if the oxytocin system is mature in the fetal lamb and disinhibited by a reduction in progesterone and allopregnanolone concentrations in late pregnancy, oxytocin may be released in the fetal brain in response to the birth process. This may be so despite a transitory increase in neurosteroid concentrations due to the stress of birth. Studies in adult male rats have shown that oxytocin release is associated with increased arousal (Lancel *et al.*, 2003). During birth this would not be a useful adaptation. However, adenosine concentrations are expected to remain high during the birth process and may even increase further due to hypoxaemia (see Chapter 1). Therefore, powerful neuroinhibition would still be present at the time when oxytocin concentrations may reach levels that may support arousal. However, once the animal is born this adenosine inhibition would be released quickly (Chapter 1) and oxytocin present in the newborn's

brain, in addition to other activating substances, may help to arouse the neonate. Would this theory be contradictory to the possible neuroinhibitory effects of residual concentrations of neuroactive steroids? Not necessarily so. It is possible that activating substances are necessary for the animal to become aroused due help overcome the residual concentrations of neuroinhibitory substances present soon after birth. These activating substances may work on a background of neuroinhibition, reducing this sufficiently to allow the animal to perform activities necessary for survival. Yet at the same time conscious perception may be modulated to a point where full conscious perception may not yet be present.

Another alternative is that an increase in localised oxytocin release, if present in the fetus/neonate in response to labour stress, may play an important part in filial attachment rather than playing a role in arousal as such.

The possible effect of other pregnane steroids

The previous sections have focussed mainly on the neuroinhibitory actions of allopregnanolone. However, other neuroactive steroids have been shown to have potent neuroinhibitory effects exerted via the $GABA_A$ receptor, including pregnanolone and tetrahydrodeoxycorticosterone (Paul & Purdy, 1992). Whether these may be present in sufficiently high concentrations to exert their effects after birth and modulate conscious perception is not known. However, tetrahydrodeoxycorticosterone concentrations in brain and plasma increase in response to stress (Purdy *et al.*, 1991) and thus may be elevated in the newborn animal, as the birth process can be seen as a very stressful event.

The possible effect of oestradiol on pregnane steroid modulation

Close to birth placental steroidogenesis switches from favouring pregnane to oestrogen production (Thorburn & Challis, 1979; Mellor & Gregory, 2003). This also means that the fetus close to birth is not only exposed to lower concentrations of pregnane steroids, but also to increased oestrogen concentrations.

A recent study has shown that oestradiol suppresses dihydroprogesterone and

allopregnanolone levels as well as gene expression of 5α-reductase in female rat brains and glioma cells (Maayan *et al.*, 2004). This may indicate that the reduction in allopregnanolone around the time of birth observed in fetal brain and plasma may not only be due to a reduction of placental precursor supply, but may also be brought about in part by the action of oestradiol on the production of pregnane steroids in the fetus. However, 5α-reductase expression in brain tissues was not seen to change around the time of birth (Nguyen *et al*., 2003a).

In addition, oestradiol has been shown to alter $GABA_A$ receptor complexes, leading, for example, to an increased binding of the receptor agonist muscimol (Maggi & Perez, 1984). However, there appear to be differences in oestradiol effects depending on the brain area investigated (O'Connor *et al.*, 1988; Schumacher *et al.*, 1989; Canonaco *et al.*, 1993). When applied in combination with progesterone, effects on GABAergic activity also seem to be less than straightforward. Some studies have reported additive effects while others have shown oestradiol and progesterone to have opposing effects (Maggi & Perez, 1984; Schumacher *et al.*, 1989). Although the effects of simultaneous exposure to oestradiol and progesterone are thus not well understood, they should, as much as possible, be taken into consideration when trying to assess the ability of residual neuroactive steroids to modulate conscious perception. Hence, should the exposure of the GABAergic system to a combination of pregnane steroids and oestradiol lead to an increase in GABAergic signalling, modulation of conscious perception may be more likely, while the opposite effect would reduce the likelihood of modulation due to a reduction in GABAergic inhibitory tone.

Further studies would be necessary to elucidate whether oestradiol has the potential to affect GABAergic signalling around the time of birth in the fetus/newborn and, if so, whether these may differ from those observed in the previous studies in which hormones were administered to adult animals rather than observing animals in a situation where elevated oestradiol and progesterone concentrations occur concurrently, such as around the time of labour.

Analgesia rather than anaesthesia or sedation: modulation of pain perception

So far, the discussion has focussed on whether residual concentrations of neuroactive

steroids in newborn lambs have the potential to modulate conscious perception. However, it may also be possible that instead of modulating conscious perception *per se*, residual neuroactive steroids could modulate the perception of particular stimuli, such as pain.

Progesterone metabolites have been reported to have analgesic properties (Kavaliers & Wiebe, 1987; Frye & Duncan, 1994) and hence modulation of pain perception by residual concentrations of neuroactive steroids in the newborn lamb appears possible.

In a study by Johnson *et al.* (2009) EEG responsiveness to castration in lightly anaesthetised lambs was shown to be reduced over the first week after birth compared to older animals, and this was most pronounced for those lambs between birth and three days of age. These results would support possible analgesic effects exerted by residual concentrations of neuroactive steroids for the first three days after birth and a waning effect over the following days. However, allopregnanolone concentrations in the present study were only elevated significantly for up to 12 hours after birth and, presumably, for a few hours beyond that, which does not fully support this line of reasoning. Additionally, the candidate did not measure allopregnanolone concentrations in lambs older than 9 days and so cannot relate any subsequent increases in responsiveness to castration to potential reductions in allopregnanolone concentrations in plasma beyond that age. However, as we do not know what the concentrations of other neuroactive steroids with analgesic properties at these times are, such an effect cannot be ruled out and further studies would be necessary to confirm whether or not the results presented by Johnson *et al*. (in press) are related to modulation of pain perception by residual neuroactive steroids.

In addition, oestradiol again may play a role in the possible modulation of pain perception in addition to residual concentrations of neuroactive steroids which act as agonists at the $GABA_A$ receptor. Frye & Duncan (1996) have shown that oestradiol potentiated the effects of neurosteroids on pain thresholds so that oestradiol-treated rats injected with allopregnanolone had longer tail-flick latencies and hence higher pain thresholds than those rats not treated with oestradiol. This, and the elevated concentrations of oestradiol during and shortly after birth (Challis & Patrick, 1981), would suggest that the combined actions of residual oestradiol, if present, and

allopregnanolone or other neuroactive steroids such as pregnanolone or tetrahydrodeoxycorticosterone, might have the potential to modulate of pain perception for some time after birth.

Other possible actions of 3α-hydroxylated steroids in the lamb around birth

Although this chapter focuses mainly on the possible hypnotic, anaesthetic and analgesic actions of residual neuroactive steroids thereby modulating conscious perception *per se* or conscious perception of impulse barrages resulting from noxious stimuli, elevated concentrations of these steroids soon after birth may also serve other functions. Some of these will be mentioned below to highlight the diversity/complexity of neuroactive steroid action.

1) Neuroprotection/anticonvulsant

During the birth process adverse conditions may threaten the health and survival of the fetus. Hence, insults, such as asphyxia, have the potential to cause brain damage or can lead to the death of the fetus during birth.

As mentioned previously, allopregnanolone is released during periods of stress, and in the case of the fetus allopregnanolone concentrations are very high even immediately before birth. The Fetal and Neonatal Research Group of Monash University in Melbourne, Australia, have investigated allopregnanolone's potential as a neuroprotective agent. Researchers from this laboratory have shown that umbilical cord occlusion during late gestation leads to elevated brain allopregnanolone concentrations as well as the expression of steroidogenic enzymes P450scc and 5α-reductase 2 (Nguyen *et al.*, 2004a). In addition, treatment of asphyxic fetuses with finasteride, a 5α-reductase 2 inhibitor, has led to an increase in asphyxia-induced brain damage in late gestation (Yawno *et al.*, 2007), supporting allopregnanolone's neuroprotective role.

2) Food intake

The similarity of the effects of neuroactive steroids and benzodiazepine receptor ligands as well as the observations that benzodiazepine receptor modulation leads to changes in

food intake, has prompted research into the effect of neuroactive steroids on food consumption (Chen *et al.*, 1996).

A study by Chen *et al*. (1996) investigated the effect of administration of pregnanolone and allopregnanolone on food intake in non-deprived male rats and found that both pregnanolone and allopregnanolone increased food intake compared to controls. However, pregnanolone was found to be more potent than allopregnanolone, being able to induce an increase in food intake at levels of 1mg/kg, while allopregnanolone was ineffective at doses below 5mg/kg.

How could this effect on food intake be useful for the newborn lamb? Colostrum intake soon after birth is essential for the newborn lamb to ensure survival. In addition, searching for the udder allows the ewe to lick the lamb and the lamb to familiarise itself with maternal olfactory clues, thereby supporting mother-young bonding. As shown in the present study, plasma allopregnanolone concentrations are elevated up to 12 hours after birth. It may well be possible that concentrations of pregnanolone are also elevated at that time, although no data appear to be available. If such were the case, and concentrations were sufficiently high, residual concentrations of allopregnanolone and pregnanolone during the early hours after birth may play a part in the onset of food intake.

3) Anxiety

Benzodiazepines, which are often used to treat anxiety in humans, enhance GABAergic signalling. Thus, it could be possible that neuroactive steroids, which are also agonists of the $GABA_A$ receptor, exhibit anxiolytic effects (Zorumski *et al.*, 2000). Indeed, several studies have supported this notion (Wieland *et al.*, 1991; Bitran *et al.*, 1993) and neuroactive steroids are considered to have anxiolytic properties.

It is therefore possible that the absence of fear-related behaviours in lambs observed over the first few days after birth, which may help to facilitate grooming and mother-young bonding (Mellor *et al.*, 2009), is related to residual concentrations of allopregnanolone, pregnanolone, tetrahydrodeoxycorticosterone, or other precursors for these metabolites.

Suggestions for improvements and future studies

- The measurement of allopregnanolone concentrations in plasma and brain between birth and 12 hours after birth would be of interest.
- Measuring the changes in the concentrations of pregnenolone sulfate in brain tissue over first the few days after birth would allow inferences to be made regarding this compounds'/metabolites' possible activating actions in newborn lambs.
- The measurement of concentrations of other pregnane steroids with neuroinhibitory properties, including pregnanolone and tetrahydrodeoxycorticosterone, could further our understanding of the possible modulation of conscious perception by neuroactive steroids soon after birth in lambs.
- For future studies, possible changes in hormone concentrations with time within stored samples should be taken into consideration or analyses should be undertaken soon after sample collection. In the present study, there was a considerable delay between sample collection and analysis (allopregnanolone 1 year; progesterone and pregnenolone 2 years), which might have affected the concentrations measured.

5.5. Conclusions

Although the present study does not inform us directly about whether modulation of conscious perception by residual concentrations of neuroactive steroids is possible in the newborn lamb, it suggests that such modulation might be present at least during the first 12 hours after birth. However, the concentrations of allopregnanolone and its precursors progesterone and pregnenolone are not the only factors that apparently need to be taken into consideration when assessing allopregnanolone's modulatory potential.

Receptor subunit changes in response to prolonged exposure to and withdrawal from progesterone and its metabolites, the actions of oestradiol around the time of birth and receptor density and steroid sensitivity also need to be considered. In addition, modulatory actions may be focussed on pain perception rather than conscious

perception *per se*.

Concentrations of other neuroactive steroids, such as pregnanolone and tetrahydrodeoxycorticosterone, which also act agonistically at the $GABA_A$ receptor, should be measured to assess their potential for the above-mentioned actions.

Residual concentrations of neuroactive steroids may not only play a role in modulation of perception, but may also be important for survival of the newborn animal by protecting the animal during and after birth from any adverse effects of the birth process, ensuring milk intake soon after birth and by reducing anxiety levels thereby assisting in mother-young bonding.

Due to the complex nature of neuroactive steroids' effects on GABAergic signalling, further studies would be necessary to elucidate the role(s) of residual neuroactive steroids for the survival and wellbeing of the newborn.

Lastly, in addition to the possible neuroinhibitory action of residual neuroactive steroids soon after birth, other factors may be involved in the changes in EEG spectra observed over the first few days after birth in the previous chapter and the responses to castration observed by Johnson *et al.* (2009). One possibility would be that milk-borne opioidergic factors or the central release of opioids in response to the sucking process are involved. However, this also would need further investigation.

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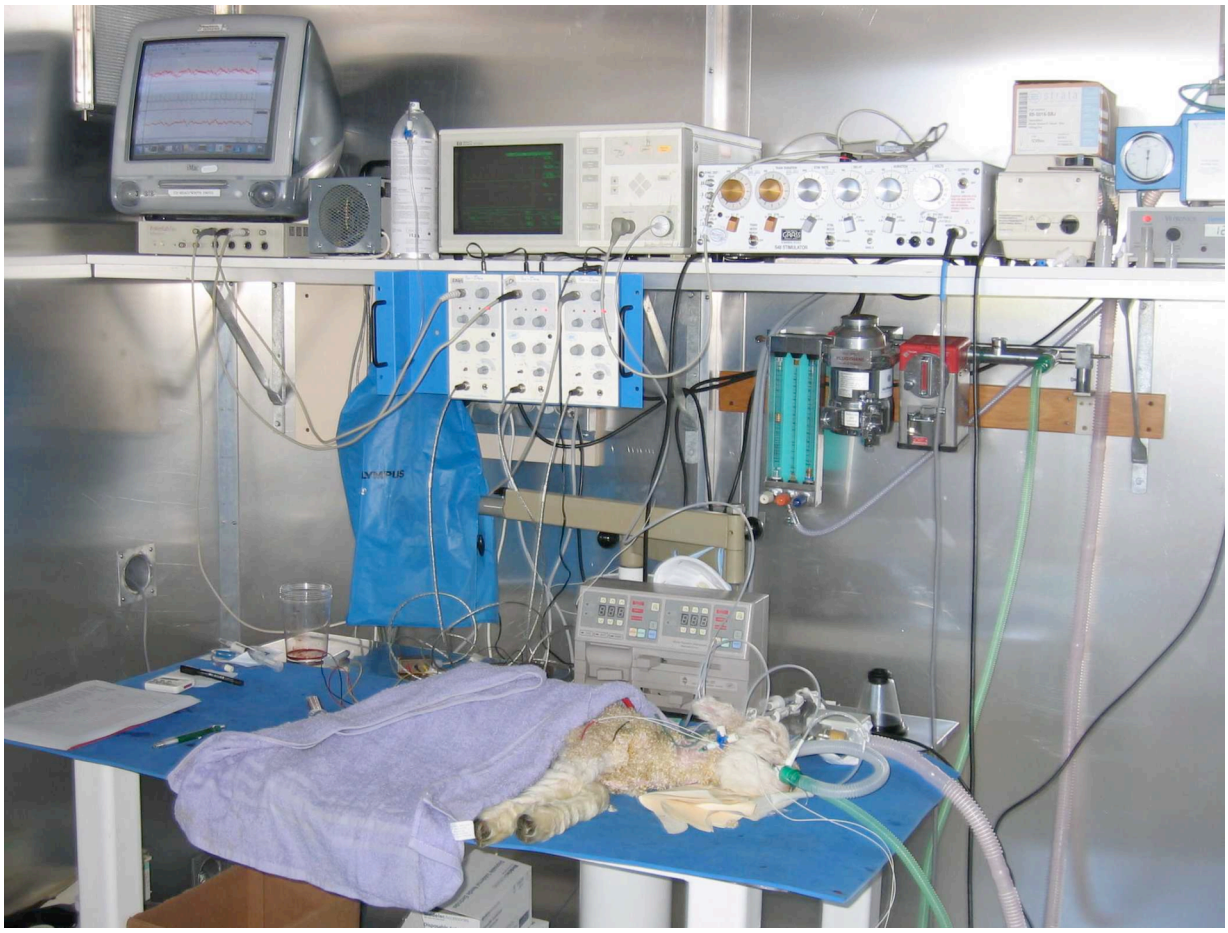
Chapter 6



Abstract

Improvement of the management of newborn farm animals is a scientific success story, as extensive research and development over the last 50 years has led to a significant reduction of mortality rates. However, concern for animal welfare is great where newborn animals do become sick and/or die. Recent evidence suggests that these animals may be protected for the first 12 hours or so by the presence of residual "*in-utero*" neuroinhibitory steroids exerting potent sedative, anaesthetic and analgesic properties, such as allopregnanolone, which appear to be involved in maintaining the fetus in sleep-like unconscious states. Were this the case it would mitigate the welfare compromise inherent in neonatal mortality. The present study aimed to clarify the effect of such neuroinhibitors on conscious perception and responsiveness to sensory inputs in the neonatal lambs. For this purpose, responses to electrical stimulation were measured in lightly anaesthetised lambs 4-24 hours or 7-11 days of age receiving an infusion of the progesterone metabolite pregnanolone, the $GABA_A$ receptor antagonist picrotoxin or a vehicle control. The onset of EEG changes in response to infusion of pregnanolone was slower in newborn than in young lambs, while there were no changes in EEG parameters in response to picrotoxin infusion in newborn lambs, suggesting a possible degree of protection by neuroinhibitory steroids. Newborn lambs (4-24 hours) of all infusion groups did not show any EEG responses to electrical stimulation, while young lambs (7-11 days) receiving pregnanolone and picrotoxin infusions did. However, young control lambs did not show any cerebrocortical responses to electrical stimulation and hence data are difficult to interpret. As expected, there were physiological differences between lambs of the two age groups, which possibly affected the functional dynamics of halothane anaesthesia as well as the pharmacokinetics for pregnanolone and picrotoxin, and thus could have affected responsiveness to electrical stimulation. Overall, the study design was very complex and, with the benefit of hindsight, had several flaws preventing meaningful interpretation of the data. These drawbacks are detailed in the discussion and possible improvements are suggested.

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EEG responses of lightly anaesthetised newborn and young lambs to an electrical stimulus: The effect of pregnanolone and $GABA_A$ receptor antagonist picrotoxin

6.1 Introduction

Without human intervention neonatal mortality rates of 30 to 50% or even more can occur in farm animals, including sheep, goats, cattle, deer and pigs (Mellor & Stafford, 2004). Extensive research and development over the last 50 years has clarified much about the causes and prevention of these naturally high mortality rates and now consistently reduces them to 10 to 25% or even less (Randall, 1992; Mellor & Stafford, 2004). Improvement of the management of newborn farm animals is therefore a scientific success story. Nevertheless, these mortality rates are still much higher than those for human infants and concern for animal welfare is great. It has been commonly believed that neonatal mortality and morbidity are accompanied by severe suffering in most cases (Mellor & Stafford, 2004). Very recent developments show that although this might be the case in animals that experience severe and protracted hunger, sickness or pain, suffering in newborn farm animals may be reduced when consciousness is obtunded by factors such as hypothermia during the hours and days after birth (Mellor & Stafford, 2004). However, it is not clear how the welfare of newborn animals would be affected were such factors not present to exert their dulling effects.

As previously discussed (Chapter 1), there is strong evidence to suggest that the presence, *in utero*, of a variety of potent neuroinhibitory substances, including the progesterone metabolites allopregnanolone and pregnanolone, are involved in keeping the late-gestation fetal sheep in unconscious sleep-like states (Mellor & Gregory, 2003; Mellor & Diesch, 2006). These progesterone metabolites have proven sedative, anaesthetic and analgesic effects (Majewska, 1992; Paul & Purdy, 1992; Lancel *et al.*, 1996; Damianisch *et al.*, 2001; Rupprecht, 2003), which they exert via the $GABA_A$ receptor by enhancing GABA-mediated inhibitory synaptic events, bringing about increased inhibition in the central nervous system (Majewska, 1992; Paul & Purdy, 1992; Compagnone & Mellon, 2000; Belelli & Lambert, 2005).

The plasma concentrations of allopregnanolone remain elevated for at least the first 12 hours after birth (see Chapter 5) and changes in electroencephalographic (EEG) spectra over the first two days after birth can be observed (Chapter 4). In addition, lightly anaesthetised lambs aged younger than 3 days had a reduced cerebrocortical response to castration than did older lambs (Johnson *et al.*, 2005a; Johnson *et al.*, 2009). Thus, a degree of analgesia and/or sedation may persist in lambs during the first day or so after birth, which corresponds to the period of highest mortality rates in neonatal animals (Mellor & Stafford, 2004). Newborn farm animals in general, and those that will become sick and/or die, may therefore have the benefits of a degree of sedation during the first few days after birth, in addition to any consciousness dulling effects of hypothermia. Were this the case it would mitigate the welfare compromise inherent in neonatal mortality. To date, however, no research has been undertaken to clarify the effect of persisting fetal neuroinhibitors on the neonatal young's consciousness and responsiveness to sensory inputs.

The aim of the present study was to assess whether the naturally occurring elevated concentrations of progesterone metabolites shortly after birth (Chapter 5) are sufficient to modulate conscious perception or whether they provide analgesia in newborn lambs and thus, whether they might have been involved in the reduced EEG responsiveness to castration in newborn lambs observed by Johnson *et al.* (2005, 2009). For this purpose, EEG responses to electrical stimulation were measured in lightly anaesthetised lambs, which were receiving an infusion of the progesterone metabolite pregnanolone, the $GABA_A$ receptor antagonist picrotoxin or a vehicle control. Lambs used for the present study were either 4-24 hours (newborn) or 7-11 days (young) old, the latter age being selected to ensure that the major transitional physiological changes occurring in the newborn animal upon first exposure to the extrauterine environment were complete.

Although presented as the last experimental chapter of this thesis, the present study was the first experiment designed and undertaken during the course of the candidate's PhD studies. The study design was very complex, making it difficult to interpret the data. Hence, this chapter, while making brief statements on the possible meaning of the results, also focuses on the lessons learnt from this study and the possible ways for improvement were the study to be repeated.

6.2 Materials and Methods

Animals

Twin lambs (Coopworth/South Suffolk X) aged 4-24 hours (4-24hr lambs n=38) and 7-11 days (7-11day lambs; n=38) and their dams were transported from the Massey University Sheep Farm at Haurongo, Palmerston North, New Zealand to the Institute of Veterinary, Animal and Biomedical Science at about 0800 hours on each study day. They were kept in an outdoor animal holding pen until returned to the farm in the evening of the same day at about 1700 hours. Only healthy lambs weighing 3.5 kilos or more were used for the study. Dams had free access to water and lambs had free access to the dam when not in the laboratory. The lambs were allowed about 30 minutes to settle after the 5 to 10 minutes of transport from the farm.

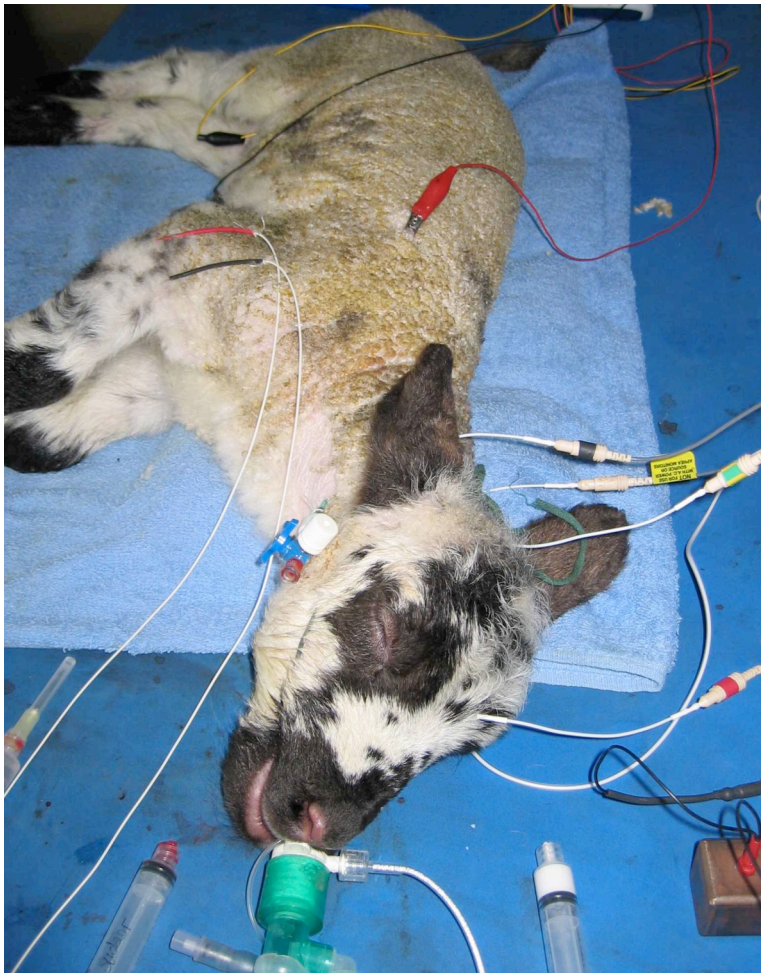
Once in the laboratory, lambs were weighed to the nearest 100g. Before the animals were returned to the farm care was taken to ensure that the ewes and lambs were well bonded and that all lambs were feeding successfully. This study was approved by the Massey University Animal Ethics Committee (Protocol # 05/65).

Preparation for experimental procedure

Anaesthesia was induced by facemask (Shoof International Ltd, Cambridge, New Zealand) with 4 to 5% halothane (Merial, Parramatta, Australia) in oxygen (100%). Once the lambs were anaesthetised sufficiently to allow endotracheal intubation, judged by loss of righting reflex, absence of general body movements and withdrawal reflex, anaesthesia was maintained with halothane in a flow of oxygen (100%) delivered through a calibrated vaporiser with intermittent positive pressure ventilation. End tidal halothane was monitored and titrated to 1.0%. End tidal CO_2 partial pressure was also monitored at several time points during the study (Figure 6.1) and maintained between 28-50mmHg for the majority of animals.

An 18G catheter (Shoof International Ltd, Cambridge, New Zealand) was placed into each jugular vein (one for infusion, one for blood sampling). Catheters were filled with heparinised saline to prevent blood clotting [5ml of 5,000IU/ml of heparin (CP Pharmaceuticals Ltd, Wrexham, UK) in 500 ml 0.9% Sodium Chloride solution (Shoof

International Ltd, Cambridge, New Zealand)].



Photograph 1: Experimental lamb with EEG, ECG and stimulating electrodes and the catheter for blood sampling in place.

Stainless steel needle electrodes (0.3mm diameter; Medelec, Oxford Instruments Medical Systems, UK) were placed subdermally at the midline over the frontal sinus (non-inverting electrode), caudal to occipital process (common electrode) and over the left and right mastoid process (inverting electrodes) (Photograph 1). This arrangement allowed bi-lateral electroencephalographic recordings.

Stainless steel crocodile clip electrodes were placed over the withers (inverting electrode), sternum (non-inverting) and caudal abdominal wall (common) for ECG

recordings (Photograph 1).

Two stainless steel electrodes were placed 1-2cm below the distal elbow and 2-3cm above the stifle for electrical stimulation. Electrodes were placed 1-2cm apart from each other (Photograph 1).

Animals were placed onto a circulated hot water blanket set at 40˚C (Gaymar, New York, USA) or a heated pad, and were covered with a cotton towel to prevent excessive heat loss during anaesthesia. Rectal temperature was recorded at the beginning of anaesthesia and at predetermined intervals throughout the study (Figure 6.1) using a digital thermometer (Becton Dickinson Ltd., Auckland, New Zealand).

Infusions

1) Pregnanolone (5β-pregnane-3α-ol-20-one)

The pregnanolone infusate was prepared by mixing 12.5mg of pregnanolone (Sigma-Aldrich Pty Ltd., Auckland, New Zealand) in 2.5ml of Intralipid® 20% (Baxter Healthcare Ltd, Auckland, New Zealand). Vigorous agitation to dissolve pregnanolone in Intralipid® was achieved using a vortex mixer for powdered pregnanolone or an ultrasonic bath for crystallised pregnanolone. A bolus injection of 2.5 or 5mg of pregnanolone in Intralipid® produces sedative effects in newborn lambs (Nicol *et al.*, 1999). These values were adopted for the present study. The present lambs received 5mg of pregnanolone in 1ml of Intralipid® administered over 5 minutes via infusion pump. As this study was conducted prior to that presented in Chapter 5, infused neurosteroid doses do not reflect concentrations measured in plasma and brain tissue.

Pregnanolone, as well as allopregnanolone, is a metabolite of progesterone containing a 3α-hydroxyl group, exerting its sedative, anaesthetic and analgesic properties via the $GABA_A$ receptor (Paul & Purdy, 1992). Although pregnanolone was used in the present study, it would have been preferable to use allopregnanolone. First, information on changes of pregnane steroid concentrations in fetal and newborn sheep is available for allopregnanolone, not pregnanolone. Second, the laboratory where plasma samples of newborn lambs could have been analysed for changes in steroid concentrations over the

course of the experiment only had RIA methods established for allopregnanolone, not pregnanolone. However, allopregnanolone was not used due to its high cost and overall financial constraints on the project.

Choice of vehicle

Intralipid® is a lipid-based solution, making it a useful solvent for lipid-soluble substances, such as steroid hormones. Intralipid® was shown to have an effect on brain electrical activity recorded in fetal lambs, decreasing high voltage ECoG activity over the first hour of infusion (Nicol *et al.*, 1999). As it is not certain what the effect of Intralipid® on brain activity would be in newborn lambs, an Intralipid® (vehicle) control group was added to the experimental design.

2) Picrotoxin

Picrotoxin can lead to seizures if given at high enough doses. Hence, it was important to determine a dose rate for infusion for the present study, which would not induce seizure activity. A dose rate of 0.3mg/kg of picrotoxin elicited behavioural arousal in fetal sheep without causing seizure activity (Nicol *et al.*, 1999). Using this information, a pilot investigation was undertaken to determine the dose rate of picrotoxin to be used for the present study. Dose rates of 0.5mg/kg and 0.3mg/kg were tested initially. Although seizures were not observed, arousal of anaesthetised lambs in response to the electrical stimulus was present for 7-11day lambs. As this was not desirable, the dose rate was further reduced to 0.1mg/kg, which did not result in arousal of anaesthetised lambs. Dose rates between 0.1 and 0.3 mg/kg were not tested due to the need to continue the study and to limited availability of lambs over the small time frame during which lambing occurred. Although 4-24hr lambs did not show arousal at the higher doses, 0.1mg/kg was used as the dose rate for both age groups to keep protocols as similar as possible between lambs of the two ages.

The picrotoxin infusate was prepared by mixing picrotoxin (0.1mg/kg) (Sigma- Aldrich Pty Ltd., Auckland, New Zealand) with physiological saline (pH 7.5) and dissolving it in an ultrasonic bath. Animals received 0.1mg/kg in 1ml of saline administered over 5 minutes via infusion pump.

As far as could be ascertained, there are no antagonists that bind specifically at the $GABA_A$ receptor binding site to which the neuroactive steroids allopregnanolone and pregnanolone bind, nor that interfere specifically with the action of these steroids at the receptor. Hence, for the purposes of this study a different antagonist had to be used. Picrotoxin is a non-competitive $GABA_A$ receptor blocker (Lancel *et al.*, 1999). As the dose-response curve for another $GABA_A$ receptor antagonist, bicuculline, was found to be very steep for fetal lambs with regard to the production of convulsive activity (Nicol *et al.*, 1999), picrotoxin was used for the present study. The drawbacks and implications for this choice of antagonist will be evaluated in the discussion.

3) Control

The control infusion consisted of 1ml Intralipid® (Baxter Healthcare, Auckland, New Zealand) infused over 5 minutes. This was the same volume of Intralipid® used for pregnanolone infusions. As this dose was a slightly higher dose than that shown to affect ECoG activity in fetal lambs (i.e. 1ml/5min compared to 10ml/60min used by Nicol *et al.*, 1999), the Intralipid® control group was added to the study design.

It was assumed that the volumes of saline to be used for infusion purposes would not be sufficient to have an effect on the EEG and hence there was no saline control group.

Experimental groups and procedure

1) Experimental groups

There were six experimental groups. These included three infusion groups, namely vehicle (control) infusion, pregnanolone infusion or picrotoxin infusion, at each of two ages, namely lambs 4 to 24 hours after birth and lambs at 7 to 11 days after birth. Lambs of either of the two ages were randomly allocated to the three infusion regimens on the day of the experiment.

2) Experimental procedure

Electroencephalographic (EEG) and electrocardiographic (ECG) recordings

Recordings were made in a Faraday cage to minimise electrical contamination. Data collection began once end tidal halothane of 1.0% was reached (usually within 10 minutes of animal preparation). Initial baseline EEG and ECG recordings were made for 15 minutes after which the protocols differed depending on the group (Figure 4.3.1).

Control group

Once the initial 15-minute baseline recordings had been completed, an electrical stimulus was presented to the lamb (2 seconds, 75V, 50Hz adapted from Murrell *et al.* (2007); Grass Product Groups, AstroMed Inc, Warwick, USA). The stimulus was followed by a recording period of 10 minutes. After a second 10-minute baseline period, the control vehicle was infused over five minutes. Before and after infusion, the infusion catheter was flushed with 0.5-1.0 ml Intralipid®. A second electrical stimulus was given to the lamb one to three minutes after completion of the infusion, followed again by a 10-minute recording period. The stimulus procedure followed by a 10-minute recording period were repeated once more thereafter.

Pregnanolone and picrotoxin groups

Once the initial 15-minute baseline recordings had been completed, lambs received infusates containing either pregnanolone or picrotoxin over five minutes. Before the infusion began, the catheter was flushed with 0.5-1.0ml of Intralipid®, the vehicle for the pregnanolone infusions, or 0.5-1.0ml of saline, the vehicle for the picrotoxin infusions. This was done to minimise the possibility of de-emulsification at the interface between heparinised saline in the catheter and the infusate once the infusion began. This was repeated after the infusion was complete to ensure that all of the infusate entered the lamb's circulation. One to three minutes after the completion of the infusion the lambs received their first electrical stimulus (2 seconds, 75V, 50Hz; Grass Product Groups, AstroMed Inc, Warwick, USA), which was followed by a 10-minute recording period. Two more stimuli were presented to the lamb thereafter, both followed by a 10-minute recording period.

Why was electrical stimulation repeated for each group?

The timing of the onset of significant neurological effects in response to the infusates was not known for newborn anaesthetised lambs. A sequence of three bouts of electrical stimulation after infusion was therefore employed to increase the likelihood that effects would be detected if the responses to infusion were not immediate. It is possible that electrical stimulation would have caused changes in the overall responsiveness to the stimulus so that hyper-responsiveness or hypo-responsiveness could have resulted. This was not controlled for, as there was no control group receiving repeated electrical stimulation without prior infusion. Thus, repeated electrical stimulation could have affected the results of the present study.

Blood sampling and other parameters monitored

Five blood samples (5ml each) were taken via syringe and transferred into 5ml sodium-heparin vacutainers (Becton Dickinson Ltd., Auckland, New Zealand) at the beginning of baseline observations, after completion of the infusion and after each observation period that followed an electrical stimulus. At the same times, rectal temperature and endtidal CO_2 partial pressure were measured and recorded. Blood glucose concentrations were measured using a glucometer (Accu-Chek Advantage System; Roche Diagnostics, Auckland, New Zealand) in the first, third and last blood sample.

Blood samples were immediately put on ice. At the end of the observation period for each lamb, all blood samples were centrifuged for 15min at 3,000rpm and plasma was withdrawn into 1.5ml Eppendorff tubes (Biolab Ltd., Auckland, New Zealand), which were kept at -20ºC until the end of the experimental day and then stored at -80ºC.

EEG and ECG recording procedures

Both the EEG and ECG were recorded continuously at a sampling rate of 1kHz using an Apple Macintosh personal computer and Powerlab 4/20 data recording system (Powerlab™ Data Acquisition System®, AD Instruments Ltd, Bella Vista NSW, Australia) with compatible recording software (Chart 5, Powerlab™ Data Acquisition System®, AD Instruments Ltd, Bella Vista NSW, Australia). Isolated biological amplifiers for both ECG and EEG recordings were set at a gain of 1000 with a low-pass

filter of 0.1Hz and a high-pass filter of 0.5kHz. Analysis of EEG and ECG was done off-line after completion of the experiment.

EEG analysis

Spectral analysis was done using a specialised Fast Fourier Transform (FFT) programme (Spectral Analyser, CB Johnson, Massey University, New Zealand, 2006). The program calculated the median frequency (F50), spectral edge frequency (F95) and total power (Ptot) for consecutive 1-second segments as well as the power for individual frequencies from 1 to 30Hz.

The *median frequency* (F50) measures the central location of the power spectrum giving the frequency below which 50% of the power spectrum resides (50^{th} percentile). The *spectral edge frequency* (F95) is a measure of the high frequencies in the power spectrum and indicates the frequency below which 95% of the power spectrum resides (95^{th} percentile) (Johnson, 2003; Tonner & Bein, 2006; Gibson *et al.*, 2007). *Total power* (Ptot) is the overall area under the power spectrum curve, and usually decreases as median frequency and spectral edge frequency increase and *vice versa* (Johnson, 2003). Here, it included the sum of EEG power present in frequencies 1 to 30Hz.

The EEG during the following periods was used for calculating the above parameters (see also Figure 6.1 for experimental outline):

- 30 seconds towards the end of initial baseline observations (baseline 1: pre stimulus 1)
- 30 seconds prior to application of the second stimulus (baseline 2: pre stimulus 2)
- 30 seconds prior to application of the third stimulus (baseline 3: pre stimulus 3)
- 30 seconds immediately after each stimulus (stimuli 1 to 3).

In addition, the EEG parameters were calculated for the last 30 seconds of the 5-minute infusion period and were compared to values of the initial baseline period (baseline 1) in order to assess whether the drugs had any effect on the EEG while the infusion was in progress.

During application of the electrical stimuli movement responses were elicited in all lambs, i.e. withdrawal of the leg to which the stimulus was presented, so that the EEGs of all lambs went off scale when the stimulus was presented and took several seconds to return to scale once the stimulus was discontinued. Therefore, the EEG periods used for calculating post-stimulus EEG parameters did not start immediately after the stimulus, which would have been desirable, but started on average nine seconds thereafter (minima of 6, 5 and 5 seconds and maxima of 28, 18 and 16 seconds for stimuli 1, 2 and 3, respectively).

For every animal the mean of each of the EEG parameters for the two EEG channels for each EEG period was calculated and used for statistical analysis. Channels were combined to get an overall representation of left and right hemispheric responses.

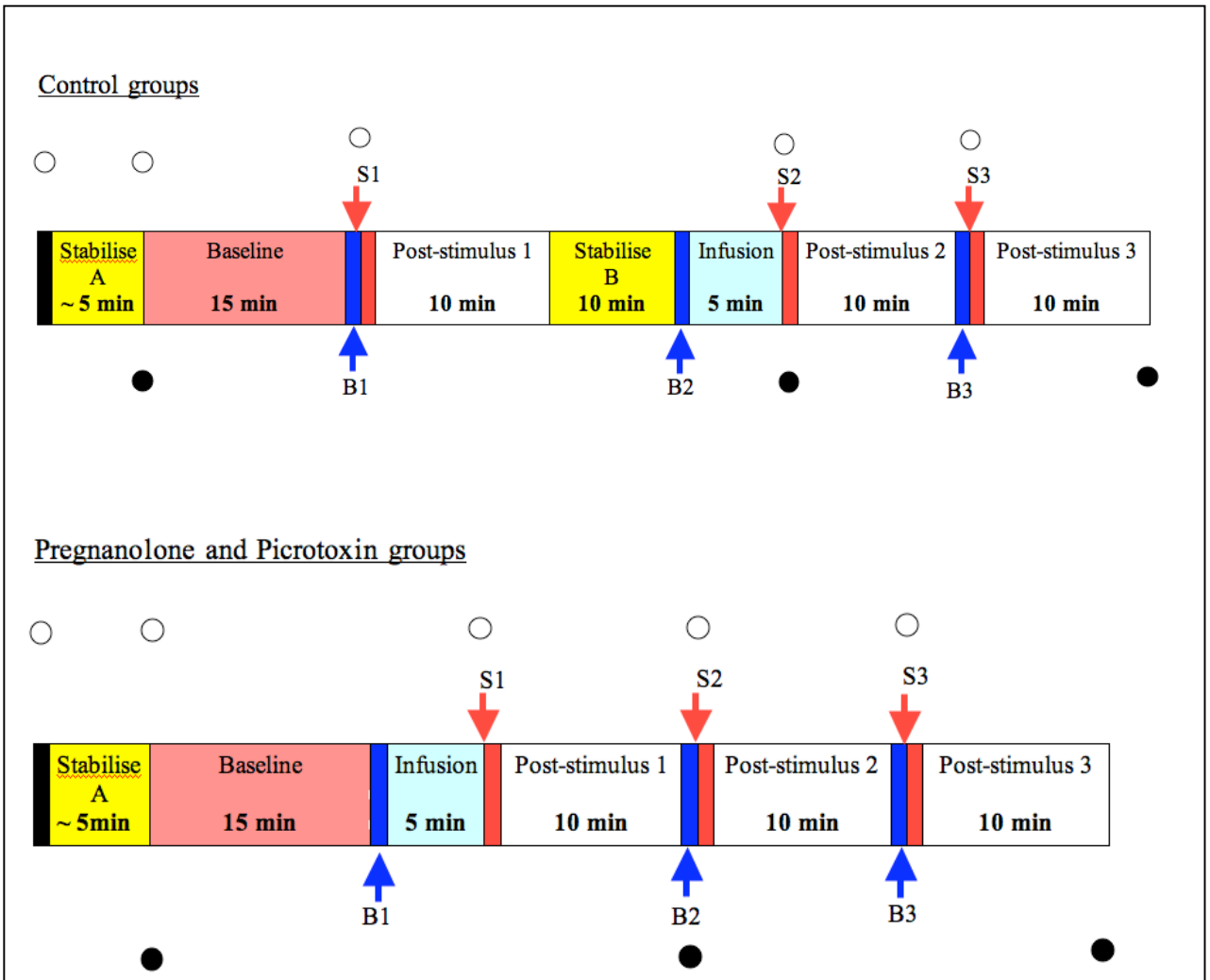
ECG analysis

The ECGs were analysed for heart rate (BPM) using Chart 5 (Powerlab™ Data Acquisition System®, AD Instruments Ltd, Bella Vista NSW, Australia). Noise thresholds were adjusted as necessary. Heart rate data were calculated per second. Time periods used for analysis included the following (see also Figure 6.1 for an experimental outline):

- one minute towards the end of initial baseline observations (baseline 1: pre stimulus 1)
- one minute prior to stimulus 2 (baseline 2: pre stimulus 2)
- one minute prior to stimulus 3 (baseline 3: pre stimulus 3)
- one minute immediately after each stimulus (stimuli 1 to 3).

Blood sample analysis

Due to insufficient funds and excessive analytical costs of other parameters, only glucose concentrations were determined in blood samples, as described above. In an ideal situation, plasma samples would have been analysed for pregnanolone and picrotoxin concentrations to determine effectiveness of infusion and metabolism of the drugs over time of the experiment.



Black strip (time 0) = time at completion of surgical preparation for study, ~20-30 min after intubation

Stabilise A = period during which end tidal halothane was titrated to 1.0%

Stabilise B = period of rest after stimulus 1 before administration of infusion in control lambs

Blue arrows and bars = periods during of which EEG/ECG data were used for pre-stimulus data
B1 = Baseline 1 = Pre stimulus 1 (30sec for EEG, 1min for ECG)
B2 = Baseline 2 = Pre stimulus 2 (30sec for EEG, 1min for ECG)
B3 = Baseline 3 = Pre stimulus 3 (30sec for EEG, 1min for ECG)

Red arrows = presentation of electrical stimulus

Red bars = periods during of which EEG/ECG data were used for post-stimulus data
S1 = Post stimulus 1 (30 sec for EEG, 1min for ECG)
S2 = Post stimulus 2 (30 sec for EEG, 1 min for ECG)
S3 = Post stimulus 3 (30 sec for EEG, 1 min for ECG)

Open circles = time points when end tidal CO_2 partial pressure and rectal temperature were measured

Filled circles = time points when glucose concentrations were measured

Figure 6.1: Experimental design for control, pregnanolone and picrotoxin groups.

Statistical analysis

SPSS 11.0 for MAC OSX (SPSS Inc., Chicago ILL, USA) and Prism 5.0 for MAC OSX (GraphPad Software Inc., San Diego CA, USA) were used for all statistical analyses. Mean and standard error of the mean (SEM) were calculated for all parameters. Data were checked for normality using the Kolmogorov-Smirnoff test with Lilliefors significance correction for samples with $n>50$ and the Shapiro Wilks test for samples with $n<50$. In addition, normal probability plots were used to assess normality of the data. Levene's test was used to assess the variances of the data where those were normally distributed. Differences were considered significant at $p<0.05$. Degrees of freedom reported (e.g. df 1, 130) represent the number of groups and overall number of data points used in individual statistical tests. Where data were not normally distributed or had unequal variances for all or some of the variables and where transformation achieved normality only in some but not all variables, non-parameteric statistical tests were performed.

1) Treatment effects

EEG response to infusions

As F50 and Ptot were not normally distributed the non-parametric Wilcoxon test for two related samples was performed to determine changes in these EEG parameters in response to the infusions in the six groups. As F95 was normally distributed and had equal variances, a paired-sample t-test was performed for this parameter.

In addition, repeated measures ANOVAs with Bonferroni post hoc tests and non-parametric Friedman's tests with Dunn's post hoc tests were performed comparing the three baseline periods within the six groups of lambs to assess the effect of infusion over the course of the experiment.

EEG and ECG responses to electrical stimulation

EEG data (F50, F95, Ptot) were not normally distributed and the non-parametric two-related sample Wilcoxon signed-rank test was performed to determine changes in EEG parameters in response to stimulation for all groups by comparing pre- and post-

stimulus EEG data for all three stimuli.

In addition, the gross differences in the patterns of distribution of the frequency spectra for frequencies of 1-30Hz for pre- and post stimulus data for the three stimuli were examined by the two-tailed Kolmogorov-Smirnoff test for all groups.

ECG data were normally distributed and the two-related sample t-test was used to determine significant changes in heart rate in response to the three stimuli for all groups by comparing pre- to post-stimulus ECG data.

General physiological information

The following tests were used to determine whether animals remained physiologically stable during the experiments. First, a repeated measures analysis of variance (ANOVA) with Bonferroni post-hoc test was performed for end tidal CO_2 partial pressure and rectal temperature for each age to investigate changes over the course of the experiment (for time points of measurements see Figure 6.1). Second, a non-parametric Friedman's test with Dunn's post-hoc tests was performed for each age group to determine changes in blood glucose concentrations over the course of the experiment (for time points of measurements see Figure 6.1).

2) Age effects

Differences in all parameters between the two age groups were assessed for data collected before infusions and electrical stimulation commenced, i.e. at the start of anaesthesia and/or during the initial baseline (baseline 1).

General information

As weight data were not normally distributed, differences in weight between age groups and genders were investigated using the non-parametric independent-sample Mann-Whitney test. Age differences for end tidal CO_2 partial pressure and rectal temperature were assessed by independent samples t-test, while those for blood glucose concentrations were assessed by non-parametric independent-sample Mann-Whitney

test.

EEG and ECG

Median frequency (F50) and total power (Ptot) data were not normally distributed and a Mann-Whitney test for independent samples was used to assess the differences in theses EEG parameters between ages. Spectral edge frequency (F95) data were distributed normally and differences between ages were assessed using an independent t-test.

In addition, a two-tailed Kolmogorov-Smirnoff test was run to assess the gross difference in the shapes of frequency spectra for each frequency between 1-30Hz between the two ages. In order to further investigate these differences statistically, a principal component (PC) analysis was run on log-transformed data (frequencies 1-30Hz). The PC loadings were investigated and the PC scores were plotted against each other in reduced dimensions to explore variation with age.

A two-tailed independent sample t-test with Welch correction for unequal variances was used to test whether there were differences in heart rate at the initial baseline (baseline 1) between the two age groups.

6.3 Results

Two lambs became hypothermic (rectal temperature below 37.0°C) and were thus excluded from the analyses.

In all, 74 lambs were used for the present study and were allocated to the six treatment groups as follows:

4-24hr control lambs n = 13
4-24hr pregnanolone lambs n = 11
4-24hr picrotoxin lambs n = 12
7-11day control lambs n = 12
7-11day pregnanolone lambs n = 13
7-11day picrotoxin lambs n = 13.

Forty-three lambs were males and 31 were females (see Table 6.1 for gender distribution according to treatment group).

Table 6.1:Gender distribution according to age and treatment group. N=number of animals

	4-24hr lambs		**7-11day lambs**	
	Male	**Female**	**Male**	**Female**
Control	**8[N]**	**5**	**8**	**4**
Pregnanolone	**6**	**5**	**5**	**8**
Picrotoxin	**8**	**4**	**8**	**5**

1) Treatment effects

Effects of vehicle control, pregnanolone and picrotoxin infusions on EEG parameters

The results of the two-related samples Wilcoxon signed rank tests (F50 and Ptot) and paired sample t-tests (F95) are presented in Table 6.2. Comparison of EEG parameters during the initial baseline period (B1) with those during the last 30 seconds of the infusion period showed that in 4-24hr lambs there were no significant differences in control, pregnanolone or picrotoxin groups. In 7-11day lambs, there was a significant decrease in F95 in response to pregnanolone infusion (20.29±0.36Hz at initial baseline *versus* 19.69±0.25Hz at the end of infusion), and a significant increase in F95 in response to picrotoxin infusion (19.59±0.24Hz at initial baseline *versus* 20.03±0.17Hz at the end of infusion).

Table 6.2: Results of the non-parametric two-related samples Wilcoxon tests and paired-sample t-tests for all treatment groups comparing pre-infusion and infusion (last 30 seconds) EEGs for 4-24hr lambs and 7-11day lambs.

	4-24hr lambs			7-11day lambs		
F50	**Control N = 13**	**Pregnan N = 11**	**Picrotox N = 12**	**Control N = 12**	**Pregnan N = 13**	**Picrotox N = 13**
Z-statistic	-1.153	-0.889	-0.549	-1.020	-0.175	-0.078
p-value	0.249	0.374	0.583	0.308	0.861	0.937
F95						
T-statistic	-1.913	0.787	0.428	-1.080	2.601	-2.337
p-value	0.080	0.450	0.677	0.303	**0.023**	**0.039**
Ptot						
Z-statistic	-0.804	-1.245	-1.726	-1.412	-1.503	0.000
p-value	0.422	0.213	0.084	0.158	0.133	1.000

The results of the repeated measures ANOVA and non-parametric Friedman tests comparing the three baselines for each group are presented in Table 6.3. Although the interpretation of these data is complicated due to the blood sampling procedures and the presentation of the electrical stimuli between the baselines, the results are presented here.

There were significant changes between the three baseline periods in F95 and Ptot in 4-24hr lambs receiving a pregnanolone infusion. The post-hoc tests showed that these differences were due to a significant increase in F95 between baseline 2 and 3 (B1 = 21.30±0.23Hz, B2 = 20.76±0.33Hz, B3 = 21.48±0.22Hz) and a significant decrease in Ptot between baseline 2 and 3 (B1 = 24.1±1.77; B2 = 24.6±1.77, B3 = 22.4±1.58).

In 7-11day lambs, significant differences between the three baseline periods were observed in F50 and F95 in pregnanolone lambs and in F95 in picrotoxin lambs. The post-hoc tests showed that these differences were due to a significant increase in F50 and F95 between baseline 1 and 3 in pregnanolone lambs (*F50:* B1 = 3.07±0.12Hz, B2

= 3.46±0.12Hz, B3 = 3.36±0.16Hz; *F95:* B1 = 20.26±0.38Hz, B2 = 20.85±0.16Hz, B3 = 21.09±0.23Hz) and a significant increase F95 between baseline 1 and 2 and baseline 1 and 3 in picrotoxin lambs (B1 = 19.62±0.25Hz, B2 = 20.68±0.27Hz, B3 = 20.58±0.29Hz).

Table 6.3: Results of the repeated measures ANOVAs for all treatment groups comparing EEG parameters of the three baseline periods (df = 2 for each comparison). * indicates Chi-Square statistic calculated by non-parametric Friedman test

	4-24hr lambs			**7-11day lambs**		
	Control	**Pregnan**	**Picrotox**	**Control**	**Pregnan**	**Picrotox**
F50	**N=13**	**N=11**	**N=12**	**N=12**	**N=13**	**N=13**
F-statistic	0.285	0.227	0.255	0.949	5.650	5.538*
p-value	0.755	0.799	0.777	0.403	**0.010**	0.063
F95						
F-statistic	0.177	5.575	1.713	1.551	8.000*	9.750
p-value	0.839	**0.012**	0.204	0.243	**0.018**	**0.001**
Ptot						
F-statistic	0.097	7.091*	3.274	2.902	0.615*	0.571
p-value	0.908	**0.029**	0.057	0.076	0.735	0.573

EEG and ECG responses to electrical stimulation

<u>*EEG*</u>

The results of the two-related sample Wilcoxon signed rank test are presented in Table 6.4, and mean and SEM for all groups in Figures 6.2 to 6.4. A summary of the results is presented in Table 6.5.

1) 4-24hr lambs

None of the three electrical stimuli presented to 4-24hr lambs elicited a significant response in the three EEG parameters investigated. This was so for control, pregnanolone and picrotoxin lambs. However, there was a tendency for F95 to increase in response to stimulus 2 and a tendency for F50 to decrease in response to stimulus 3 in

pregnanolone lambs, although these changes only approached significance.

2) 7-11day lambs

Stimulus 1

For 7-11day lambs, there was a significant increase in F50 and F95 in response to stimulus 1 for pregnanolone and picrotoxin lambs, (Figure 6.2 and 6.3, respectively).

Stimulus 2

In response to stimulus 2 Ptot decreased significantly in picrotoxin lambs (Figure 6.4).

Stimulus 3

F50 and F95 increased significantly in both pregnanolone and picrotoxin lambs in response to stimulus 3 (Figures 6.2 and 6.3, respectively). There was a decrease in Ptot in control, pregnanolone and picrotoxin lambs in response to S3, which approached significance in control and pregnanolone lambs and was significant for picrotoxin lambs (Figure 6.4).

Table 6.4: Results of the non-parametric two-related samples Wilcoxon test for F50, F95 and Ptot for all treatment groups comparing pre-stimulus and post-stimulus EEGs for all stimuli.

		4-24hr lambs			7-11day lambs		
		Control	**Pregnan**	**Picrotox**	**Control**	**Pregnan**	**Picrotox**
F50		**N=14**	**N=11**	**N = 12**	**N=12**	**N=13**	**N=13**
B1 – S1	*Z-Statistic*	-0.743	-1.334	-0.784	-1.560	-2.97	-1.223
	p-value	0.463	0.182	0.433	0.117	**0.003**	0.221
B2 – S2	*Z-Statistic*	-0.105	0	-0.549	-0.784	-0.384	-0.943
	p-value	0.917	1.000	0.583	0.433	0.701	0.345
B3 – S3	*Z-Statistic*	-0.175	-1.956	-0.235	-0.078	-2.481	-2.411
	p-value	0.861	**0.050**	0.814	0.937	**0.013**	**0.016**
F95							
B1 – S1	*Z-Statistic*	-1.433	-1.867	-0.314	-1.567	-1.083	-2.691
	p-value	0.152	0.062	0.754	0.117	0.279	**0.007**
B2 – S2	*Z-Statistic*	-0.874	-1.956	-0.784	-1.020	-1.852	-1.642
	p-value	0.382	**0.050**	0.433	0.308	0.064	0.101
B3 – S3	*Z-Statistic*	-1.153	-0.178	-1.098	-1.098	-2.481	-3.180
	p-value	0.249	0.859	0.272	0.272	**0.013**	**0.001**
Ptot							
B1 – S1	*Z-Statistic*	-1.083	-0.800	-1.334	-0.941	-0.524	-1.572
	p-value	0.279	0.424	0.182	0.347	0.600	0.116
B2 – S2	*Z-Statistic*	-1.013	-0.178	-0.863	-1.569	-1.363	-2.132
	p-value	0.311	0.859	0.388	0.117	0.173	**0.033**
B3 – S3	*Z-Statistic*	-0.734	-0.089	-0.549	-1.961	-1.922	-3.18
	p-value	0.463	0.929	0.583	**0.050**	**0.055**	**0.001**

B1 = baseline 1 (initial baseline, pre stimulus 1)

B2 = baseline 2 (pre stimulus 2)

B3 = baseline 3 (pre stimulus 3)

S1 = stimulus 1

S2 = stimulus 2

S3 = stimulus 3

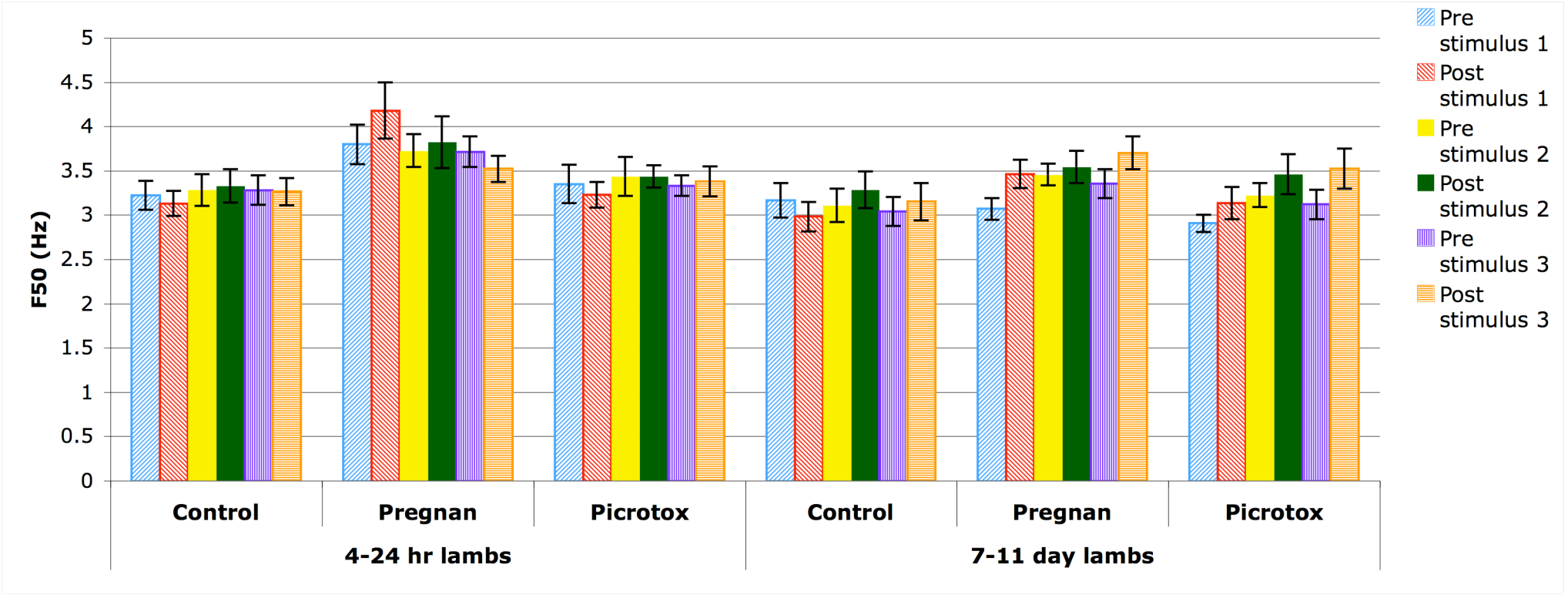


Figure 6.2: Mean and standard error of the mean (SEM) of F50 for control, pregnanolone and picrotoxin lambs comparing pre- and post-stimulation periods.

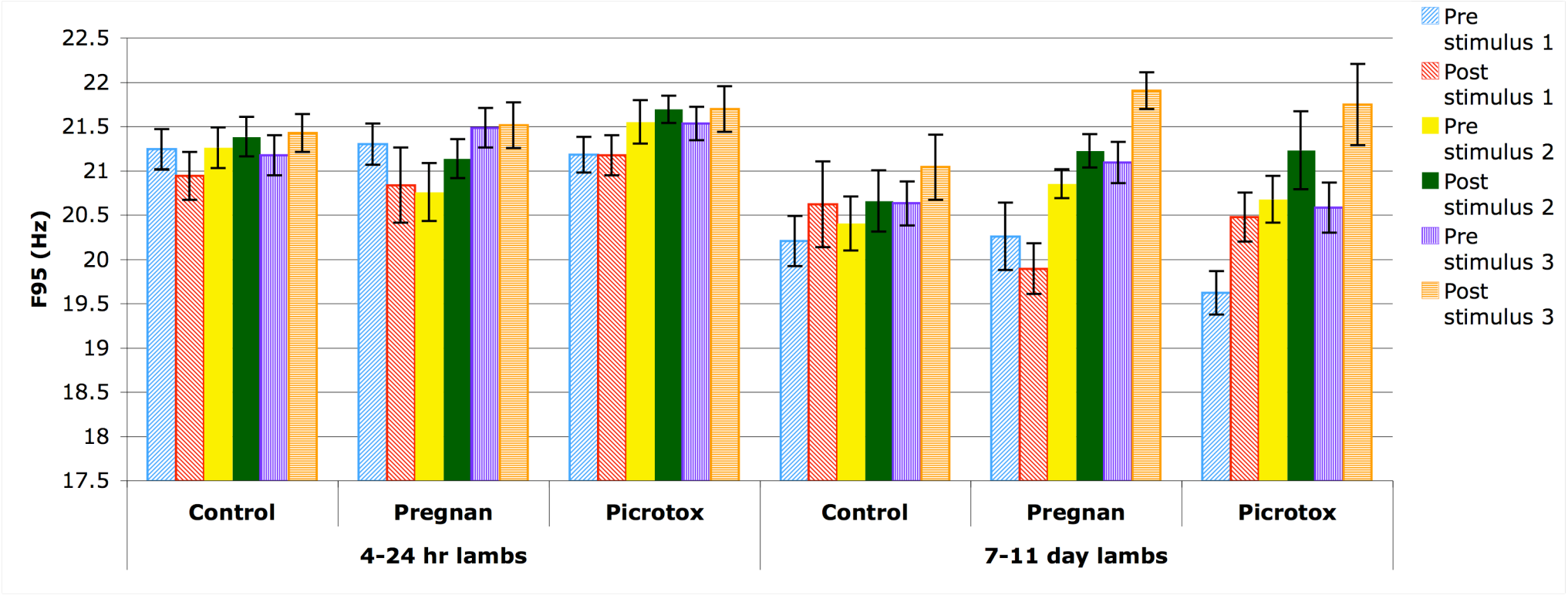


Figure 6.3: Mean and standard error of the mean (SEM) of F95 for control, pregnanolone and picrotoxin lambs comparing pre- and post-stimulation periods.

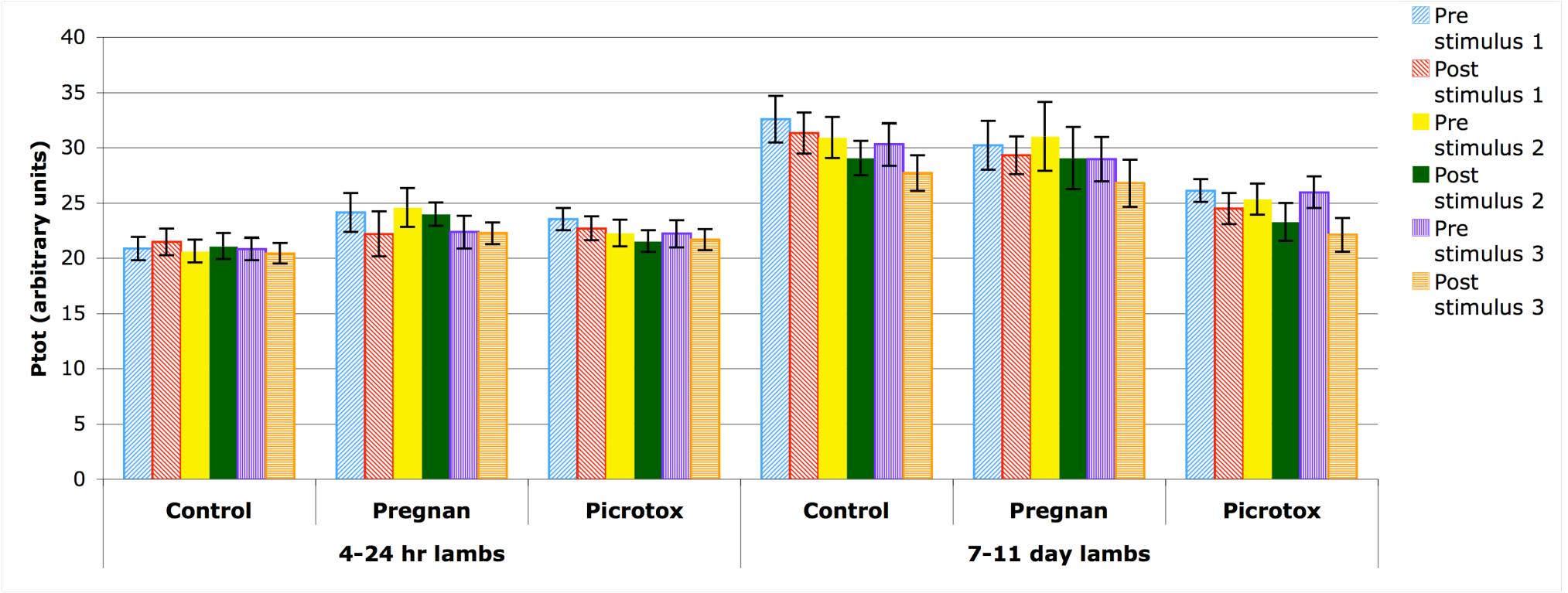


Figure 6.4: Mean and standard error of the mean (SEM) of Ptot for control, pregnanolone and picrotoxin lambs comparing pre- and post-stimulation periods.

Table 6.5: Summary of the results of the paired-sample Wilcoxon signed rank tests showing significant changes in EEG parameters for the different groups in response to the three stimuli (+ denotes an increase in a parameter, while - denotes a decrease in a parameter).

4-24hr lambs	**Control**	**Pregnanolone**	**Picrotoxin**
Stimulus 1	No response	No response	No response
Stimulus 2	No response	No response	No response
Stimulus 3	No response	No response	No response
7-11day lambs	**Control**	**Pregnanolone**	**Picrotoxin**
Stimulus 1	No response	F50 +	F95 +
Stimulus 2	No response	No response	Ptot -
Stimulus 3	No response	F50 + F95 +	F50 + F95 + Ptot -

Table 6.6: Results of the two-tailed Kolmogorov-Smirnoff test comparing frequency spectra (1-30Hz) pre- and post-stimulation for the three stimuli for control, pregnanolone and picrotoxin lambs of both ages.

4-24hr lamb									
Frequency (Hz)	**Control**			**Pregnanolone**			**Picrotoxin**		
	B1 vs. S1	*B2 vs. S2*	*B3 vs. S3*	*B1vs. S1*	*B2 vs. S2*	*B3 vs. S3*	*B1 vs. S1*	*B2 vs. S2*	*B3 vs. S3*
1				0.023			0.046		
7-11day lamb									
Frequency (Hz)	**Control**			**Pregnanolone**			**Picrotoxin**		
	B1 vs. S1	*B2 vs. S2*	*B3 vs. S3*	*B1vs. S1*	*B2 vs. S2*	*B3 vs. S3*	*B1 vs. S1*	*B2 vs. S2*	*B3 vs. S3*
1									0.046
7							0.046		
10							0.046		

B1 = Baseline 1 (initial baseline, pre stimulus 1)

S1 = Stimulus 1

B2 = Baseline 2 (Pre stimulus 2)

S2 = Stimulus 2

B3 = Baseline 3 (Pre stimulus 3)

S3 = Stimulus 3

Direct comparison of frequency spectra (1-30Hz) means of pre- and post-stimulus data suggested that there were no significant differences in response to electrical stimulation in any group. This is supported by the results of the two-tailed Kolmogorov-Smirnoff tests (Table 6.6) for 4-24hr and 7-11day lambs, which showed that significant differences between frequency spectra were only present for 4-24hr pregnanolone and picrotoxin lambs at the frequency of 1Hz (baseline 1 *versus* stimulus 1). In 7-11day picrotoxin lambs significant differences were observed at frequencies of 1Hz (baseline 3 *versus* stimulus 3) and 7 and 10Hz (baseline 1 *versus* stimulus 1).

Due to this overall lack of significant differences in the power of frequency spectra 1-30Hz, Principal Component (PC) Analyses, which have been performed in the previous chapters of this thesis at this stage of analysis, have not been performed.

ECG

The mean, SEM and percentage change from pre-stimulus values for heart rate data for 4-24hr lambs and 7-11day lambs are presented Figure 6.5 and Figure 6.6, respectively, and the results of statistical analyses for both in Table 6.7.

Heart rate of 4-24hr control lambs did not change in response to any of the electrical stimuli. In contrast, a significant decrease in heart rate was observed in 7-11day control lambs for all three stimuli.

A significant increase in heart rate was observed in 4-24hr pregnanolone lambs in response to stimuli 1 and 2 and in 4-24hr picrotoxin lambs in response to stimulus 1. The 7-11day pregnanolone and picrotoxin lambs responded to stimuli 2 and 3 with a significant decrease in heart rate. They did not however respond to stimulus 1.

Table 6.7: Results of the paired sample t-test for heart rate for 4-24hr and 7-11day lambs of all treatment groups [control, pregnanolone (pregnan) and picrotoxin (picrotox)], comparing pre-stimulus and post-stimulus ECGs for the three stimuli.

		4-24hr lambs			**7-11day lambs**		
		Control	**Pregnan**	**Picrotox**	**Control**	**Pregnan**	**Picrotox**
BPM		**N=13**	**N=11**	**N=12**	**N=12**	**N=13**	**N=13**
B1 – S1	*T-statistic*	-0.051	-4.051	-3.244	4.371	-0.635	1.246
	p-value	0.960	**0.002**	**0.008**	**0.001**	0.537	0.236
B2 – S2	*T-statistic*	-1.513	-2.265	-1.121	2.917	5.303	4.546
	p-value	0.156	**0.047**	0.286	**0.014**	**<0.001**	**0.001**
B3 – S3	*T-statistic*	0.129	-1.744	-0.492	5.384	3.604	7.091
	p-value	0.899	0.112	0.632	**<0.001**	**0.004**	**<0.001**

B1 = baseline 1 (initial baseline pre stimulus 1)

B2 = baseline 2 (pre stimulus 2)

B3 = baseline 3 (pre stimulus 3)

S1 = stimulus 1

S2 = stimulus 2

S3 = stimulus 3

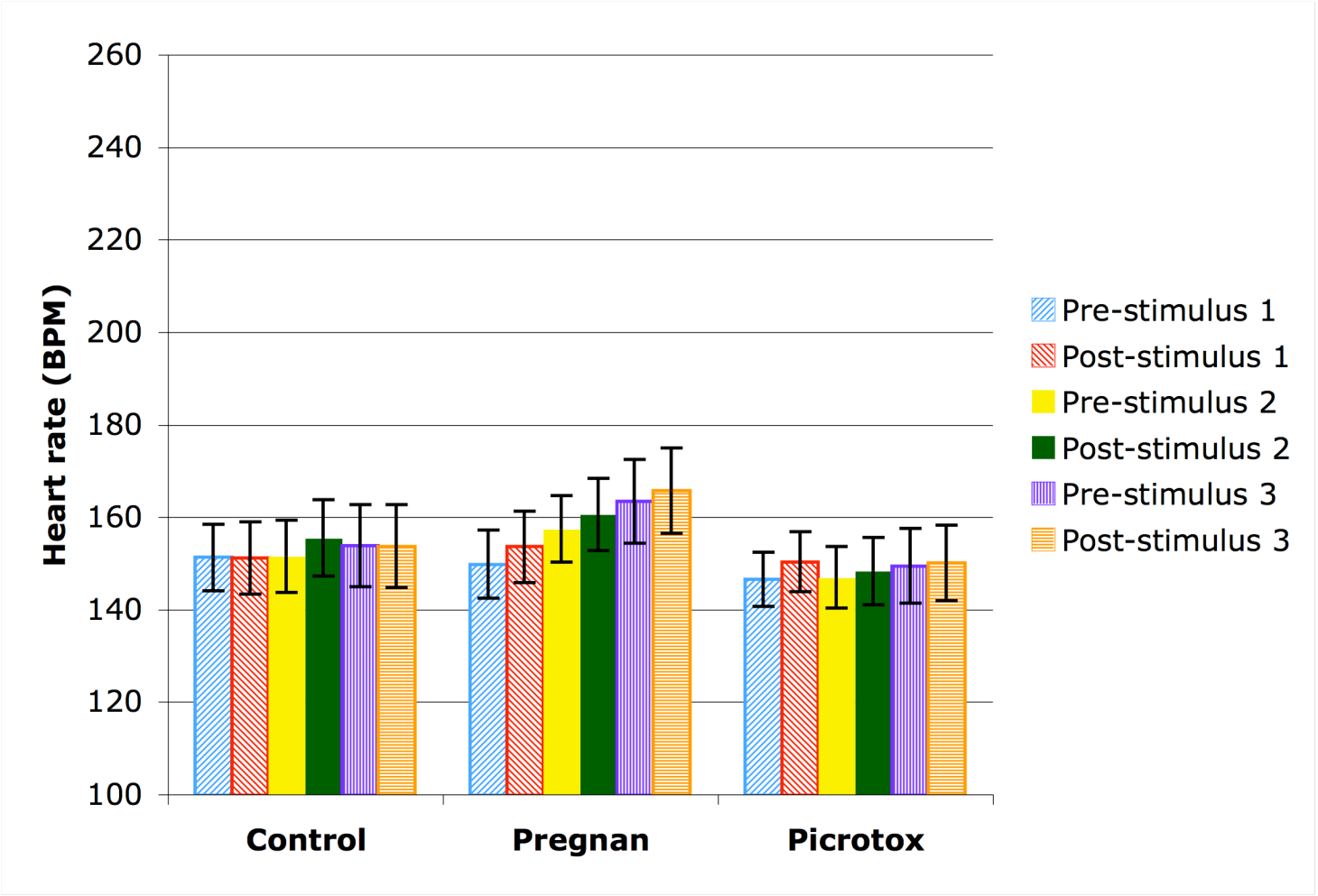


Figure 6.5: Means and standard error of the mean (SEM) of heart rate for pre- and post-stimulus ECGs for the three electrical stimuli for control, pregnanolone and picrotoxin lambs 4-24 hours after birth. The mean percentage change in heart rate between pre- and post stimulus is also presented.

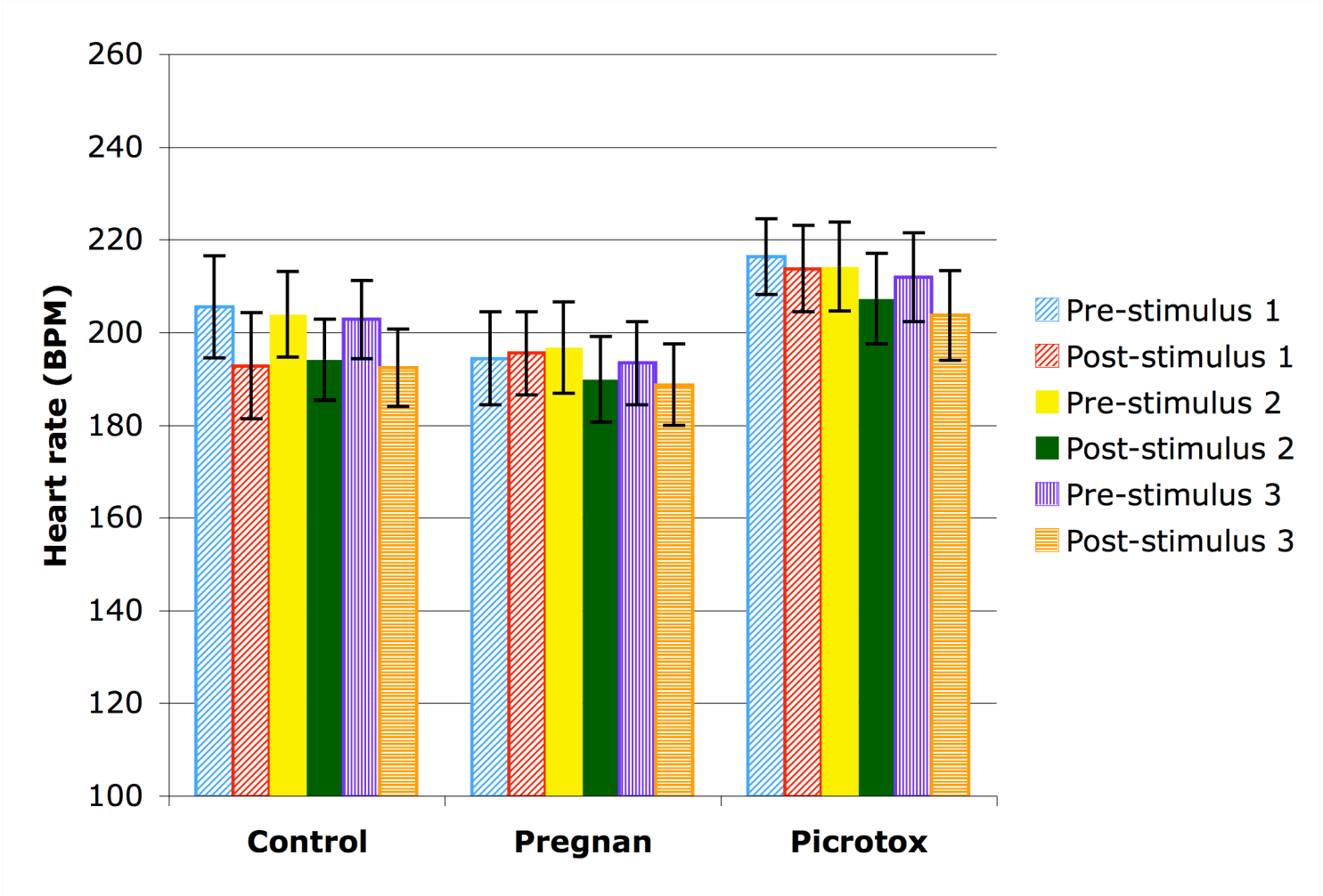


Figure 6.6: Means and SEM for heart rate from pre- and post-stimulus ECGs for the three electrical stimuli for control, pregnanolone and picrotoxin lambs 7-11 days after birth. The mean percentage change in heart rate between pre- and post stimulus is also presented.

General physiological information

There was a significant decrease in rectal temperature during the experiment (see Table 6.8 for means and SEMs) for both age groups (4-24hr lambs: $p<0.001$, df 4, 35, $F=26.859$; 7-11day lambs: $p<0.001$, df 4, 36, $F=32.549$). This was due mainly to a significant drop in rectal temperature between the initial reading at the start of anaesthesia and the subsequent readings, rather than to a progressive decline in rectal temperature over the whole study (Figure 6.7). In three 4-24hr lambs rectal temperatures of around 37.5°C were observed. This however only included the last recording (i.e. immediately before presentation of stimulus 3) and therefore these animals were not excluded from the study.

The mean and SEM for end tidal CO_2 partial pressure have also been presented in Table 6.3. In 4-24hr lambs end tidal CO_2 partial pressure changed significantly ($p<0.001$, df 4, 35, $F=8.009$), showing, after an initial drop at baseline, an increase prior to stimulus1 after which it remained stable. In 7-11day lambs there were no significant changes in end tidal CO_2 partial pressure ($p=0.085$, df 4, 35, $F=2.097$).

Glucose concentrations decreased significantly as the experiment progressed in 4-24hr lambs ($p<0.001$, df 2, 28, Chi-Square 14.42), but remained stable in 7-11day lambs ($p = 0.193$, df 2, 37, Chi-Square = 3.292) (see Table 6.8 for means and SEMs). In six animals, five of which were 4-24hr lambs, blood glucose concentrations dropped to below 2.0mmol/L, with 1.0mmol/L being the lowest value recorded.

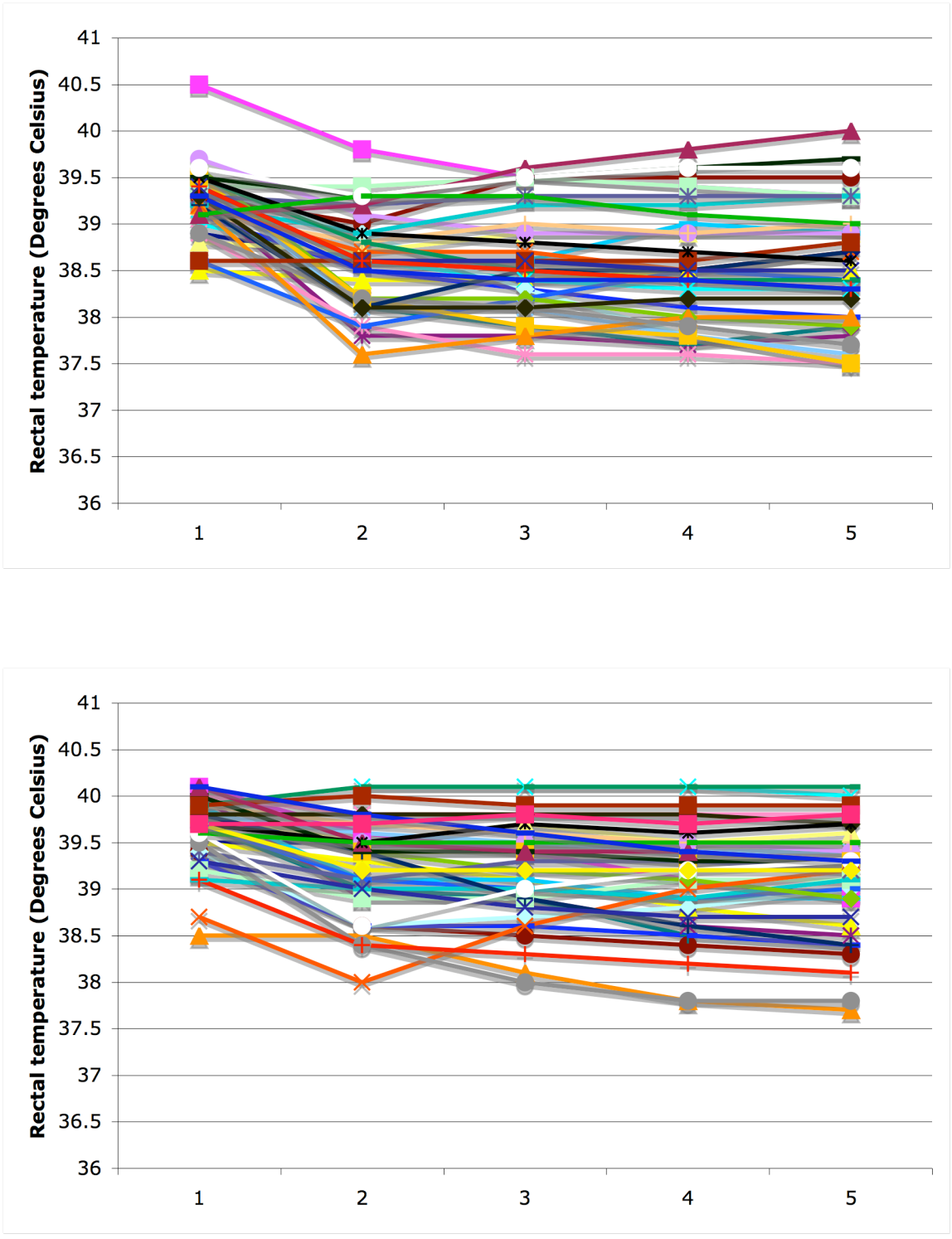


Figure 6.7: Rectal temperature (°C) of individual 4-24hr (top) and 7-11day (bottom) lambs at the five sampling periods (1 = start of anaesthesia; 2 = at initial baseline, 3 = pre stimulus 1, 4 = pre stimulus 2; 5 = pre stimulus 3).

Table 6.8: Mean, SEM and number of animals (N) for endtidal CO_2 partial pressure, rectal temperature and blood glucose concentrations for 4-24hr lambs and 7-11day lambs during the course of the study. Approximate timing of measurements is given in brackets in minutes from time 0 (start of stabilisation period, see Figure 6.1).

	Start of anaesthesia	**Start of baseline 1 (~5min)**	**Pre stimulus 1 (~20-25)**	**Pre stimulus 2 (~35-45)**	**Pre stimulus 3 (~45-55)**
Endtidal CO_2 (mmHg)					
4-24hr	40.5^{Mean}	39.1	42.3	43.3	43.4
	1.31^{SEM}	1.25	1.45	1.24	1.18
	36^{N}	36	36	36	36
7-11day	47.8	50.4	48.0	45.5	46.2
	1.26	1.39	1.49	1.39	1.47
	36	37	38	38	38
Rectal temperature (°C)					
4-24hr	39.3	38.6	38.6	38.6	38.6
	0.06	0.09	0.10	0.11	0.12
	36	36	36	36	36
7-11day	39.6	39.2	39.2	39.1	39.1
	0.05	0.08	0.09	0.09	0.10
	37	38	38	38	38

Glucose concentration (mmol/L)	**Start of baseline 1 (~5min)**	**Pre stimulus 2 (~35-45)**	**End of Study (~55-65)**
4-24hr	4.12	3.58	3.45
	0.22	0.30	0.33
	31	29	31
7-11day	5.31	5.43	5.42
	0.12	0.16	0.19
	38	38	38

2) Age effects

General information

As expected, 4-24hr lambs weighed less than 7-11day lambs ($p<0.001$; df 73; $Z= -6.538$; 5.0±0.1 kg and 6.8±0.1 kg, respectively). Weight did not differ between genders at either age ($p=0.469$; df 73; $Z=-0.724$).

End tidal CO_2 partial pressure was lower in 4-24hr lambs than 7-11day lambs at the start of anaesthesia ($p<0.001$, df 71, $t=-3.940$; 40.7±1.29mmHg *versus* 47.8±1.26mmHg) as well as at the initial baseline (B1) ($p<0.001$, df 72, $t=-6.004$; 39.3±1.22mmHg *versus* 50.4±1.39mmHg). This was also the case for rectal temperature at start of baseline ($p<0.001$, df 72, $t=-4.236$; 39.3±0.06°C *versus* 39.6±0.06°C) and at the initial baseline (B1) ($p<0.001$, df 73, $t=-5.146$; 38.6±0.09°C *versus* 39.2±0.08°C).

As expected, blood glucose concentrations at the initial baseline (B1) were also lower in 4-24hr lambs than 7-11day lambs ($p<0.001$, df 68, $Z=-4.252$; 4.15±0.22mmol/L *versus* 5.31±0.12mmol/L).

EEG and ECG

Lamb 4-24hr after birth had higher F50 values at the initial baseline (B1) than did 7-11day lambs ($p=0.016$, df 1,73, $Z=-2.406$; 3.44±0.08Hz *versus* 3.04±0.08Hz, respectively), and this was also the case for F95 ($p<0.001$, df 1, 73, $t=-5.496$; 21.24±0.12Hz versus 20.02±0.18Hz, respectively). In contrast, Ptot was lower in 4-24hr lambs than 7-11day lambs ($p<0.001$, df 1, 73, $Z=-4.650$; 22.7±0.76 *versus* 29.6±1.10).

The two-tailed Kolmogorov-Smirnoff test showed that there were significant differences in frequency spectra between the two age groups (Table 6.9). These differences were highly significant in the lower frequencies (1 to 9Hz).

The factor analysis identified four Principal Components (PCs) (Eigenvalues > 1) that contributed 88.8% of the overall variation in the data (Table 6.10). As Components 3 and 4 only contributed 4.5% and 3.7%, respectively, they have not been discussed further in any detail.

Component loadings showed that Component 1 (69.5%) strongly correlated with all frequencies (most loadings >0.8) and could thus be seen to represent overall EEG power. Component 2 (11.1%) correlated with frequencies in the range of 1 to 11Hz. There were no contrasts (positive *versus* negative loadings) in Component 1, but a contrast between frequencies of 1 to 11Hz (positive) and 14 to 29Hz (negative) was observed in Component 2.

As can be seen from Figure 6.8, 4-24hr lambs scored lower on both axes than 7-11day lambs. This indicates that overall EEG power was lower in 4-24hr lambs than 7-11day lambs, although the distinction between the two ages along Component 1 was not very strong. In contrast, Component 2 was able to distinguish between the ages well. Hence, 4-24hr lambs had lower power in frequencies between 1 to 11Hz and higher power in frequencies between 14 to 29Hz compared to 7-11day lambs.

At the initial baseline (B1) heart rate was lower in 4-24hr lambs than 7-11day lambs ($p<0.001$, df 1, 73, $t=-8.191$; 149±3.9BPM *versus* 205±5.7BPM, respectively).

Table 6.9: P-values and Kolmogorov-Smirnoff test statistic (K-statistic) calculated by two-tailed Kolmogorov-Smirnoff test comparing frequency spectra between 4-24hr and 7-11day lambs at the initial baseline.

Frequency (Hz)	K-statistic	P-value
1	2.188	<0.001
2	2.917	<0.001
3	1.968	0.001
4	2.433	<0.001
5	2.426	<0.001
6	2.785	<0.001
7	2.219	<0.001
8	2.194	<0.001
9	1.829	0.002
10	1.439	0.032
11	1.307	0.065
12	1.213	0.105
13	0.981	0.291
14	0.572	0.899
15	0.49	0.97
16	0.616	0.842
17	0.66	0.776
18	0.503	0.962
19	0.616	0.842
20	0.704	0.705
21	0.603	0.86
22	0.534	0.938
23	0.509	0.958
24	0.754	0.62
25	1.069	0.204
26	0.981	0.291
27	1.207	0.109
28	1.333	0.057
29	1.113	0.168
30	1.571	0.014

Table 6.10: Eigenvalues and Component scores for all frequencies for Principal Components (PCs) 1 to 4 calculated by Principal Component analysis for age comparison of baseline EEG spectra.

Eigenvalues	**PC1**	**PC2**	**PC3**	**PC4**
Total	20.86	3.33	1.33	1.11
Percentage of variance	69.5%	11.1%	4.5%	3.7%
Frequency (Hz)	**PC1**	**PC2**	**PC3**	**PC4**
1	0.578	0.450	0.194	0.026
2	0.664	0.593	0.135	0.205
3	0.622	0.561	-0.015	0.353
4	0.715	0.560	-0.025	0.274
5	0.771	0.518	-0.011	0.119
6	0.803	0.491	-0.017	0.005
7	0.824	0.450	-0.053	-0.049
8	0.872	0.351	-0.146	-0.064
9	0.884	0.268	-0.166	-0.125
10	0.886	0.218	-0.098	-0.158
11	0.912	0.138	-0.172	-0.153
12	0.915	0.034	-0.183	-0.184
13	0.924	-0.085	-0.182	-0.141
14	0.902	-0.165	-0.230	-0.099
15	0.828	-0.303	-0.361	0.017
16	0.777	-0.365	-0.437	0.101
17	0.781	-0.391	-0.328	0.267
18	0.762	-0.425	-0.222	0.367
19	0.808	-0.368	0.096	0.353
20	0.803	-0.337	0.249	0.309
21	0.841	-0.291	0.313	0.176
22	0.856	-0.245	0.328	0.078
23	0.874	-0.227	0.292	-0.039
24	0.883	-0.120	0.337	-0.032
25	0.898	-0.178	0.213	-0.134
26	0.904	-0.198	0.163	-0.160
27	0.899	-0.131	0.125	-0.214
28	0.895	-0.137	0.129	-0.225
29	0.902	-0.133	-0.004	-0.255
30	0.893	-0.049	0.090	-0.185

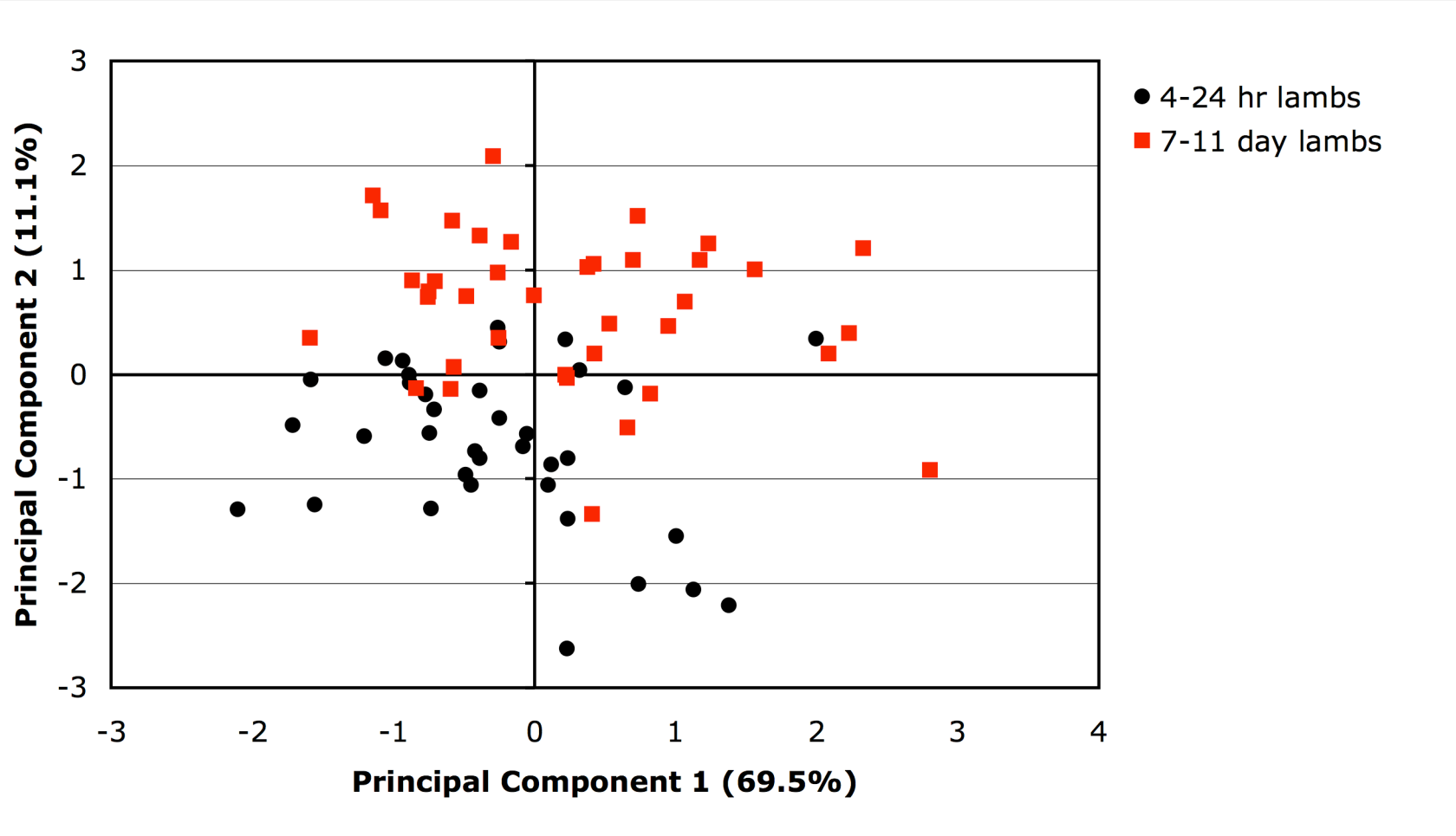


Figure 6.8: Factor scores plotted against the 1st and 2nd principal component axes showing separation according to age (4-24hr lambs versus 7-11day lambs.

6.4 Discussion

The aim of this study was to determine whether the natural neuroinhibitors present during the first day after birth in lambs have the potential to alleviate suffering by reducing noxious sensory input or awareness of it.

The main findings were as follows. First, there were no immediate effects in response to infusion of either vehicle control, pregnanolone or picrotoxin in newborn lambs (i.e. 4-24 hours) whereas immediate EEG changes in response to infusion of pregnanolone and picrotoxin were observed in young lambs (i.e. 7-11 days). Second, in both newborn and young lambs changes in EEG between successive baseline measurements were observed in pregnanolone and picrotoxin lambs. Third, electrical stimulation *per se* did not apparently lead to EEG changes in vehicle control lambs of both ages. Fourth, EEGs of young, but not newborn, pregnanolone and picrotoxin lambs responded to electrical stimulation. Fifth, electrical stimulation in newborn control lambs did not result in significant changes in heart rate, while in young control lambs changes in heart rate could be observed for all infusion groups. Fifth, newborn lambs were physiologically and neurologically different from young lambs. Sixth, overall variation in the data set was small indicating that electrical stimulation and/or infusions did not lead to pronounced changes in EEG parameters. Last, the study design had several study design flaws, thereby preventing meaningful interpretation of the data.

EEG infusion effect

There were no significant changes in any of the EEG parameters investigated between successive baseline measurements in control lambs of the two ages, suggesting that Intralipid® did not have EEG effects. Changes in EEG parameters in response to infusion as well as across the three baselines were assessed to determine whether pregnanolone and picrotoxin were infused at sufficiently high concentrations to have effects on the EEG.

1) Immediate infusion effect

It is interesting that the EEG parameters of newborn pregnanolone and picrotoxin lambs

did not show any significant changes in immediate response to infusion, while there were significant EEG changes in young pregnanolone as well as picrotoxin lambs. With regard to pregnanolone, it is possible that the residual elevated concentrations of allopregnanolone and/or pregnanolone present in the newborn lambs (see Chapter 5) delayed the EEG response, as neuroinhibition by these factors presumably was already present and, if so, additional input via infusion may not have led to further neuroinhibition unless reaching a certain threshold concentration. The presence of residual neuroinhibitory steroids may have protected the newborn animals' CNS from any picrotoxin effects (possibly by reducing excitatory activity in the brain or competitive binding via the receptor) as no EEG responses to picrotoxin infusion were observed in the newborn lambs. However, picrotoxin doses used were small and this, rather than any protective effect, may have been the cause for the absence of EEG responses to picrotoxin infusion. This may appear more likely, as allopregnanolone concentrations have been shown to decrease rapidly after birth (Chapter 5) and thus may only have affected the EEG responsiveness in the present study in those animals younger than 12 hours. With regard to both pregnanolone and picrotoxin, the absence of immediate EEG changes in response to infusion could also be explained by differences in pharmacokinetics between lambs of the two ages (see below). However, at this point the data of the present study do not allow more than speculation about the possible mechanisms involved.

2) Comparison of the three baselines

In contrast to immediate infusion effects, pregnanolone infusions did lead to EEG changes in newborn as well as young lambs when EEGs were compared across the three baselines. However, in newborn lambs these effects were only seen between baselines 2 and 3, while those for young lambs were detected between baselines 1 and 2 and baseline 1 and 3. These data, in addition to those representing immediate responses to infusion, suggest that the possible delay of EEG effects of pregnanolone infusion in newborn lambs was due to elevated background concentrations of neuroinhibitory steroids (Chapter 4), or differences in pharmacokinetics between the two age groups.

The absence of picrotoxin effects in newborn lambs during the entire experiment supports the idea that the residual neuroinhibitory steroids protect newborn lambs from

the activating effects of picrotoxin, as mentioned above. However, picrotoxin infusions at a dose rate of approximately 0.3mg/kg led to an increase in fetal arousal in non-anaesthetised fetuses (Nicol *et al.*, 1999). Therefore, it is possible that the dose rate used in the newborn lambs of the present study (0.1mg/kg) was not sufficiently high to overcome the neuroinhibitory effects exerted by residual concentrations of allopregnanolone and other neuroactive steroids possibly present at the time. This however would not explain the difference between the results of the present study and that by Nicol *et al*. (1999), as fetal lambs are exposed to even higher concentrations of endogenously produced allopregnanolone and pregnanolone than newborn lambs (Chapter 1), so that one would have anticipated a smaller arousal response in the fetus compared to the neonate. Thus, it is possible that the combined actions of residual neuroinhibitory steroids and the differential effects of halothane anaesthesia related to the age of lambs prevented an EEG response in newborn lambs. This is also supported by the fact that, in a pilot study, an anaesthetised newborn lamb did not show an arousal response to an electrical stimulus when infused with a higher dose of picrotoxin (0.5mg/kg), while young lambs did.

It has to be acknowledged that the changes in EEG parameters observed between the baselines could have been affected by general changes in response to electrical stimulation over time, blood sampling procedures or exposure to continuing halothane anaesthesia with time. However, as EEG changes were not observed in control lambs, this explanation does not appear likely.

3) Direction of changes in EEG parameters

Studies in anaesthetised animals and humans have shown that a reduction of arousal with onset of anaesthesia is associated with well-defined changes in spectral edge frequency (F95) (Gajraj *et al.*, 1998). Thus, a reduction in arousal is associated with a decrease in F95, while arousal is associated with an increase in F95 (Schwender *et al.*, 1996).

It is interesting to note that the direction of change in F95 in immediate response to infusion was different between young pregnanolone and picrotoxin lambs (i.e. decrease and increase, respectively), while that between the three baselines was the same for

young pregnanolone and picrotoxin lambs (i.e. increases in both). An initial decrease in F95 in pregnanolone lambs in response to the infusion of pregnanolone may be associated with an increase in neuroinhibition. However, there was an increase in F95 between baselines 1 and 3. It is possible that this increase between baselines was due to clearance of pregnanolone, gradually reducing the neuroinhibition in the lamb. The increase in F95 in response to picrotoxin infusions suggests increased arousal, as picrotoxin antagonises GABAergic activity. The increase in F95 between baselines 1 and 2 and baseline 1 and 3 supports this idea. However, as F95 did not change significantly between baselines 2 and 3 it appears that the concentrations of picrotoxin present in young lambs had reached their maximum effect by the time of baseline 2.

4) Pharmacokinetics

Differences in EEG responsiveness to drug infusions in the present study are possibly related to changes in the pharmacokinetics of pregnanolone and picrotoxin between the two age groups, if such changes do occur over the first week after birth in the newborn lamb. The pharmacokinetics for drugs in the neonate are different from those of older animals and adults, as developmental changes after birth alter the processes of distribution, metabolism and excretion (Nouws, 1992; Schwark, 1992; Alcorn & McNamara, 2003). Differences in drug distribution are related to newborn animals having a higher water content, lower fat content, lower skeletal mass, a more permeable blood-brain barrier, and greater tissue binding affinity as well as lower plasma protein binding (Nouws, 1992; Schwark, 1992; Alcorn & McNamara, 2003). In addition, differences in hemodynamic factors, such as cardiac output, tissue perfusion and membrane permeability are likely to play a role. Moreover, metabolism and excretion pathways may be inefficient soon after birth thus leading to longer elimination half lives of drugs (Nouws, 1992; Schwark, 1992; Alcorn & McNamara, 2003).

EEG and ECG responses to electrical stimulation

1) EEG

EEG responses to electrical stimulation were not observed in any newborn lambs. There are several possible explanations for this. First, residual concentrations of neuroactive steroids exerting their sedative, anaesthetic or analgesic effects may have prevented

EEG responses to electrical stimulation in newborn lambs. Second, nociceptive processes in the newborn lamb may be different from those of older lambs despite neurological maturity at birth. Third, halothane anaesthesia may have affected newborn lambs differently than older lambs due to the physiological adjustments to the extrauterine environment occurring at the time. Fourth, there may be other factors present around the time of birth that may affect processing of noxious stimuli. Fifth, a combination of the above may have been involved.

In young lambs, electrical stimulation resulted in an increase in F50 and F95 in pregnanolone and picrotoxin lambs as well as a decrease in Ptot in picrotoxin lambs. Previous studies within our laboratory, investigating the electroencephalographic response to noxious stimulation, have shown that an increase in F50 and F95 as well as a decrease in Ptot were associated with nociception (Johnson *et al.*, 2005b; Gibson *et al.*, 2007; Murrell *et al.*, 2007; Johnson *et al.*, 2009). Thus, the changes in EEG parameters observed in young infusion lambs seem to indicate that a response to noxious stimulation was present. However, as there were no significant changes in EEG parameters in control lambs, these responses are difficult to explain. In addition, the fact that EEG responses to both infused agents followed the same direction (i.e. increase in F50 and F95) is contrary to what would have been expected seeing that pregnanolone and picrotoxin were anticipated to have opposing effects.

However, as neither newborn nor young control lambs showed EEG responses to electrical stimulation it is possible that the electrical stimulus presented was not an effective one or that halothane anaesthesia was too deep thereby preventing EEG responses. As all lambs received an infusion as well as electrical stimuli, the absence of a response in control lambs makes it difficult to determine the extent to which EEG responses observed were due to the drugs infused or the electrical stimulus presented.

In addition, blood samples taken prior to stimulation could have affected responsiveness of the EEG. But as already stated, if this had been the case, changes in control lambs would have been expected, as these lambs also had blood samples taken.

2) ECG

Heart rate was recorded to assess responsiveness of the autonomic nervous system to stimulation of lambs of the different treatment groups. Autonomic responses have previously been shown to be elicited by noxious stimulation in a characteristic manner (Livingston & Chambers, 2000).

The newborn control lambs of the present study did not show a response in heart rate to any of the electrical stimuli. This is in contrast to young control lambs, in which a significant drop in heart rate was observed in response to all three electrical stimuli. There are several possible explanations for the absence of a response in heart rate in the newborn control lambs. First, newborn lambs' nociceptive thresholds may be higher and hence an autonomic response was not elicited. Second, the present newborn lambs may still have been adjusting to extrauterine life physiologically and hence autonomic responsiveness to stimulation may have been immature. Third, the continuing postnatal production of neuroinhibitors may have exerted analgesic and/or sedative effects at spinal cord or higher brain levels in newborn lambs thereby reducing the impact of electrical stimulation, preventing a significant response.

In contrast to newborn control lambs, an increase in heart rate was observed in newborn pregnanolone and picrotoxin lambs in response to stimulus 1 and in newborn pregnanolone lambs in response to stimulus 2. On the other hand, a reduction in heart rate was evident for young pregnanolone and picrotoxin lambs in response to stimulus 2 and stimulus 3.

Previous studies have variously shown that heart rate may increase or decrease in response to noxious stimulation. It appears that the response depends on the timing after the stimulus, so that recordings made several minutes to hours after the stimulus show an increase in heart rate (Lay *et al.*, 1992a; Lay *et al.*, 1992b; Peers *et al.*, 2002), while those within seconds of the stimulus show a decrease in heart rate (Haga & Ranheim, 2005; Gibson *et al.*, 2007; Johnson *et al.*, 2009). As a decrease in heart rate was also observed in response to routine immunisation in non-anaesthetised infants (Johnston & Strada, 1986), an effect of anaesthesia in the different responses to noxious stimulation does not seem likely. While the increase in heart rate in response to noxious stimulation is possibly due to an increase in sympathetic stimulation, the bradycardia observed in

other studies has been suggested to be a result of reflex increases in vagal activity or to be mediated indirectly by alterations in respiratory function or other responses (Johnson *et al.*, 2009).

However, in the present study the recording periods for heart rate were the same for newborn and young lambs and therefore do not explain the differences in response between the two age groups. It may be possible that bradycardia was present initially in newborn lambs, but that this was short-lived compared to young lambs and thus did not lead to a significant change when taking the whole minute of observation into consideration. Alternatively, the physiological adjustments occurring in newborn lambs are likely related to the differences in autonomic responsiveness between the two age groups, judged by the different responses observed in control lambs at the two ages. Thus, differences in responsiveness in pregnanolone and picrotoxin lambs of the two ages may be due to physiological adjustments occurring soon after birth, in addition to the resultant differential effect of halothane on the lambs of the different ages.

It is interesting that heart rate changed significantly in response to stimuli 1 and 2 in newborn pregnanolone and picrotoxin lambs, but did so in response to stimuli 2 and 3 in young pregnanolone and picrotoxin lambs. This is not easy to explain, especially considering the fact that heart rate of newborn control lambs did not show any changes in response to stimulation and that heart rate of young control lambs did show a significant decrease in response to stimulus 1 only. Again, differences in pharmacokinetics between the two ages, residual concentrations of neuroinhibitory factors, halothane anaesthesia or a combination of these factors may be involved.

Physiological and neurological differences between ages

1) Physiological changes occurring after birth

Around the time of birth, the newborn animal undergoes a variety of physiological changes ensuring adaptation to the extrauterine environment. The physiological changes of the major organ systems and processes, including the respiratory, cardiovascular and nervous systems, as well as the renal and hepatic processes, energy metabolism and heat production have been briefly been outlined in Chapter 4.

2) Age effects in the present study

Age effects were assessed by comparing all parameters measured at the start of anaesthesia or at the initial baseline between the two age groups. Newborn lambs differed physiologically and neurologically from young lambs in that they had lower body weights, rectal temperature, plasma glucose concentrations, endtidal CO_2 partial pressure, heart rate and Ptot as well as higher F50 and F95 frequencies. These differences are likely due to the newborn lambs undergoing significant physiological adjustments to the extrauterine environment.

The differences in heart rate between the two age groups in the present study are interesting, as another study has not reported a change in heart rate with age over the first 10 days after birth in non-anaesthetised lambs (Piccione *et al.*, 2007). It is therefore possible that the present heart rates differences were due to differential effects of halothane anaesthesia on lambs at the different ages. Myocardial contractility appears to be more easily depressed by halothane in younger animals and newborns (Cook *et al.*, 1981) and young animals seem to be more susceptible to the haemodynamic effects of halothane than adult animals are (Gregory *et al.*, 1983; Friesen *et al.*, 2000).

The progressively decreasing plasma glucose concentrations in newborn lambs of the present study are likely related to the interruption of colostrum intake. In the newborn lamb over the first day after birth, availability and mobilisation of liver glycogen and conversion of lactose into glucose as a result of the digestion of colostrum, are the main sources of circulating glucose (Mellor & Cockburn, 1986). The balance between these two mechanism is very delicate so that a delay or an interruption to colostrum intake over the first 24 hours after birth is more likely to reduce circulating glucose concentrations than would be observed in an older lamb (Mellor & Cockburn, 1986). It is therefore not surprising that the newborn lambs of the present study exhibited lower blood glucose concentrations as the study progressed, as colostrum intake was not sustained during the period of the study itself and it is not certain when lambs had their last feed before the onset of anaesthesia.

The lower EEG power observed in the newborn lambs, in addition to higher F50 and

F95, could either be related to continuing neurological maturation after birth, an effect of endogenous neuroinhibitory factors on the EEG in newborn lambs or differential effects of halothane anaesthesia on the lambs at different ages (see next section). It seems more likely however, that a combination of these factors would be involved. As EEG power in non-anaesthetised lambs up to 30 minutes after birth was shown to be lower than that of lambs 1 to 2 days of age (Chapter 4), it is possible that increases occur into the second week of life and that these are indeed associated with continuing neurological maturation or changes in the exposure to neuroinhibitory substances. Nevertheless, we cannot discount the possible direct or indirect effects of halothane anaesthesia.

Halothane anaesthesia in the newborn

The minimum alveolar concentration (MAC) is an estimate of anaesthetic requirement and indicates the alveolar concentration of an anaesthetic at which half of the patients do respond to a stimulus (reflex movement) (Quasha *et al.*, 1980). One factor that greatly influences MAC is age. An inverse relationship between age and anaesthetic requirement has previously been reported (Gregory *et al.*, 1969; Cook *et al.*, 1981; Lerman *et al.*, 1983). For example, the MAC of both newborn and young rats has been shown to be higher than that of adult rats (Orliaguet *et al.*, 2001). Various reasons for this difference in MAC have been suggested including compositional/structural differences in the brain (Gregory *et al.*, 1969), differences in physiological variables including cerebral blood flow, cerebral oxygen consumption and neuronal density (Quasha *et al.*, 1980) or water content (Cook *et al.*, 1981). In addition, changes in receptor numbers, including those of the $GABA_A$ receptor (Harris *et al.*, 1994), around the time of birth and thereafter may be involved.

In contrast, studies looking at MAC in lambs over the first 12 hours after birth have shown that MAC is lower initially and increases thereafter (Gregory *et al.*, 1983). It is possible that exposure to progesterone and its metabolites and/or endorphins soon after birth may be responsible (Lerman *et al.*, 1985; LeDez & Lerman, 1987; Grobin & Morrow, 2001). The greater degree of neurological maturity of lambs compared to rat pups at birth may also have contributed to this difference.

Thus, it is likely that MAC of animals 4-24 hours after birth differs from that of 7-11 day lambs. Whether this difference would be sufficient to have affected the results of the present study is not clear. It also has to be acknowledged that differential effects of halothane anaesthesia on physiological systems other than the nervous system could have been involved.

Drawbacks of experimental design

1) General issues

There are several flaws in the design of the present study which could have impacted on the results, making them difficult to interpret.

First, as it was not possible to determine whether EEG changes in response to electrical stimulation were due to electrical stimuli *per se*, or to blood sampling before the stimuli, drug concentrations or a combination of these factors, it may have been beneficial to include a sham control group for the purposes of the present study. This would have been a group receiving the various infusions without electrical stimulation or blood sampling procedures.

Second, as with the studies reported in Chapters 2 and 3, the fact that lambs were anaesthetised could have confounded the results of the present study. This may have been particularly so when comparing newborn and young lambs, as the physiological differences between these age groups could have affected the impact of halothane and therefore the results of the present study. Conducting a study where anaesthesia could have been maintained at a multiple of MAC tailored to each individual animal may have controlled for age-related differences in anaesthesia-related effects. The small magnitude of apparent effect and similar direction of change in the EEG with both pregnanolone and picrotoxin infusions suggest that the minimal anaesthesia model may be flawed.

Third, it appears that electrical stimulation was not effective, as there were no EEG responses to electrical stimulation in control lambs of either age. Thus, it would have been prudent to use a stimulus which had previously been shown to work, such as

castration. Alternatively, a pilot study could have been undertaken to assess the most suitable electrical stimulus in terms of duration and strength. It has to be noted that electrical stimuli have serious drawbacks. For example, intense electrical stimulation non-differentially excites all peripheral fibres, including those not directly implicated in nociception (i.e. large diameter fibres; Le Bars *et al.*, 2001).

In the control group, a noxious stimulus was presented before infusion in the baseline period. However, in the experimental groups the stimulus was given only after infusion. It may have been interesting to also conduct a study where pre-infusion responsiveness to noxious stimulation was conducted.

Fourth, pregnanolone was used in the present study as an alternative to allopregnanolone, which was too costly to use. However, as a recent *in vitro* study has shown, 3α-5α-steroids may interact with different binding sites from those for 3α-5β-steroids (Mennerick *et al.*, 2004). Hence, the results of the present study for pregnanolone may not reflect the situation for allopregnanolone.

Fifth, the choice of antagonist was not ideal for the purposes of the present study and this is discussed in the next section. In addition, picrotoxin doses were relatively low.

2) Choice of antagonist

Picrotoxin was used in the present study to antagonise neuroinhibitory GABAergic effects of residual concentrations of allopregnanolone. However, in hindsight, this may not have been the best choice of antagonist.

First, as no convulsions were observed in the present study in response to picrotoxin infusion, it can be concluded that only a part of the $GABA_A$ receptors were blocked by the dose used in the present study (Lancel *et al.*, 1999).

Second, although picrotoxin and progesterone (and hence progesterone metabolites) have previously been found to antagonise each other's effects, this does not necessarily have to occur at the level of the $GABA_A$ receptor (Lancel *et al.*, 1999). Indeed, picrotoxin does not only act via the $GABA_A$ receptor, but also via glycine receptors,

serotonin receptors and $GABA_C$ receptors (Dong & Werblin, 1996; Rajendra *et al.*, 1997; Das *et al.*, 2003; Wang *et al.*, 2006).

Third, $GABA_A$ receptors are very complex (see introduction and discussion Chapter 5) and the action of picrotoxin may affect not only those receptors sensitive to neuroactive steroid action, but also others. Thus, picrotoxin action is not specific to receptors through which allopregnanolone and pregnanolone exert their effects and hence changes in the responsiveness to noxious stimulation after picrotoxin infusion, if observed, are not necessarily indicative of previous residual neuroactive steroid action.

One alternative to picrotoxin would have been to pre-treat lambs with a 5α-reductase inhibitor, such as finasteride, thereby preventing the conversion of residual progesterone to allopregnanolone. Alternatively, indomethacin, a blocker of 3α-hydroxysteroid dehydrogenase (oxidoreductase) which catalyses the conversion of 5α-dihydroprogesterone and 5β-dihydroprogesterone into allopregnanolone and pregnanolone respectively, could have been used (Penning *et al.*, 1985). However, indomethacin is also a non-steroidal anti-inflammatory drug (NSAID) and therefore has antinociceptive effects (Hu *et al.*, 1994; Steen *et al.*, 1995), which, as the present study concentrated on responsiveness to noxious stimulation, would not have been desirable. NSAIDs inhibit the production of prostaglandins from arachidonic acid via inhibition of the enzyme cyclo-oxygenase (COX) (Nishida *et al.*, 2006). Prostaglandins have been shown to play a role in general cardiovascular regulation and cerebral blood flow in fetal (Hohimer *et al.*, 1985) and adult animals (Schumann *et al.*, 1996). Hence, treatment with indomethacin could lead to changes in cerebral blood flow thereby adversely affecting study results. In addition, both finasteride and indomethacin treatment would not have addressed the residual concentrations of neuroactive steroids present. Giving allopregnanolone/pregnanolone antibodies to mop up any circulating drug in lambs prior to the study, in addition to treatment with finasteride, may have been a way to address this problem, although it is not clear to the candidate whether this would have been a feasible protocol.

It has to be acknowledged that any treatment is likely to lead to physiological side effects, which could have the potential to adversely affect the data investigated.

6.5 Conclusions

Due to the complex nature and several study design flaws, the results of the present study were difficult to interpret. Further studies would be necessary to confirm whether residual concentrations of neuroactive steroids, such as allopregnanolone present over the first 12 hours or so after birth, have the potential to affect conscious perception and pain perception soon after birth in animals born neurologically mature and thus, whether they have the ability to mitigate any welfare compromise newborn animals may experience when they become sick and/or die.

6.6 References

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Chapter 7

Parts of this Chapter have been presented in the following publications *(see Appendix 2 for copies)*

Diesch TJ, Mellor DJ, Johnson CB & Lentle RG. (2008). Responsiveness to painful stimuli in anaesthetised newborn and young animals of varying neurological maturity (lambs, rat pups and wallaby joeys). Proceedings of the Sixth World Congress on Alternatives and Animal Use in the Life Sciences, Tokyo, Japan, 2007. *Alternatives to Animal Testing and Experimentation* **14**, 79-83.

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General Discussion

For any animal to *experience* pain and to suffer from it, two prerequisites have to be met (Mellor and Diesch, 2006). First, the animal has to be sentient. This means that the animal's nervous system has to be sufficiently developed to relay sensory inputs (e.g. electrical impulses) from the periphery to the higher centres of the brain (i.e. cerebral cortex), where such impulses can then be interpreted. Second, the animal has to be conscious, as unconscious animals cannot experience pain, for instance during general anaesthesia.

The developmental stage at which animals are capable of experiencing pain therefore depends on *when* they become sentient and consciously aware, i.e. when they are capable of consciously perceiving sensations. While research over the past few decades has vastly improved our knowledge about neurological development in a variety of species, we do not yet know clearly when conscious perception emerges in most species. Therefore, the aim of the present thesis was to investigate when, after birth, conscious perception may emerge, how the timing may differ between species born with varying degrees of neurological maturation, and whether modulation of conscious perception was present for some time after birth in animals born neurologically mature.

7.1 The onset of conscious perception after birth: major findings and conclusions of the present thesis

The major findings and conclusions of the present thesis are presented below according to animal species. To keep this document succinct only the main conclusions are summarised here. Again, the reader is reminded that the results of the present study in addition to published information on neurological, EEG and behavioural development have been used to make tentative suggestions about the onset of conscious perception in the three species assessed in the present thesis.

Chapter 2: Extremely immature at birth - wallaby joeys

Marsupial pouch young are extremely immature at birth and most neurological

development occurs postnatally while they are in the mother's pouch (Tyndale-Biscoe & Janssens, 1988).

The development of the EEG and its responses to a toe clamp in anaesthetised tammar wallaby joeys between 95 and 260 days after birth were investigated. The EEG in these animals remained isoelectric until about 120 days of in-pouch age and became continuous by 150 to 160 days. Overall, there was an increase in EEG power in all frequencies investigated (1-30Hz) between 140 to 180 and 187 to 260 days, indicating maturation of cerebral functioning. Also, the cerebrocortical response to a toe clamp was relatively smaller in joeys aged 140 to 180 days compared to those aged 187 to 260 days.

These results, in addition to published information on joey development and behaviour collated in Chapter 2, suggest that tammar wallaby joeys are not capable of conscious perception, and hence cannot experience pain, before about 120 days after birth. Behavioural signs of conscious perception are apparent by 160 to 180 days, at which stage we would expect joeys to be able to *experience* pain and, if it is noxious enough, to suffer. The period between 120 and 160 to 180 days therefore is a transitional period during which major developmental changes in the nervous system occur. However, we cannot be sure when during this period conscious perception may become possible.

Chapter 3: Moderately immature at birth - rat pups

Rat pups are born neurologically moderately immature. The EEG is isoelectric, intermittent or continuous but undifferentiated at birth. REM-non-REM differentiation occurs between postnatal days 12 to 18 (Ellingson & Rose, 1970) and evidence for conscious perception is not apparent before this.

EEG responses to tail clamping in anaesthetised rat pups between 5 and 22 days after birth – i.e. before, during and after REM-non-REM differentiation – were examined in order to determine when after birth rat pups may become capable of experiencing pain, and hence when they may capable of conscious perception. The EEG of anaesthetised pups aged 5 to 7 days (before REM-non-REM differentiation) was isoelectric and hence did not show a cerebral response to tail clamping. Overall, there was an increase in EEG

power in all frequencies investigated (1-30Hz) between 12 to 14 and 21 to 22 days of age. Pups aged 12 to 14 days (around which age REM-non-REM differentiation occurs) showed a moderate change in some EEG parameters investigated when their tail was clamped, while those aged 21 to 22 days (after REM-non-REM differentiation is complete) showed a marked response in all parameters investigated in response to clamping. These results, in addition to published information on REM-non-REM differentiation, behaviour and general neurological development discussed in Chapter 3, suggest that rat pups older than 18 days are normally capable of conscious perception and hence are able to experience pain and that conscious perception would not normally occur earlier than about 10 to 12 days after birth. Moreover, the differences between the EEG responses of 12 to 14 and 21 to 22 day-old rat pups might suggest that the pain experience would be qualitatively different between the two ages.

Chapters 4-6: Mature at birth - Lambs

Lambs are neurologically very mature at birth. REM-non-REM differentiation occurs after about 80% of pregnancy has elapsed but, as discussed in Chapter 1, the fetal lamb is maintained in unconscious sleep-like states by a variety of *in utero* neuroinhibitory factors (Mellor & Gregory, 2003; Mellor *et al.*, 2005; Mellor & Diesch, 2006). However, conscious perception appears soon after birth, as judged by the lambs' volitional responses to maternal and environmental stimulation and the presence of established sleep-wake cycles within a day or so of birth (Mellor & Gregory, 2003; Mellor & Stafford, 2004).

Although conscious perception evidently develops rapidly after birth in lambs, it is not likely to be an "on-off" phenomenon. Rather, behavioural observations suggest that its onset is gradual, such that its initial manifestations take at least some minutes to appear, and other features are not apparent for several hours (Mellor *et al.*, 2009). This is supported by the results of the present studies in lambs, showing maturation of the EEGs of unanaesthetised lambs (i.e. an increase in EEG power) between birth and 1 to 2 days after birth. Moreover, anaesthetised lambs at 1 to 2 days after birth show reduced EEG responses to castration compared to lambs at one week of age and older (Johnson *et al*., 2009). As assessed in Chapter 5, this may in part be due to the slow postnatal waning of the secretion of hormonal factors with anaesthetic, sedative and analgesic

properties that are synthesised before birth by the fetal brain (Mellor & Diesch, 2006; Johnson *et al.*, 2009).

Overall conclusions

From the results of the present thesis, tentative conclusions relating to the onset of conscious perception can be drawn. First, the onset of conscious perception does not seem to follow an "on-off" phenomenon; rather it appears to develop gradually, even in those species that are neurologically mature at birth. This view is supported by the increases in overall EEG power with age observed in all three species reported on here and the concomitant increases with postnatal age in their responsiveness to potentially painful stimulation. Second, although conscious perception, and hence pain experience, may be qualitatively different in younger animals than in older and/or more mature animals, on the basis of the precautionary principle, when significantly invasive procedures are planned, pain relief should be provided from those postnatal ages when pain may first be perceived – i.e. from about 120 days in the tammar wallaby joey, about 10 days in the rat pup and from close to birth in the lamb.

7.2 Implications for animal welfare

The prevention or alleviation of negative mental states, including anxiety, fear, distress, pain, breathlessness, sickness, nausea, extremes of hunger and thirst, and boredom (Wemelsfelder, 1993; Mellor & Reid, 1994; Mellor & Stafford, 2001; Gregory, 2004; Mellor & Stafford, 2004; Mellor *et al.*, 2009), is essential if we want to ensure good animal welfare (Mellor & Reid, 1994). For any living animal to suffer, however, it has to be sentient and conscious (Mellor & Reid, 1994; Mellor & Stafford, 2004; Mellor & Diesch, 2006). Thus, once newborns become capable of conscious perception, whenever that may occur (see above), they have the potential to experience noxious sensations (Mellor & Stafford, 2004).

It could be argued that analgesia and/or anaesthesia in young animals undergoing invasive or other potentially noxious procedures (in the laboratory, on the farm or in the veterinary practice) are not necessary if these animals have not yet attained the capacity for conscious perception (e.g. wallaby joeys, rat pups), or if they have (e.g. lambs), but

still have the benefit of continuing modulation of conscious perception by "*in utero*" neuroinhibitors, as they will not be able to experience pain at all or would to a lesser degree. However, there is merit in being cautious. Even if such newborn and young animals are not capable of *experiencing* pain, invasive procedures will nevertheless stimulate pain receptors and will elicit impulse barrages in those nerve fibres that have developed by the time the procedures are undertaken. This in turn will lead to physiological and behavioural responses, including the withdrawal of the stimulated body parts, stress hormone release and vocal reflexes, which are mediated spinally or by lower brain centres (Lee *et al.*, 2005; Mellor *et al.*, 2005; Mellor & Diesch, 2007). Such reflex responses not only have the potential to negatively affect the invasive procedure to be undertaken (i.e. movement during surgery), but are also likely to compromise the physiological data to be collected.

Recent evidence suggests that exposure to noxious stimulation and stress during the early postnatal period can lead to changes in pain thresholds and an increased vulnerability to stress disorders and anxiety-related behaviours in later life (Taddio *et al.*, 1997; Anand *et al.*, 1999; Eckstein Grunau, 2000; Lin & Al-Chaer, 2003; Page *et al.*, 2005; Sternberg *et al.*, 2005). This appears to be so, at least regarding pain thresholds, for animals in which neurological maturation at birth is not fully complete, such as the newborn rat and mouse, as well as the somewhat more mature human infant (pre- and full term). Whether long lasting effects in response to noxious stimulation are also present in animals born neurologically mature, such as lambs, is currently being investigated in our laboratory, but initial data suggest that this may be so (McCracken *et al.*, 2006). The possible neurophysiological changes which may occur in response to noxious procedures and other stressful situations, could therefore impact on the future welfare of experimental animals should these be kept alive following exposure to such procedures.

On the other hand, it has to be acknowledged that animals that are born neurologically immature may be more susceptible to long-lasting adverse effects of analgesia and/or anaesthesia. Thus, exposure to these agents may lead to histopathological changes in the central nervous system (Mellon *et al.*, 2007) and temporary changes in nociceptive sensitivity and thresholds (Zhang & Sweitzer, 2008). Thus, dosing regimens and the types of drug to be used should be selected carefully in order to ensure the best possible

outcomes for both the animal and the research results.

7.3 Experimental design and limitations

General

As experimental design and limitations for each study have already been outlined in the previous chapters, this section gives a brief overview of the main limitations relevant for this thesis in general.

The present thesis attempted to assess changes in higher neurological function with age by assessing EEG responses to noxious stimulation in different age groups. However, it has to be acknowledged that comparisons of responses to treatments between age groups are never straightforward due the potential confounding effects of age and associated maturational changes on the functional capacities of the physiological systems of interest. Hence, potential major maturational effects on the responses need to be considered when arriving at even tentative conclusions about age-related changes (Mellor & Murray, 1989). For example, as stated, interpretation of the present age-related changes in EEG responses is confounded by possible differential effects of halothane on the physiological systems of animals at the different ages studied.

In addition, it has to be acknowledged that the absence of changes in spectral parameters in response to noxious stimulation in some animals may not have been representative of actual EEG responsiveness. Changes may have occurred in response to stimulation, but may not have been registered as such if they occurred in different frequencies simultaneously thereby preventing an overall change in spectral parameters (i.e. F50, F95 or Ptot). This problem however was addressed by including data on individual EEG frequencies. Although no biological significance was ascribed to individual frequencies in the present thesis, their inclusion in the analyses was thus warranted, giving a more accurate account on EEG changes in response to noxious stimulation. Whether changes in individual EEG frequencies in response to noxious stimulation are representative of activity changes in particular brain areas is, as far as the candidate is aware, not known. Changes in frequencies were quite small in the present studies and this would imply small biological significance. However, consistent

changes were observed in response to the treatments and hence these small changes in individual frequencies should not be discounted too quickly.

Minimal anaesthesia model critique

The minimum anaesthesia model uses halothane as an anaesthetic agent, as it is said to cause less depression in the cerebral cortex of adult animals than other anaesthetic agents (Murrell & Johnson, 2006; Murrell *et al.*, 2008). However, the findings of the present thesis suggest that this may not be the case in neurologically immature young early in postnatal development. Although other factors might have been involved, it appears that halothane anaesthesia, as used in the present thesis, had depressive effects on the cerebral cortex, leading to a higher proportion of isoelectric periods. This is not desirable from an analytical point of view, as the Fast Fourier Transform requires data to be stationary. In addition, such depression of cortical function is likely to affect responsiveness to stimulation and thus would have a major impact on research outcomes. However, the results of the present thesis show that a response to a potentially noxious stimulus can nevertheless be detected. Thus, the model may still be useful for investigating potentially painful procedures in immature animals at ages at which sufficiently long EEG periods can be obtained. However, age comparisons could nevertheless pose a problem, due to differences in the underlying anatomy, physiology and biochemistry between age groups, the impact of neuroactive factors such as opioids or steroid hormones, and/or age-related differences in the effects of halothane and/or associated factors on the immature nervous system. Further research would be necessary to validate this model for the use in immature animals, including the assessment of correct halothane doses to prevent or reduce isoelectric periods (i.e. excessive cortical depression). .

7.4 Where do we go from here: Future studies

As suggestions for improvements for the studies presented in this thesis have already been discussed in the previous chapters, this section focuses on studies that could follow on from the present work.

The present thesis has, in the candidate's opinion, opened up some possible new

avenues for further research. First, studies in fetal animals investigating the indirect evidence presented in Chapter 1 regarding the maintenance of the fetus in unconscious sleep-like states would be warranted to further support this line of thought. In particular, EEG responsiveness to potentially noxious stimulation *in utero* should be investigated. This is in light of the persistence of the opinion that fetuses in the second half of gestation, when neurological systems are mature enough to support conscious perception and hence pain perception in prematurely born individuals, are capable of experiencing pain (Anand, 2007; Peisker *et al.*, 2008).

Second, detailed studies of the effect of halothane on developing animals are suggested. This would allow the minimal anaesthesia model to be validated properly for this group.

Third, it would be of interest to determine whether opioidergic pre-cursors are present in colostrum and milk of various animal species and whether these factors reach the brain of the newborn animal via the circulation once colostrum/milk intake has occurred. Should this be the case, further studies could investigate whether these factors have the potential to affect pain perception, or conscious perception in general, in newborn animals. Fourth, the activation of the opioidergic system by sucking and/or contact with the mother may be another avenue by which newborn animals may derive a certain degree of protection from the potential negative impact of noxious stimulation once conscious perception is possible. Studies in newborn and young animals where husbandry practises are a common procedure may focus on whether sucking may help mitigate some of the noxiousness of these procedures.

Fifth, the fact that progesterone metabolites are apparently involved in maintaining the late-gestation fetus in unconscious sleep-like states and may also affect conscious perception in maturely born species for sometime after birth, suggests that these neuroinhibitors may have potential benefits for the management of prematurely born human infants. Prematurely born infants are regularly exposed to a variety of clinical manipulations, which are often invasive (Grunau, 2000; Fitzgerald & Beggs, 2001), and to a range of sensory environmental stimuli, which are potentially different in character, magnitude and intensity from those a fetus of the same post-conception age would experience *in utero*. The virtually continuous sensory "over-stimulation" to which very premature infants are exposed is of concern, especially as there is the potential for such

stimulation to cause abnormal patterns of neurological development or heightened sensitivity to noxious stimulation (Grunau, 2000; Fitzgerald & Beggs, 2001). Many aspects of brain development depend on experience for normal development. However, developmentally inappropriate experience can significantly alter CNS structure and function (Grunau, 2002). Thus, although some forms of stimulation apparently help to enhance some features of pre-term infant development (Brandon *et al.*, 2002; White-Traut *et al.*, 2002), overall brain growth and complexity are significantly reduced (Ajayi-Obe *et al.*, 2000; Huppi *et al.*, 2001). In addition, pre-term infants often exhibit neurological problems in infancy, childhood and adolescence including cognitive deficits, learning disorders and poor motor performance (Grunau, 2000).

Due to the loss of the placenta at birth the prematurely born human infant is no longer exposed to the high concentrations of placentally-derived neuroinhibitory factors which were present before birth, although this might be a gradual waning of neuroinhibition over a few days due to immature metabolism and excretion. It would be of interest to test whether exposure to progesterone or its metabolites could be beneficial for prematurely born infants by reducing negative side effects of exposure to an overstimulating environment.

If it were possible to develop a viable model to study prematurely born lambs, they could be studied to assess possible adverse effects of premature birth and subsequent environmental stimulation on the developing nervous system. Once such effects had been well understood, further studies could be designed during which prematurely born lambs would be regularly treated with progesterone or progesterone metabolites to assess whether such treatment had prevented adverse effects of premature birth and environmental stimulation.

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Appendix 1: Laboratory Protocols

Permission has been granted by Prof David Walker, Physiology Department, Monash University, Clayton, Melbourne, Australia, to include the laboratory protocols in this thesis.

Files of the full protocols can be found on the CD attached to the back of this thesis.

1) Allopregnanolone extraction from plasma and brain tissue
Allopregnanolone RIA

2) Progesterone extraction from plasma and brain tissue
Progesterone RIA

3) Pregnenolone and pregnenolone sulfate extraction from plasma
Pregnenolone and pregnenolone sulfate RIA

Appendix 2: Full publications related to the present thesis

The candidate participated fully in the literature research, experimental planning and execution, and in the writing of the publications listed below. Full copies of these publications can be found on the CD attached to the back of this thesis.

Diesch TJ, Mellor DJ, Johnson CB & Lentle RG. (2008). Responsiveness to painful stimuli in anaesthetised newborn and young animals of varying neurological maturity (lambs, rat pups and wallaby joeys). *Alternatives to Animal Testing and Experimentation* **14,** 549-552.

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