ANATOMY OF THE SLEEP-WAKE SYSTEMS IN FOUR SPECIES OF EQUID

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Master's Thesis

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DECLARATION

I, Alexis Sarah Chaumeton, declare that this Dissertation (by research) is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

_____

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_____ day of_____2017 at_____

The opportunity to pursue knowledge becomes an even greater privilege when inspired by one's parents.

To
Claudia Boffard
and
Baxter Brown

ABSTRACT

Within the order Perissodactyla the physiological measurable parameters of sleep have been investigated in a number of species, however no studies exist that describe the neuronal organisation and morphology of the sleep-wake systems in any of its members. The central aim of this dissertation is to address this gap by providing the first complete description of the somnogenic systems in the basal forebrain, diencephalon, midbrain and pons of four equid species; the donkey, horse, plains and mountain zebra. By means of standard immunohistochemical techniques the cholinergic, catecholaminergic, serotonergic, orexinergic and GABAergic systems were identified and qualitatively described in each of the four species. The results revealed that, for the most part, the nuclear organisation and morphology of the sleep-wake systems did not differ between the species examined, and displayed the typical mammalian organisational plan. However, two novel findings were noted: 1) the presence of tyrosine hydroxylase neurons in the predominantly GABAergic thalamic reticular nucleus; 2) the presence of a medial cluster of parvocellular orexinergic neurons within the hypothalamus. It is proposed that the population of tyrosine hydroxylase neurons in the thalamic reticular nucleus likely play a role in postural maintenance during standing rapid eye movement sleep and potentially contribute to memory consolidation in mammals with short sleep times. Additionally, the parvocellular cluster of orexin neurons is hypothesised to balance short sleep time and appetite drive in larger animals with high-energy demands and a low trophic status. The data produced from this dissertation extends the pre-existing phylogenetic database and offers further opportunity for reliable comparisons across mammals towards a more complete definition of the phenomenon of sleep.

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ABBREVIATIONS

III: oculomotor nucleus

IV: trochlear nucleus

Vmot: trigeminal motor nucleus

Vmes: fifth mesencephalic nucleus

Vmes.tr: fifth mesencephalic tract

Vs: trigeminal sensory nucleus

3V: third ventricle

4V: fourth ventricle

5n: trigeminal nerve

7n: facial nerve

8vn: cochlear nucleus, ventral

A5: fifth arcuate nucleus

A6d: diffuse portion of locus coeruleus

A7d: nucleus subcoeruleus, diffuse portion

A7sc: nucleus subcoeruleus, compact portion

A8: retrorubral nucleus

A9: substantia nigra

A9l: substantia nigra, lateral

A9m: substantia nigra, medial

A9pc: substantia nigra, pars compacta

A9v: substantia nigra, ventral

A10: ventral tegmental area

A10c: ventral tegmental area, central

A10d: ventral tegmental area, dorsal

A10dc: ventral tegmental area, dorsal caudal

A11: caudal diencephalic group

A12: tuberal cell group

A13: zona incerta catecholaminergic nucleus

A14: rostral periventricular nucleus

A15d: anterior hypothalamic group, dorsal division

A15v: anterior hypothalamic group, ventral division

ac: anterior commissure

B9: supralemniscal serotonergic nucleus

bic: brachium of inferior colliculus

C: caudate nucleus

ca: cerebral aqueduct

cic: commissure of the inferior colliculus

CLi: caudal linear nucleus

Diag.B: diagonal band of Broca

DRc: dorsal raphe nucleus, caudal division

DRd: dorsal raphe nucleus, dorsal division

DRif: dorsal raphe nucleus, interfascicular division

DRl: dorsal raphe nucleus, lateral division

DRp: dorsal raphe nucleus, peripheral division

DRv: dorsal raphe nucleus, ventral division

DT: dorsal thalamus

EWpg: Edinger-Westphal nucleus, preganglionic

f: fornix

GC: central grey matter

GiCRt: gigantocellular of the reticular formation

GP: Globus pallidus

Hbl: habenular nucleus, lateral

Hbm: habenular nucleus, medial

Hyp: hypothalamus

Hyp.d: dorsal hypothalamic cholinergic nucleus

Hyp.l: lateral hypothalamic cholinergic nucleus

Hyp.v: ventral hypothalamic cholinergic nucleus

IC: inferior colliculus

ICP: inferior cerebellar peduncle

ic: internal capsule

ILG: intralaminar cholinergic group

IP: interpeduncular nucleus

IPc: interpeduncular nucleus, central division

IPl: interpeduncular nucleus, lateral division

Is.CALL/TOL: islands of Calleja and olfactory tubercle

LDT: laterodorsal tegmental nucleus

lfp: longitudinal fasciculus of the pons

LOT – lateral olfactory tract

LSO: lateral superior olivary nucleus

LV: lateral ventricle

LVe: lateral vestibular nucleus

MB: mammillary bodies

Mc: main cluster of orexinergic neurons

mcp: middle cerebellar peduncle

MGB: medial geniculate body

mlf: medial longitudinal fasciculus

MnR: median raphe nucleus

N.Acc: nucleus accumbens

N.Bas: nucleus basalis

OC: optic chiasm

OT: optic tract

OTc: optic tract cluster of orexinergic neurons

P: putamen nucleus

PBg: parabigeminal nucleus

Pc: parvocellular cluster of orexinergic neurons

PC: cerebral peduncle

PCRt: parvocellular of reticular formation

PPT: pedunculopontine tegmental nucleus

Pta: pretectal nucleus, anterior

Rmc: red nucleus, magnocellular division

RMg: raphe magnus nucleus

RtTg: reticulotegmental nucleus

S: septal nuclear complex

SC: superior colliculus

scp: superior cerebellar peduncle

Sep.L: lateral septal nucleus

Sep.M: medial septal nucleus

SON: supraoptic nucleus

Son: superior olivary nucleus

Sp5: spinal trigeminal tract

STN: subthalamic nucleus

Tz: Trapezoid body

TOL: olfactory tubercle

TRN: thalamic reticular nucleus

VPO: ventral pontine nucleus

VTCG: ventral tegmental cholinergic group

XSCP: decussation of the superior cerebellar peduncle

zi: zona incerta

Zic: zona incerta cluster of orexinergic neurons

CHAPTER ONE: Introduction and Chapter Outline

1.1 Introduction

Among the many examples of sleep research across extensive phylogeny in mammals, neuroanatomical data is often generated to supplement physiological or behavioural studies, and while sleep research in domestic equids is no different, wild equids have enjoyed no such privilege. Additionally, nothing is known of the sleep-wake neural systems in either domestic or wild equids and to date, sleep research in the order Perissodactyla has focused on physiological studies of the domestic horse, donkey, pony and black rhinoceros (Ruckebusch et al., 1970; Ruckebusch, 1962, 1963, 1972; Dallaire and Ruckebusch, 1974a and b; Hale and Huggins, 1980; Belling, 1990; Moehlman, 1998, Santymire et al., 2012). While this information has made a valuable contribution to data on sleep in mammals in general, laboratory-based physiological studies in domestic perissodactyls falls short of providing multi-disciplinary and scientifically rigorous research that contributes to how equids sleep or why mammals would choose sleep over valuable arousal time (Duncan, 1985; Tobler 1995; Siegel, 2008).

Physiologically, sleep varies considerably between species. That said, the neuronal systems controlling sleep remain, for the most part, conserved across species (Manger, 2005). It would thus be of interest to determine whether changes in the organisation and morphology of the sleep-wake systems can accurately predict how sleep manifests physiologically in a given species or order. To this end, this study proposes a comprehensive neuroanatomical investigation of the somnogenic nuclei in the brains of four equids.

A recent physiological study conducted by Williams et al., (2008) has both reinforced and contradicted studies done by Ruckebusch during the 1960s and 1970s suggesting that while recorded sleep times for the domestic horse remain short, this animal is capable of entering brief periods of rapid eye movement (REM) sleep while remaining standing. This interesting physiological phenomenon would suggest that equids might have a supplementary or variable expression of somnogenic nuclei or chemical substrates during rapid eye movement (REM) sleep onset that would ensure

postural integrity during standing and effect the suppression of the atonic phase of REM sleep.

A further benefit of a neuroanatomical investigation in equids would be to contribute to the growing evidence that cetartiodactyls are not the only order of mammals that possess a parvocellular cluster of orexin neurons located in the medial hypothalamus. Cetartiodactyls demonstrate a large body mass, with evidence of low energy-value diets that may compel them to sacrifice sleep time to fulfil appetite drive (Dell et al., 2012, 2016a−c). This hypothesis has been further reinforced with the identification of this unusual parvocellular cluster in an animal outside of the order Cetartiodactyla. The African elephant of the order Proboscidea (Maseko et al., 2013) is a large mammal that is recorded as having the shortest sleep time of all land mammals (Gravett et al., 2017). Further, REM sleep has not formally been identified in the elephant and this animal has been observed to endure extended periods of arousal showing little evidence of sleep rebound (Gravett et al., 2017). Equids are mammals with a high body mass index. They graze on preferred grasses and demonstrate short sleep times (Ruckebusch et al., 1970; Belling, 1990). In 1984, Duncan completed a study on free-roaming Camargue horses in which he observed that these animals tended to spend more time sleeping (lying down) during the spring months when they had access to grasses with a higher protein content (Duncan, 1985). This suggests that during other months, appetite drive increases, sleep time decreases, and that a neural mechanism such as the medial parvocellular cluster of orexin neurons may act as a homeostatic mechanism that balances sleep time against appetite drive.

Sleep is dependent on many exogenous factors (Siegel, 2005) and as such it would be of value and interest to investigate neuroanatomical differences that may exist between both domestic and wild species of equids, and whether their sleep habits as a function of their contrasting behaviour and environment, may help to explain any divergence in their neural morphology. Further, it is hoped that the completion of neuroanatomical studies in the domestic horse and donkey and the plains and mountain zebra, will drive further stereological and comparative studies in this and closely related orders in an attempt to further facilitate our understanding of the mechanisms of sleep and waking and the function of sleep as a whole.

1.2 Aims and objectives

The global aim of the dissertation is to provide, for the first time, a complete qualitative description of the nuclear organisation and morphology of the systems in Perissodactyla, and in doing so, contribute to a growing database on the evolution and function of sleep in this order.

The primary objective of this dissertation is to qualitatively examine the somnogenic systems in the domestic donkey (*Equus africanus asinus*) and horse (*Equus caballus*), the plains zebra (*Equus quagga*) and the Cape mountain zebra (*Equus zebra zebra*) by means of immunohistochemistry and microscopy. The study investigates the cholinergic, catecholaminergic, serotonergic, orexinergic and gamma aminobutyric acid (GABAergic) systems located in the basal forebrain, midbrain, diencephalon and pons. The data collected provides a series of insights that contribute to valid comparisons between species within the same order and closely related orders.

This dissertation thus contributes to the evident lack of neuroanatomical studies in the mammalian order Perissodactyla, and hypothesises the possibility of both order and species-specific features and potential similarities that may exist with the closely related sister group Cetartiodactyla, and other mammals in general.

1.3 Chapter outline

This dissertation is organised into six chapters:

Chapter One: Introduction

This chapter includes an introduction to the dissertation subject, the aims objectives and hypothesis.

Chapter Two: Literature review

This chapter provides a summary of the physiology of sleep and a more comprehensive description of the mammalian sleep-wake neurotransmitters and systems. The typical mammalian neuroanatomical organisation and morphology of the sleep-wake systems and the proposed functions of sleep are discussed. An extensive literature review is undertaken on the four species of equid examined.

Chapter Three: Materials and Methods

A detailed description of the perfusion, brain fixation and immunohistochemical protocols followed in this study are provided. The enzymes (choline acetyltransferase and tyrosine hydroxylase), neurotransmitter (serotonin), neuropeptide (orexin A) and calcium binding proteins (calbindin, calretinin and parvalbumin) used in the investigation of the sleep-wake systems in the donkey, horse and plains and mountain zebra, are described and an overview of the data analysis is provided.

Chapter Four: Results

The results provide a qualitative description of the organisation and morphology of sleep-wake-related nuclei in the four species of equid studied. The sleep-wake nuclei comprise those of the basal forebrain, the medial, lateral and posterior hypothalamus, the midbrain and the pons.

Chapter Five: Discussion

This chapter critically and comparatively discuss the results of the current study in relation to members of the same order, the closely related order of Cetartiodactyla, and mammals in general. Anomalies discovered in the thalamic reticular nucleus (TRN) and the hypothalamic orexinergic system are discussed in relation to other mammals that show similar variations in these nuclei. Hypotheses regarding functional implications of these anomalies are proposed and discussed.

Chapter Six: Conclusion

This chapter concludes the study and summarises the implications and significance of its findings for further investigations; more specifically, the unusual findings of both the TRN and the orexin system that, it is hoped, may further contribute to the field of sleep research.

CHAPTER TWO: Literature Review

2.1 The physiology of sleep

Sleep is a universal recurring state that is observed across the animal kingdom. It is fundamental to the wellbeing of all living organisms, despite being considered remarkably variable in its architecture and puzzling in its function. In general, mammalian sleep consists of alternating cycles of non-rapid eye movement (NREM) and (REM) sleep, with slow waves dominating the NREM sleep stage (Zepelin et al., 2005; Capellini et al., 2008a). NREM sleep is considered to be quiet sleep, or more specifically an inactive phase of sleep, while REM sleep is defined as a dynamic state during which both body and mind are more active despite the prevailing unconscious state (Elgar et al., 1988; Capellini et al., 2008a).

The physiological study of sleep, discriminating between NREM and REM sleep and stage transitions, is greatly enhanced by data provided by the electroencephalogram (EEG), the electro-oculogram (EOG), and the electromyogram (EMG). While the EEG is considered the single most important tool for the scientific study of sleep (Siegel, 2004), all three measures are considered essential for accurate phasing and analysis of sleep. Wakefulness shows the greatest variability in EEG, EOG and EMG patterns (Harris, 2005). Being awake means being aroused and aware. The former is physiological in nature, the latter psychological (Passer and Smith, 2004), and the two make up what is known as a state of consciousness. Wakefulness is recorded as depolarisation or high-firing on the EEG (Mignot, 2008) with high frequency beta waves ranging from 8-40 Hz, and low voltage amplitude ranges of 5-50 μV depending on the level of arousal being recorded (Siegel, 2004a; Harris, 2005; Fuller et al., 2006). EMG demonstrates a high voltage. NREM sleep differs from REM sleep in both frequency and amplitude on a hypnograph (Siegel, 2004a; Harris, 2005; Capellini et al., 2008a). NREM EEG activity is characterised by synchronised periods of neuronal depolarisation and hyperpolarisation (Mignot, 2008), with delta frequencies predominating the deeper stages of sleep (Fuller et al., 2006). EEG amplitude thus increases (100-400 μV) while frequency decreases. EOG activity is mostly absent during NREM sleep, and EMG activity is low to moderate in nature (Harris, 2005). REM sleep is the dream-state of sleep and is also called paradoxical sleep because the EEG patterns recorded during this phase are like those found during

periods of wakefulness (Siegel, 2004a; Fuller et al., 2006). REM sleep is associated with a desynchronised EEG and increased hippocampal theta activity (Mignot, 2008). EEG amplitude is characteristically low and frequency is high. During the tonic phase of REM sleep, EMG readings demonstrate muscle atonia, with the exception of the diaphragm. The phasic period of REM sleep is characterised by muscle twitches and rapid eye movements (Mignot, 2008), and EOG and EMG readings are accompanied by saw-tooth waveforms (2-4 Hz) on the EEG (Harris, 2005). Thus, depending on the sub-phase of REM sleep being recorded, it is not unusual for hypnogram readings to show both high and low amplitudes with slow or faster frequencies.

The past decade has seen many important breakthroughs in the refinement of polysomnography. This has helped to categorise sleep and contextualise it as an evolving circadian "rest activity" (Tobler, 1995, p. 35). Above all, polysomnography has lent a much-needed hand in describing sleep despite the heterogeneity and uncertainty surrounding this phenomenon. Analytical research has shown, or at least strongly indicates that sleep in animals is identified as varying stages of dormancy (apart from coma, hypothermia and hibernation) unique to the character of each species, both physically and biologically, and is easily reversible through self-regulation (Fuller et al., 2006; Mignot, 2008).

As previously mentioned, animal sleep shows great variation in structure, duration and placement over a 24-hour period (Lyamin et al., 2008; Rattenborg et al., 2009). Domesticated donkeys (*Equus asinus*) sleep no more than three hours a day (Ruckebusch, 1963). In contrast, the little pocket mouse (*Perognathus longimembris*) spends more than 20 hours slumbering (Elgar et al., 1988). Specifically, somnogenic architecture has been shown to differ significantly between species and across orders, with these cycles varying greatly in phasing and length (Tobler, 1989; Lesku, 2009). Moreover, variation in sleep architecture is influenced by population and resources, migration and environment, genetic predisposition and changing physiological or mental states. These influences fall within the context of the evolving seasons and lifespan changes to influence individual and group inactivity patterns (Moehlman et al., 1998a; Harris, 2005; Capellini et al., 2008a; Moser et al., 2009).

Much of the research done on mammalian sleep evolution demonstrates analyses conducted across species and orders that display different morphologies and show unusual forms of sleep (Nicolau et al., 2000; Kavanau, 2002; Lesku et al., 2006, 2008; Capellini et al., 2008a). To date, no single sleep study, behavioural, electrophysiological or neuroanatomical has been done on any of the zebra, and limited physiological and no neuroanatomical studies have been undertaken on the donkey and horse. While polysomnographic studies seem to be in favour, neuroanatomical research cannot be ignored as it has the potential to provide valuable insights into the mechanisms responsible for sleep, which in turn can lead to a better understanding of the function of mammalian sleep. This dissertation thus sets out to address this gap by providing the first complete description of the neuroanatomical systems involved in the generation and maintenance of the sleep-wake cycle in the donkey, horse and zebra. This data may help explain variance in sleep habits within the Perissodactyla order, and may be used as a valid comparison with other closely related orders such as Cetartiodactyla, thereby contributing to the limited existing neuroanatomical somnogenic database.

2.2 The neuroanatomy of sleep

The sleep-wake nuclei considered typically mammalian extend from the basal forebrain (BF) through the diencephalon and midbrain to the pontine region of the brainstem. They include the cholinergic nuclei of the basal forebrain and pons that are responsible for cortical upregulation, and thus fire maximally during wakefulness and REM sleep (Harris, 2005; Jones, 2005). Cholinergic nuclei discharge acetylcholine, synthesised from choline acetyltransferase (ChAT). In the brain, acetylcholine behaves as a neurotransmitter and a neuromodulator.

Catecholaminergic nuclei secrete classic monoamines such as dopamine, epinephrine and norepinephrine, the latter of which is located in the locus coeruleus and is most active during arousal (Jones, 2005). Serotonin, a midbrain monoamine of the class tryptamine, fires during quiet periods of reduced arousal, is responsible for habits such as grooming and introversion (Davimes, 2017) and is considered a neuromodulator and neurotransmitter like all monoamine action in the brain. Orexinergic nuclei of the hypothalamus are responsible for the maintenance of arousal via extensive projections to histaminergic, norepinephrine and serotonergic systems

(Siegel, 2004b). Orexin A (OxA) behaves as a neuropeptide in the central nervous system.

Gamma-aminobutyric acid (GABA) promotes sleep onset and maintenance via inhibitory projections to all nuclei responsible for arousal. The GABAergic cell groups are mostly, but not exclusively, located in the basal forebrain and preoptic-anterior hypothalamus (Gritti et al., 1994). Certain groups of GABAergic interneurons fire maximally during different phases of the sleep cycle to ensure the integrity and completion of the phase, and of the sleep cycle as a whole (Siegel, 2004b; Davimes, 2017). Although classed as an amino acid, GABA is considered to be the primary inhibitory neurotransmitter of the mammalian central nervous system.

2.2.1 Cortical up-regulators

2.2.1.1 Acetylcholine

The reticular formation located in the midbrain and pons, and its rostral neural pathways known as the ascending reticular activating system (ARAS), are responsible for cortical arousal (Siegel, 2004a; Jones, 2005; Fuller et al., 2006; Datta and MacLean, 2007). Further research based on the work of nineteenth and twentieth century pioneers like Von Economo and Moruzzi and Magoun, uncovered specific nuclei and pathways within the ARAS (Siegel, 2004a). Most notably at the level of the pontomesencephalic junction, two prominent cholinergic nuclei known as the pedunculopontine and laterodorsal tegmental nuclei (PPT and LDT), collectively known as the parabrachial nuclei, surround the superior cerebellar peduncles. Acetylcholine is the main neurotransmitter of this ascending arousal system (Siegel, 2004a; Jones, 2005, Fuller et al., 2006). Two ascending pathways emanate from these nuclei: a dorsal and a ventral projection. The more direct glutamatergic dorsal projection courses to the intralaminar and midline nuclei of the thalamus, and then on to the cortex (Siegel, 2004b; Jones, 2005). The more indirect ventral projection synapses initially in the tuberomammillary nucleus where histamine is released, and then continues projecting to the entire cortex. It then courses on to the thalamus and basal forebrain, where it synapses with acetylcholine neurons that extend to the cortex (Siegel, 2004a, Jones, 2005). These cholinergic neurons of the BF are responsible for behaviours that define arousal and attention, such as memory and learning, decision-making and sensory processing (Datta and MacLean, 2007; Deurveilher and Semba,

2011). This said, cholinergic neurons make up a total of only 5% of the cell population of the BF: 35% are GABAergic and the remaining 60% are posited to be glutamatergic (Deurveilher and Semba, 2011). While acetylcholine is classed as a neurotransmitter that promotes wakefulness, the role of cholinergic neurons in the BF is more complex and depends on whether they synapse with GABAergic or glutamatergic neurons (Deurveilher and Semba, 2011). Caudal projections from the ARAS are mediated by glutamate, and are mostly responsible for the maintenance of muscle tone and posture (Jones, 2005). Thus, acetylcholine and glutamate play a critical role in maintaining wakefulness (Siegel, 2004a; Jones, 2005), along with other key arousal neurotransmitters such as histamine and orexin (Jones, 2005; Blanco-Centurion et al., 2007).

2.2.1.2 Dopamine

Dopaminergic neurons of the ARAS are found in the substantia nigra (pars compacta, SNc) and the ventral tegmental area (VTA). This neurotransmitter plays an indirect role in wakefulness – firing rates of these neurons seem to remain stable across the sleep-wake cycle (Datta and MacLean, 2007). However, recent studies implicate this neurotransmitter in REM sleep, perhaps during moments of reward or pleasure while dreaming, and in the maintenance of arousal evinced by the action of dopamine antagonists that reduce arousal and promote NREM sleep. (Datta and MacLean, 2007)

2.2.1.3 Norepinephrine

Norepinephrine (noradrenalin) located predominantly in the locus coeruleus (LC), projects to the cortex, hippocampus, amygdala, basal forebrain, thalamus and hypothalamus. Norepinephrine activates and intensifies awareness (Siegel, 2004b; Jones, 2005; Datta and MacLean, 2007; Mignot, 2008). Like histamine, discharge is reduced during periods of NREM sleep, and absent during periods of REM sleep (Motchizuki et al., 1992; Jones, 2005). However, there is a difference in how these two monoamines maintain arousal. Histamine works directly on the forebrain to maintain wakefulness, while norepinephrine is implicated in maintaining muscle tone during periods of arousal (Siegel, 2004b; Jones, 2005; Datta and MacLean, 2007; Mignot, 2008). This is evident in narcoleptic patients that experience cataplexy (loss of muscle tone) during periods of arousal. In this case, histaminergic neurons are still

active, while norepinephrine is completely deactivated (Brown et al., 2001, Huang et al., 2001).

2.2.1.4 Orexin

Orexin (hypocretin) cells are located in the hypothalamus tucked between the rostral GABAergic sleep-inducing neurons and the more caudal histaminergic group of neurons responsible for arousal. This neuropeptide interacts with norepinephrine and serotonergic cell groups (Siegel, 2004a; Jones, 2005) and collaborates particularly with histaminergic neurons to regulate arousal (Jones, 2005; Datta and MacLean, 2007). In humans that suffer from narcolepsy accompanied by cataplexy, it has been shown that more than 90% of the orexinergic neuron population is compromised or not present (Siegel, 2004b). Orexin is a dual action neuropeptide (Siegel, 2004b). When it acts on a particular area of the brain and provokes a discharge of the amino acid glutamate, it plays an excitatory role. REM sleep induction and resolution is an example of glutamate mediated orexinergic action (Jones, 2005). However, when orexin acts on an area that releases calcium buffers (calbindin, calretinin and parvalbumin) of the gamma-aminobutyric acid neurotransmitter, it behaves indirectly in an inhibitory capacity. This is demonstrated in cases of REM sleep where muscle tone is preserved. It would thus seem that the role orexin plays in excitation and inhibition is a fine-tuned balance that can cause gross sleep pathology when disturbed (Siegel, 2004b).

2.2.2 Cortical up- and down-regulators

2.2.2.1 Serotonin

Serotonin, a class of tryptamine neurotransmitter is located in the midline raphe nuclei (passing from the midbrain rostrally to the medulla caudally), particularly the dorsal and median raphe areas (Datta and MacLean, 2007; Lapierre et al. 2013). The serotonergic nuclei are implicated in arousal and like histamine and norepinephrine, are stimulated by orexin (Portasa et al., 2000; Fuller et al. 2006) and inhibited by the GABAergic system (Datta and MacLean, 2007). Serotonergic activity is substantially reduced during SWS and inactive during REM (Siegel, 2004b; Jones, 2005; Lapierre et al., 2013). Serotonin is however presumed to play a role in the maintenance of muscle tone during phasic REM sleep (Siegel, 2004b) and serotonin activity in the dorsal raphe nucleus during this phase of sleep may account for REM

sleep without atonia syndrome (Lapierre et al., 2013). Serotonin restricts ponto-geniculo-occipital spikes (PGO) and when inactive results in an open gateway allowing these waves to propagate from the pons rostrally to the thalamus and cortex (Lapierre et al., 2013) inducing associated REM sleep muscle shivers and saccades (Siegel, 2004b).

Several studies have demonstrated that serotonin cells in the raphe nucleus (RN) fire maximally during quiet wakefulness to encourage the onset of sleep and that the pharmacological class of selective serotonin reuptake inhibitors (SSRI) play an active role in inhibiting hypothalamo-cortical and basalo-cortical arousal systems via the suppression of cholinergic cells of the basal forebrain (Datta et al, 1997). Other studies have shown that serotonin cells are not sensitive to changes in sleep-wake states; however, this does not rule out the possibility that serotonin contributes to the maintenance of sleep or wakefulness, only that it may not contribute to the initiation of either of these states or their phases (Datta et al., 1997; Datta and MacLean, 2007).

Lesion studies on the RN demonstrate an increase in wakefulness and a decrease in SWS (Petitjean et al., 1978; Sakai and Croched, 2001). The above evidence thus suggests that serotonin plays a role in both arousal and sleep, but that the specifics of its action on both states may depend on the post-synaptic receptor targeted, interaction with other neurotransmitters (Portsas et al., 2000, Boutrel et al., 2002) and/or a specific functional topographical distribution within the RN (Sakai and Crochet, 2001).

2.2.2.2 Neurotransmitter interaction during REM sleep induction and resolution

Further, it seems that the induction and resolution of REM sleep is controlled by brainstem structures such as the parabrachial nuclei that show higher rates of firing activity during onset and offset of REM sleep. Single cell recordings of the PPT have revealed that the cholinergic neurons of the PPT promote REM sleep. During REM sleep, cholinergic activity levels are at 65% of normal arousal levels and are reduced significantly during NREM (Lee et al., 2005; Datta and MacLean, 2007). A similar pattern is observed in the cholinergic firing rates of the LDT (Lee et al, 2005). Offset of REM sleep and re-induction of NREM sleep is accompanied by activity of norepinephrine and serotonin cells in the locus coeruleus and raphe nuclei

respectively (Jones, 2005). Norepinephrine and serotonergic neurons thus have an inhibitory effect on the cholinergic neurons of the PPT and LDT (Jones, 2005). REM-on and REM-off could thus be considered as a set of sub-switches within a larger circuit that act by negative feedback (Jones, 2005; Mignot, 2008).

2.2.3 Cortical down-regulators

2.2.3.1 Gamma-aminobutyric acid

The single most important structure responsible for the regulation of sleep length is the hypothalamus (Siegel, 2004b). GABAergic neurons of the anterior hypothalamus (ventrolateral and median preoptic areas) and basal forebrain (most active during NREM sleep), are responsible for deactivating the neurotransmitters of wakefulness, specifically cholinergic neurons in the BF and histaminergic neurons of the tuberomammillary nucleus, located in the posterior hypothalamus (Mignot, 2008). Direct deactivation of cholinergic neurons in the BF by GABAergic neurons, indirectly deactivates the cortex and produces sleep. Antihistamines that cross the blood-brain barrier mimic the role that GABA plays in inducing drowsiness by suppressing histaminergic activity in the posterior hypothalamus (Siegel, 2004b; Jones, 2005).

The TRN is a GABAergic structure that forms part of the diencephalon (Guillery et al., 1998; Fitzgerald et al., 2012). This nucleus is a relay station for many sensory, motor and limbic cortico-thalamo-cortical projections that pass through it (Pinault, 2004; Ferrarelli and Tononi, 2011). These projections give off side branches that send glutamatergic mediated excitatory discharges to the TRN, that in turn send inhibitory GABAergic mediated discharges back to the thalamus (Guillery et al., 1998). During sleep, this mechanism is said to prevent information from being relayed to the cortex, hence maintaining the integrity of the somnogenic state (Guillery et al., 1998). Once these projections have reached their destination in the cortex, they double back through the TRN to their corresponding nuclei in the thalamus. This structure therefore controls thalamic nuclei discharge rate to the cortex (Fitzgerald et al., 2012). Functionally this means that the TRN is able to filter out the "background" noise created by cortical activity during wakefulness and process more unusual auditory, visual and tactile experiences (Fitzgerald et al., 2012). Moreover, Crabtree and Isaac (2002) have shown that all nuclei of the dorsal thalamus inter-

compete via the TRN, to "switch each other off", albeit momentarily, with the ultimate goal of allocating an organism's limited attention to the most important aspects of its environment. In short, this structure is responsible for what is deemed salient (Guillery et al., 1998).

The pattern of firing in the TRN is either tonic or burst discharge (Ferrarelli and Tononi, 2011; Halassa et al., 2014). Single or tonic discharge predominates during arousal and REM sleep, while burst-spike firing is confined to NREM sleep and is responsible for the synchronised inception, maintenance and propagation of fast oscillating sleep spindles that are characteristic of this phase of sleep (Steriade et al., 1993; Ferrarelli and Tononi, 2011; Halassa et al., 2014).

2.2.4 Organisation, distribution and morphology of the sleep-wake systems in mammals

The sleep-wake systems most commonly investigated in mammals include the cholinergic, putative catecholaminergic, serotonergic, GABAergic and orexinergic systems that are found in the basal forebrain, hypothalamus, midbrain and pons of the mammalian brain. Organisation and distribution of neuronal systems that are involved in the generation of the sleep-wake cycle are largely conserved between mammalian species. However, differences do occur. They are rare and the ever-growing repository of neuroanatomical data has led Manger (2005) to suggest that mammals of the same order will display the same or similar complements of neuronal structures, while any variation in such structures is usually seen between orders and not between species of the same order (Manger, 2005; Maseko et al., 2007; Dell et al., 2010; Calvey et al., 2013).

Neuroanatomical studies describing the organisation, distribution and morphology of the sleep-wake systems in Perissodactyla are lacking, however several studies completed in the closely related Cetartiodactyla have revealed unique differences in the orexinergic and cholinergic systems of this order. A stereological comparative study by Dell et al., (2012) investigating orexinergic neuronal organisation in two cetartiodactyls with similar brain masses, the giraffe (*Giraffa camelopardus*) and the harbour porpoise (*Phocoena phocoena*), revealed that the harbour porpoise possesses a supernumerary population of orexinergic neurons in the

parvocellular and magnocellular divisions of the hypothalamus, while orexinergic neurons in the giraffe in these two areas of the hypothalamus are larger in volume, area and length. In addition, a significant parvocellular cluster of orexinergic neurons in the medial hypothalamus was observed in both species. Further studies undertaken in Cetartiodactyla demonstrated a similar parvocellular cluster of orexinergic neurons in the Arabian oryx (*Oryx leucoryx*), hippopotamus (*Hippopotamus amphibius*), African elephant (*Loxodonta africana*) and minke whale (*Balaenoptera acutorostrata*) (Maseko et al., 2013; Dell et al. 2016a, 2016b, 2016c; Davimes et al., 2017).

Bux et al. (2010) and Dell et al. (2016c) reported that the cholinergic neurons of the laterodorsal tegmental (LDT) and pedunculopontine tegmental (PPT) nuclei in the hippopotamus and giraffe, demonstrated a qualitative difference in cell size. The neurons of the LDT nucleus were larger than those located in the PPT nucleus. Mahady et al. (2016) studied the Goettingen mini pig (*Sus scrofa domesticus*) and found the opposite; the PPT demonstrated larger cell bodies than the LDT. The Arabian oryx showed a similar cholinergic nuclei pattern to the Goettingen mini pig (Davimes et al., 2017). This soma size variation appears to be unique to the artiodactyls, as in most other species studied to date there is homogeneity in neuronal cell sizes of the LDT and PPT nuclei (Bux et al., 2010).

The neuroanatomical anomalies seen between species are of interest as they potentially provide a basis for understanding differences in the physiological manifestation of sleep. Thus, despite compelling statistical evidence that certain sleep architectural traits are inherited (Lesku et al., 2009), it cannot be assumed that species showing phenotypic or genotypic resemblance automatically display similarities in sleep behaviour (Siegel, 2004a, 2005, Capellini et al., 2008).

2.3 The proposed functions of sleep

It has been proposed that sleep and wakefulness are controlled by a series of "flip-flop" switches ensuring that these two states occur as a cycle and not at the same time (Mignot, 2008). Both Jones (2005) and Mignot (2008) suggest that a maximal threshold of chemical substrates responsible for arousal is reached at a particular point in the day-night cycle; the substrate is then metabolised or withdrawn, and the

individual sleeps. Thus, while sleep and arousal are defined as two separate states, they are not mutually exclusive but rather mutually inhibitory (Mignot, 2008), and their co-ordinated cyclical nature entails an integrated appreciation of both states to better understand the biological role of sleep (Tobler, 1995).

Several teleological explanations for the function of sleep have been proposed, but are yet to be determined with any certainty (Fuller et al., 2006). Sleep seems to have a general homeostatic and restorative effect; more specifically, sleep probably has a role to play in cellular repair, macromolecular activity, energy demands and conservation, brain development, cognition and memory (Siegel, 2004a; Capellini et al., 2008a and b; Mignot, 2008). These criteria are related not only to genetic makeup and environment, but to the specific phylogenetic adaptation that an organism undergoes to become ecologically suited to its environment (Lima et al., 2005; Roth II et al., 2010). A fair example of this is predation pressure that forces prey species to sleep very differently to predators (Mignot, 2008). Groups of ponies tend to always make use of a guard who remains standing while the rest of the group is recumbent. When an exchange occurs, the sentry relieved of duty does not lie down until the new sentry has been standing for at least five minutes (Belling, 1990)

It is generally accepted that after extended periods of wakefulness, animals with a cerebral cortex need a period of rest to consolidate and "make space" for subsequent periods of arousal (Mignot, 2008). The obvious evidence for this theory is that humans work less efficiently when sleep deprived (Passer and Smith, 2004). Coordination and memory become compromised and they are less likely to make effective and sensible decisions. However, this does not account for a full explanation of why all organisms sleep, specifically those like drosophila, which do not possess a forebrain (Kilduff, 2000), and thus rely more on non-glutamatergic mediated plasticity and learning, but still display rest and sleep activity (Mignot, 2008). The obvious corollary to this idea is that the criteria for rest are not the same for all animals.

In attempting to understand the function of sleep (for which a consensual theory is lacking) researchers have selectively removed both NREM and REM sleep in experimental subjects (Siegel, 2004a; Mignot, 2008). Rechtschaffen and colleagues

(1989) deprived rats of sleep that resulted in death within two to three weeks, and four to five weeks when REM sleep was interrupted. This research suggests that a cascade of physiological events such as lowered immune response, weight loss, increased appetite and poikilothermia colluded in the death of the rats, rather than specifically a lack of sleep (Rechtschaffen et al., 1989). This emphasises the view that sleep cannot be separated from organism homeostasis and the universal chronobiological feature of life – the circadian rhythm (Siegal, 2004a). Sleep deprivation results in a strong tendency to make up for sleep deficits, a rebound response – the exceptions being migratory birds and nursing cetacea (Mignot, 2008). Evolution's response has thus been to favour adaptation over elimination and to create variety rather than conformity in mammalian sleep (Siegel, 2004a; Fuller et al., 2006; Mignot, 2008).

2.4 Perissodactyla

The order Perissodactyla originated from early mammals known as Phenacodontid condylarths in the Palaeocene (65 Ma to 56 Ma) of the Cainozoic era (Radinsky, 1969). The beginning of the Eocene (60 Ma to 40 Ma) saw the diversification of the perissodactyls into three families, two of which, the Equidae and Tapiridae remain extant today (Radinsky, 1969). The family of Rhinocerotidae seems to have appeared in the late Eocene as a branch of the Tapiridae family (Radinsky, 1969). Towards the end of the Eocene and through the Oligocene (34 Ma to 23 Ma), 10 of the 13 superfamilies of the order Perissodactyla underwent rapid extinction (Trifonov et al., 2008). The tapir and equid family karyotypes then proceeded to evolve at an accelerated rate (Trifonov et al., 2008). This high rate of genetic reshuffling, especially in the donkey and zebra, could explain the radical narrowing of the order into three highly speciated families: equids, tapirs and rhinoceros (Trifonov et al., 2008; Price and Bininda-Emonds, 2009).

The family Equidae has one surviving genus: *Equus*. Tapirs and rhinoceros hail from the subgenus Ceratomorpha, while the equids are classed as Hippomorpha (Trifonov et al., 2008) with one exception, Grévy's zebra (*Equus grevyi*), the sole remaining member of the subgenus Dolichohippus. Grévy's zebra is the largest of the wild equids and is mainly confined to the plains of Kenya and Ethiopia. The above exception determines whether this genus is considered to comprise eight or nine sub-species that fall within the three species categories; *Equus caballus* (horse), *Equus*

africanus asinus (donkey), and *Equus quagga* (zebra) (Trifonov et al., 2008). The first two species can be categorised as either wild or domesticated, while the last, the zebra and its subspecies, despite various attempts at domestication, remain classified as wild animals.

2.4.1 Experimental animals

2.4.1.1 The horse

The earliest ancestor of the horse is the Eohippus (Dawn Horse). This primitive equid existed in the Eocene, was the size of a fox, and possessed four toes on each front foot and three on the hind-feet. Unlike the donkey, numerous fossils exist today that suggest that this odd-toed ungulate underwent a quantitative adaptation in population and behaviour prior to its domestication (Beja-Pereira et al., 2004; Blench, 2004). The horse was thus domesticated in its natural environment, and this may be one of the reasons why there is very little behavioural difference observed between the feral horse (*Equus ferus caballus*) and its domesticated counterpart (*Equus caballus*) (Houpt et al., 1978; Christensen et al., 2002; Goodwin, 2002; Krueger and Heinze, 2008; Ransom and Cade, 2009). Today, domestic and feral horses are found on all continents of the world except the Antarctica, with the largest communities of feral horses found in Australia and North America (Ransom and Cade, 2009).

The horse shows little sexual dimorphism, and domestication has led to a large range of breed-specific characteristics. Average body mass ranges from 227 to 900 kg, with some larger breeds weighing in at over 1 000 kg. Height and length is again greatly variable, with averages ranging between 900 to 1 700 cm and 220 to 280 cm respectively (Animal Diversity Web, 2015). As with body mass, size has potential outliers for minimum and maximum values. Colouring, pattern of coat and thickness of pelt varies greatly and is breed- and environment-dependent. Domesticated horses (*Equus caballus*) eat hay and lucerne and in the summer months are put out to graze, but rarely have the opportunity to browse. The lifespan of the horse ranges from 21 to 30 years and is dependent on breed, environment and treatment (Houpt, 1980; Goodwin, 2002).

Horses are social animals and their domestication seems to have enhanced this trait. Pair-bonding between individuals is prevalent (Ransom and Cade, 2009). During the breeding season, females go into oestrous every 21 days for an average of 6.5 days. The female ovulates in the last two days of this period, and produces one foal a year, usually in spring and at night. Gestation is breed-dependent and lasts on average 335 days, however, values range from 287 to 419 days. Foals are precocial and weigh between 20 and 40 kg, domesticated breeds having a greater birth weight than feral foals (Animal Diversity Web, 2015; San Diego Zoo Global, 2009).

The first physiological sleep studies (using EEG) on the domestic horse were completed by Ruckebusch in the 1970s. In the first study, two juveniles of unknown sex were examined (1970) and in the second study (1972), three adult stallions were investigated. A total sleep time (TST) value of 4.37 hours with a REM to NREM ratio of 0.83 to 3.54 hours was established for the first experiment. The second established an inferior TST value of 2.88 hours and an inferior REM to NREM ratio of 0.79 to 2.09 hours. Behavioural studies came later and as Belling (1990) notes, such studies demonstrate that sleep patterns in the horse are enormously dependent on circumstance: type and size of confinement, age, health and food, grouping, familiarity and stimuli, degree of tameness, time of day and weather patterns. The so-called drowsy stance of a horse is standing, eyelids half closed, head hanging halfway down. Horses drowse for two hours during the day and sleep no more than three hours at night (Houpt, 1980), and are thus classified as diurnal polyphasic sleepers (Dallaire and Ruckebusch, 1974a and b; Belling, 1990). Drowsing time during the day may increase in warmer weather (Houpt, 1980). At night, if the animal is comfortable in its surroundings, it may recline. There are numerous stances of recumbency, but mostly the animal is sternally recumbent as it enters NREM sleep (Dallaire and Ruckebusch, 1974b; Houpt, 1980). Following this, the horse may wake to stretch its hind legs before going back into NREM followed by a period of REM sleep during which it is usually laterally recumbent (Houpt, 1980; Belling, 1990). Often a horse will go through a cycle of NREM sleep (five minutes), REM sleep (five minutes), another bout of NREM (five minutes) and then wake and stand up for 45 minutes before going back to sleep (Dallaire and Ruckebusch, 1974b; Houpt 1980; Belling, 1990). In foals, Houpt (1980) observed that approximately half of a 24-hour period was spent asleep and that adult values for TST were reached by the age of seven months.

In summary, Ruckebusch and Dallaire have created a significant body of information on sleep in domestic equids, spanning a period beginning in the 1960s through the 1980s; most notably, several physiological studies conducted by Ruckebusch and Ruckebusch et al. on the horse, and by Dallaire and Ruckebusch on the Pottock pony (Ruckebusch et al., 1970; Ruckebusch, 1962, 1963, 1972; Dallaire and Ruckebusch, 1974a and b). Despite incongruences in measurement standards as discussed earlier, their literature is mostly relevant and has formed a solid foundation for further studies in equine sleep (Bertone, 2015).

In 1980, Hale and Huggins conducted a physiological study on sleep stages in a group (three females and one male) of "grade ponies" (the term "grade" refers to equids of unknown parentage or sufficiently "mixed" that they cannot be registered as a particular breed). Their results for TST, REM to NREM sleep ratio and recognised stages (awake, drowsy, NREM, REM) were in significant agreement with previous studies conducted by Ruckebusch and Dallaire in the 1970s. Further, Hale and Huggins were able to identify irregular cardiac patterns during NREM and REM sleep, a phenomenon first identified by Dallaire and Ruckebusch in 1974. This may help to explain why equids are reluctant to remain in sternal recumbency for long periods, and mostly avoid all lateral recumbency except when dust rolling (Dallaire and Ruckebusch, 1974b; Hale and Huggins, 1980). More recently, Williams et al. (2008) performed polysomnographic studies accompanied by ECG (electrocardiogram) on five adult horses. The authors observed similar sleep times and habits to previous studies and confirm instances of second-degree atrioventricular block during the slow wave sleep phase of NREM sleep (Williams et al., 2008). This is the first study in equids to confirm brief standing REM sleep episodes with EEG data (Williams et al., 2008).

Finally, there is a small body of observational work done by Houpt (1980) on sleep mannerisms in the domestic horse and by Belling (1990) on sleep patterns, but nothing on its feral counterpart. Further, following an extensive literature search, it would seem that no neuroanatomical studies have been undertaken on this species and its many subspecies.

2.4.1.2 The donkey

The donkey (*Equus africanus asinus*) is indigenous to Africa and thought to be descended from the domestication of the Nubian (*Equus africanus africanus*) and the Eritrean (*Equus africanus dianae*) wild ass (Murray, Byrne and D'Eath, 2013), while the Nubian wild ass is generally considered to be the ancestor of the present-day feral ass (Blench, 2004).

Little sexual dimorphism exists in donkeys, and domesticated adults have an average body mass of 275 kg, a head/body length of 200 cm and a shoulder height that ranges between 80 cm and 150 cm (Moehlman, 1998; San Diego Zoo Global, 2009). Tail length is approximately 41 cm, and donkeys have upright manes with disproportionately large ears (200 mm). Markings are geographically dependent and domestic breeds tend to have shorter legs than their wild counterparts (San Diego Zoo Global, 2009). These animals have strong immune systems and survive easily on protein-poor fodder and little water (Yilmaz, 2012). A jenny's first oestrus occurs at 12 months of age and gestation lasts approximately 365 days. The domesticated donkey is a more social animal than its feral counterpart, and thrives in smaller groups or single pairs. Pair-bonding between same and different sex individuals is commonplace (Murray et al., 2013). Donkeys separated from a chosen companion or a small group become highly distressed and demonstrate pining and loss of appetite (Murray et al., 2013). Average life expectancy ranges between 27 and 40 years (Murray et al., 2013), and is mostly environment-dependent for the feral ass and treatment-dependent for the domesticated donkey (Yilmaz, 2012).

The donkey, both feral and domesticated, can sleep in a standing or reclining position. Adults tend to remain standing, especially in hotter climates (Moehlman, 1998). They tend to sleep in the heat of the day and close to midnight, when they may recline, while other members of the herd remain standing and on guard (Yilmaz, 2012). The typical sleep-standing stance is neck just lower than the horizontal, eyes closed and ears down to the sides of the head (Moehlman, 1998). Foals tend to sleep in a recumbent position close to their mothers, while adults recline mostly when they want to roll (Moehlman, 1998). In 1963, Ruckebusch undertook three 24-hour EEG recordings on three donkeys and reported a TST of 3.1 hours with a REM value of 0.4 hours to a NREM value of 2.7 hours. As with the horse, sleep was found to be

dependent on both environmental and physiological factors affecting the animal. This said, the donkey is considered a diurnal polyphasic sleeper with TST and recognised phases of sleep similar to the horse (Ruckebusch, 1970). Belling (1990) estimates that donkeys tend to spend more time recumbent (31% in sternal recumbency and 5% in lateral recumbency of TST) than horses. Sleep distribution in the donkey is dependent on climate, with more sleep bouts occurring during the day in hotter climates (Moehlman, 1998).

In summary, the donkey has similar total sleep times to the horse and some breeds of pony (Campbell and Tobler, 1984). This similarity has done little to encourage further physiological sleep studies in both feral and domesticated donkeys. To date, a single physiological study exists, completed by Ruckebusch in 1963. In this study a cohort of donkeys was observed to sleep mostly at night and for a slightly longer period than the horse. Ruckebusch's EEG data suggests that sleep onset is far quicker when the donkey is recumbent than when standing, the position of the ears being a good measure of the depth of sleep (Ruckebusch, 1970). Sleep time in recumbency is estimated at between seven to eight minutes with extremes of two to 15 minutes (Ruckebusch, 1970). During periods of recumbency (approximately four to five mins), Ruckebusch was able to identify paradoxical sleep in the horse, observing saccades, shallow respiration and bruxism, intermittent cutaneous shivers, occasional tachycardia and erect ears followed by sudden arousal, standing and vigilance (Ruckebusch, 1970). This behaviour was, however, not observed in the donkeys during periods of recumbent sleep, and Ruckebusch's study thus concluded that while paradoxical sleep waves were seen on the EEG, other physiological indicators recorded in the horse, were not seen in the donkey (Ruckebusch, 1970). Further paradoxical sleep in domestic equids seems to be a phenomenon that is present only when the animal lies down (Ruckebusch, 1970; Hale and Huggins, 1980) and as such, a degree of muscular relaxation must be attained before the animal can enter REM sleep. Recumbency is dependent on how comfortable the animal is in its surrounding environment (Hale and Huggins, 1980) and with this in mind, REM sleep times for wild equids may possibly be very different. This study's literature search found no neuroanatomical studies done on the sleep-related nuclei in the brain of the donkey.

2.4.1.3 The zebra

The plains and mountain zebra, subgenus Hippotigris, is a well-dispersed southern African species of grazers belonging to the genus Equidae. It is generally accepted that the zebra has two distinct species clades: the plains zebra (*Equus quagga*) (Gray, 1824) and the mountain zebra (*Equus zebra*). The plains zebra, formerly known as Burchell's zebra or the common zebra, is the most well-known and has a large geographical spread from as far north as southern Ethiopia, to the south-eastern regions of South Africa. The plains zebra has six extant subspecies and one well-known extinct group, the quagga. The two sub-species of mountain zebra, the Cape mountain zebra (*Equus zebra zebra*) and Hartmann's mountain zebra (*Equus zebra hartmannae*) are geographically limited to South Africa, Namibia and southern Angola.

Cape mountain zebras show sexual dimorphism with stallions being slightly larger than mares, whereas the plains zebra show little sexual dimorphism (Smithers, 1983). The Cape mountain zebra have an average body mass of 250 to 260 kg with an average shoulder height of 1.3m. The plains zebra (average body mass: 290 to 340 kg, average shoulder height: 1.3 m) tend to be more compact and thickset than mountain zebra (Stuart and Stuart, 2001). Osteologically, the skull of the plains zebra, in particular the occiput, is the smallest and narrowest of the species (Groves and Bell, 2004). The plains zebras of southern Africa are stouter than their northern counterparts; striping tends to be narrower in northern groups, with none of the brown shadowing that is characteristic of the southern plains species (Bennett, 1980).

The Cape mountain zebra can be distinguished from the plains zebra morphologically, possessing a prominent dewlap and on average larger ears (Smithers, 1983; Skinner and Chimimba, 2005). Mountain zebra also have a more definite striping that resembles a "gridiron", with no brown shadowing (Stuart and Stuart, 2001). Striping does not usually extend onto the stomach in mountain zebra, and the underside is white with a single median longitudinal black stripe (Smithers, 1983; Stuart and Stuart, 2001). The muzzle of a mountain zebra is black with orange-brown hair extending upward to the eye region. Plains zebra have black muzzles with no orange shading (Smithers, 1983; Stuart and Stuart, 2001).

Distribution of the Cape mountain zebra is recorded as having extended from the mountains around Paarl in the south west of South Africa, following the Cape frontal ranges eastward to where this range merges with the Amatola, Nuweveld, Suurberg and Stormberg ranges of the Great Escarpment of the Eastern Cape. In the 1930s, this sub-species was threatened with extinction. Today, they are confined to the Mountain Zebra National Park, Cradock district, the Kouga and Baviaanskloof all in the Eastern Cape province, and the Gamka and Kamanassie reserves of the Western Cape province, where they are protected (Smithers, 1983).

Burchell's or plains zebra are more widely distributed and are reported to occur right across southern Africa from southern Angola/northern Namibia, northern Botswana, Zimbabwe, Mozambique and as far north as southern Ethiopia. The south-eastern distribution occurs throughout Mozambique, Swaziland and northern Kwazulu-Natal (Smithers, 1983).

Cape mountain zebra prefer the protection of cliffs and rocky ridges. In the colder months, harems may descend to lower altitudes. Plains zebra prefer woody grassland with no trees and plentiful waterholes. Zebra prefer grazing to browsing, are particularly fond of rooigras (*Themeda triandra*) and couch grass (*Cynodon dactylon*), and will actively search it out (Smithers, 1983; Stuart and Stuart, 2001). Plains zebra are less territorial than mountain zebra (Smithers, 1983), and herds often overlap geographically, their home range extending from 111 km^2 to over 200 km^2 (Smithers, 1983).

Social organisation of the zebra is designed around two groups. A harem, or family, being the most stable social structure (lasting from months to years), and is characteristically made up of one sexually mature stallion and several, often pregnant or lactating females and their offspring (Fischhoff et al., 2007; Smithers, 1983). Many harems come together to form a herd. Herds seem to be less stable and more democratic than the hierarchical harem, and their structure and population can change hourly to daily (Rubenstein, 1986). Foaling takes place at any time of year, with concentrated peaks from October to March, peaking in December and January (Smithers, 1983). All foals are precocial, and plains zebra foals weigh between 30 and 35 kg at birth (Smithers, 1983; Stuart and Stuart, 2001). Gestation takes 360 to

390 days, with an average of 372 days reported (Klingel, 1965, 1972; Smithers, 1983).

Zebra are diurnal animals (Smithers, 1983), and are most active in the two hours after dawn, for a brief period in the late morning, and then late in the afternoon (Smithers, 1983; Stuart and Stuart, 2001). They spend their active time grazing or guarding, and inactive time in standing somnolence. Foals tend to lie down in sternal recumbency. Being a member of the equid family, the zebra possesses the knee-joint locking ligaments that make it easier for the animal to rest in a standing position. This occurs because the ligament locks the joint with minimal effort from the surrounding muscles (Moehlman, 1998; Yilmaz, 2012). Additionally, the ligamentum nuchae comprises elastin that helps reduce the need for muscular tonus (Moehlman, 1998), unlike in humans where this structure arises from the surrounding muscular aponeuroses of the upper trapezius, the splenius capitis and rhomboid minor, and dense connective tissue (Mercer and Bogduk, 2003). These anatomical structures suggest that this wild animal may spend most of its sleep time in the standing position as adaptive behaviour to the risk of predation (Moehlman, 1998; Yilmaz, 2012).

A series of observational studies on zebra in their natural habitats, concluded by Klingel, Joubert, Penzhorn and Smuts during the 1960s, 1970s and 1980s, have contributed considerably to the foundation of a growing body of literature in many areas such as taxonomy, reproduction, tooth development and home range migration (Klingel, 1965, 1972; Smuts, 1972, 1976; Joubert, 1975; Penzhorn, 1975, 1984). To date, however, none of the literature addresses how zebra sleep, and it is thus the view of this author that no behavioural, physiological or anatomical studies on sleep in zebra have been undertaken. It remains to be investigated as to whether the zebra sleep architecture resembles that of other perissodactyls in the wild, such as rhinoceros, or domesticated equids, and what, if any neuroanatomical differences prevail.

CHAPTER THREE: Materials and Methods

3.1 Experimental animals

The brains of four adult males of the order Perissodactyla, family *Equidae* were used for this current study. The domestic donkey (*Equus asinus africanus*) has an average body mass of 180-225 kg (Huggins, 2002), and the specimen acquired for this study, a brain mass of 398.5 g. The domestic horse (*Equus caballus*) has an average body mass range of 300-400 kg (Goodwin, 2002), and the specimen acquired for this study a brain mass 613.7 g. The plains zebra (*Equus quagga*), has an average body mass of 290-340 kg (Smithers, 1983; Stuart and Stuart, 2001), and the specimen used in this study, a brain mass of 538.7 g. The mountain zebra (*Equus zebra*) has an average body mass of 250-260 kg (Smithers, 1983; Stuart and Stuart, 2001) and the specimen used for this study, a brain mass of 443.5 g. All animals were obtained from a licenced dealer and were treated and studied in accordance with the guidelines of the University of the Witwatersrand Animal Ethics Screening Committee, which parallel those of the NIH for the care and use of animals in scientific experimentation (Ethics Clearance Number: 2015/06/23/A).

3.2 Brain perfusion and extraction

All animals were anaesthetised using weight appropriated doses of ketamine and xylazine and euthanised with an overdose of sodium pentobarbital administered intraperitoneally (200 mg sodium pentobarbital/kg). Once in cardiopulmonary arrest, the heads were cut free from each animal's body, anterior to the upper limb and posterior to the larynx. The heads were then perfusion fixed with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH = 7.4) using cannulae with 4 mm inner diameters inserted and secured to the common carotid arteries. The brains were extracted from the crania, weighed and post-fixed in a solution of 4% paraformaldehyde in 0.1 M PB and stored at 4°C for 48 hours. The brains were then submerged in a solution of 30% sucrose in 0.1 M PB at 4°C for a period of approximately two weeks during which time equilibration took place. The brains were then placed in an anti-freeze storage solution (30% glycerol, 30% ethylene glycol, 30% distilled water and 10% 0.244 M PB) in the fridge overnight, after which they were stored in a freezer at -20°C until histological processing began.

3.3 Tissue selection and immunostaining

For the immunohistochemical identification of the sleep related neuronal systems, a block of brain tissue extending from the basal forebrain through the pons to the rostral medulla oblongata was dissected from the brains of all four equids and equilibrated in 30% sucrose in 0.1 M PB solution at a temperature of 4°C prior to sectioning. For sectioning, the block of brain tissue was mounted to an aluminium stage, frozen in crushed dry ice and sectioned at 50 µm, in the coronal plane, using a sliding microtome.

A 1:20 series of serial sections (1:9 series used for this project; 1:11 in storage for future projects) was made for Nissl and myelin (for the identification of architectonic borders), choline acetyltransferase (ChAT – for the identification of the cholinergic system), tyrosine hydroxylase (TH – for the identification of the catecholaminergic system), serotonin (5-HT – for the identification of the serotonergic system), orexin (OxA – for the identification of the orexinergic system), calbindin (CB), calretinin (CR) and parvalbumin (PV) (for the identification of the calcium binding proteins of the GABAergic system). Sections used for the Nissl were mounted on 0.5% gelatine-coated glass slides, dried and cleared overnight in a solution of 1:1 chloroform and methanol and stained with 1% cresyl violet. Myelin sections were stored in 5% formalin for two weeks at 4°C and then mounted on 1.5% gelatine-coated slides and stained with a silver solution to reveal the myelin sheaths (Gallyas, 1979).

For the immunohistochemical staining each section was treated with endogenous peroxidase inhibitor (49.2% methanol: 49.2% 0.1 M PB: 1.6% of 30% H_2O_2) for 30 minutes and subsequently subjected to three 10-minute 0.1 M PB rinses. Sections were then pre-incubated for 2 hours, at room temperature, in a blocking buffer solution containing 3% normal goat serum (NGS, Chemicon) for TH, 5-HT, OxA, CB, CR and PV sections, 3% normal rabbit serum (NRS, Chemicon) for the ChAT sections, plus 2% bovine serum albumin (BSA, Sigma) and 0.25% Triton X-100 (Merck) in 0.1 M PB. This was followed by three 10-minute rinses in 0.1 M PB. Sections were then placed in a solution that contained the appropriately diluted primary antibody in blocking buffer. Anti-choline acetyltransferase (AB144P,

Millipore, raised in goat) at a dilution of 1:3000 was used to reveal cholinergic neurons. Anti-tyrosine hydroxylase at a dilution of 1:3000 (AB151, Millipore, raised in rabbit) was used to reveal catecholaminergic neurons of the dopaminergic and noradrenergic types. Serotonergic neurons were revealed using anti-serotonin (AB938, Millipore, raised in rabbit) at a dilution ratio of 1:5000. Orexinergic neurons were revealed using anti-Orexin A at a dilution of 1:3000 (AB3704, Millipore, raised in rabbit). Calcium binding neurons and terminal networks were revealed using anti-calbindin (CB38, SWant, raised in rabbit), anti-calretinin (7699/3H, SWant, raised in rabbit) and anti-parvalbumin (AB 11427, Abcam, raised in rabbit) at a dilution of 1:1000. All sections were incubated for a period of 48 hours at a temperature of 4°C under gentle shaking. This was followed by three 10-minute rinses in 0.1 M PB. Following the primary antibody incubation, all sections underwent a further 2-hour incubation in a secondary antibody solution, under gentle shaking at room temperature. TH, 5-HT, OxA, CB, CR and PV sections were incubated at a dilution of 1:1000 in a solution containing biotinylated anti-rabbit IgG (BA-1000, Vector Labs), 3% NGS, 2% BSA and 0.1 M PB. ChAT sections were incubated at a dilution of 1:1000 in a blocking buffer solution containing biotinylated anti-goat IgG (BA-5000, Vector Labs), 3% NRS, 2% bovine serum BSA and 0.1 M PB. Following this all sections underwent three 10-minute rinses in 0.1 M PB. The sections were then incubated for 1 hour in a solution containing avidin-biotin complex (ABC, Vector Labs) and 0.1 M PB, followed by a further three 10-minute rinses in 0.1 M PB. Sections were then placed in a solution of 0.05% diaminobenzidine (DAB) in 0.1 M PB (2 ml/section) for 5 minutes, followed by the addition of 3 µl of 30% hydrogen peroxide to each 1 ml of solution in which each section was immersed. Chromatic precipitation was visually monitored and verified under a low power stereomicroscope. Staining was allowed to continue until the background stain was at a level that accommodated architectural reconstruction without obscuring the immunopositive neurons and terminal networks. Development was arrested by placing sections in 0.1 M PB, followed by two more rinses in this solution. To check for nonspecific staining from the immunohistochemistry protocol, we omitted the primary antibody and the secondary antibody in selected sections, which produced no evident staining. Sections were mounted on 0.5% gelatine-coated glass slides, dried overnight, dehydrated in a graded series of alcohols, cleared in xylene and cover-

slipped with Depex.

3.4 Analysis of results

3.4.1 Neuroanatomical analysis and drawings

A qualitative description of the sleep related neuronal systems in the brain of the donkey, horse, the plains and mountain zebra was undertaken using low and high power stereomicroscopy. The sections were viewed with a low power stereomicroscope, and the architectonic borders were traced according to the Nissl and myelin-stained sections using a camera lucida. The immunostained sections were then matched to these drawings, and the immunopositive neurons were marked for ChAT, TH, OxA and 5-HT. Due to the often-high number and density of the calcium binding proteins of the GABAergic system, PV, CB and CR neurons and terminal networks were matched to these drawings, the relative densities and terminal networks were noted and photographed under higher powered light microscopy. The drawings were then scanned and redrawn using the Canvas 8 (Deneba) drawing programme. Nomenclature used for the cholinergic nuclei was taken from Woolf (1991), Manger et al. (2002a), Maseko and Manger (2007), Maseko et al. (2007), Bhagwandin et al. (2008) and Gravett et al. (2009). For the catecholaminergic system in this study nomenclature was taken from Dahlström and Fuxe (1964), Hökfelt et al. (1984), Smeets and González (2000)), Manger et al. (2002b), Maseko and Manger (2007), Maseko et al. (2007), Moon et al. (2007), Dwarika et al. (2008), Bhagwandin et al. (2008) and Gravett et al. (2009). Serotonin nomenclature was referenced from Törk (1990), Bjarkam et al. (1997), Manger et al (2002c), Maseko and Manger (2007), Maseko et al. (2007), Moon et al. (2007), Dwarika et al. (2008), Bhagwandin et al. (2008) and Gravett et al. (2009). Nomenclature for the orexinergic system was taken from Bhagwandin et al. (2008), Dell et al. (2012).

CHAPTER FOUR: Results

In mammals, the initiation, maintenance and regulation of the sleep-wake cycle and its stages are mediated by a variety of somnogenic nuclear systems located in the basal forebrain, hypothalamus and the brainstem. Most often, these systems remain preserved across species and throughout orders (Bhagwandin et al., 2013; Dell et al., 2012, 2016; Davimes et al., 2017). Nuclei are considered to have a similar function based on their location and their presiding neurotransmitter or neuropeptide. Further, the primary unit of the nucleus, the neuron, may be described in terms of its shape, number of cytoplasmic projections, dendritic orientation and density – these being strikingly and repeatedly alike across species and orders. The same is often true of cell population size and terminal network density. This said, differences in nuclear arrangement and neuronal morphology do occasionally occur, the former less common than the latter (Bhagwandin et al., 2013; Dell et al., 2016; Davimes et al., 2017). This study found an atypical population of tyrosine hydroxylase neurons in the TRN and a medial parvocellular cluster of orexinergic OxA+ neurons, in all four of the equids studied. The sleep-related nuclei described, unless otherwise stated, apply to all four equids investigated in this study. Any differences found were recorded per species.

4.1 Cholinergic nuclei of the basal forebrain (telencephalon)

Five sleep-related cholinergic nuclei were immunohistochemically examined in the basal forebrain of all four equids. The medial septal nucleus (Sep.M), diagonal band of Broca (Diag.B), islands of Calleja within the olfactory tubercle (Is.CALL/TOL), and the nucleus basalis (N.Bas) were identified as containing ChAT+ neurons (Fig. 1.1A – C).

The Sep.M was found to be a bilateral mid-line nucleus situated medial to the lateral ventricle, inferior to the septum pellucidum and superior to the most rostral extension of the hypothalamus, and superomedial to the Diag.B. Neurons were mostly oval, a few being spherical, multipolar and showed no specific dendritic orientation. ChAT+ neuronal density was moderate to low for all species except the horse, which demonstrated a moderate to high density (Fig. 1.1A).

Figure 1.1

Diagrammatic reconstructions of six coronal sections through the basal forebrain and diencephalon of the domestic horse, illustrating the location of neurons forming nuclei immunopositive for choline acetyltransferase (ChAT, closed circles), tyrosine hydroxylase (TH, closed squares), and orexin A (OxA, parvocellular neurons – light grey open stars, magnocellular neurons – closed black stars). Figure A is the more rostral section, F the more caudal. The drawings are approximately 3000 μm apart. Each symbol represents a single cell body. Reconstructions represent a single horse brain. Medial is to the left, dorsal is to the top. Scale bar = 5 mm and applies to all diagrams. See list for abbreviations.

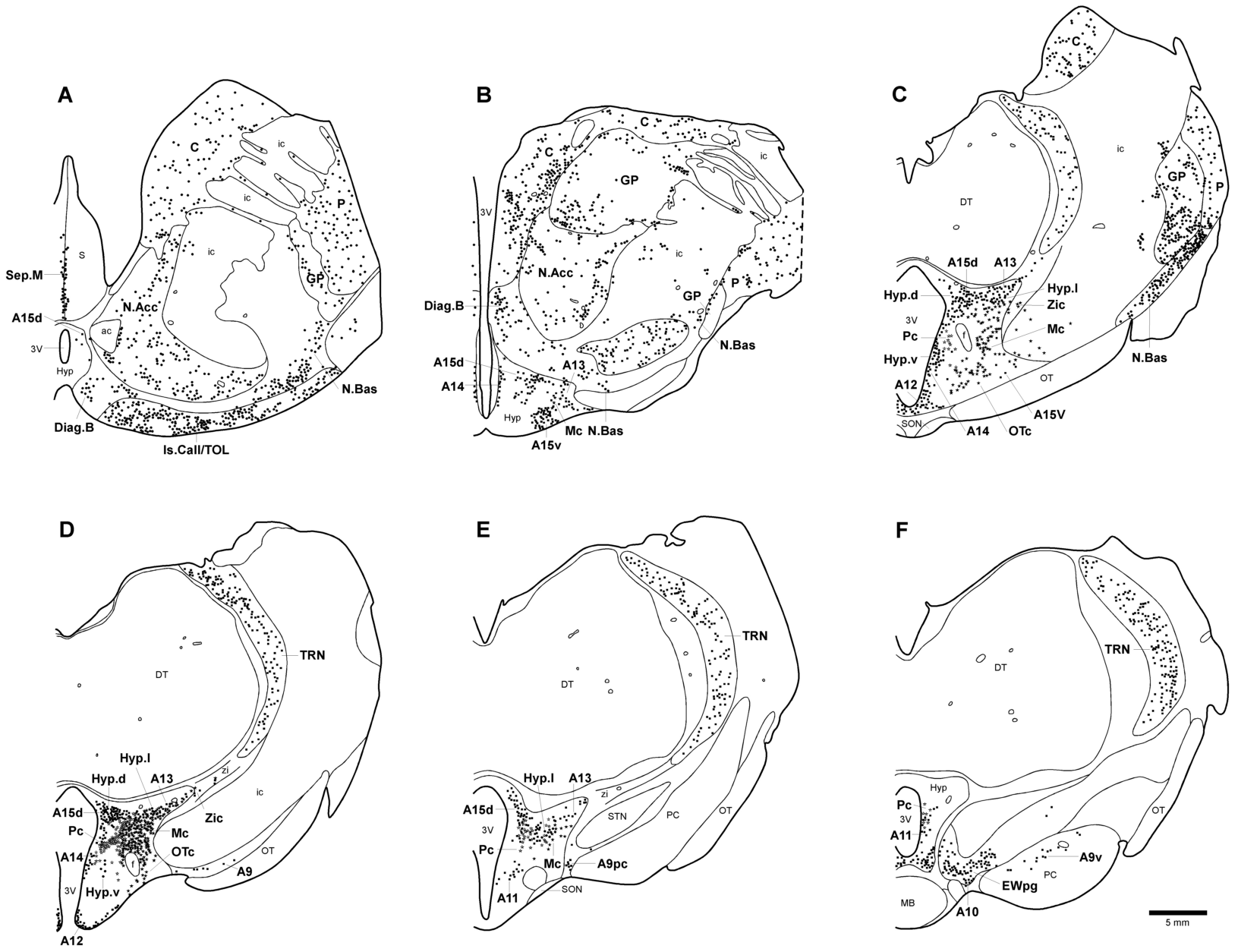
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Figure 1.2

Diagrammatic reconstructions of six coronal sections through the diencephalon of the domestic horse, illustrating the location of neurons forming nuclei immunopositive for choline acetyltransferase (ChAT, closed circles) and tyrosine hydroxylase (TH, closed squares). Figure G is the more rostral section, L the more caudal. The drawings are approximately 3000 μm apart. Each symbol represents a single cell body. Medial is to the left, dorsal is to the top. Reconstructions represent a single horse brain. Scale bar = 5 mm and applies to all diagrams. See list for abbreviations.

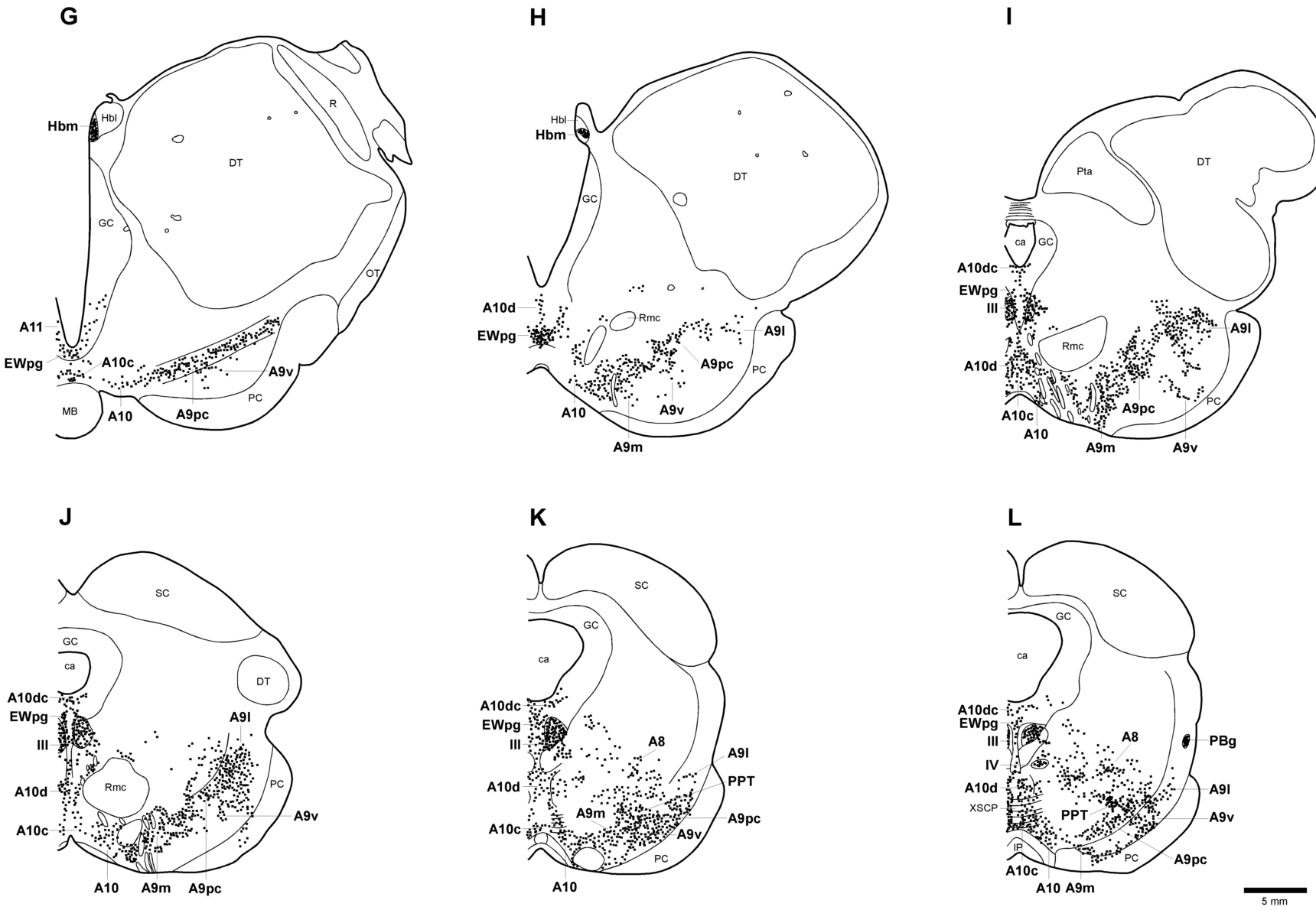
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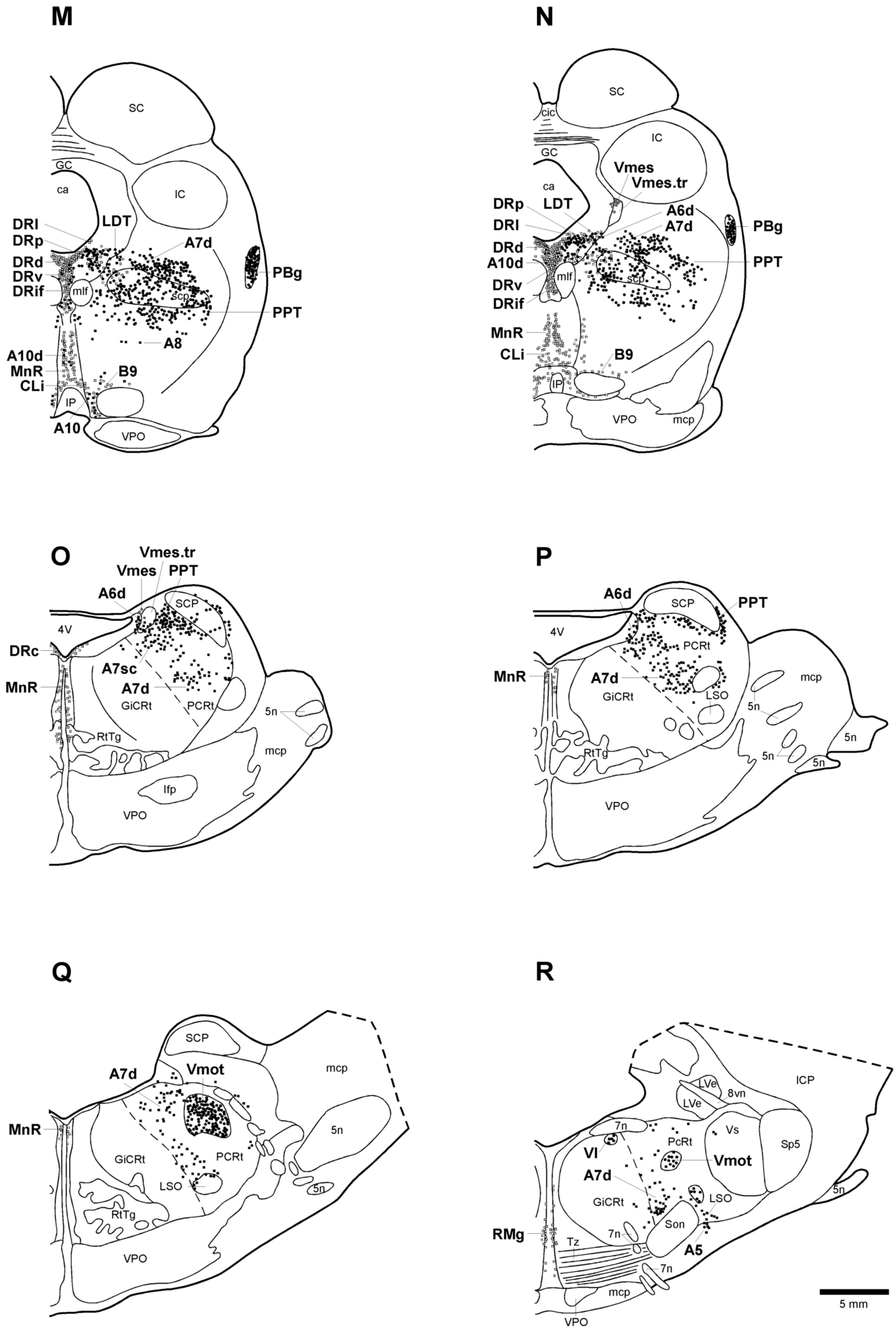
Figure 1.3

Diagrammatic reconstructions of six coronal sections through the midbrain, pons and medulla oblongata of the domestic horse, illustrating the location of neurons forming nuclei immunopositive for choline acetyltransferase (ChAT, closed circles), tyrosine hydroxylase (TH, closed squares), and serotonin (5-HT, open squares). Figure M is the more rostral section, R the more caudal. The drawings are approximately 3000 μm apart. Each symbol represents a single cell body. Medial is to the left, dorsal is to the top. Reconstructions represent a single horse brain. Scale bar = 5 mm and applies to all diagrams. See list for abbreviations.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DRc
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PCRt
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lfp
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A7d
mcp
GiCRt
LSO
5n
RtTg
5n
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SCP
A7d
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mcp
MnR
5n
GiCRt
PCRt
LSO
5n
RtTg
VPO
R
ICP
LVe
8vn
LVe
7n
Vs
Sp5
PcRt
Vl
Vmot
A7d
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Tz
A5
7n
mcp
VPO
5 mm

The Diag.B was found to be a rostral nucleus located in the ventromedial cerebral hemisphere, inferior and slightly lateral to the septal nuclear complex and the anterior commissure. It extended anterolaterally from the rostral hypothalamus. The neurons of this nucleus were oval and multipolar. ChAT+ neurons of this complex were more immunoreactive (as indicated by darker staining), tended to cluster, and demonstrated a moderate to high density with no specific dendritic orientation (Fig. 1.1A and B).

The Is.CALL/TOL, a striatal group of ChAT+ neurons was found in the floor of the cerebral hemisphere, extending laterally from the Diag.B, inferolateral to the septal area and ventral to the nucleus accumbens. This complex had a rostro-caudal extension from the anterior horn of the lateral ventricle to the level of the anterior commissure. Neuronal density was moderate to high throughout the olfactory tubercle (TOL) and the high-density ChAT+ clusters located more rostrally (less distinct in both the plains and mountain zebra), were recognised as the islands of Calleja (Is.CALL). Neurons were ovoid in shape, multipolar, and demonstrated no specific dendritic orientation (Fig. 1.1A and B).

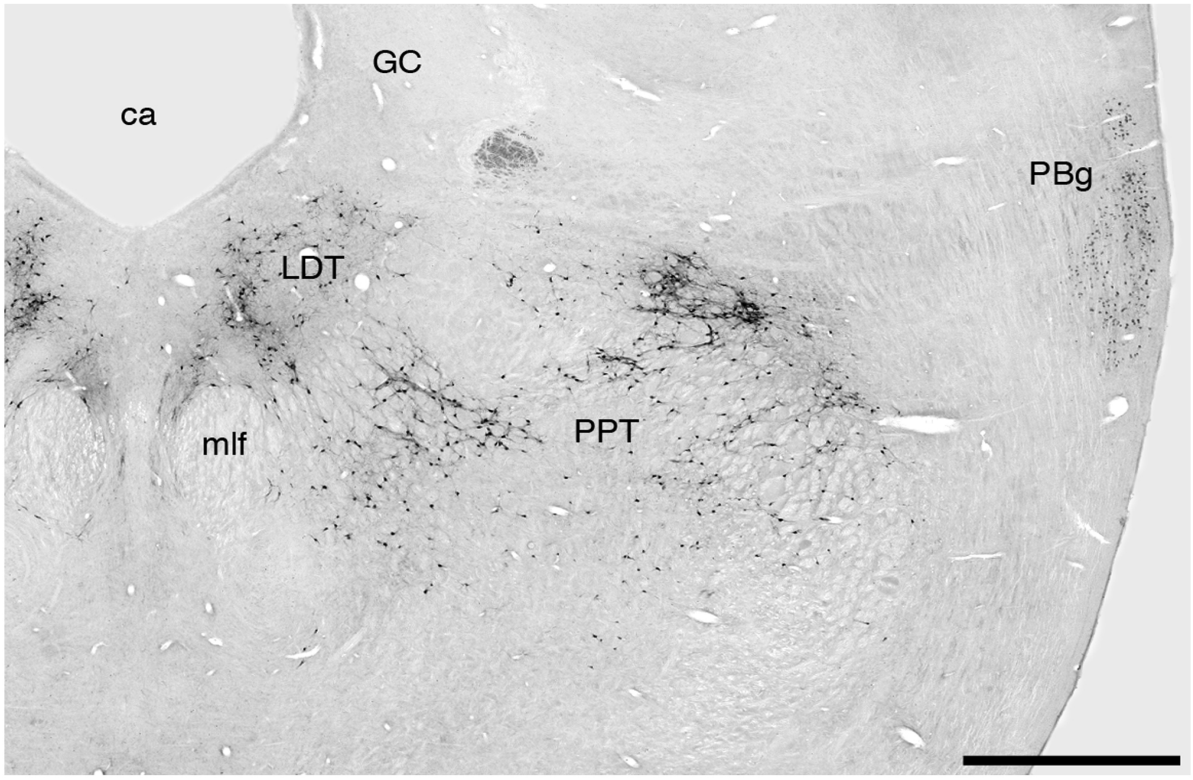
The N.Bas was identified in a ventrolateral position superior to the TOL, inferolateral to the internal capsule and nucleus accumbens, and inferior to the globus pallidus. Neuronal density was observed to be moderate, with a slightly higher density seen in the plains zebra. Neurons were oval, multipolar with no specific dendritic orientation, and particularly immunoreactive in the donkey and the horse (Fig. 1.1A and B).

4.2 Cholinergic nuclei of the pontomesencephalic nuclear complex and the ventral tegmental nucleus

The pedunculopontine tegmental nucleus (PPT) and the laterodorsal tegmental nucleus (LDT) of the pontine region lay adjacent to each other, and while the PPT extended slightly more rostrally than the LDT, both generally ran rostrally from the cerebral aqueduct at the level of the oculomotor nucleus (III), caudally to the level of the trigeminal motor nucleus (Vmot) and to the rostral part of the reticular formation (Figs. 1.2 K – L, 1.3 P, 2).

Figure 2

Low magnification photomicrographic montage of the pons showing the cholinergic laterodorsal (**LDT**) and pedunculopontine (**PPT**) tegmental nuclei in the mountain zebra. Note that the cholinergic neurons forming the **LDT** are limited in their distribution to within the periaqueductal grey matter (**GC**) surrounding the cerebral aqueduct (**ca**), while those of the **PPT** around found in the lateral pontine tegmentum. The parabigeminal nucleus (**PBg**) is also seen in the dorsal lateral aspect of the pontine tegmentum. Scale bar = 2500 μm.


GC
ca
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PPT

The LDT was located within the peri-acqueductal and periventricular grey matter, and evinced a few scattered magnocellular neurons, a few of which were more immunoreactive. The neuronal density of this complex was moderate with no specific dendritic orientation. The neurons were ovoid and multipolar (Figs. 1.3M and N, 2).

The PPT nucleus lay in a ventrolateral position in relation to the LDT and extended laterally towards the outer margin of the midbrain and pontine regions. In its more rostral position it lay inferolateral to the superior cerebellar peduncle, extending towards the parabigeminal nucleus. In the donkey and mountain zebra, this nucleus comprised a majority of parvocellular neurons found around the periphery of a smaller magnocellular group, the latter of which was more centrally positioned with more immunoreactive cell bodies. Intermingling of the two cell types occurred and as such, no clear border was discernible. The horse had a similar nuclear morphology; however, there was a distinct division between the parvocellular and magnocellular groups, with a small cluster of parvocellular neurons that lay separate from and lateral to the magnocellular group within the midbrain. In the plains zebra, both parvo- and magnocellular groups were highly immunoreactive. Rostrally, the magnocellular cluster migrated laterally, but as it progressed caudally, this cluster migrated inferiorly. The parvocellular cluster migrated laterally towards the periphery in its caudal extent. Neuronal morphology was the same for all species, with both parvo- and magnocellular groups displaying typical ovoid and multipolar cell bodies with no specific dendritic orientation. Both groups demonstrated a moderate to high neuronal density (Figs. 1.2K – L, 1.3M – P, 2).

4.3 Putative catecholaminergic nuclei of the locus coeruleus

For the putative catecholaminergic system (tyrosine hydroxylase, TH+ neurons), three nuclei of the locus coeruleus complex in the pons were identified and described: A7sc (subcoeruleus compact), A7d (subcoeruleus diffuse), A6d (locus coeruleus diffuse) (Figs. 1.3M – R, 3). The fifth arcuate nucleus (A5) was not described.

The more caudal complex of A7sc was located ventrolateral to the periventricular grey matter and ventromedial to the superior cerebellar peduncle. It extended rostrally from Vmot in the inferior pons to within the parvocellular cluster

of the reticular formation caudally. Neurons were ovoid and multipolar. Neuronal density was moderate with a few high-density clusters scattered throughout this nucleus for both the plains and mountain zebra. Additionally, both species of zebra demonstrated a small population of bipolar fusiform neurons with a weak lateromedial orientation. Dendritic orientation was non-specific in all species (Figs. 1.3O, 3).

A7d lay inferior to the superior cerebellar peduncle as a superolateral and less dense radiation of A7sc. Neurons of this cluster were ovoid and multipolar with no specific dendritic orientation. Neuronal density was moderate (Figs. 1.3M – R, 3).

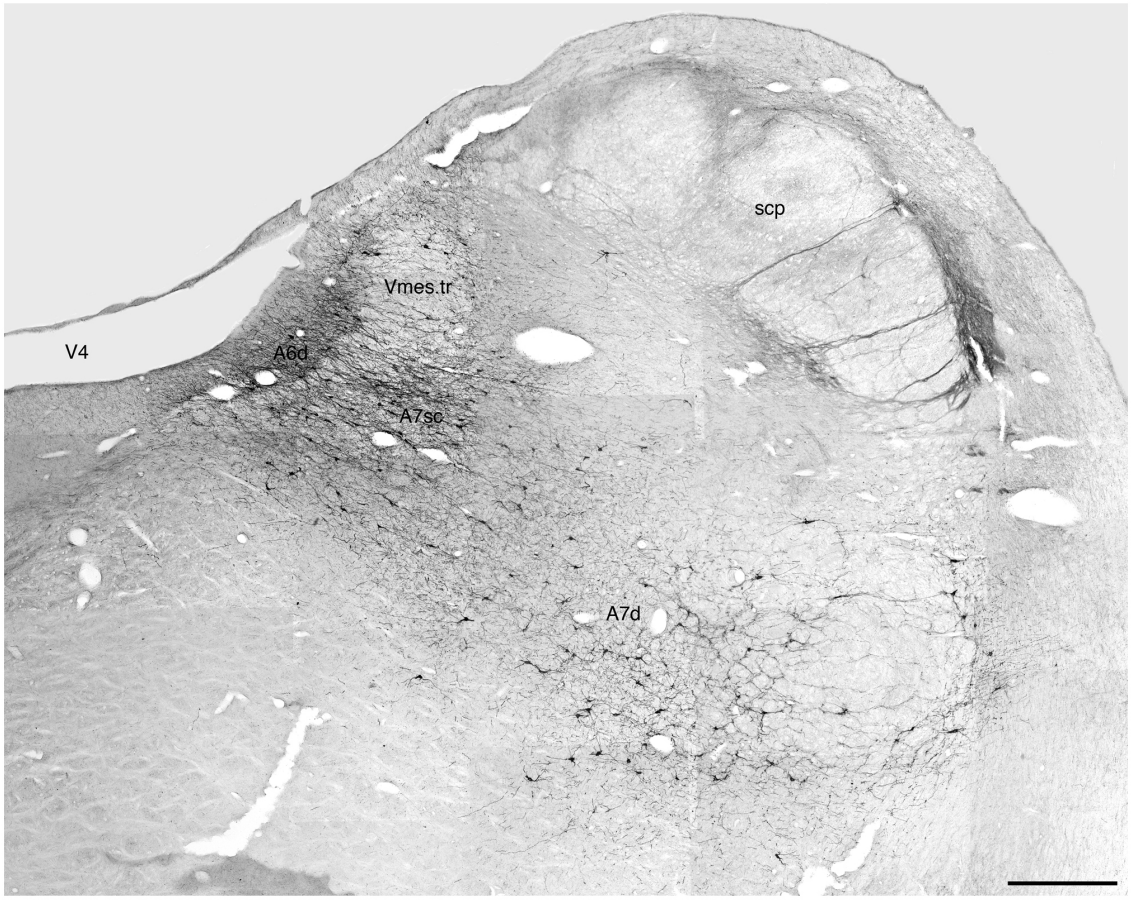
The A6d cluster lay within the periventricular grey matter abutting its ventrolateral margin, superolateral to the LDT and adjacent to the superior cerebellar peduncle. Its caudal limit was found close to the fibres of the facial nucleus (VII), resting on the superior margin of the parvocellular complex of the reticular formation (PCRt). Neurons were oval, multipolar and showed no specific dendritic orientation. Neuronal density was low for the donkey and horse and moderate for both species of zebra (Figs. 1.3N – P, 3)

4.4 Serotonergic nuclei of the dorsal raphe nuclear complex

The dorsal raphe complex, a midline serotonergic (5-HT) brainstem structure was found to extend caudally from the midbrain tegmental area at the level of the oculomotor nucleus (III), to the pontine tegmental area at the level of Vmot. Six nuclei of the rostral subdivision of the dorsal raphe complex were identified and described: the dorsal raphe interfascicular (DRif), the dorsal raphe ventral (DRv), the dorsal raphe dorsal (DRd), the dorsal raphe peripheral (DRp), the dorsal raphe lateral (DRl) and the dorsal raphe caudal (DRc) nuclei. The neurons of the DRif were found to be located in the ventral midline, inferior to the cerebral aqueduct of the midbrain. This nucleus lay between the bilateral medial longitudinal fasciculi, with the median raphe nucleus located inferiorly. The neurons were fusiform in shape, mostly bipolar, with some multipolar, and displayed a dorsoventral dendritic orientation. Neuronal density was moderate (Figs. 1.3M – O, 4).

Figure 3

Low magnification photomicrographic montage of the pons showing three subdivisions of the locus coeruleus complex in the domestic horse. Within the periventricular grey matter a small cluster of tyrosine hydroxylase immunoreactive cells represent the diffuse portion of the locus coeruleus (**A6d**), while in the adjacent pontine tegmentum, just ventral and lateral to the fifth mesencephalic trigeminal tract (**Vmes.tr**), lies the compact portion of the subcoeruleus (**A7sc**). Spreading laterally and ventrally from the A7sc is the diffuse portion of the subcoeruleus (**A7d**), with cells reaching the ventrolateral edge of the pontine tegmentum, and at different anteroposterior levels surrounding the superior cerebellar peduncle (**scp**). Scale bar = 1 mm. **V4** – fourth ventricle.


scp
Vmes.tr
V4
A6d
A7sc
A7d

The DRv lay in a more dorsal position in relation to the central canal caudally, and migrated to a more ventral position inferior to the DRd in its more rostral extent. The DRv lay in the midline, superior to the DRif, within the periaqueductal grey matter. More rostrally, the neurons lay adjacent to the oculomotor nucleus (III) and the superior border of the median longitudinal fasciculus. Neuronal density was moderate for all the species and neurons were oval, multipolar and showed no specific dendritic orientation (Figs. 1.3M – O, 4).

The DRd originated more rostrally in the brainstem at the level of the trochlear nucleus (IV). The DRd included all neurons found immediately inferior to the ventral border of the cerebral aqueduct, and superior to the DRv. Neuronal density was high and the dendritic orientation was nonspecific. Neurons were ovoid and multipolar for all four equid species (Figs. 1.3M – O, 4).

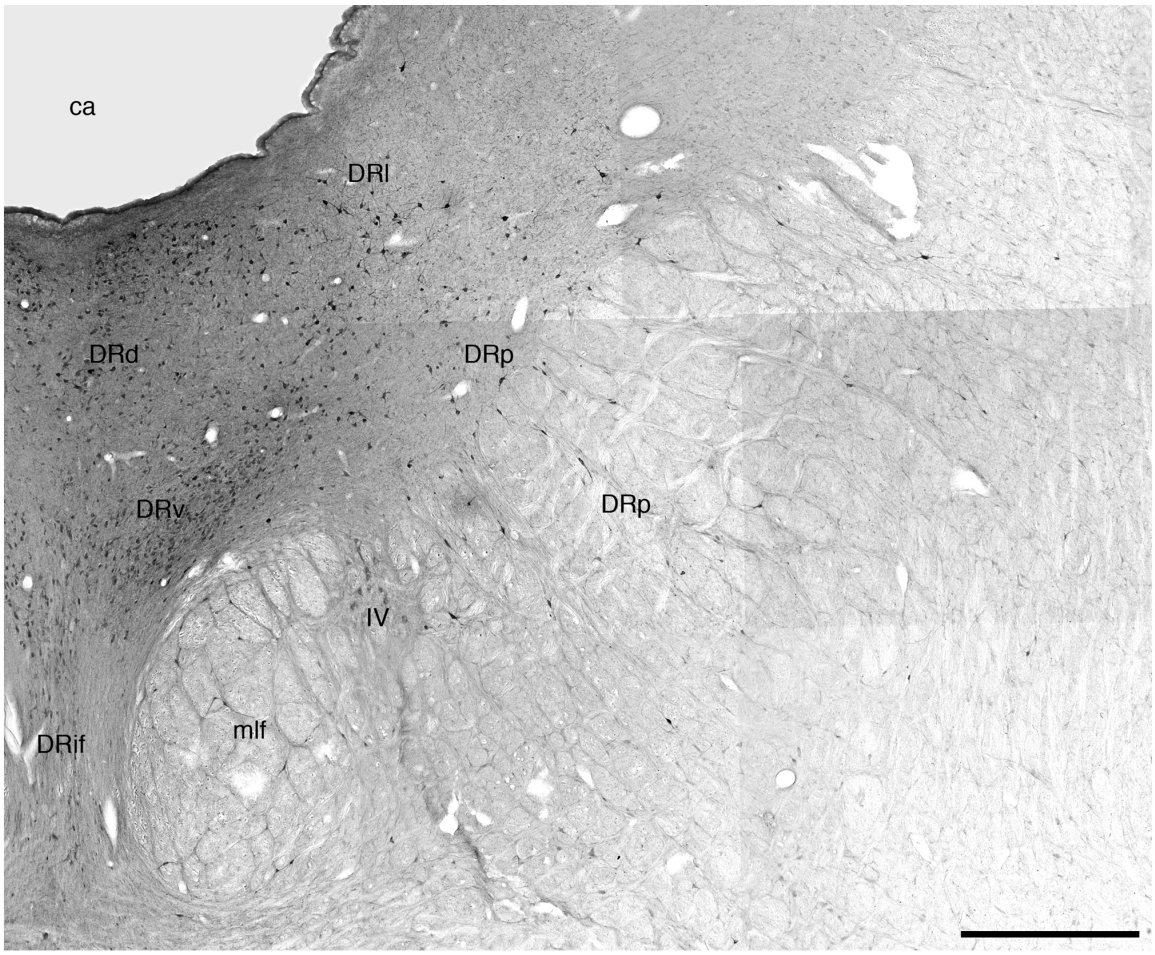
The DRl was found to be a more rostral complex, and lay adjacent to the ventrolateral border of the cerebral aqueduct and dorsolateral to the DRd. The cell bodies radiated laterally from the peri-aqueductal grey matter. This nuclear complex demonstrated a high neuronal density, with ovoid multipolar neurons that showed no specific dendritic orientation. The cell bodies were large and similar in size to those found in the DRp (Figs. 1.3M – O, 4).

The DRp was located inferolateral to the DRd and inferior to the DRl. The DRp was located superolateral to the median longitudinal fasciculus and the oculomotor nucleus (III). Neuronal density for this complex was moderated and the neuronal morphology was consistent with that described for the DRl (Figs. 1.3M – O, 4).

The DRc was a caudal continuation of the DRl (Dell et al., 2016) and lay within the periventricular grey matter abutting the ventrolateral border of the fourth ventricle (4V). It lay medial to the LDT and superolateral to the median raphe nucleus. The trochlear nucleus (IV) was located more laterally and lay inferior to the fifth mesencephalic nucleus. Neurons displayed the same morphology as the DRl and DRp with neuronal density moderate in the plains zebra and donkey, low in the mountain zebra and horse (Figs. 1.3M – O, 4).

Figure 4

Low magnification photomicrographic montage of the ventral and lateral periaqueductal grey matter and adjacent midbrain tegmentum showing five subdivisions of the dorsal raphe nuclear complex in the domestic donkey. Straddling the midline beneath the cerebral aqueduct, the dorsal (**DRd**), ventral (**DRv**) and interfascicular (**DRif**) divisions of the dorsal raphe can be observed. Within the periaqueductal grey matter, dorsal and lateral to the **DRd**, the lateral division (**DRl**) was observed. The peripheral (**DRp**) division was observed lateral to the **DRv** within the periaqueductal grey matter, extending into the adjacent tegmentum. Medial is to the left of the image, dorsal to the top. Scale bar = 1 mm. **IV** – trochlear nucleus, **mlf** – medial longitudinal fasciculus.


ca
DRl
DRd
DRp
DRv
DRp
IV
mlf
DRif

4.5 Orexinergic nuclei of the hypothalamus

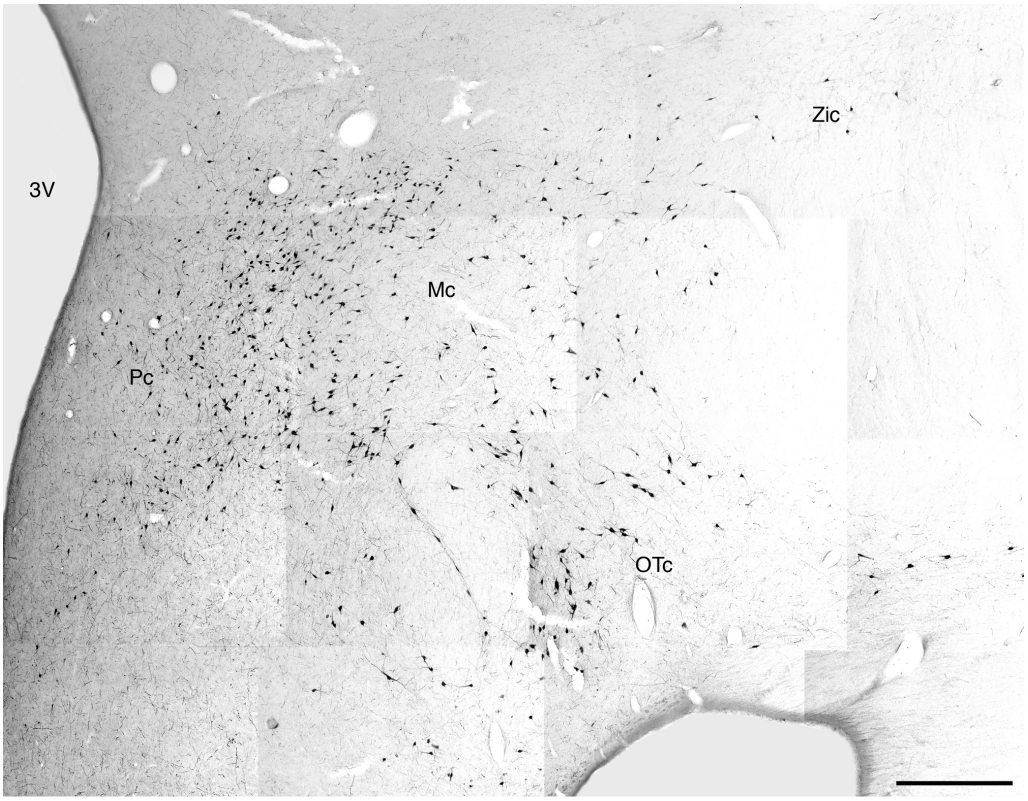
Orexinergic neurons (OxA+) of the hypothalamus were found to be confined to two main groups: a medial parvocellular complex (Pc) and a more lateral magnocellular complex. The latter could be further divided into three sub-divisions: the main cluster (Mc), the zona incerta cluster (Zic), and the optic tract cluster (OTc). Both parvo- and magnocellular complexes demonstrated a similar rostro-caudal extension, beginning rostrally at the caudal extent of the olfactory tubercle (TOL), the N.Bas and the putamen, where the caudate nucleus transitioned from its body to the superior part of its tail. These complexes extended caudally to the level of the mammillary bodies and the habenular nucleus. All divisions were identified and described in all four equids investigated in this study (Figs. 1.1C – F, 5, 6).

The Pc radiated supero- and inferomedially from the main cluster following the lateral border of the third ventricle (3V) and approximated the medial border of the fornix. Neurons were less immunoreactive (as indicated by lighter staining), mostly multipolar, with some being bipolar, and mainly ovoid in shape with some fusiform. In general, there was no specific dendritic orientation, with a weak parallel affiliation to the walls of the 3V and border of the fornix, as cell bodies approximated these structures. Neuronal density was found to be moderate for the donkey, horse and plains zebra, with a moderate to high density for the mountain zebra (Figs. 1.1C – F, 5, 6).

Located lateral to the border of the 3V slightly above the fornix, the Mc was heterogeneous. This magnocellular group predominated and was more immunoreactive. As the cell bodies radiated supero- and inferolaterally from the Mc, they became smaller and slightly less immunoreactive in all species except the plains zebra, where the cell bodies remained magnocellular. Neurons were mostly oval shaped and multipolar with some fusiform bipolar cell bodies (mainly found around the superior border of the fornix). Dendritic orientation was non-specific and the neuronal density was high, while all radiations demonstrated a more moderate compact pattern (Figs. 1.1C – E, 5, 6).

Figure 5

Low magnification photomicrographic montage of the region of the hypothalamus of the plains zebra containing orexin-A immunopositive neurons. Note the four specific clusters, medially the parvocellular cluster (**Pc**), in the perifornical region the main cluster (**Mc**), in the dorsolateral region of the hypothalamus the zona incerta cluster (**Zic**) and in the ventrolateral region of the hypothalamus the optic tract cluster (**OTc**). Cell sizes in the **Mc**, **Zic** and **OTc** are larger than those in the **Pc**. Scale bar = 1 mm.


3V
Zic
Mc
Pc
OTc

The OTc was found to be a moderate radiation of OxA+ neurons that ran rostrally along the ventrolateral border of the fornix adjacent to the optic tract, and closer to the cerebral peduncle more caudally. In the donkey, cell bodies were fusiform and mostly bipolar, evincing a dorsoventral dendritic orientation. There were a few ovoid multipolar neurons that showed no specific dendritic orientation. Neuronal density in the donkey was moderate. In the horse and both species of zebra, neurons were mostly ovoid and multipolar with no specific dendritic orientation. In contrast to the donkey there were a few bipolar fusiform neurons showing a dorsoventral dendritic orientation and neuronal density was observed as moderate to high (Figs. 1.1C and D, 5, 6).

The Zic cluster was a superolateral extension of the Mc and lay inferior to the dorsal thalamus. It radiated superior to the cerebral peduncle and towards the inferior extent of the TRN. Neuronal density was moderately low becoming very low as the neurons migrated more laterally. Neurons were ovoid and multipolar with no specific dendritic orientation (Figs. 1.1C and D, 5, 6).

4.6 Tyrosine hydroxylase neurons of the thalamic reticular nucleus

This nucleus was found lateral to the dorsal hypothalamus and medial to the internal capsule and optic tract. As it migrated inferiorly, arching medially towards the 3V and fornix, it was seen to lie superior to the cerebral peduncle. This nucleus was observed extending rostrally from the optic chiasm to the mammillary bodies caudally, and shortened superiorly in its rostral extent. TH+ neurons were of a variety of shapes: ovoid, fusiform and spherical, mostly multipolar with no dendritic orientation. In the donkey, density was moderate to high and neurons clustered in reticulated groups. As the nucleus radiated inferomedially, neurons became slightly less immunoreactive. The horse and mountain zebra showed reticulated clusters of a moderate density. The plains zebra showed a low-density reticulated pattern. Neuronal cell body size varied considerably between species throughout this nucleus with magnocellular neurons scattered amongst larger populations of parvocellular groups (Figs. 1.1D – F, 7 – 10).

Figure 6

High magnification photomicrographs of the parvocellular (**A**, **C**, **E**, **G**) and magnocellular (**B**, **D**, **F**, **H**) orexinergic neuron types in the hypothalamus of the domestic donkey (**A**, **B**), domestic horse (**C**, **D**), mountain zebra (**E**, **F**) and plains zebra (**G**, **H**). Note the consistent difference in size between the two cell types across the four species of equids. Scale bar in **H** = 50 μm and applies to all.

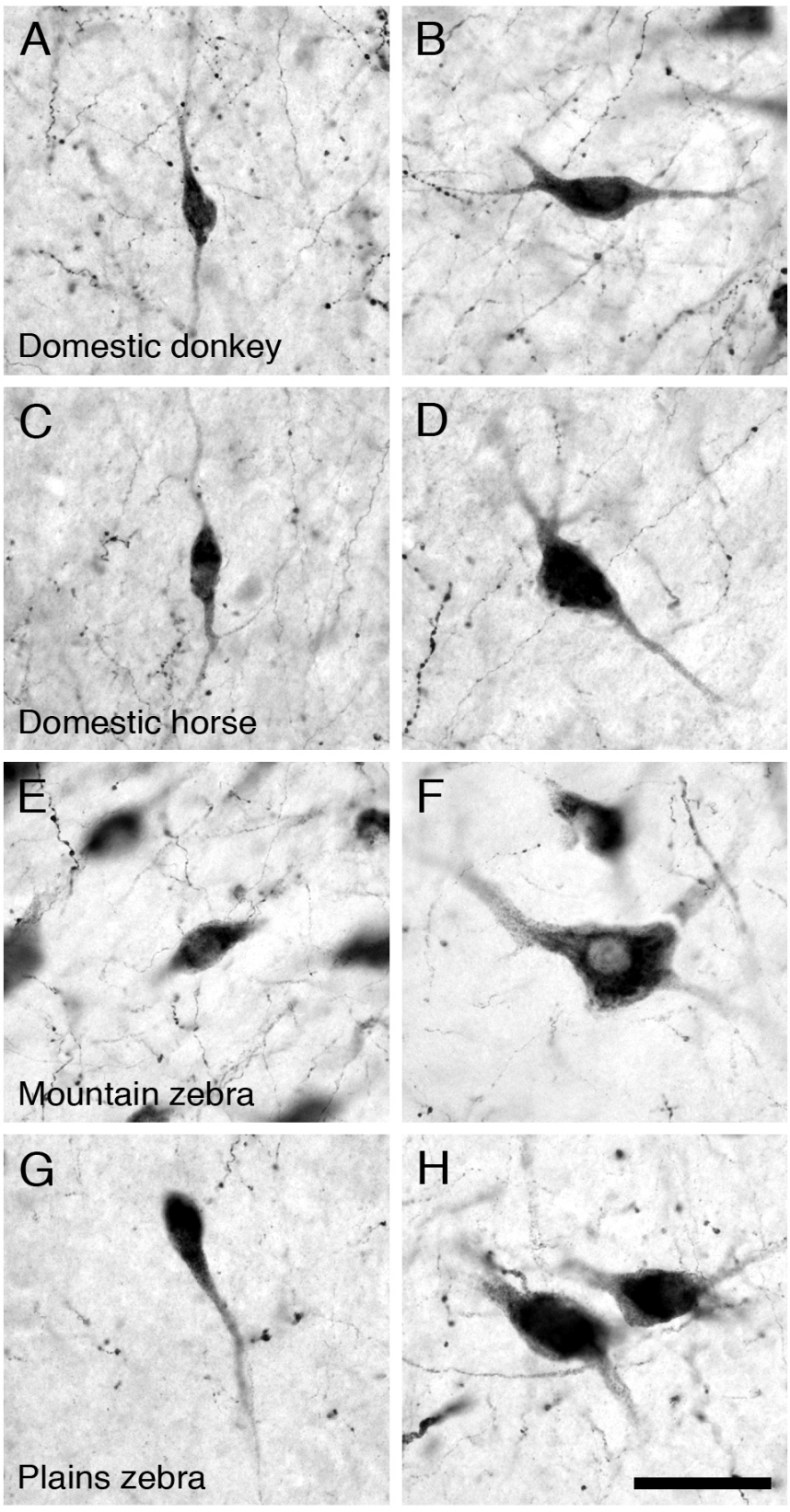

A
Domestic donkey
B
C
Domestic horse
D
E
Mountain zebra
F
G
Plains zebra
H

Figure 7

Low power photomicrographs of the thalamic reticular nucleus (**TRN**) of the domestic donkey in the coronal plane stained for Nissl bodies (**A**), myelin (**B**) and immunostained for tyrosine hydroxylase (**C**). Note the typically mammalian location of the thalamic reticular nucleus, surrounding the lateral edge of the nuclear mass that forms the dorsal thalamus (**DT**); however, the neurons of the thalamic reticular nuclear are immunopositive for tyrosine hydroxylase, indicating that in addition to being GABAergic neurons, the neurons of the thalamic reticular nucleus in equids are also dopaminergic. **NSB** – nigrostriatal bundle. Scale bar in **C** = 5 mm and applies to all.

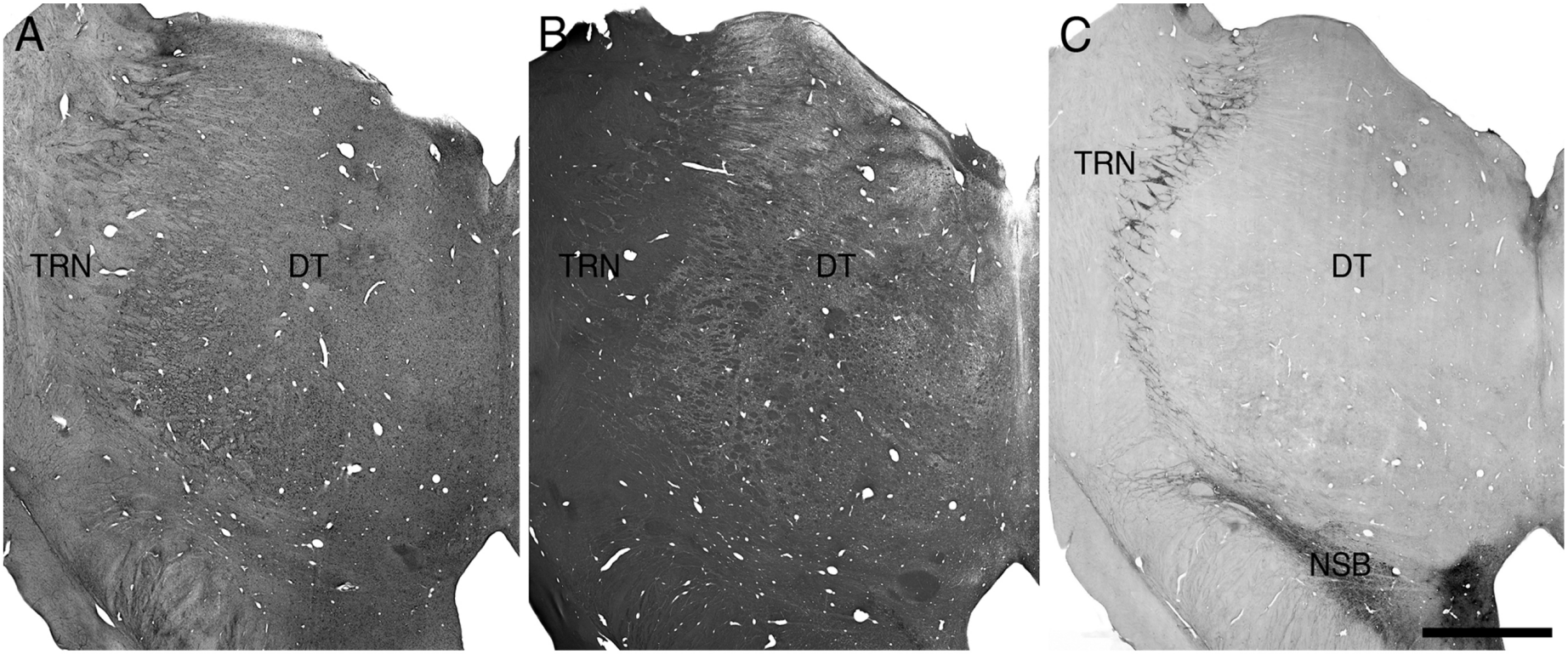

A
TRN
DT
B
TRN
DT
C
TRN
DT
NSB

Figure 8

Photomicrographs of a portion of the thalamic reticular nucleus of the domestic donkey (**A**, **C**, **E**) and domestic horse (**B**, **D**, **F**) stained for Nissl bodies (**A**, **B**) with cresyl violet, immunostained for tyrosine hydroxylase (**C**, **D**), and immunostained for parvalbumin (**E**, **F**). Note the atypical presence of tyrosine hydroxylase immunoreactive neurons in the thalamic reticular nucleus of both species, which appear to be the same neurons as those typically immunoreactive in mammals for parvalbumin. In all images dorsal is to the top and medial to the left. Scale bar in **F** = 500 μm and applies to all.

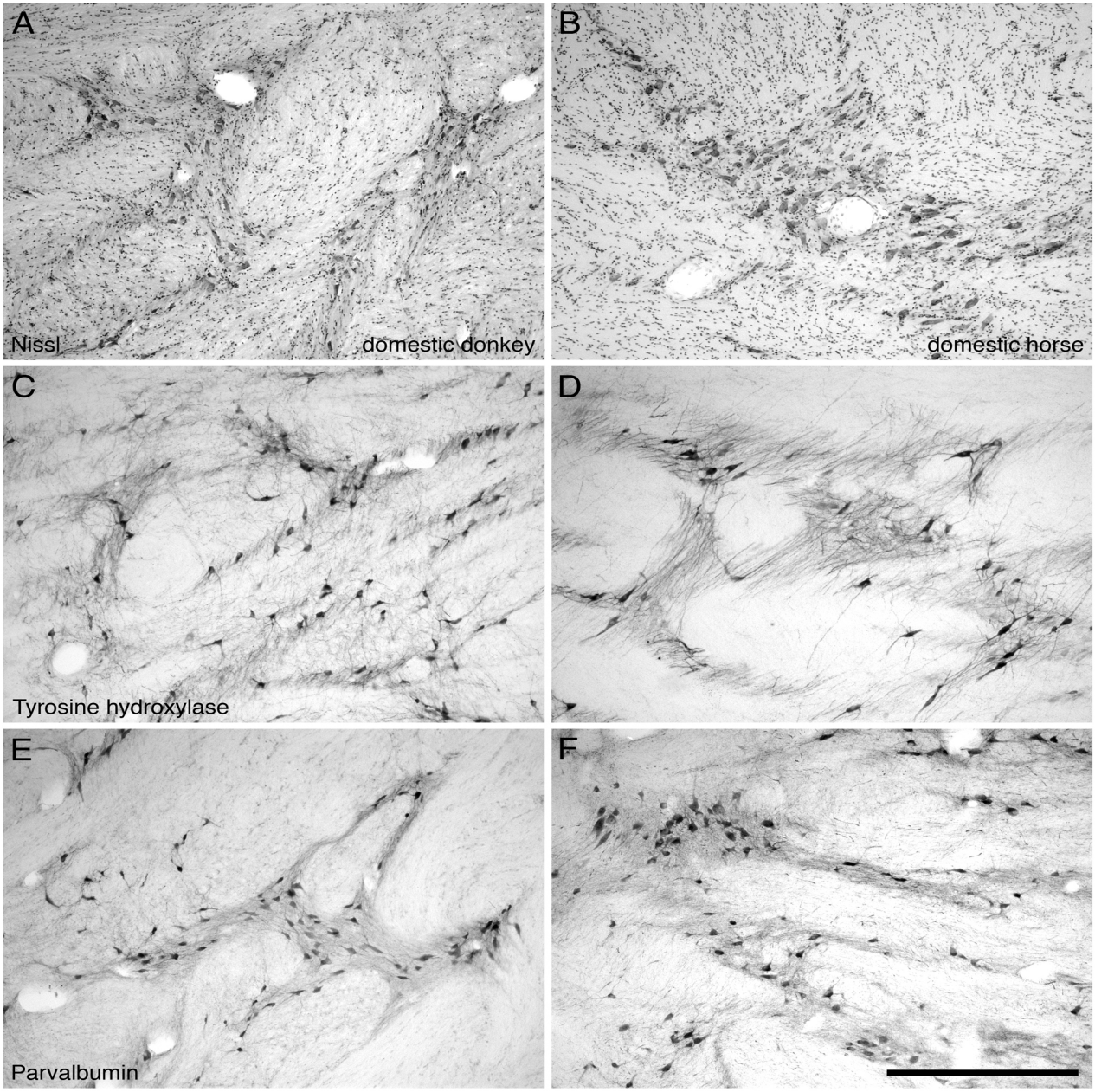

A
Nissl
domestic donkey
B
domestic horse
C
Tyrosine hydroxylase
D
E
Parvalbumin
F

Figure 9

Photomicrographs of a portion of the thalamic reticular nucleus of the mountain zebra (**A**, **C**, **E**) and plains zebra (**B**, **D**, **F**) stained for Nissl bodies (**A**, **B**) with cresyl violet, immunostained for tyrosine hydroxylase (**C**, **D**), and immunostained for parvalbumin (**E**, **F**). Note the atypical presence of tyrosine hydroxylase immunoreactive neurons in the thalamic reticular nucleus of both species, which appear to be the same neurons as those typically immunoreactive in mammals for parvalbumin. In all images dorsal is to the top and medial to the left. Scale bar in **F** = 500 μm and applies to all.

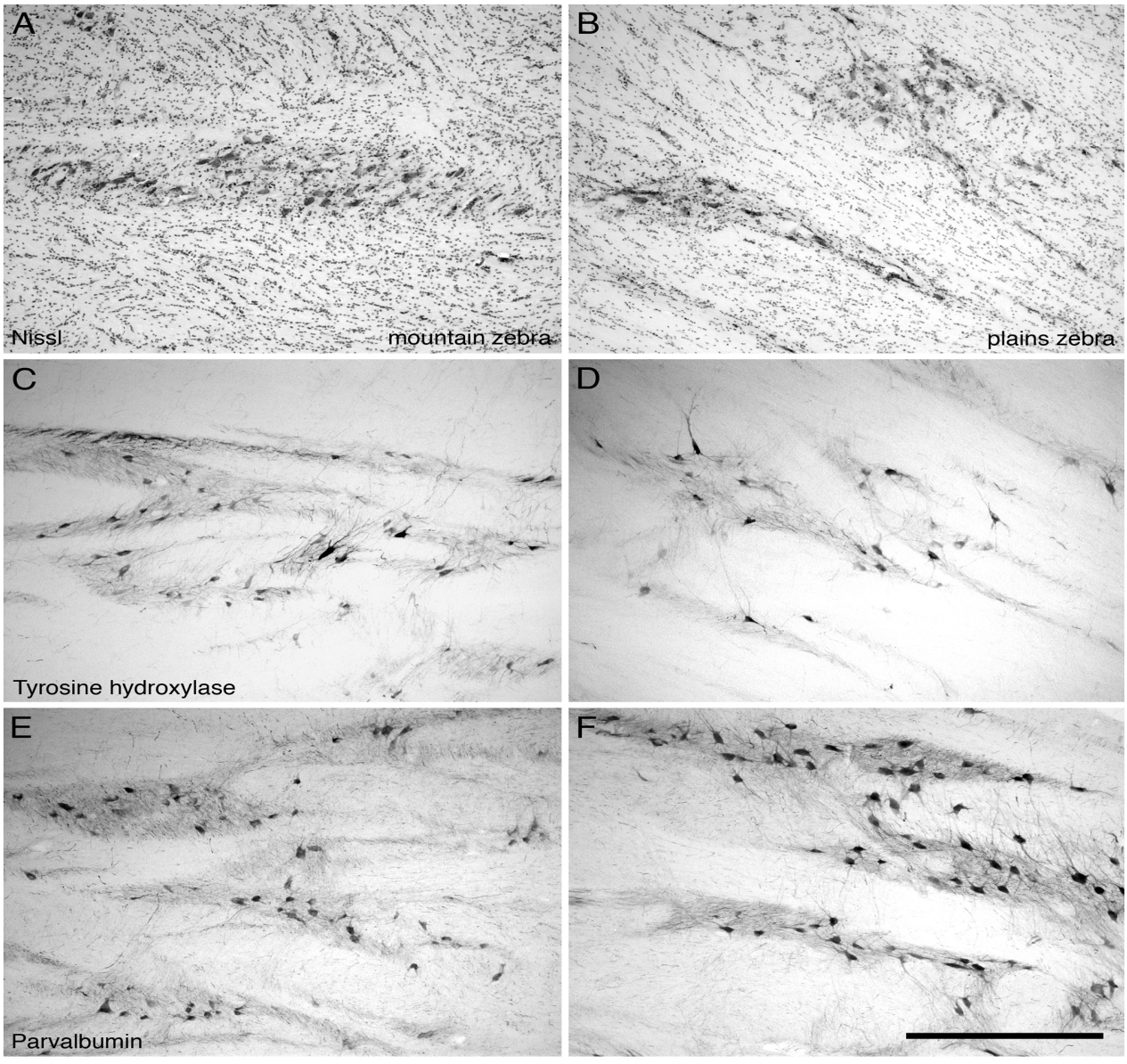
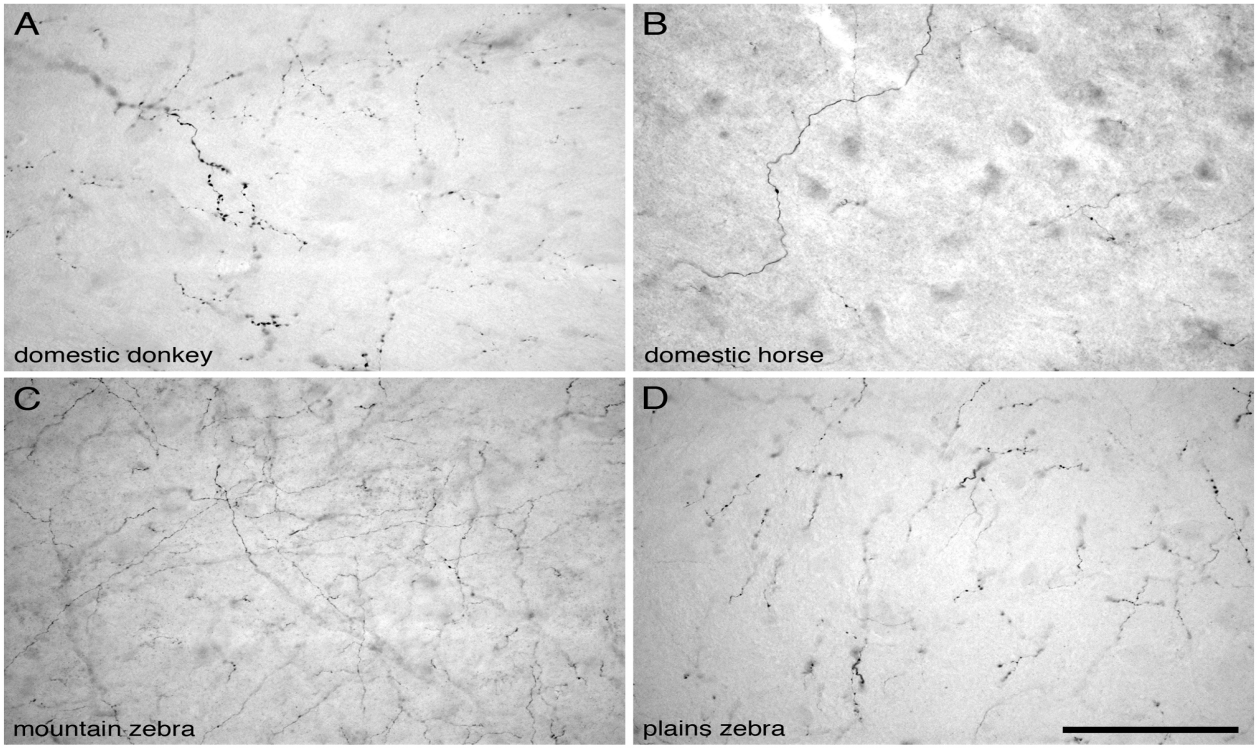

A
Nissl
mountain zebra
B
plains zebra
C
Tyrosine hydroxylase
D
E
Parvalbumin
F

Figure 10

Photomicrographs of tyrosine hydroxylase immunoreactive axons and bouton terminal networks within the grey matter mass that forms the dorsal thalamus in the domestic donkey (**A**), domestic horse (**B**), mountain zebra (**C**) and plains zebra (**D**). These axons appear to emanate from the tyrosine hydroxylase immunoreactive neurons found in the thalamic reticular nucleus of these species, although hodological studies would be needed to confirm this. In all images dorsal is to the top and medial to the left. Scale bar in **D** = 100 μm and applies to all.


A
domestic donkey
B
domestic horse
C
mountain zebra
D
plains zebra

4.7 Neurons and terminal networks containing calcium binding proteins

Immunohistochemical staining of Calbindin (CB), Calretinin (CR) and Parvalbumin (PV) revealed that these calcium binding proteins behaved as immunoreactive tags for different types of GABAergic neurons (Bhagwandin et al., 2013; Dell et al., 2016). A qualitative description of these populations of CB+, CR+ and PV+ neurons corresponding to the sleep-wake nuclei of the basal forebrain, hypothalamus and brainstem as previously described in this chapter was undertaken. Neuron density and co-localised terminal network density (neuropil), as summarised in Tables 1, 2 and 3 (see end of chapter, pg. 76), were described for all four species of equid and any differences found were recorded per species.

4.7.1 Neurons and terminal networks of the basal forebrain containing calcium binding proteins

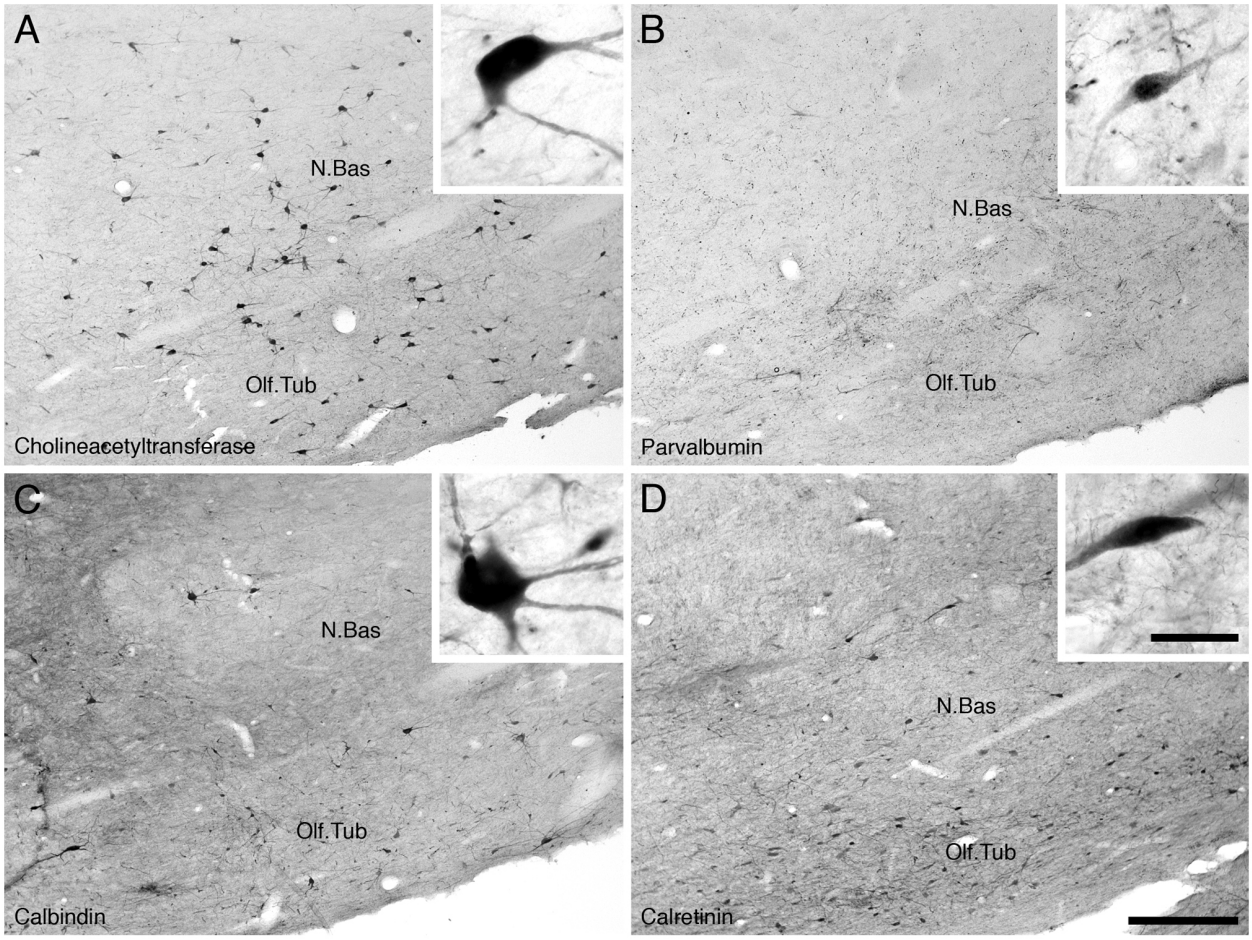
The Sep.M contained a low density of CB+ neurons and a low to moderately dense neuropil for all four species. CR+ neuronal density of the Sep.M for the donkey and horse was low, and low to moderate in both the plains and mountain zebra. The corresponding neuropil for the donkey and horse was absent or very low, for the plains zebra low, and for the mountain zebra low to moderate. PV+ neurons of the Sep.M showed an absent to low pattern for both neuronal density and neuropil in all four animals (Fig. 11).

The Diag.B contained a low to moderate density of CB+ neurons for the donkey, a moderate to high neuronal density for the horse and plains zebra, and a moderate neuronal density for the mountain zebra. The neuropil for the donkey and both zebra was moderate and moderate to high for the horse. The CR+ neuron density and neuropil were moderate for all the species, except for the mountain zebra, which evinced a moderate to high neuronal density and neuropil. PV+ neuron density was moderate for the donkey, low to moderate for the horse, and low for both species of zebra. The neuropil was moderate for the donkey, low to moderate for the horse, low for the plains zebra, and absent to low for the mountain zebra (Fig. 11).

CB+ neuron density and neuropil in the Is.CALL/TOL was moderate for all four species. CR+ neuron density was moderate to high for all the species, with a neuropil ranging from moderate for the donkey, moderate to high for the horse and

Figure 11

Low (main image) and high (inset) magnification photomicrographs of the olfactory tubercle (**Olf.Tub**) and nucleus basalis (**N.Bas**) cholinergic neurons in the basal forebrain of the domestic horse. **A**. Neurons immunoreactive for choline acetyltransferase showing the olfactory tubercle (**Olf.Tub**) and part of nucleus basalis (**N.Bas**). **B**. Parvalbumin immunoreactivity, note the low density parvalbumin immunoreactive cells and terminal networks in this region of the horse brain. **C**. Calbindin immunoreactivity, note the low to moderate density of cells and terminals in this region of the brain. **D**. Calretinin immunoreactivity, note the moderate density of cells and terminals in this region of the brain. Scale bar in **D** = 500 μm and applies to all, scale bar in **D** inset = 50 μm and applies to all insets. In all images medial is to the left and dorsal to the top.


A
N.Bas
Olf.Tub
Cholineacetyltransferase
B
N.Bas
Olf.Tub
Parvalbumin
C
N.Bas
Olf.Tub
Calbindin
D
N.Bas
Olf.Tub
Calretinin

plains zebra, to high for the mountain zebra. The donkey and horse demonstrated a low to moderate PV+ neuronal density, while the plains and mountain zebra evinced a low neuronal density. The PV+ neuropil for this nucleus was high for the donkey, moderate to high for the horse, low for the plains zebra, and moderate for the mountain zebra (Fig. 11).

The N.Bas showed a low to moderate CB+ neuronal density and a moderate to high neuropil across the board in all four species. The donkey and the plains zebra showed a moderate CR+ neuronal density, while the horse and the mountain zebra demonstrated a low CR+ neuronal density. The neuropil was moderate for all the species except the donkey, which showed a moderate to high CR+ terminal network density. PV+ neuron density and neuropil was low for all animals with the exception of a moderate neuronal density for the plains zebra (Fig. 11).

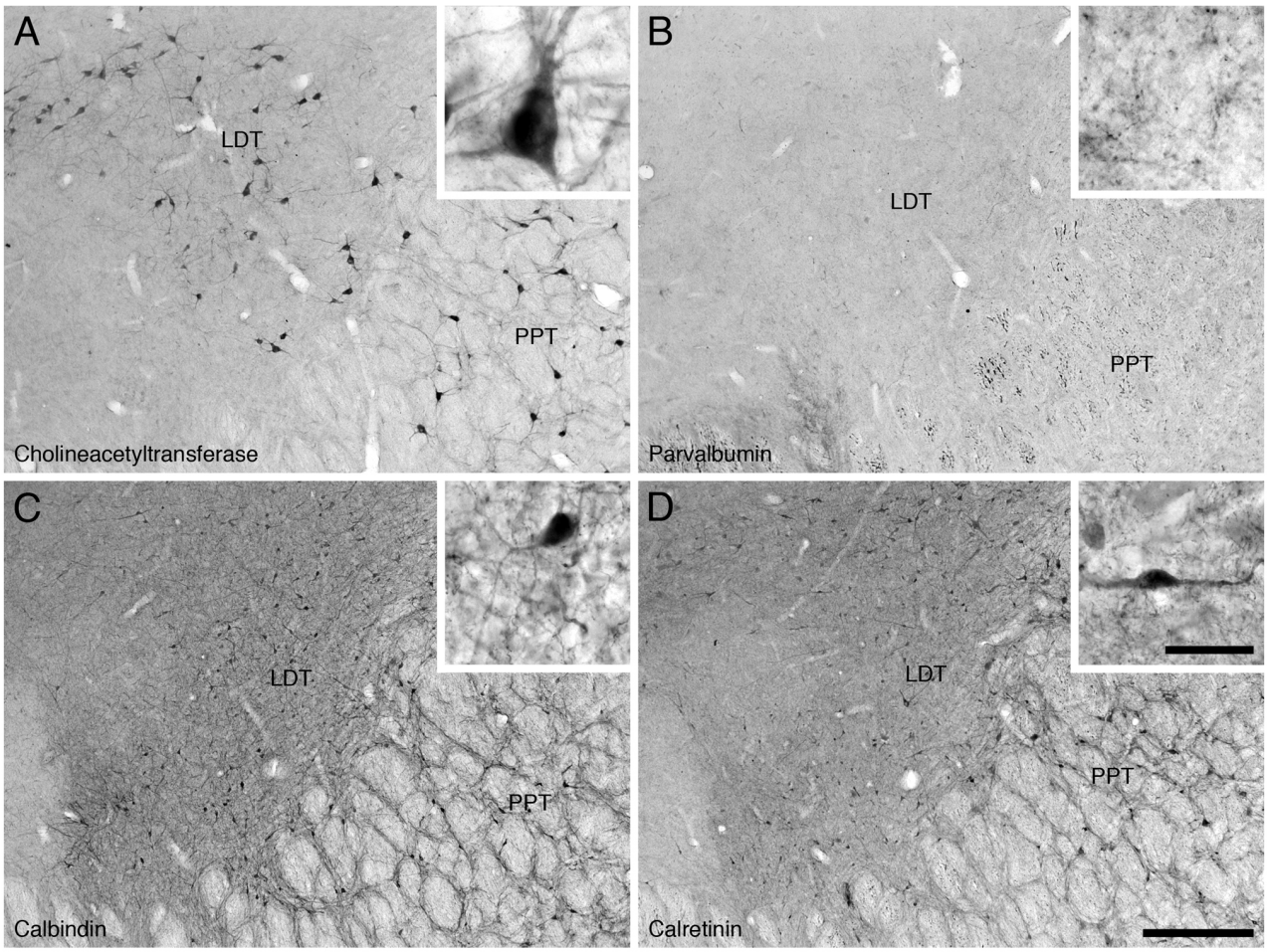
4.7.2 Neurons and terminal networks of the pontomesencephalic complex containing calcium binding proteins

CB+ neuron and terminal network density of the PPT were moderate for all the species bar the donkey, which displayed a low to moderate neuronal density for this protein. CR+ neuronal density and neuropil were moderate for all species with the exception of neuronal density in the horse, which was considered to be moderate to high. PV+ neuronal density in the PPT was low to moderate for the donkey, low for the horse, and absent to low for both species of plains zebra. The neuropil was low for both donkey and horse, and absent to low for both zebra (Fig. 12).

CB+ neuronal density in the LDT was moderate, and the neuropil was moderate to high for all four animals. The LDT had a more varied CR+ pattern with a moderate to high neuronal density in the donkey and horse, a moderate neuronal density in both species of zebra, and a moderate to high neuropil for all the species except the donkey, which demonstrated a high neuropil. PV+ neuronal density for the horse, and both zebra was absent or low, and moderate for the donkey. The neuropil for the horse and mountain zebra was absent or low, and low for the plains zebra and donkey (Fig. 12).

Figure 12

Low (main image) and high (inset) magnification photomicrographs of the laterodorsal tegmental (**LDT**) and pedunculopontine tegmental (**PPT**) cholinergic nuclei in the pons of the domestic horse. **A**. Neurons immunoreactive for choline acetyltransferase showing the laterodorsal tegmental nucleus (**LDT**) and part of the pedunculopontine tegmental nucleus (**PPT**). **B**. Parvalbumin immunoreactivity, note the absence of parvalbumin immunoreactive cells, but a moderately dense parvalbumin immunoreactive terminal network in these nuclei. **C**. Calbindin immunoreactivity, note the moderate density of cells and terminals in these nuclei. **D**. Calretinin immunoreactivity, note the moderate density of cells and terminals in these nuclei. Scale bar in **D** = 500 µm and applies to all, scale bar in **D** inset = 50 µm and applies to all insets. In all images medial is to the left and dorsal to the top.


A
LDT
PPT
Cholineacetyltransferase
B
LDT
PPT
Parvalbumin
C
LDT
PPT
Calbindin
D
LDT
PPT
Calretinin

4.7.3 Neurons and terminal networks of the locus coeruleus complex containing calcium binding proteins

The CB+ neuron density of the subcoeruleus complex pars compacta (A7sc) was absent to low in both the donkey and horse. Neuronal density in both zebra was low to moderate. The neuropil for the donkey, horse and mountain zebra was moderate while the plains zebra was moderate to high. CR+ neuronal density was more varied, being low for the donkey, moderate for the horse and plains zebra, and moderate to high for the mountain zebra. The neuropil was moderate for the donkey and mountain zebra, and low to moderate for the horse and plains zebra. The PV+ neuronal density was moderate for the donkey and plains zebra, low to moderate for the horse and absent to low in the mountain zebra. The neuropil was moderate for the donkey, absent to low for the horse and mountain zebra, and low to moderate for the plains zebra (Fig. 13).

CB+ neuronal density of the subcoeruleus pars diffuse (A7d) was low for both the donkey and horse. In the plains zebra neuronal density was moderate to high, and in the mountain zebra, neuronal density was low to moderate. The neuropil was moderate for the donkey, horse and mountain zebra. The plains zebra demonstrated a moderate to high neuropil. CR+ neuronal density was low to moderate in the donkey, moderate in the horse, and moderate to high in both species of zebra. The neuropil was low to moderate in the donkey and horse and moderate to high for both species of zebra. PV+ neuron density in the donkey was low, absent or low in the horse and mountain zebra, and moderate in the plains zebra. The neuropil was low to moderate in the donkey, absent to low in the horse and mountain zebra, and low in the plains zebra (Fig. 13).

The locus coeruleus pars diffuse (A6d) showed a low CB+ neuronal density for the donkey, horse and plains zebra, and an absent to low density for the mountain zebra. The neuropil was moderate to high for all four species except for a moderate neuropil recorded for the plains zebra. Neuronal density for CR+ in the donkey and horse was low, absent to low in the plains zebra, and moderate in the mountain zebra. The neuropil was moderate to high throughout. PV+ neuronal density was moderate for the donkey, low to moderate for the horse, low for the plains zebra, and absent or

low for the mountain zebra. The neuropil was moderate for the donkey, moderate to high for the horse, high for the plains zebra and low for the mountain zebra (Fig. 13).

4.7.4 Neurons and terminal networks of the dorsal raphe complex containing calcium binding proteins

The DRif had a low to moderate CB+ neuronal density for all the species bar the mountain zebra. This species demonstrated a more elevated density of moderate to high. The neuropil was moderate to high for all species with the zebra being the exception. This animal showed a moderate neuropil. CR+ neurons were absent or very low for both the donkey and the plains zebra, low for the mountain zebra, and low to moderate for the horse. The neuropil was moderate for the donkey and plains zebra, and moderate to high for the horse and mountain zebra. PV+ neuronal density was absent in all the species, with a low neuropil for the donkey and horse, and an absent or very low neuropil for both zebra (Fig. 14).

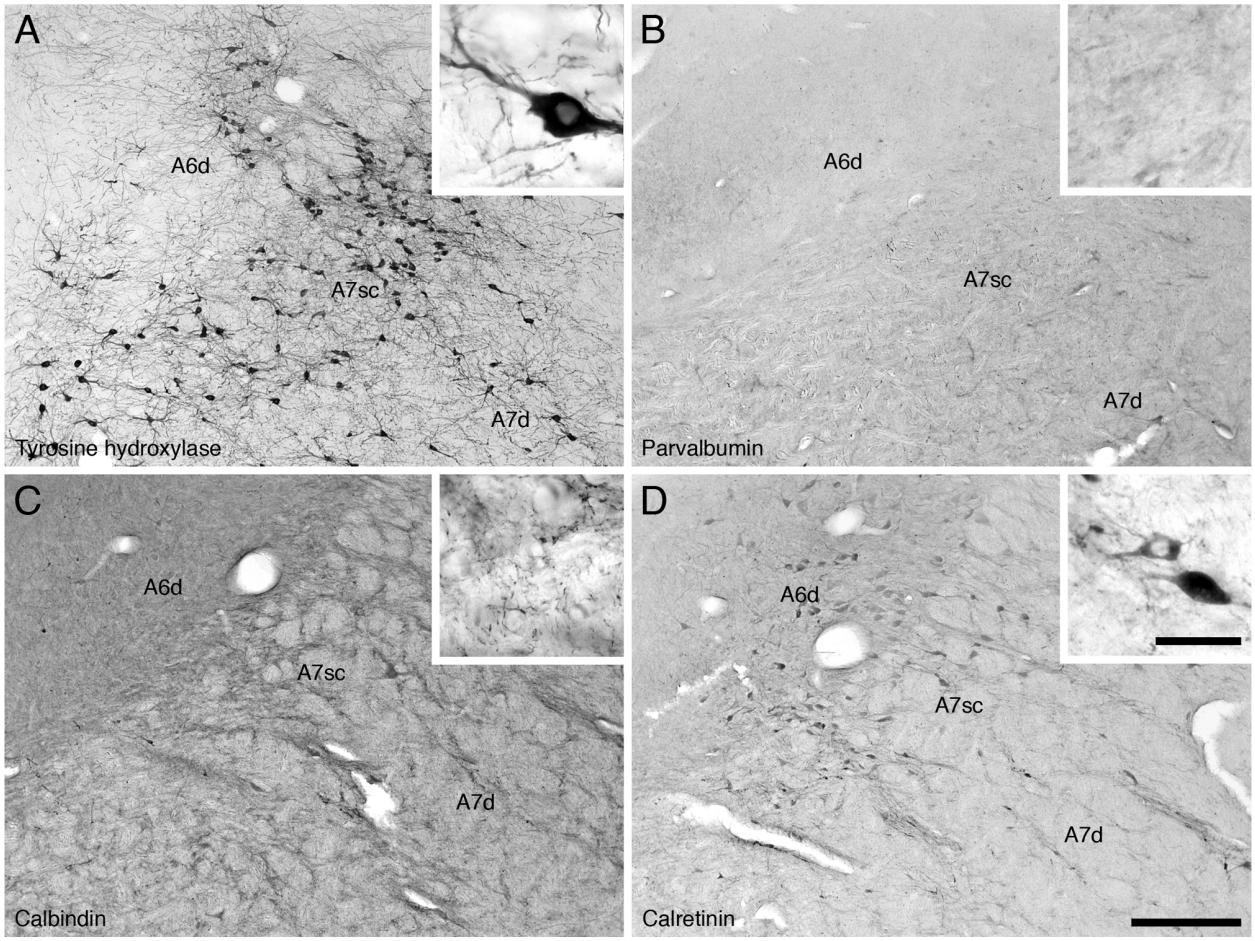
CB+ neuron density in the DRv was low to moderate for all the species with the exception of the mountain zebra. This animal demonstrated a moderate to high neuronal density. The neuropil was moderate to high for all species. CR+ neuron density was absent to very low in the donkey, moderate to high in the horse, and low to moderate for both zebra. The neuropil was moderate in the donkey, and moderate to high for the other three species. PV+ neuron density and neuropil were the same in DRv as observed in the DRif (Fig. 14).

The DRd showed the same CB+ neuronal density and neuropil pattern as the DRv in all four species. The CR+ neuron densities and neuropil in the DRd was the same as seen in the DRv except for the neuron density of the mountain zebra that was moderate. PV+ neuronal densities and neuropil remained the same as the DRif and DRv (Fig. 14).

The CB+ neuronal density was moderate in the donkey, moderate to high in the horse and mountain zebra, and low to moderate in the plains zebra in the DRl. The neuropil was moderate to high across the board. CR+ neuron density in the DRl was low to moderate in the donkey and the plains zebra, moderate in the horse, and absent

Figure 13

Low (main image) and high (inset, from the compact portion of the subcoeruleus) magnification photomicrographs of the locus coeruleus complex in the pons of the plains zebra. **A**. Neurons immunoreactive for tyrosine hydroxylase showing the diffuse portion of the locus coeruleus (**A6d**), the compact portion of the subcoeruleus (**A7sc**) and the diffuse portion of the subcoeruleus (**A7d**). **B**. Parvalbumin immunoreactivity, note the absence of parvalbumin immunoreactive cells and terminal networks in these nuclei. **C**. Calbindin immunoreactivity, note the lack of immunoreactive cells, but the presence of a low to moderate density of terminals in these nuclei. **D**. Calretinin immunoreactivity, note the low to moderate density of cells and terminals in these nuclei. Scale bar in **D** = 500 μm and applies to all, scale bar in **D** inset = 50 μm and applies to all insets. In all images medial is to the left and dorsal to the top.


A
A6d
A7sc
A7d
Tyrosine hydroxylase
B
A6d
A7sc
A7d
Parvalbumin
C
A6d
A7sc
A7d
Calbindin
D
A6d
A7sc
A7d
Calretinin

to low in the mountain zebra. The neuropil was moderate to high for the donkey, horse and mountain zebra, and moderate for the plains zebra. PV+ neuronal density and neuropil remained the same as observed in the DRif, DRv and DRd for the four species (Fig. 14).

The DRp had a moderate to high CB+ neuron density and neuropil for all animals except for the neuropil for the donkey that demonstrated a moderate density. CR+ neuron density was moderate in the donkey and horse, low to moderate in the plains zebra, and low in the mountain zebra. The neuropil was moderate bar the donkey that showed a moderate to high neuropil. As described for the other dorsal raphe nuclei, PV+ neurons were absent in all species and the neuropil was low for the donkey and horse, and absent to low for both species of zebra (Fig. 14).

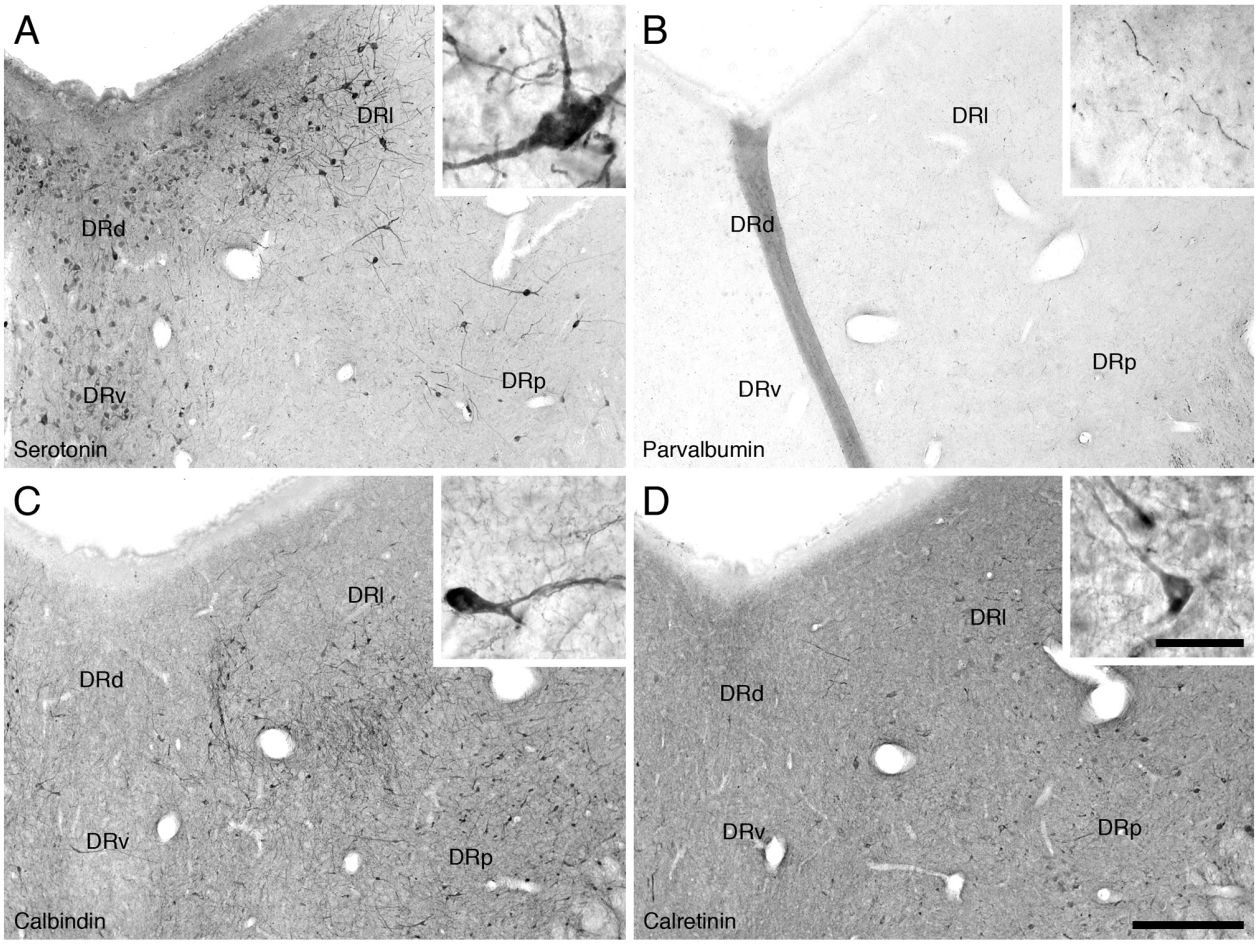
The DRc had a moderate CB+ neuronal density in the donkey, a low density in the horse and mountain zebra, and a low to moderate density in the plains zebra. The neuropil was moderate to high for the donkey and plains zebra, high for the horse, and low to moderate for the mountain zebra. CR+ neuronal density was absent to low in the donkey and plains zebra, moderate in the horse, and low in the mountain zebra. The neuropil was moderate to high in the donkey and horse, moderate in the plains zebra, and high in the mountain zebra. PV+ neurons were again absent in all species with a low neuropil for the horse and donkey and an absent to low neuropil for both zebra (Fig. 14).

4.7.5 Neurons and terminal networks of the hypothalamic orexinergic complex containing calcium binding proteins

The Pc cluster showed a moderate CB+ neuronal density and a moderate to high neuropil in all species. The CR+ neuronal density was low to moderate in the donkey and plains zebra, absent to low in the horse, and moderate to high in the mountain zebra. The neuropil was varied, being moderate in the donkey, low to moderate in the horse, low in the plains zebra, and moderate to high in the mountain zebra. PV+ neuronal density was absent for all the species. The neuropil was absent to low in all species (Fig. 15).

Figure 14

Low (main image) and high (inset) magnification photomicrographs of some divisions of the dorsal raphe nuclear complex in the midbrain of the mountain zebra. **A**. Neurons immunoreactive for serotonin showing the dorsal (**DRd**), ventral (**DRv**), lateral (**DRl**) and peripheral (**DRp**) divisions of the dorsal raphe complex. High power inset shows a neuron from the **DRl**. **B**. Parvalbumin immunoreactivity, note the absence of parvalbumin immunoreactive cells, but a low density parvalbumin immunoreactive terminal network, especially so in the **DRl** (inset). **C**. Calbindin immunoreactivity, note the moderate density of cells and terminals in these divisions, although the density of calbindin immunopositive structures appears lower in the **DRd** and **DRv** divisions. Inset from the **DRl**. **D**. Calretinin immunoreactivity, note the low to moderate density of cells and terminals in these nuclei, which again appears lower in the **DRd** and **DRv**. Scale bar in **D** = 500 μm and applies to all, scale bar in **D** inset (all taken from the **DRl**) = 50 μm and applies to all insets. In all images medial is to the left and dorsal to the top.


A
DRl
DRd
DRv
DRp
Serotonin
B
DRl
DRd
DRv
DRp
Parvalbumin
C
DRl
DRd
DRv
DRp
Calbindin
D
DRl
DRd
DRv
DRp
Calretinin

The Mc showed a moderate to high CB+ neuron density and neuropil in all the species with the exception of the neuropil for the donkey, which was moderate. The CR+ neuron density was moderate for the donkey, horse and plains zebra and moderate to high in the mountain zebra. The neuropil was moderate for the donkey and horse, moderate to low for the plains zebra, and moderate to high for the mountain zebra. PV+ neuronal densities and neuropil were the same as observed in the Pc for all four animals (Fig. 15).

CB+ neuron density in the OTc was moderate to high for the donkey, low to moderate for the horse, and moderate for both zebra. The neuropil was moderate to high throughout. The CR+ neuronal density and neuropil was moderate for all the species bar the neuronal density in the plains zebra, which was recorded as low to moderate. PV+ neuronal densities and neuropil was the same as observed in the Pc and Mc for all species (Fig. 15).

The Zic CB+ neuronal density was low to moderate for the donkey and both zebra, and moderate in the horse. The neuropil was moderate in the donkey and mountain zebra, moderate to high in the horse, and low to moderate in the plains zebra. CR+ neuron density was low to moderate in all the species except the donkey, which showed a moderate to high density. The neuropil was moderate to high for all the species. PV+ neuron density and neuropil were moderate to high across all four species (Fig. 15).

4.7.6 Neurons and terminal networks of the thalamic reticular nucleus containing calcium binding proteins

There was an absence of CB+ and CR+ neurons in the TRN and the neuropil for both was low to moderate in all four species. PV+ neuronal density was high, and the neuropil was moderate to high in all four animals (Figs. 7 – 10).

Figure 15

Low (main image) and high (inset, from main cluster) magnification photomicrographs of the parvocellular (**Pc**) and main (**Mc**) clusters of orexinergic neurons in the hypothalamus of the domestic donkey. **A**. Neurons immunoreactive for orexin-A showing the parvocellular (**Pc**) and main (**Mc**) clusters. **B**. Parvalbumin immunoreactivity, note the absence of parvalbumin immunoreactive cells, but a low-density parvalbumin immunoreactive terminal network is observed (see inset). **C**. Calbindin immunoreactivity, note the low to moderate density of cells and high density of terminals in this region of the hypothalamus. **D**. Calretinin immunoreactivity, note the low to moderate density of cells and moderate density of terminals in this region of the hypothalamus. Scale bar in **D** = 500 μm and applies to all, scale bar in **D** inset = 50 μm and applies to all insets. In all images medial is to the left and dorsal to the top.

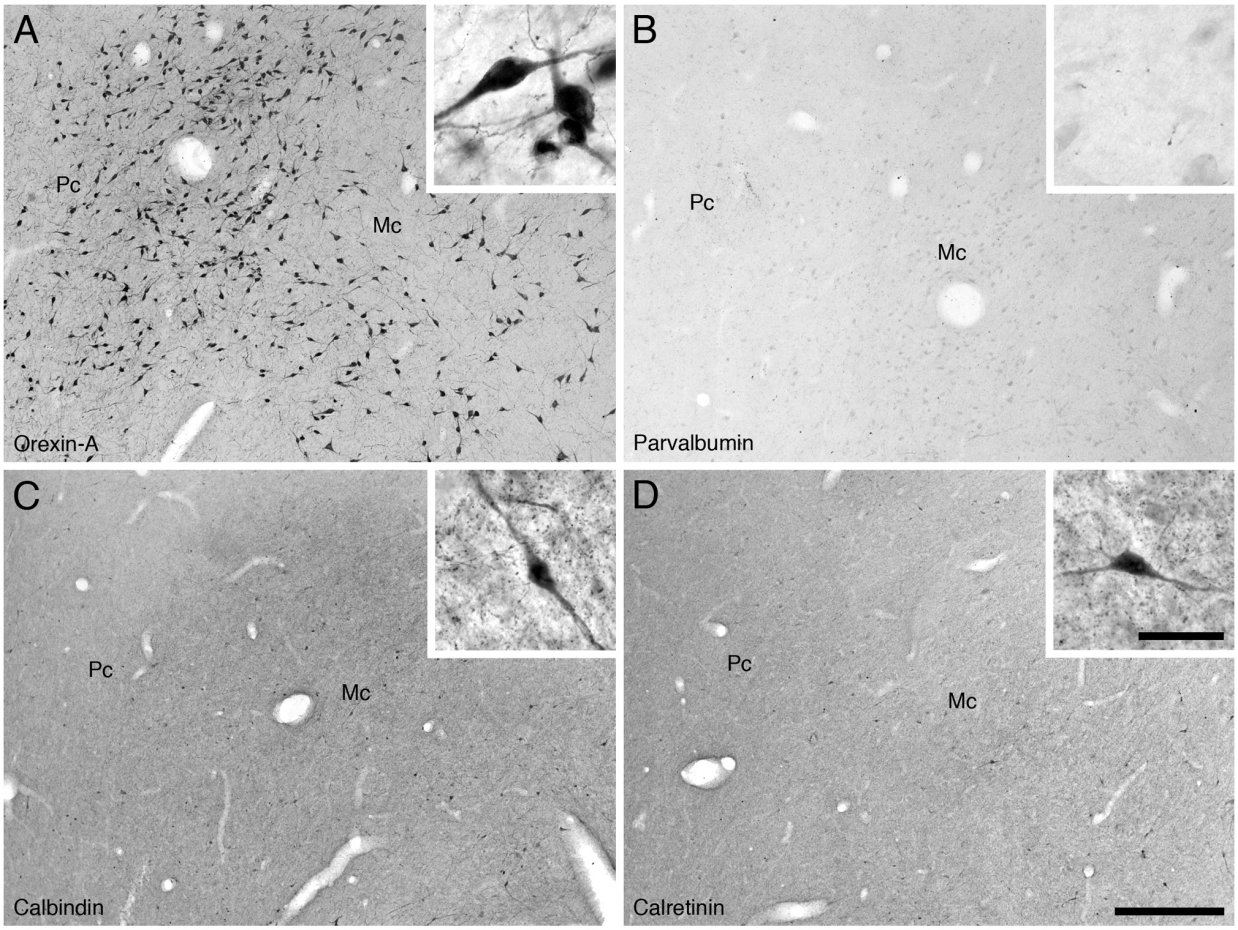

A
Pc
Mc
Orexin-A
B
Pc
Mc
Parvalbumin
C
Pc
Mc
Calbindin
D
Pc
Mc
Calretinin

Table 1. Neuron and terminal network density for the calcium binding protein calbindin (CB) of the GABAergic system
Key: (-) absence. (+) low density. (++) moderate density. (+++) high density.

Sleep-Wake Related Nuclei	Donkey (EAA)		Horse (ECF)		Plains Zebra (EQQ)		Mountain Zebra (EZZ)	
	Neurons	Neuropil	Neurons	Neuropil	Neurons	Neuropil	Neurons	Neuropil
Cholinergic nuclei								
Sep.M	+	+ / ++	+	+	+	+ / ++	+	+ / ++
Diag.B	+ / ++	++	++ / +++	++ / +++	++ / +++	++	++	++
IS.Call and TOL	++	++	++	++ / +++	++	++	++ / +++	++
N.Bas	+ / ++	++ / +++	+ / ++	++ / +++	+ / ++	++ / +++	+ / ++	++ / +++
PPT	+ / ++	++	++	++	++	++	++	++
LDT	++	++ / +++	++	++ / +++	++	++ / +++	++	++ / +++
Catecholaminergic nuclei								
A7sc	- / +	++	- / +	++	+ / ++	++ / +++	+ / ++	++
A7d	+	++	+	++	++ / +++	++ / +++	+ / ++	++
A6d	+	++ / +++	+	++ / +++	+	++	- / +	++ / +++
Serotonergic nuclei								
DRif	+ / ++	++ / +++	+ / ++	++ / +++	+ / ++	++	++ / +++	++ / +++
DRv	+ / ++	++ / +++	+ / ++	++ / +++	+ / ++	++ / +++	++ / +++	++ / +++
DRd	+ / ++	++ / +++	+ / ++	++ / +++	+ / ++	++ / +++	++ / +++	++ / +++
DRl	++	++ / +++	++ / +++	++ / +++	+ / ++	++ / +++	++ / +++	++ / +++
DRp	++ / +++	++	++ / +++	++ / +++	++ / +++	++ / +++	++ / +++	++ / +++
DRc	++	++ / +++	+	+++	+ / ++	++ / +++	+	+ / ++
Orexinergic nuclei								
Pvc	++	++ / +++	++	++ / +++	++	++ / +++	++	++ / +++
Mc	++ / +++	++	++ / +++	++ / +++	++ / +++	++ / +++	++ / +++	++ / +++
OTc	++ / +++	++ / +++	+ / ++	++ / +++	++	++ / +++	++	++ / +++
Zic	+ / ++	++	++	++ / +++	+ / ++	+ / ++	+ / ++	++
Thalamic reticular nucleus	-	+ / ++	-	+ / ++	-	+ / ++	-	+ / ++

Table 2. Neuron and terminal network density for the calcium binding protein calretinin (CR) of the GABAergic system
Key: (-) absence. (+) low density. (++) moderate density. (+++) high density.

Sleep-Wake Related Nuclei	Donkey (EAA)		Horse (ECF)		Plains Zebra (EQQ)		Mountain Zebra (EZZ)	
	Neurons	Neuropil	Neurons	Neuropil	Neurons	Neuropil	Neurons	Neuropil
Cholinergic nuclei								
Sep.M	+	- / +	+	- / +	+ / ++	+	+ / ++	+ / ++
Diag.B	++	++	++	++	++	++	++ / +++	++ / +++
IS.Call and TOL	++ / +++	++	++ / +++	++ / +++	++ / +++	++ / +++	++ / +++	+++
N.Bas	++	++ / +++	+	++	++	++	+	++
PPT	++	++	++ / +++	++	++	++	++	++
LDT	++ / +++	+++	++ / +++	++ / +++	++	++ / +++	++	++ / +++
Catecholaminergic nuclei								
A7sc	+	++	++	+ / ++	++	+ / ++	++ /+++	++
A7d	+ / ++	+ / ++	++	+ / ++	++ / +++	++ / +++	++ / +++	++ / +++
A6d	+	++ / +++	+	++ / +++	+ / ++	++ / +++	++	++ / +++
Serotonergic nuclei								
DRif	- / +	++	+ / ++	++ / +++	- / +	++	+	++ / +++
DRv	- / +	++	++ / +++	++ / +++	+ / ++	++ / +++	+ / ++	++ / +++
DRd	- / +	++	++ / +++	++ / +++	+ / ++	++ / +++	++	++ / +++
DRl	+ / ++	++ / +++	++	++ / +++	+ / ++	++	- / +	++ / +++
DRp	++	++ / +++	++	++	+ / ++	++	+	++
DRc	- / +	++ / +++	++	++ / +++	- / +	++	+	+++
Orexinergic nuclei								
Pvc	+ / ++	++	- / +	+ / ++	+ / ++	+	++ / +++	++ / +++
Mc	++	++	++	++	++	+ / ++	++ / +++	++ / +++
OTc	++	++	++	++	+ / ++	++	++	++
Zic	++ / +++	++ / +++	+ / ++	++ / +++	+ / ++	++ / +++	+ / ++	++ / +++
Thalamic reticular nucleus	-	+ / ++	-	+ / ++	-	+ / ++	-	+ / ++

Table 3. Neuron and terminal network density for the calcium binding protein parvalbumin (PV) of the GABAergic system

Key: (-) absence. (+) low density. (++) moderate density. (+++) high density.

Sleep-Wake Related Nuclei	Donkey (EAA)		Horse (ECF)		Plains Zebra (EQQ)		Mountain Zebra (EZZ)	
	Neurons	Neuropil	Neurons	Neuropil	Neurons	Neuropil	Neurons	Neuropil
Cholinergic nuclei								
Sep.M	- / +	- / +	- / +	- / +	- / +	- / +	- / +	- / +
Diag.B	++	++	+ / ++	+ / ++	+	+	+	- / +
IS.Call and TOL	+ / ++	+++	+ / ++	++ / +++	+	+	+	++
N.Bas	+	+	+	+	++	+	+	+
PPT	+ / ++	+	+	+	- / +	- / +	- / +	- / +
LDT	++	+	- / +	- / +	- / +	+	- / +	- / +
Catecholaminergic nuclei								
A7sc	++	++	+ / ++	- / +	++	+ / ++	- / +	- / +
A7d	+	+ / ++	- / +	- / +	++	+	- / +	- / +
A6d	++	++	+ / ++	- / +	+	+	- / +	- /+
Serotonergic nuclei								
DRif	-	+	-	+	-	- / +	-	- / +
DRv	-	+	-	+	-	- / +	-	- / +
DRd	-	+	-	+	-	- / +	-	- / +
DRl	-	+	-	+	-	- / +	-	- / +
DRp	-	+	-	+	-	- / +	-	- / +
DRc	-	+	-	+	-	- / +	-	- / +
Orexinergic nuclei								
Pvc	-	- / +	-	- / +	-	- / +	-	- / +
Mc	-	- / +	-	- / +	-	- / +	-	- / +
OTc	-	- / +	-	- / +	-	- / +	-	- / +
Zic	++ / +++	++ / +++	++ / +++	++ / +++	++ / +++	++ / +++	++ / +++	++ / +++
Thalamic reticular nucleus	+++	++ / +++	+++	++ / +++	+++	++ / +++	+++	++ / +++

CHAPTER FIVE: Discussion

The aim of the current dissertation was to provide the first full description of the nuclear organisation and morphology of the somnogenic systems in four species of the Perissodactyla order. The results revealed that the four species examined demonstrated the same complement of basal forebrain cholinergic nuclei as well as midbrain and pontine cholinergic, catecholaminergic and serotonergic nuclei. The calcium binding proteins of the GABAergic system were also consistent across the four species, as was the hypothalamic orexinergic system. This latter system included a medial parvocellular cluster of orexin neurons present in all four animals. Furthermore, this study also revealed a novel population of tyrosine hydroxylase cell bodies within the predominantly GABAergic TRN.

5.1 Similarities and differences within nuclei responsible for cortical up-regulation

The four species showed similar complements and topographical distribution of cholinergic, catecholaminergic, orexinergic and serotonergic neurons when compared with other orders and mammals in general (Manger, 2005). Perissodactyl and cetartiodactyl cell size, shape and density were mostly alike, with small variances that occurred between animals of the same order and those across orders. The typical parcellation of the parabrachial complex showing the PPT and LDT was present in all equids, with a small magnocellular cluster seen in the LDT, and a larger population of parvocellular neurons present in the PPT. Interestingly, the PPT of all four equids demonstrated a central magnocellular cluster surrounded by a more parvocellular and at times less immunoreactive group of neurons. Past neuroanatomical studies on the parabrachial nuclei have shown that the giraffe and hippopotamus demonstrate a magnocellular LDT and a parvocellular PPT (Bux et al., 2010; Dell et al., 2016c), while the Goettingen mini pig (Mahady et al., 2016) and the Arabian oryx (Davimes et al., 2017) show the opposite, a magnocellular PPT and a parvocellular LDT. This study thus shows that perissodactyls, like cetartiodactyls, also show a variance in cell size of this nuclear complex and that this is therefore not only a cetartiodacyl-specific feature (Davimes et al., 2017). It is thus likely that functionally, the variance in soma cell size is related to the mechanism that induces REM sleep in this species. The parabrachial nuclei are widely responsible for arousal and REM sleep onset, but not

REM maintenance (Siegel, 2004; Datta and Maclean, 2007), suggesting that the presentation of REM sleep in this species is perhaps different to other species and that polysomnographic studies would be of great benefit as an adjunct to the neuroanatomical data.

Finally, while equids have short sleep times like the elephant (Gravett et al., 2017), neuroanatomical investigations of the locus coeruleus complex show a difference in parcellation in these two animals. The four equids in this study reveal no evidence of a novel medial cluster located in the periventricular grey matter of the pons of the elephant (Maseko et al., 2013). Gravett et al., (2017) surmise that this supplementary cluster, seen in the elephant, may contribute to this animal's short sleep. This study suggests that while equids show the classic complement of LC nuclei, they may have projections from this complex to other nuclei that help maintain arousal and contribute to short sleep times in this species.

5.2 Similarities and differences within nuclei responsible for cortical down-regulation

In general, GABAergic nuclei remain conserved across species with little variation seen between mammalian orders (Manger, 2005). Perissodactyla show standard mammalian expression of these calcium binding proteins when compared to species of the order Cetartiodactyla. Small differences were seen in the TRN where parvalbumin is the predominant calcium-binding protein expressed. However, the harbour porpoise (Dell et al., 2016a) and the minke whale (Dell et al., 2016b) showed moderated expression of calbindin and calretinin neurons, while the equids showed no staining for these calcium binding proteins. These small variations may be related to a need for increased cortical awareness due to the harbour porpoise and minke whale's aquatic environment and their unusual unihemispheric slow wave sleep patterns. Small variances in the expression of the central nervous system's primary inhibitory neurotransmitter may indicate differences in sleep phase change, time spent in a particular sleep phase, and how long it takes an animal to become fully awake. These neuroanatomical variations would be an interesting addition to future physiological and naturalistic behavioural studies done in wild equids. This data could then be compared with existing studies in domestic breeds of this order (Ruckebusch et al.,

1970; Ruckebusch, 1962, 1963, 1972; Dallaire and Ruckebusch, 1974a and b; Hale and Huggins, 1980; Belling, 1990; Moehlman, 1998) and other species.

5.3 The orexinergic system in Perissodactyla

The results of the present study revealed that the topography of the perissodactyl orexinergic system is largely typical of mammals. This study employed the same fixation protocol and antibodies as described in Dell et al. (2012) to stain for OxA in the brains of the donkey, horse, plains and mountain zebra. All four species of equids exhibited the typical orexinergic magnocellular tri-parcellation scheme as seen in other mammals, thus further supporting the notion that the magnocellular parcellation is largely preserved across mammalian orders (Manger, 2005), apart from certain species of hamsters and microbats that lack the optic tract cluster (Mintz et al., 2001; McGranaghan and Piggins, 2001; Khorooshi and Klingenspor, 2005; Vidal et al., 2005; Kruger et al., 2010).

5.3.1 Clade evolution of the medial parvocellular cluster

Notably, all four species of this study demonstrated the unusual and novel medial parvocellular cluster, initially presumed to manifest only in Cetartiodactyla, and which led Dell et al., (2012, 2016a – c) to propose that the orexin system reaches a highly-evolved state in this superorder. Further studies conducted on animals of other eutherian clades have also identified this orexinergic parvocellular cluster in several species, clearly indicating that while the orexinergic system appears highly evolved, this medial cluster is not a cetartiodactyl-specific feature as proposed by Dell et al. (2012). Moreover, we would suggest that this specialisation may have evolved when Eutheria branched into Atlantogenata and Boreoeutheria. This division in mammalian evolution led to the development of Afrotheria and Laurasiatheria. Afrotheria evolved to the further clade of Paenungulata*,* as the last common ancestor of the Proboscidea. Laurasiatheria developed the branch Scrotifera that gave rise to perissodactyls and cetartiodactyls (Tabuce et al., 2008). Thus, the last common ancestor of the orders Perissodactyla, Cetartiodactyla and Proboscidea would have existed where Atlantogenata and Boreoeutheria diverged (Tabuce et al., 2008), and as such, further investigations into the origin of this parvocellular cluster are not possible from this angle. However, this hypothesis could be further tested by isolating those closest living relatives of the three orders that have not yet been investigated for this

parvocellular cluster of orexin neurons. The pangolin of the order Pholidota, is a close relative of both the perissodactyls and the cetartiodactyls. The dugong of the order Sirenia, is a close clade relative of the elephant and hyrax (Tabuce et al., 2008). Therefore, a study of the pangolin and dugong would bridge the gap in neuroanatomical studies that presently exists. The dugong is a marine herbivore, whereas the pangolin is a terrestrial insectivore that is nocturnal with poor eyesight. Both these animals have a wide variance in physiology, more specifically body mass, habitat, feeding and sleeping habits. Comparative neuroanatomical studies of animals that demonstrate these differences in physiology and habit would greatly add to our understanding of the parvocellular cluster as an adaptive addition to certain animals, and the function of the orexinergic system as a whole.

Additional neuroanatomical investigations of the orexinergic system in three other closely related orders to perissodactyls and cetartiodactyls have been undertaken in Chiroptera, Hyracoidea and Eulipotyphla (Kruger et al., 2010b; Bhagwandin et al., 2011; Gravett et al., 2011; Dell et al., 2013). Conversely, microbats (Kruger et al., 2010b), megabats (Dell et al., 2013) and mole rats (Bhagwandin, 2011) showed no evidence of a parvocellular cluster of OxA neurons. Similarly, the rock hyrax (Gravett et al., 2011), closely related to the elephant, showed no medial orexin group either (Gravett et al., 2011). Despite the distant common ancestry in all animals studied to date, their great variability makes it difficult to pinpoint a common feature that would suggest the reason why some have a medial parvocellular cluster of orexin neurons, while others don't. However, those that do possess this parvocellular cluster, tend to spend a great deal of their day feeding to fulfil an energy deficit either due to a large body mass (Siegel, 2005; Gravett et al, 2017) or as a consequence of energy-demanding environmental conditions such as extreme temperatures (Dell et al., 2012; Davimes et al., 2017). All mammals studied possess orexin-discharging neurons in the hypothalamus, but only some have an extra medial group that is proposed to be more evolved, and as such, must have further implications for the function of this neuropeptide (Dell et al., 2012).

5.3.2 Orexin as a regulator of appetite and sleep time

Orexin is homeostatic in nature and regulates locomotion, maintains arousal and at times increases it, through neuroendocrine-based functions such as blood

pressure, respiration, temperature, energy output and appetite drive (Ebrahim et al., 2002; Dell et al., 2012; Davimes et al. 2017). Specifically, the medial hypothalamus is involved in the initiation of circadian behaviours such as aggression related to territory and copulation, feeding, body weight and energy consumption (Fitzgerald et al., 2012). The results of this study suggest that the parvocellular cluster of orexinergic neurons evolved as part of an adaptive trade-off between sleep time and feeding behaviours. Larger animals that consume food of a lower energetic value, will be driven to feed for longer periods of their waking hours to fulfil the deficits in energy demanded of their higher body mass. Quite simply, animals need to be awake long enough to meet energy demands, satisfy appetite drive, and feel satiated (Capellini et al., 2008a and b; Lesku et al., 2006, 2009). Thus, while appetite drive is a function of food intake and nutrient quality, it is also dependent on energy output and environment.

A fair example of this would be the eating habits of both plains and mountain zebra. These animals enjoy grazing on particular species of grass, and will travel distances of up to 100 km to find preferred food sources (Smithers, 1983; Stuart and Stuart, 2001). Grazers do not enjoy a diet that is as nutrient-rich as browsers, and are presumed to spend more time searching for enough food (Dell et al., 2012). The Arabian oryx survives in extremely harsh desert temperatures, and during the summer months, as food becomes increasingly scarce, it adjusts its basal metabolic rate for greater digestive efficiency (Davimes et al., 2017). Thus, both animals may make use of extended arousal times, albeit in different ways, to balance appetite drive with energy demands and cope with environmental factors. Axiomatically, when hay is substituted for a more nutrient-rich food like oats in the housed pony, or alternatively, the animal is left to fast, TST and total recumbency times increase, the latter by approximately 20%. Both NREM and REM sleep increase incrementally and the animals spend less time awake or in a drowsy state (Dallaire and Ruckebusch, 1974a). This reinforces the argument that when satiated the animal may rest, and when hungry with no access to food, the animal must conserve energy by resting. In short, based on the accepted paradigm that arousal uses up energy, and that energy depletion drives the organism to sleep (Schmidt, 2014), the orexinergic system may play a vital role in balancing two of the organisms most important biological investments in survival: food and sleep.

5.3.3 Proposed interaction between orexin, leptin and ghrelin

Another possible function of this medial parvocellular cluster of orexinergic neurons may be related to two other metabolic hormones that influence and interact with orexin. Leptin is a peripheral anorectic protein that is secreted in response to fluctuating levels of adipocytes (Willie et al., 2001). It has been shown to have a direct and indirect inhibitory influence on orexin feeding pathways via the arcuate nucleus of the medial hypothalamus (Willie et al., 2001). Furthermore, there is considerable evidence to suggest that orexin plays a role in coordinating both orexinergic and anorectic pathways of the arcuate and ventromedial nuclei to increase appetite drive and supress leptin secretion (Willie et al., 2001). Fischhoff et al. (2007) posited that pregnant or lactating female plains zebra are the initiators and drivers of group migration to better grazing areas. Pregnant mammals, specifically during the first two trimesters of pregnancy, have increased amounts of adipose tissue, with higher serum leptin levels (Brunton and Russell, 2008). Increased leptin levels are not only due to a higher body fat content, but also to placental secretion (Brunton and Russel, 2008). While the results reported in this dissertation were obtained from male zebras, physiological changes in body mass index, such as an increase in body fat, would provoke higher serum levels of leptin, regardless of gender. An increase in leptin engenders suppression of orexin with resultant appetite loss (Willie et al., 2001; Brunton and Russell, 2008).

This study suggests that the parvocellular group of orexin neurons may thus supress high levels of leptin in these females (or males that have a higher fat content) in order to increase appetite drive and arousal so that pregnant females, who endure a long gestation period of 360 to 390 days, with an average of 372 days reported (Klingel, 1965, 1972; Smithers, 1983), obtain enough food for themselves and the growing foetus. Additionally, a long gestation period means that mares are often found to lactate in the early stages of their following pregnancy, an especially energy demanding activity for all mammals, let alone those with parturition so close to a second gestational period (Bartošová et al., 2011). Neuroanatomical studies on leptin in post-parturition and lactating females would help elucidate this hormone's interaction with orexin, and why these mammals do not seem to develop leptin resistance in the way humans do. Additionally, gender comparative studies on cell

size in both the magno- and parvocellular clusters of orexinergic neurons in zebra may lend further valuable insights to this proposed theory.

Furthermore, impetus for the existence of a parvocellular group of orexin neurons may be found in a comparison of sleep deprived animals that do not demonstrate this medial cluster, and those animals such as wild roaming equids, and elephants that display a low TST with little to no sleep rebound (Gravett et al., 2017). Animal experiments show that rats (these animals do not demonstrate a parvocellular cluster of orexinergic neurons) deprived of sleep, show increased appetite and energy expenditure, followed by weight loss and ultimately, death (Berger and Phillips, 1995). It is proposed that weight loss and death are not in fact due to acute sleep deficits, but to the stress related to this event (Taheri et al., 2004). In humans, short sleep duration is related to a decrease in leptin and an increase in ghrelin, leading to an overall increase in body mass index (BMI). Low leptin and high ghrelin levels are associated with an increase in appetite, and a desire for foods rich in fat in both humans and animals (Berger and Phillips, 1995; Taheri et al., 2004). Rats fed a high fat diet when sleep deprived, showed an increase of up to 250% in appetite (Taheri et al., 2004). Thus, it may not be unreasonable to propose that the medial parvocellular cluster found in cetartiodactyls, perissodactyls, and the elephant, may interact with both leptin and ghrelin in animals that have an established short sleep baseline habit, high energy demands, and a somewhat variable to low trophic level (Gravett et al., 2017).

Many perissodactyls, particularly wild equids such as zebra, are subject to predation and to environmental changes that leave them nutritionally depleted (Lesku et al., 2008). The plains zebra has a home range that extends from 111 km^2 to over 200 km^2 (Smithers, 1983), and alertness and good memory are indispensable to ward off dangers, recognise other members of large herds that do not pose a territorial threat, and locate alternative feeding grounds or watering holes (Willie et al., 2001; Siegel, 2005; Gravett et al., 2017). The cascade of symptoms related to sleep deprivation displayed in other animals that do not possess a parvocellular cluster, such as depression, obesity and anorexia nervosa among others, is not evolutionarily adaptive to animals that need to extend periods of arousal in order to survive. The parvocellular cluster may thus contribute to an added level of sleep plasticity vital to

the survival of animals living in the wild. Plains and mountain zebra move to more habitable areas during the winter months, thus their environment changes seasonally. Add to this the sustained threat of predation and the unstable nature of herds that constantly evolve as harems that break off and re-join (Rubenstein, 1986; Pluháček et al., 2006). It is evident that animals living within such a fluctuating ecosystem, would not survive long if they could not alter their sleep habits to accommodate this flux.

5.4 The thalamic reticular nucleus in Perissodactyla

Immunohistochemical staining of GABAergic neurons in the TRN of the four equids in this study revealed that CB and CR neuron populations stained immunonegatively, indicating an absence of these calcium binding proteins, while PV stained immunopostively. Although TRN expression of CB and CR varies across species, PV is undoubtedly the most prevalent calcium binding protein in this nucleus (Bhagwandin et al., 2013; Maseko et al., 2013; Dell et al., 2016 a – c). It is thus widely accepted that PV is the driving calcium binding protein for GABA expression in the TRN. Further, the gross morphology and cellular arrangement of the TRN as reinforced by numerous immunohistochemical studies (Bhagwandin et al., 2013; Maseko et al., 2013; Dell et al., 2016 a – c) has largely been shown to remain constant, with few variations of no real significance (Manger 2005).

This study revealed an uncharacteristic and novel population of cell bodies that stained positively for tyrosine hydroxylase (TH) in this nucleus. An extensive examination of the literature revealed that the Syrian hamster (*Mesocricetus auratus*), a nocturnal mammal with extended sleep bouts similar to that of the golden hamster of 14.4 hours (60.1%) of a 24-hour period (Campbell and Tobler, 1984), is the only other mammal that demonstrates this unusual population of TH cells in the TRN (Asmus et al., 1993). In contrast, equids are diurnal and exhibit much shorter total sleep times (Ruckebusch, 1974b; Houpt 1980; Campbell and Tober, 1984; Belling, 1990), suggesting that further analysis of the co-expression of TH and PV may provide more insight into the excitation and inhibition of neuronal networks and their function in the TRN during sleep and arousal.

5.4.1 Tyrosine hydroxylase as a postural aid during standing REM sleep

An unusual physiological feature of equid sleep, is the horse's ability to enter a brief period of REM sleep of no more than 16 – 23 seconds while standing (Williams et al., 2008). The recent discovery heralding the African elephant as the mammal with the shortest TST with little proof that REM sleep exists in this animal (Gravett et al., 2017), has led the authors to propose that elephants, like horses, may enter a short burst of REM sleep while standing. The authors further suggest that this may be possible in the elephant due to a novel medial cluster of norepinephrine cells in the LC that remains depolarised during this short REM phase, providing this animal with just enough muscle tone to remain upright. This unusual medial subdivision of the LC has not been identified in equids, however perissodactyls possess the stay apparatus that locks the limb joint demanding little help from surrounding muscles, tendons or other ligaments (Moehlman, 1998; Yilmaz, 2012). It is thus possible that posture in equids is maintained as a combination of minimal muscular activity and a maintained level of proprioceptive awareness during sleep. Cortical awareness of proprioception is mediated by somatosensory thalamo-cortico-thalamic loops via the TRN, and during a short burst of REM sleep, may be maintained by the D4 receptors that receive projections from the LC in contrast to direct LC involvement, as is the proposed mechanism in elephants (Gravett et al., 2017).

Numerous studies have demonstrated a significant population of D4 receptors in the TRN innervated by projections that originate in the substantia nigra pars compacta (Florán et al., 2004). This strongly suggests that the final catecholamine released in the TRN may indeed be dopamine (Floràn et al., 2004). However, the precise interaction these receptors have on PV calcium binding neurons in this nucleus remains unclear (Florán et al., 2004; Ferrarelli and Tononi, 2011). GABA plays an important role in the inhibition of muscle contractions (Datta and MacLean, 2007), while dopamine is vital for the initiation and follow-through of smooth motor action, (Datta and MacLean, 2007; Herrera-Solís et al., 2016). If D4 receptors inhibit GABAergic action in the TRN (Floràn et al., 2004), perhaps the unusual cell population of TH (essential for the rate-limiting phase of dopamine synthesis) seen in the TRN plays a pivotal role in maintaining muscle tone during short episodes of standing REM sleep when GABAergic neurons would normally be contributing to the

maintenance of muscle atonia (Siegel, 2004a and b; Datta and MacLean, 2007; Mignot, 2008). Thus, the presence of a monoamine such as dopamine or norepinephrine in the TRN, may serve an added advantage to an order that is prone to greater predation risk than elephants. Equids, more specifically zebra, may spend most of their sleep time in the standing position as an adaptive behaviour to avoid predation (Moehlman, 1998; Yilmaz, 2012) and being able to become aware more rapidly is adaptive for survival.

Finally, equids are susceptible to pulmonary stasis in dorsolateral recumbency and when rolling, reach a pivotal point during rotation when abdominal organs become prone to gut twist (Belling, 1990). It is suggested that equids sleep very carefully in lateral recumbency (Williams et al., 2008), demonstrating a typical pose with the lower front leg flexed at the knee and pastern and the upper leg completely straight (Wilson, 1970; Belling, 1990). TH expression in the TRN may safeguard against total loss of muscle tone during standing REM sleep. This mechanism may encourage the animal to spend more time in standing sleep than lateral recumbency thereby limiting exposure to REM sleep muscle atonia, observed in recumbent stances, and the resultant risk of pulmonary stasis and abdominal complications.

5.4.2 Tyrosine hydroxylase as a possible memory enhancer

PV is assumed to play a role in the production of oscillatory spindles during NREM sleep that arise in the TRN (Steriade et al., 1993; Traub et al., 2005). These spindles have been implicated in the filtering of sensory information during NREM sleep and the consolidation of sleep-dependent memories (Halassa et al., 2014). One of the unusual features of equid sleep is its brevity. The sleep cycle lasts no more than fifteen minutes, with a five-minute NREM sleep phase followed by a five-minute REM sleep phase, and then another five-minute NREM sleep phase (Houpt, 1980). Similarly, the sleep cycle of the hamster is fairly short 10 – 12 minutes (Tobler, 1995). Deboer et al., (1994) provide a NREM:REM ratio for the Djungarian hamster as 7:1.8 minutes, thus suggesting that while hamsters may sleep far longer than the equids, their REM to NREM episodes are extremely short. It would be of interest to test whether this novel population of TH cells plays any role in oscillatory spindle memory formation during NREM sleep. A starting point would be to "quieten" these oscillatory spindles by conducting a repeat experiment using ketamine-xylazine

anaesthesia on a population of Syrian hamsters. This has been shown to synchronise slow waves and prevent cortical processing during NREM sleep (Chauvette et al., 2011). Reversal of anaesthesia, and a period of recovery should be followed by a memory test such as the Morris water maze to ascertain whether the animal suffers from anaesthesia-induced amnesia (Chauvette et al., 2011) as a function of loss of slow wave oscillatory spindles. If memory loss is evident, the experiment would suggest that the novel population of TH neurons in the TRN has no influence on memory consolidation during sleep. This experiment is suggested because plains zebra have very large migratory home ranges in terrain that is fairly featureless and flat (Smithers, 1983). It is thus surmised that the zebra may have a large hippocampus, and because the TRN is implicated in the transporting of memories from the hippocampus to the cortex (Roth II et al., 2010), there may be direct involvement of this novel parcellation of TH neurons in memory consolidation in this animal that demonstrates a very short daily sleep time.

CHAPTER SIX: Conclusion and Future Directions

6.1 Conclusion

Mammalian sleep is a prominent component within the field of neuroscience (Siegel, 2005), and research concentrates on contributing to the growing body of literature that brings us closer to a scientific explanation of why sleep is presumed a universal constant across all mammals. Of the approximate 4 500 identified mammalian species, very few have been investigated comprehensively with regard to sleep (Siegel, 2008). Most physiological and behavioural studies have concentrated on an insufficient group of domestic mammals in the laboratory setting, and the adjunct of neuroanatomical investigations is infrequent (Lesku et al., 2006, 2009; McNamara et al., 2008). Thus, while neuroanatomical studies may be limited, the identification of the sleep related nuclei in the mammalian brain has shown a quantifiable stability in structure that provides a sound scientific foundation for further comparative research.

The equids in this study showed consistency in the neuroanatomical organisation, parcellation and morphology of the sleep-wake systems. The neuronal population density and cytoplasmic projections were also fairly constant and only showed small variations when compared with other mammals. Two unusual findings were noted. The TRN demonstrated a prolific population of neurons that stained positively for TH, and the orexinergic system included an additional medial parvocellular cluster previously considered to be a cetartiodactyl-specific feature. The former finding may provide clues to the unusual ability that the horse demonstrates in physiological studies (Williams et al. 2008) in its ability to enter REM sleep while standing. Additionally, this population of TH neurons may play a role in the processing of sensory information that consolidates declarative memory during sleep. This is crucial to wild animals that need to recall good grazing areas, reliable water supply and safe birthing grounds. The latter finding of a parvocellular cluster of orexin neurons is suggested to play a role in larger animals with high energy expenditure that have developed reduced sleep times as an evolutionary adaptation so that more time is allocated to arousal and devoted to feeding and satisfying a strong appetite drive.

The qualitative uniformity observed in these mammals provides us with a common starting point for quantitative studies such as stereology, and further comparative behavioural and physiological studies. Emerging studies on wild mammals in their natural settings such as the African elephant (Gravett et al., 2017) and the Black rhinoceros (Santymire et al., 2012) have revealed the intriguing nature of sleep and its variability as a function of the animal's environment. Additionally, novel findings such as those in this study, of a population of TH cells in the TRN of equids, provides the field of sleep research with challenges in seeking out similar examples in mammals of different orders.

6.2 Limitations

This study undertook a full neuroanatomical investigation of a sample group of four male equids using a protocol that has been shown to work well on other animals (Kruger et al., 2010 a and b; Bhagwandin et al., 2012, 2013; Dell et al., 2010, 2012, 2013, 2016 a – c; Maseko et al., 2013 and Gravett et al., 2013). The study has thus fulfilled its mandate as a qualitative neuroanatomical investigation. However, it is suggested that the addition of a further immunohistochemical study would formally complete the dataset provided by the present research. Future immunohistochemical runs should include a greater sample group of both males and females of all four species. This would help elucidate any differences that may exist in gender and confirm the small variances seen between species in this order.

Further, a solid context in which to place these results would accomplish a more complete study. Apart from a small contribution in laboratory-based physiological and behavioural studies in domestic equids (Ruckebusch et al., 1970; Ruckebusch, 1962, 1963, 1972; Dallaire and Ruckebusch, 1974 a and b; Hale and Huggins, 1980; Belling, 1990; Moehlman, 1998), there is little data for comparative research. As such, it is suggested that one of the limits of this dissertation is the lack of quantitative data. Future stereological investigations would help elucidate qualitative findings, improve conjectural hypotheses of variations, and provide comparable data, especially with the sister order Cetartiodacyla, in which such studies have already been completed (Dell et al., 2012).

6.3 Future directions

Schilder and Boer (1987) observed that zebra stallions tend to spend more time managing their harem; they seem to feed less and are more alert and on the lookout for predators and possible competition from other stallions. It would therefore be of interest to examine sleep in wild male zebra in their natural habitat, in order to provide quantitative data to support these behavioural observations. It is well known that various ecological factors influence sleep, and by examining sleep in the zebra in the wild, the relationship between factors such as predation pressure, weather, food availability and social structure on sleep architecture should be investigated.

Further, a behavioural comparative gender study would be of importance in determining whether male or female zebras sleep longer. This study has shown that female zebras take the lead in harem and herd movements in order to seek out good food and water sources. Male zebras feed less because they invest their time in other behaviours such as mating and protection and maintenance of their harems. A gender study may reveal what energy-consuming habits of arousal tend to prioritise sleep.

The actiwatch and GPS collar for remote monitoring were used with considerable success in the elephant to investigate their sleep behaviours and the environmental effects on sleep architecture (Gravett et al., 2017). Actigraphy data gained from a naturalistic and mostly non-invasive study could then be compared to data generated from a polysomnographic study. This would provide two benefits: Actigraphy's advantage is its ability to record sleep continuously and over a far longer period than polysomnography (Ancoli-Israel et al., 2003). Polysomnography identifies sleep times and phasing using more accurate criteria than simple periods of activity and inactivity (Ancoli-Israel et al., 2003; Tryon, 2003). Correlation of statistics generated by both devices would help validate sleep times in these animals (Tryon, 2003).

The neuroanatomical data produced by this study, used in conjunction with the results obtained from physiological studies, would provide added impetus for a stereological study. Cell counts and soma size measurements conducted in equids would have enhanced meaning, specifically in relation to the parvocellular cluster of

orexinergic neurons in the hypothalamus and the cholinergic nuclei of the LDT and PPT.

Finally, while this study has isolated TH cells in the TRN, the immunohistochemical staining does not tell us anything about the final catecholamine produced by these cells (Asmus et al., 1993; Florán et al., 2004; Ferrarelli and Tononi, 2011). Even if TH mRNA is expressed prolifically in these neurons, this does not infer that there are stable or significant levels of the TH protein in these cells (Asmus et al., 1993). As such, further immunocytochemical studies need to be completed to establish whether these nuclei have enough functional TH protein available and if so, whether it is aromatic amino acid decarboxylase, L-Dopa or dopamine that is produced as a result.

Thus, there is still a great deal to be done in the field of sleep research, and our present achievements and rank of knowledge permit the assumption that sleep in animals is more an observation than a certainty. This phenomenon is obvious in some animals, less so in others, at the same time strikingly similar and astoundingly different. Above all, it is not easy to present as a neatly packaged concept.

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APPENDIX

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2015/06/23/A

APPLICANT: Mr A Bhagwandin

SCHOOL: School of Anatomical Sciences
DEPARTMENT:
LOCATION:

PROJECT TITLE: Aspects of the neuroanatomy of Equids: Horse - Equus caballus, and Donkey - Equus asinus

Number and Species

3 Equus caballus, 3 Equus asinus

Approval was given for the use of animals for the project described above at an AESC meeting held on 2015/06/30. This approval remains valid until 2017/07/26.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

None

Signed: _____ (Chairperson, AESC) Date: 28 July 2015

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 28 July 2015

cc: Supervisor:
Director: CAS

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Please note that only typewritten applications will be accepted. Should additional space be required for section "I" and/or "j", please use the back of this form.

ANIMAL ETHICS SCREENING COMMITTEE

MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Adhil Bhagwandin

b. Department: Anatomical Sciences

c. Experiment to be modified / extended **AESC NO:**

Original AESC number	2015/06/23/A
Other M&E's : not applicable	

d. Project Title: Aspects of the neuroanatomy of Equids: Horse – *Equus caballus*, and Donkey – *Equus asinus*.

e.	Number and species of animals **originally approved**:	6	3 horses, 3 donkeys
f.	Number of additional animals **previously allocated on M&Es**:	0	
g.	Total number of animals **allocated** to the experiment to date:	6	3 horses, 3 donkeys
h.	Number of animals **used** to date:	6	3 horses, 3 donkeys

i. Specific modification / extension requested:

In comparing the hippocampal volume of the horse and donkey to that observed in the plains zebra, we find that the plain's zebra has a far larger hippocampal volume than either the horse or donkey, or indeed other perissodactyls we have examined. However, we have only examined a single plains zebra ourselves (obtained from the Copenhagen zoo) and have one record from the literature. In order to test whether the plains zebra has a truly uniquely large hippocampus, I would like to examine the brains of 3 adult plains zebra (*Equus quagga*) and 3 adult mountain zebra (*Equus zebra*). These animals will likely be obtained from Wildlife Assignments International, or directly from the suppliers to this company. It is possible that I might also obtain some specimens from hunters, but this is uncertain at the moment. The animals will be treated as per the original application, unless they are obtained from hunters, where the animals will be shot through the heart.

j. Motivation for modification / extension: see above

Date: 18 March, 2016 Signature: