Interactions between hypothalamic and brainstem arousal circuits: 
Anatomical, electrophysiological, and behavioural studies

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Abstract

Hypothalamic orexin and melatonin-concentrating hormone (MCH) neuropeptide signalling systems act as fine-tuning mechanisms in the control of arousal and motivated behaviours. Orexin systems promote arousal through several different neural pathways in response to internal and external cues. One such pathway that was investigated in the current studies was the orexinergic/glutamatergic neurons that project to the rostroventrolateral medulla (RVLM). Neurophysiological studies revealed these neurons were activated by hypoglycaemia, which in turn, activated glutamatergic projections to adrenal sympathetic preganglionic neurons (SPN). As a consequence, this activation elevated adrenal sympathetic nerve activity (ASNA) and release of adrenaline to restore blood glucose. These findings were observed after electrical stimulation of the perifominal hypothalamus, and orexin/glutamate antagonist microinjection into the RVLM or the intermediolateral cell column of the spinal cord (IML).

Other pathways examined in the current studies were the putative orexin and MCH projections to the brainstem, nucleus incertus (NI). Previous studies revealed that the NI receives an innervation from orexinergic neurons in the lateral hypothalamus (LH) and NI neurons express orexin type 1 and 2 receptors (OX1/2R) (OX2R >> OX1R). In vitro whole-cell recordings, NI neurons were mainly depolarised via OX2R activation. In the current studies, the behavioural effects of orexin infusion into the NI were assessed, as well as the diurnal regulation of orexin inputs to NI, using quantitative immunofluorescence. Furthermore, the inputs to, and effects of MCH on, NI neurons was also investigated. Arousal in awake rats was assessed using protocols of feeding and locomotor activity. Microinfusion of orexin-A (600 pmol) into the NI of conscious, satiated rats during the light phase, increased locomotor activity recorded in automated locomotor cells (interaction between treatment and time variables: F(41, 205) = 1.863, P = 0.0026; n = 6/group) and increased cumulative food intake (interaction between treatment and time variables: F(3, 33) = 3.983, P = 0.0156; n = 12/group), compared to vehicle-treated control rats.

In contrast, intra-NI infusion of MCH (600 pmol) only produced a trend towards decreased locomotor activity (interaction between treatment and time variables: F(41, 588) = 0.059, P > 1.0, n = 7-9/group) and food intake (interaction between treatment and time variables: F(3, 21) = 0.35,
Compared to vehicle-treated rats. Interestingly, in rats fasted for 6 h, prior to testing during the early dark phase, MCH (600 pmol) infusion increased food intake during the first 2 h post-infusion ($P < 0.01$, n = 7/group, with no significant interaction between time and treatment: $F_{(3, 18)} = 1.22; P = 0.33$), with no effect on locomotor activity (interaction between treatment and time variables: $F_{(44, 450)} = 0.11, P > 1.0$; n = 6/group). To better understand the expression and distribution of orexin and MCH receptors within the NI, RNAscope in situ hybridisation was used to characterise OX1R, OX2R and MCH1R mRNA distribution and colocalisation, relative to the GABA neuron marker, vesicular GABA transporter (vGAT) mRNA, in different neuron populations. A distinct and heterogenous mRNA colocalisation profile was observed. Using immunofluorescence to better understand the orexin and MCH inputs to the NI, a diurnal regulation of putative MCH axonal segments and terminations was observed, while orexin immunofluorescence appeared unchanged in the light vs dark phase. Conversely, studies of the reciprocal nature of NI and relaxin-3 projections to orexin and MCH neurons of the lateral hypothalamus revealed a minor projection directed to orexin/MCH neurons, but a stronger projection to a neighbouring calretinin-positive, GABA neuron population in the ventral part of the lateral hypothalamus.

These studies have characterised novel aspects of the orexin/OX2R and MCH/MCH1R systems and their involvement in autonomic and behavioural responses related to appetite/metabolism and arousal. These findings provide direct evidence for orexin involvement in brainstem RVLM regulation of ASNA and blood glucose, and an involvement of orexin and MCH in regulating brainstem NI activity and associated arousal and feeding.

Future studies will focus on functional and electrophysiological characterisation of the target neurons in lateral hypothalamus and NI in in vivo systems. The contribution of these circuits to arousal can be identified by selective activation or inhibition of target neurons in forebrain and brainstem, using optogenetic or chemogenetic techniques. The further identification of how these circuits contribute may provide a new understanding of the ascending reticular arousal system (ARAS) and sleep-wakefulness control, as well as further insights into the aetiology of disorders due to malfunction of neuromodulatory signalling systems in dementia, Parkinson’s disease, sleep disorders, and vegetative states after head trauma or coma.
Declaration

This is to certify that:

(1) the thesis comprises only my original work towards the degree of Doctor of Philosophy, except those studies indicated in the preface,

(2) due acknowledgment has been made in the text to all other material used, and

(3) the thesis is fewer than 100,000 words in length, exclusive of tables, bibliography and appendices.

A. Sabetghadam

Azadeh Sabetghadam Esfahani
Preface

I am grateful to the people who provided me with their support during my thesis studies.

I received intellectual/technical support from my supervisors, Professor Andrew Gundlach and Doctor Sherie Ma, for all aspects of my studies. I also received intellectual/technical support from Associate Professor Anthony Verberne and Doctor Willian Korim for the studies described in Chapter 3, and from Doctor Hanna Kastman for studies in Chapter 4. Doctor Verena Wimmer and Doctor Ellie Cho provided technical training for the studies in Chapter 5.

The studies described in Chapter 3 have been published in a first-author article in the peer-reviewed journal, *Autonomic Neuroscience*. The studies described in Chapter 4 have been submitted for publication as a first-author article in the peer-reviewed journal, *Neuropharmacology*. The studies described in Chapter 5 are being prepared for submission to a peer-reviewed neuroanatomy journal.

To the best of my knowledge, this thesis contains no previously published material or material written by another person, except where reference is made in the text of the thesis.
Acknowledgements

I would like to express my genuine gratitude to people who were kind and generous enough to dedicate time for coaching, helping and advising me in many intellectual and practical ways. First of all, I would like to thank my principal supervisor, Professor Andrew Gundlach, for not only being an amazing and knowledgeable supervisor, but for his generosity in helping me through the turmoil amid my PhD journey. I am grateful for all his useful feedback, patience and timely responses to my frequent requests and enquiries. The completion of this thesis would not have been possible without his valuable supervision.

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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine (serotonin)</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotrophic hormone</td>
</tr>
<tr>
<td>AgRP</td>
<td>agouti-related peptide</td>
</tr>
<tr>
<td>AP</td>
<td>anterior-posterior</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAT</td>
<td>brown adipose tissue</td>
</tr>
<tr>
<td>CART</td>
<td>cocaine- and amphetamine-regulated transcript</td>
</tr>
<tr>
<td>CCK</td>
<td>cholecystokinin</td>
</tr>
<tr>
<td>Ca\textsubscript{v}2</td>
<td>caveolin 2</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>DREADD</td>
<td>designer receptor exclusively activated by designer drug</td>
</tr>
<tr>
<td>DV</td>
<td>dorsoventral</td>
</tr>
<tr>
<td>EEG</td>
<td>electroencephalogram</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G-protein-coupled receptors</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamic-pituitary-adrenal (axis)</td>
</tr>
<tr>
<td>Hz</td>
<td>hertz</td>
</tr>
<tr>
<td>i.c.v</td>
<td>intracerebroventricular</td>
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</tbody>
</table>
IgG  immunoglobulin G
i.v.  intravenous
KiR3  inward rectifying potassium channel
KO  knock-out
M  molar
MΩ  milliohm
mA  milliamp
mg/kg  milligram per kilogram of body weight
ml  millilitre
ML  mediolateral
mmol  millimolar
mRNA  messenger ribonucleic acid
ms  millisecond(s)
μl  microlitre
NHS  normal horse serum
NPY  neuropeptide Y
PE  polyethylene
pmol  picomole
PTSD  post-traumatic stress disorder
RT-PCR  reverse transcription polymerase chain reaction
RXFP3  relaxin family peptide receptor 3
SNA  sympathetic nerve activity
TRH  thyrotropin-releasing hormone
vGAT  vesicular GABA transporter
vGlut  vesicular glutamate transporter
Publications and Communications

The following original articles and communications have resulted from this research.

Peer-reviewed articles


Abstracts (presenting author)


Scholarship and Awards

Florey Studentship, Florey Institute of Neuroscience and Mental Health, Melbourne, Australia, 2017 Jan-June

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Melbourne International Research Scholarship, The University of Melbourne, Australia, 2013-2016

Austin Medical Research Foundation Scholarship, Austin Health, Melbourne, Australia, 2013-2016

Graduate Student Travel Award, Department of Medicine (Austin Health), The University of Melbourne, Australia, Apr 2015

Graduate Student Travel Award, 34th Annual Meeting of Australasian Neuroscience Society (ANS), Adelaide, Australia, Feb 2014
Chapter 1 - GENERAL INTRODUCTION
1.1. Preface

Arousal is a physiological and psychological state of being awoken and perceptive, which involves coordinated activity of the reticular-activating, autonomic nervous and endocrine systems. While arousal is crucial for attention, information processing and consciousness, it is also a vital process underlying motivated behaviours, such as motivation-related mobility, seeking nourishment, stress responses such as fight or flight response, as well as sexual functions and emotion (Stellar, 1994).

It is now well-known that arousal imbalance can affect task performance in humans and also influences emotional stability and personality (Pfaff, 2006). For example, the extrovert personality is generally characterised with under-stimulated arousal seeking and therefore these people actively seek arousing stimuli, whereas the introvert personality is characterised with already over-stimulated arousal and as such these people generally avoid arousal stimuli. In addition, detection, retention and retrieval of information to process and shape long or short-term memories are dependent on level on individual arousal. Therefore, emotionally arousing information can lead to better information coding, and hence better retention and retrieval. Preference for familiar over unfamiliar environments is a condition requiring less arousal, which leads an individual to decide whether increased arousal is favourable or not. This phenomenon is called arousal/avoidance conflict (Pfaff, 2006).

In addition to an involvement in shaping personality and emotion, arousal is also related to psychological disorders. For example, depression is associated with suppressed arousal and an inability to be aroused by stimuli, whereas there is a complex relationship between arousal and anxiety (Kemp and Felmingham, 2008). Individuals with anxiety disorders have distorted and exaggerated perceptions that lead to hyper-arousal, which leads to increase avoidance behaviour to constrain arousing stimuli (Kemp and Felmingham, 2008). Other pathological conditions likely associated with altered arousal state, such as alcohol withdrawal and acute encephalitis (Kattimani and Bharadwaj, 2013), coma induced by head trauma (Cossu, 2014); (Huey et al., 2017), rabies (Senthilkumaran et al., 2011), hemisphere lesions caused by stroke (Goldfine and Schiff, 2011; Vazquez-Marrufo et al., 2014), require further research to examine the contribution of arousal systems to pathology, which may shed new light on therapeutic targets for these disorders.
The research described in this thesis explored the anatomical and functional interactions of arousal centres in hypothalamus and brainstem. The findings obtained provide direct evidence for a novel hypothalamic-brainstem circuit in the regulation of adrenal sympathetic nerve activity and blood glucose, and in regulating arousal and feeding in rats. These findings provide a basis for further targeted research for better treatments for diabetes, sleep and eating disorders, stress-related mental disorders and other arousal-related conditions.

1.2. The ascending reticular-activating system and arousal

In ethology, arousal refers to “the overall state of responsiveness of the animal, as indicated by the intensity of stimulation necessary to trigger a behavioural reaction. It moves animals toward readiness for action from the state of inactivity” (Immelmann and Beer, 1989). Central nervous system (CNS) arousal is fundamental to all cognitive and emotional functions. In other words, arousal leads to a “fixed action pattern” in response to a stimulus manifested by instinctive behaviour or learned goal-oriented behaviour. Therefore, generalised arousal is higher in animals/humans who are alert to sensory stimuli, more active physically and more reactive emotionally, all of which are measurable by behaviour, electroencephalogram (EEG) recordings of brain activity and readouts from the autonomic nervous system (Levy, 2013; Del Percio et al., 2017). Based on information processing theories by Claude Shannon (Shannon and Weaver, 1963), it was concluded that unpredictable and uncertain environments promote arousal, which led to the establishment of various behavioural paradigms designed to provide novel, inconsistent and salient environments to study arousal using animal models (Mousavian et al., 2016).

The Ascending Reticular-Activating System (ARAS), also known as the ascending arousal system was first identified during the encephalitis lethargica epidemic of 1915-1926 that spread through Europe and North America, affecting ~5 million people with 1.5 million deaths (Dickman, 2001). The Viennese neurologist, Baron Constantin von Economo, noticed affected patients with symptoms of consciousness, though they were not fully-awake. These patients would sit motionless/speechless with a lack of energy, emotion, and motivation, and were unresponsive to events around them. Some patients were reported to go without sleep for days, whereas others reported more than 20 h of sleep and profound sleepiness. However, both these group had normal
cognitive function (Triarhou, 2006). Although Economo could never identify the cause of this syndrome, which he later called von Economo’s sleeping sickness, he was able to identify the lesion in specific brain areas post-mortem. He reported that lesions in the junction of midbrain and diencephalon were associated with prolonged sleepiness, whereas lesions in anterior hypothalamus were associated with insomnia. He hypothesised that the region between these lesioned areas was the origin of the ascending arousal system and projection of this system to forebrain promoted wake (Triarhou, 2006). Since then, research has further established that this system begins in rostral pons, coursing through the midbrain reticular formation to forebrain. Thus, the system was renamed as the ARAS, which is now known for its mediation of pathological disorders such as dementia, improper sleep-wake transitions, and occurrence of vegetative state in progressive cases.
Figure 1.1. A schematic drawing showing some key components of the ascending arousal system.

A major input to the relay and reticular nuclei of the thalamus (red pathway) originates from cholinergic (ACh) cell groups in the upper pons, the pedunculopontine (PPT) and laterodorsal tegmental nuclei (LDT). These inputs facilitate thalamocortical transmission. A second pathway (blue) activates the cerebral cortex to facilitate the processing of inputs from the thalamus. This arises from neurons in the monoaminergic cell groups, including the tuberomammillary nucleus (TMN) containing histamine (His), the A10 cell group containing dopamine (DA), the dorsal and median raphe nuclei containing serotonin (5-HT), and the locus coeruleus (LC) containing noradrenaline (NA). This pathway also receives contributions from peptidergic neurons in the lateral hypothalamus (LHA) containing orexin (ORX) or melanin-concentrating hormone (MCH), and from basal forebrain (BF) neurons that contain γ-aminobutyric acid (GABA) or ACh (Saper and Fuller, 2017).
The ARAS has two major neural pathways (Figure 1.1). The first originates from fast-firing pedunculopontine nucleus (PPT) and laterodorsal tegmental nucleus (LDT) neurons during wakefulness and rapid eye movement (REM) sleep, and projects to thalamus to activate relay neurons crucial for information relay to cortex (Van Dort et al., 2015). The second pathway is mainly monoaminergic originating from noradrenergic neurons in the locus coeruleus (LC), serotoninergic neurons in the dorsal raphe (DR) and median raphe (MR), dopaminergic neurons in the periaqueductal gray (PAG), and histaminergic neurons in the tuberomammillary nucleus (TMN), all of which send projections that bypass the thalamus to basal forebrain (BF) and lateral hypothalamus (LH) (Blandina et al., 2012). Projections to the cortex are augmented by fast-firing peptidergic neurons in LH, which express the neuropeptides orexin and MCH (Chometton et al., 2014), as well as cholinergic and GABAergic neurons in BF (Zaborszky et al., 2015). Furthermore, GABAergic and galaninergic neurons located in ventrolateral preoptic nucleus (VLPO), which are mainly active during sleep, also project to arousal centres in TMN (Uschakov et al., 2007) and LC/DR (Schwartz and Roth, 2008). These VLPO neurons receive inhibitory inputs from noradrenergic, GABAergic and serotoninergic neurons, and therefore is reciprocally inhibited by the same arousal centres to inhibit sleep processes during wake (Samuels and Szabadi, 2008; Saper et al., 2010). This system is referred to as the ‘flip-flop switch’, which implies a neural loop consisting of mutually inhibited regions that form a self-reinforcing circuit where activity of one region inhibits the inhibitory inputs from another region, hence reinforcing overall inhibition (Saper et al., 2005). This results in no transitional state in between sleep-wake, the transition is abrupt or ‘flip-flop’ in nature. During wake, LH orexin neurons reinforce monoaminergic tone to stabilise inhibition of the VLPO (Sakurai, 2005b). However, there is no orexinergic projection to VLPO, and orexin receptors are not found in VLPO, suggesting that this action is mediated indirectly (Sakurai et al., 1998). In contrast, there are inhibitory VLPO projections to orexin neurons, which are active during sleep in addition to projections to TMN, LC and DR (Saper et al., 2005). Inputs from four sensory systems (vestibular, somatosensory, auditory and taste) can influence the ARAS output by providing some projections to the brainstem origin of this system whereas the remaining two sensory systems (smell and vision) have inputs via different pathways; the olfactory system converges on the basal forebrain (amygdala), while visual signals travel to the superior colliculi in midbrain and to the pulvinar nucleus in thalamus, they form the ascending
arousal system (Pfaff, 2006). Thereafter, the ascending arousal system conveys sensory information to the cortex through cholinergic and glutamatergic projections. Importantly, different components of the ascending arousal system have distinct anatomical connections that are not point-to-point (i.e. some projections form a ‘ladder’ of connections, resulting in amplification of arousal signals, and all components are highly redundant both neurochemically and functionally. An example of this connection pattern can be seen in the projection from ventral medullary neurons to LC and then to the nucleus of solitary tract (NTS) in order to amplify the transmission via the noradrenergic LC neurons (Van Bockstaele and Aston-Jones, 1992). In addition to ascending projections and effects on cortical arousal, brainstem regions also have descending projections to the spinal cord to mediate autonomic responses via the sympathetic and parasympathetic systems (Kop et al., 2011; Kyle and McNeil, 2014).

The ascending arousal system also involves the septohippocampal system, which is important for hippocampal-dependent attention and spatial memory (Broadbent et al., 2004), the mesolimbic dopaminergic system, which is important for motivation/reward (Bromberg-Martin et al., 2010), the corticotropin-releasing factor (CRF) system, which is important for stress-responses (Smith and Vale, 2006), and hypothalamic feeding centers (Stuber and Wise, 2016). Indeed, the forebrain septal area is a key node for regulating of arousal (Morgane et al., 2005). Ascending projections from the medial septum (MS) to the hippocampus provide a ‘pacemaker’ for theta rhythms/oscillations produced in hippocampus (Kang et al., 2015), whereas descending projections from the lateral septum (LS) to various subcortical regions mediate a wide range of visceral and metabolic functions such as aggression, social and sexual behaviour as well as circadian rhythms (Olucha-Bordonau et al., 2012). Theta rhythm is slow sinusoidal activity of the hippocampus and/or other areas, and is easily identified in EEG, being first reported by Jung and Kornmuller (1938). This brain oscillation has been correlated with arousal, goal-orientation, attention, exploration, learning and memory, motivational drive and emotion, and voluntary movement (Buzsaki, 2005). It is also hypothesised that theta rhythm might underlie information selection, processing and recording in memory (Duncan et al., 2005).
Research using new electrophysiological techniques, such as juxtacellular labelling, has enhanced knowledge of neuronal properties and responses *in vivo* (Pinault, 2011). Furthermore, genetically-modified mice, such as those expressing green fluorescent protein (GFP) under control of a glutamic acid decarboxylase (GAD) promotor to label GABAergic neurons, have revealed the importance of GABAergic systems in regulating arousal and sleep-wake cycle (Chen et al., 2010; Bergman et al., 2014). These and other markers such as red fluorescent proteins (tdTomato or m-Cherry) used recently for labelling the vesicular GABA transporter (vGAT) (Anaclet et al., 2018) or parvalbumin (PV), a calcium-binding protein expressed in a population of fast-firing GABAergic neurons (McKenna et al., 2013) that has been used to advantage to generate transgenic Cre recombinase expressing mice for optogenetic and DREADD studies, have revealed the physiological properties of wakefulness and arousal (de Lecea et al., 2012; Brown and McKenna, 2015; Taylor et al., 2016).

1.3. Neuromodulation underlying behaviour and arousal

Neurons communicate with each other in various ways to relay information related to the environment, to moving through the environment, and to provide responses to actions within the environment. Unlike the classical concept of fast (millisecond time-scale), point-to-point and simple excitation or inhibition properties of neural transmission, it is now universally accepted that neural communication is far more complex. Thus, the term ‘neuromodulation’ has broadened the concept of neurotransmission, whereby neuromodulators or ‘tuning’ transmitters are generally slow-release transmitters (over hundreds of milliseconds to minutes) (Katz, 1999). Sensory inputs to the CNS can be influenced by neuromodulators by altering their sensitivity and sensory coding (Márquez et al., 2013). Neuromodulators are conventionally categorised as monoamines, peptides and hormones, however recently, it has been demonstrated that even GABA, glutamate or nitric oxide can act as neuromodulators (Marder, 2012). They are released from neurons to bind to receptors on pre/postsynaptic neurons (axo-axonal synapses) to gate the release of fast transmitters (Brezina, 2010), cause presynaptic inhibition or facilitation (Marder and Thirumalai, 2002; Vanden Pol, 2010), or diffuse from the synaptic cleft into the extracellular space, blood or cerebral...
spinal fluid (CSF), to act via what is described as volume transmission (Sarter et al., 2009; Fuxe et al., 2013).

Volume transmission can also lead to termination of synaptic actions by removing transmitters from the synaptic cleft or relay signals to neighbouring neurons/glial cells via calcium waves (Hirase et al., 2014; Monai et al., 2016). A neuron generally releases a fast-acting neurotransmitter along with one or more neuromodulators, which allows the neuron to act over various time- and spatial- scales (Werner and Mitterauer, 2013). However, different transmitters and neuromodulators are present in different neurons (Bucher and Marder, 2013; Marder et al., 2014).

Neuromodulators generally activate metabotropic receptors (G-protein-coupled receptors (GPCRs)), rather than ionotropic receptors activated by fast neurotransmitters, and activate second messenger cascades that activate kinases and phosphorylation of ligand- or voltage-gated ion channels (Civelli, 2012) (Figure 1.2). The G-proteins have been classified into four main categories based on the sequence similarity of their Ga subunits: GaS, GaI/O, Gaq/11 and Ga12/13 (Hanlon and Andrew, 2015). The classification defines both receptor and effector specificity as the Ga and Gq have two well-defined effector pathways, respectively (Billington and Penn, 2003). Although the role of Ga subunits as a major component for GPCRs activation pathways are established, the Gb subunit can also mediate signal transduction by interacting with different effectors including AC, PI3 kinase, proteins of the MAPK pathway, and several ion channels (Billington and Penn, 2003; New and Wong, 2007; Eishingdrelo and Kongsamut, 2013; Kamal et al., 2017). Ion channels modulated by GPCRs include Cav2 calcium channels, which trigger neurotransmitter release, and fast synaptic transmission in the mammalian central nervous system (CNS) is mediated by several voltage-dependent Ca2+ channels (Altier, 2012; Zamponi and Currie, 2013). The N type and the P/Q-type Ca2+ channels are common types of CNS Ca2+ channels regulated by GPCRs, and have significant roles in neuronal communication (Ishikawa et al., 2005; Dolphin, 2016). For example, these channels have a major role in synaptic modulation mediated by both endogenous hormones as well as exogenous GPCRs agonists (Jewell and Currie, 2013; Inanobe and Kurachi, 2014). GPCRs also modulate a number of K+ channels including the K+-inward rectifier Kir3 subfamily, e.g. Kir3.1 and Kir3.4 (Hibino et al., 2010). Inward rectifier K+
channels (Kᵢ₃, or G-protein inward rectifier K⁺ channels (GIRK)) play a key role in maintaining the resting membrane potential by regulating the action potential duration and membrane excitability. Following stimulation of Gᵢₒ-coupled receptors, a low basal level of activity is dramatically increased via the Kᵢ₃ channels. These channels are activated when Gᵦ subunits bind directly to the N- and C-terminal regions of each subunit. The channel can also be activated by phosphatidylinositol bisphosphate (PIP2) which is essential for the proper gating of the channel following Gᵦᵦ activation. Muscarinic receptors, P2Y receptors, somatostatin receptors, dopamine receptors and GABA_B receptors are among the GPCRs which act via Kᵢ₃ channels (Hibino et al., 2010).

![Figure 1.2. Neuromodulation of ion channels and membrane excitability.](image)

Different intracellular signaling pathways activated upon GPCR agonist stimulation. Following agonist stimulation of GPCRs, the Ga and Gβγ subunits disassociate and either subunit can modulate a diverse array of effectors. + indicates activation, - indicates inhibition (Gohar, 2014).
Functionally, GPCR signalling reconfigures the firing pattern of the neuron and the synaptic interactions with other neurons in a nonlinear dynamic manner (Tian et al., 2017). Consequently, changing the output of hard-wired connectivity of neural networks allows for behavioural adaptability, which is crucial for survival. Previously, it was believed that the neuromodulatory centres in brain consisted of the raphe nuclei, substantia nigra and LC, comprised of small clusters of phenotypically similar neurons projecting throughout the brain and transmitting aminergic signals (e.g. serotonin, dopamine and noradrenaline, respectively) (Saper et al., 1976). However, it is now accepted that all transmitters, except for glycine, can act on metabotropic receptors and ionotropic receptors (Marder, 2012).

Neuromodulators can act at the cellular, synaptic, and network level and their actions can be supportive or, surprisingly, opposing in terms of net physiological effect. It was recently hypothesised that this opposing effect at various levels can provide a system of ‘checks and balances’ to stabilise and regulate the new modulated status (Harris et al., 2010). Thus, neuromodulation is essential for the control of information flow in the brain. A deficit in these systems can have pathological consequences, such as impaired conscious perception and motor abilities in schizophrenia (Dokucu, 2015) and Parkinson’s disease (DeLong and Wichmann, 2015), which are caused by interrupted dopamine neuromodulation. A better understanding of these systems will very likely lead to better treatment of mental disorders.

1.4. The orexin and orexin receptor (OX1R and OX2R) system

Orexins (or hypocretins) are highly conserved neuropeptides, orexin A (OXA) and orexin B (OXB), which bind to two GPCRs - orexin 1 (OX1R) and orexin 2 (OX2R) (de Lecea et al., 1998; Sakurai et al., 1998) (Figure 1.3). OXA, a 33 amino-acid peptide with two disulfide bridges, binds and activates both OX1R and OX2R though displays a higher binding affinity for OX2R (O’Leary, 2014). In contrast, OXB is a linear, non-lipophilic 28 amino-acid peptide that has equal binding affinity for both orexin receptors. Both OXA and OXB are encoded from the same exonic region of the prepro-orexin gene, which is abundant in the lateral hypothalamus (LH) (de Lecea and Sutcliffe, 1999). The OXA gene is conserved in rat, mouse, pig, dog and human, and the rat and mouse OXB sequence differs by only one amino acid (S18N) from the porcine, canine and human
OXB (Sakurai, 2005a). The orexin receptors also exhibit high structural and functional homology between rodent and human, which confirms the value of using rodent models to understand the functions of orexins in human (Boss and Roch, 2015).

There are only a small number of orexin-producing neurons (~3,000 neurons in rat and ~70,000 neurons in human) located in the LH, specifically in the dorsomedial hypothalamus (DMH) and perifornical area (PeF) (Nambu et al., 1999), whereas orexin receptors are widely expressed throughout the CNS indicating the broad influence of orexins in various physiological functions (Scammell and Winrow, 2011). Importantly, the densest orexin-containing projections are found descending to arousal centres of the brainstem, such as the TMN, LC and DR, which also express the highest densities of orexin receptors (either OX1R and OX2R or both) (Marcus et al., 2001), suggesting that sub-populations of orexin neurons may regulate distinct functions. Substantial receptor expression is also found in regions important for vigilance (Nishino, 2011), stress and reward processing and addiction (Aston-Jones et al., 2009; Johnson et al., 2012). Further to these functions, via different circuits the orexin system orchestrates other physiological processes, such as energy homeostasis and feeding (Gao and Horvath, 2016) (Figure 1.4.), thermogenesis (Madden et al., 2012), sensory modulation (Razavi and Hosseinzadeh, 2017), locomotion (Tsujino and Sakurai, 2013; Luna et al., 2017), cognition (Song et al., 2015), and endocrine and visceral functions regulated by autonomic system (e.g. adipose tissue, endocrine pancreas, adrenal gland, reproductive organs and haematopoietic cells) (Leonard and Kukkonen, 2014; Li et al., 2014a; Verberne et al., 2014) (Figure 1.5.). Thus, modulation of this system can have broad effects on many pathophysiological disorders, including sleep-wake cycle disturbance, addiction, obesity, stress and anxiety disorders, and pain processing (Boss and Roch, 2015).
Figure 1.3. Orexin peptides, receptors and projections.

(A) Orexin A and orexin B are derived from a common precursor peptide, prepro-orexin. Orexin A is a 33 amino acid peptide and is completely conserved among several mammalian species (human, rat, mouse, cow, sheep, dog and pig). Orexin B is a 28 amino acid with several species differences, although overall orexin B is highly conserved and is similar in some part with orexin A (blue portions). The actions of orexins are mediated by two GPCRs: orexin receptor type 1 (OX1R) and orexin receptor type 2 (OX2R) (A). OX1R has one order of magnitude greater affinity for orexin A over orexin B, whereas OX2R binds both ligands with similar affinities.

(B) Orexin neurons, of which there are ~3,000 in the rat brain and ~70,000 in the human brain are localized exclusively in the hypothalamus, including the lateral hypothalamic area, perifornical area and posterior hypothalamus, but project widely in the brain. Projections to monoaminergic and cholinergic nuclei (green, blue and yellow circles) in the brainstem, where OX1R and OX2R are differentially expressed in these areas, are particularly dense (Sakurai, 2014).
Although orexin-A acts on both OX1R and OX2R, orexin B exclusively acts on OX2R (Sakurai et al., 1998) (Figure 1.3). As mentioned, despite the restricted distribution of orexinergic neurons in LHA, they have widespread projections throughout the brain, which is matched by the widespread but differential distribution of OX1R and OX2R (Peyron et al., 1998). *In situ* hybridisation studies of the distribution of orexin receptors demonstrate that OX1R mRNA is mainly located in hippocampus, paraventricular thalamic nucleus (PVN), ventromedial hypothalamic nucleus (VMH), DR, and LC, whereas OX2R mRNA is present in other areas including the cerebral cortex, hippocampus, ventral premammillary nucleus, PVN, DR and TMN (Marcus et al., 2001). Orexin knockout mice display a narcolepsy-like phenotype (Chemelli et al., 1999) similar to narcoleptic dogs deficient in the expression of OX2R (Lin et al., 1999). In addition, the regions expressing OX1R, such as PVN and arcuate nucleus (Arc) are enriched in the hypothalamus and are strongly implicated in the modulation of feeding and reward. Based on the divergent distribution of orexin projections and related receptors, it was suggested that orexin neurons in the non-LH regions including dorsomedial hypothalamus (DMH) and perifornical area (PFA) control arousal, while those in the LH contribute more in reward seeking behaviour (Harris and Aston-Jones, 2006), however this hypothetical functional separation has not been universally observed.

The orexin system also regulates wakefulness and attention. Wakefulness is a binary state of either wake or sleep, whereas attention (active wake or arousal) is an analogue state that is continuous and variable (Oken et al., 2006). Orexin contributes to stabilising attention, and its effects on feeding and locomotor activity are not a ‘by-product’ of increased wakefulness (Wright et al., 2012; Korotkova and Ponomarenko, 2017). Hence the behavioural response of orexin is concentration-dependent and not merely ‘on or off’. Interestingly, animals and humans with narcolepsy are still capable of being awake despite fragmented sleep/wake patterns (Sato et al., 2014), suggesting that the role of orexin in arousal is not exclusive but, in fact, orchestrates arousal and vigilance in response to external stimuli.
Figure 1.4. Orexin neurons in the regulation of feeding.

Orexin neurons receive input from neuropeptide Y (NPY) neurons in the arcuate nucleus and can sense circulating hormones and nutrients, such as leptin, ghrelin and glucose. Inputs from the limbic system, including the bed nucleus of the stria terminalis (BNST) and nucleus accumbens (NAc) (in yellow), might convey emotive components that are associated with food recognition. Orexin neurons project to the paraventricular nucleus of the hypothalamus (PVN), dorsomedial hypothalamus (not shown), nucleus of the solitary tract (NTS), area postrema (AP) and NAc (in yellow) to increase food intake. Note that functional excitation might include disinhibition, such as inhibition of GABAergic projections. GR, glucoreceptor; DA, dopamine; VTA, ventral tegmental area (Sakurai, 2014).
Figure 1.5. Orexin neurons in the regulation of autonomic function.

Input areas are shown in yellow, output areas are shown in blue. Salient emotional stimuli evoke autonomic responses and arousal through neural connections between the amygdala and lateral hypothalamus (LH), in which orexin neurons are located. Orexin neurons receive innervations from limbic regions, including the nucleus accumbens (NAc), bed nucleus of the stria terminalis (BNST) and central amygdala (CeA), and send projections to the premotor autonomic centres, including the periaqueductal grey (PAG), parabrachial nucleus (PBN), nucleus of the solitary tract (NTS), paraventricular nucleus of the hypothalamus (PVN), rostral ventrolateral medulla (RVLM) and rostral ventromedial medulla (RVMM). The activity of orexin neurons is further influenced by CO2 and pH, which also reflect the autonomic state (Sakurai, 2014).
1.5. The melanin-concentrating hormone (MCH) and MCH1R receptor system

Melanin-concentrating hormone (MCH) is a highly conserved neuropeptide that was first discovered in fish teleost (Kawauchi et al., 1983) and rat hypothalamus in the 1980s (Nahon et al., 1989). In rodents, it has been shown that MCH has an inhibitory role at presynaptic and postsynaptic levels, via its GPCRs MCH1R and MCH2R (Guyon et al., 2009), with the latter found only in ‘high-order’ mammals (Gao, 2009) (Figure 1.6). In addition, MCH directly modulates the activity of hypothalamic neurons by inhibiting voltage-dependent ion channels (Gao and van den Pol, 2001; Gao, 2009).

Figure 1.6. Structure and brain distribution of the rat MCH and MCH-R1.

(A) Structure of the rat MCH (cyclic peptide) and (B) rat MCH-R1 (7TM GPCR), (C) sagittal sections of the rat brain showing the distribution of MCH neurons and projections and (D) MCH-R1 mRNA expression. Black dots indicate MCH neuron perikarya, and lines correspond to fibres. Brain areas with expression of MCH-R1 mRNA are noted by white dots, redrawn, respectively (Presse et al., 2014).
The MCH neuropeptide family consists of MCH and two other neuropeptides, known as neuropeptide E-I (NEI) and neuropeptide G-E (NGE), and all peptides originate from the precursor pro-MCH gene, expressed in the hypothalamus (Nahon et al., 1989). Almost 96% of MCH neurons co-express NEI (Bittencourt et al., 1992). Studies have revealed interactions among MCH, NEI and NGE, as well as with other related systems, such as melanin-stimulating hormone (α-MSH) and proopiomelanocortin (POMC) receptors (Diniz and Bittencourt, 2017), however the functions of NGE and NEI are still unclear. MCH is orexigenic in mammals (MacNeil, 2013) despite its sole function in adaptive pigmentation in fish (Logan et al., 2006), thus much research has examined a putative role of MCH in obesity (Ludwig et al., 2001; Cheon, 2012). The MCH neuropeptide in different mammalian species is localised predominantly in LHA and zona incerta (ZI). However, it is not co-localised with orexin, but instead co-localises with cocaine- and amphetamine-regulated transcript (CART) (Elias et al., 2001). In addition, small populations of MCH-immunopositive and orexin-immunopositive cells co-express dynorphin (Chung et al., 2009).

The hypothalamic area containing MCH-immunopositive and preproMCH mRNA-positive neurons is divided into seven regions: IHy, the anterior, tuberal, and posterior subdivisions of the LH (LHAa, LHAt, and LHAp), anterior periventricular nucleus, dorsolateral part of the zona incerta, and the dorsomedial part of the tuberomammillary complex (Saper et al., 1979; Bittencourt, 2011). In addition to these subdivisions, a number of MCH neurons are also located in the medial hypothalamus, between the dorsomedial nucleus of the hypothalamus (DMH) and ventromedial nucleus of the hypothalamus (VMH), which is referred to as the inter-nuclear region (Swanson, 1998; Bittencourt, 2011). In addition to these diencephalic sites, MCH is also expressed in the olfactory tubercle, within the boundaries of the islands of Calleja, and in the pons, in an unmapped region within the paramedian reticular formation (Bittencourt et al., 1998). In female lactating rats, there are additional sites of prepro-MCH mRNA expression, such as the ventromedial preoptic nucleus, periventricular preoptic nucleus, and the rostral PVH (Rondini et al., 2010). More recent studies suggested that MCH might inhibit reproductive function and behaviours during lactation via this pathway, based on a significant increase in the MCH innervation of the ventrolateral subdivision of the ventromedial hypothalamic nucleus and the ventral premammillary nucleus (Naufahu et al., 2013). These MCH neurons do not co-express
oxytocin and interestingly, prepro-MCH mRNA and MCH-immunoreactivity are absent in non-lactating rats (Knollema et al., 1992). In other areas, such as the caudal laterodorsal tegmental nucleus (LDT), MCH neurons were detected that were independent of the lactation cycle and co-expressed the GABA neuron marker, GAD67 (Rondini et al., 2007).

Despite their restricted distribution, MCH neurons project broadly throughout the brain to the median eminence (ME), hippocampal formation (HF), prefrontal cortex (PFC), PAG, pars lateralis of the medial mammillary nucleus (ML), nucleus accumbens shell (AcbSh), MS and the interoanterodorsal nucleus of the thalamus (Bittencourt et al., 1998). MCH neurons receive mainly inhibitory inputs from neurons expressing dopamine, serotonin, acetylcholine, noradrenaline, GABA, α-MSH, CART, and other MCH neurons (Gonzalez et al., 2016), in addition to excitatory glutamatergic and orexinergic inputs (Konadhode et al., 2015). As MCH neurons express leptin receptors, it has been hypothesised that these neurons are involved in energy balance for integrating leptin signals from the arcuate nucleus (Arc) or subfornical organ, which both lack a blood-brain barrier, and relays this information to VMH, PVH, and LH (Leinninger, 2011). In addition, the arrangement of the MCH neurons in the LH/IHy is ideal for providing descending projections to pro-encephalic nuclei, brainstem nuclei or to the spinal cord (Bittencourt, 2011). Thus, there is a hub of peptidergic systems in the LH/IHy (MCH, orexin and CART), with putative functions in motivated behaviours, sleep-wake cycle, circadian rhythm control, stress, emotion, and autonomic control, movement, hunger/thirst, attention, reward, learning and memory, and neuroendocrine regulation, and their study should provide a broader insight into MCH function beyond just food intake behaviour (Bittencourt, 2011; Diniz and Bittencourt, 2017).

1.6. Physiological interactions between orexin and MCH systems

The first studies that suggested interactions between orexin and MCH systems were by Guan et al. (2002), who used immunocytochemistry and electron microscopy to observe that orexin and MCH neurons made synaptic contacts with each other (Guan et al., 2002). Several studies have since confirmed the reciprocal or complementary interactions between orexin and MCH systems, as well as interactions with other arousal centres (Konadhode et al., 2015).
1.6.1. The flip-flop switch model of sleep/wakefulness

Recorded by electroencephalography (EEG), sleep is composed of 4 stages including one stage of rapid-eye movement (REM) sleep, and three stages of non–rapid eye movement (NREM) sleep. The timing and regulation of REM and NREM is affected by both circadian and homeostatic conditions, such as sleep pressure during wakefulness (Morris et al., 2012). Sleep is influenced by the circadian internal clock-like rhythm, involving the suprachiasmatic nucleus (SCN), which is entrained by cyclic environment cues, such as sunlight (Wurts and Edgar, 2000).

Transition between wake, NREM and REM is associated with ARAS activation, as most ARAS brain regions are either wake- or sleep- (NREM or REM) promoting. Two thalamic circuits are separately involved in these transitions, which are activated by the pedunculopontine tegmental (PPT) and laterodorsal tegmental nuclei (LDT) (Schwartz and Kilduff, 2015). Although the thalamic pathway is more important for REM and NREM sleep, there is an extra-thalamic pathway that consists of DR, LC, LH, TMN, vPAG, and VLPO that is important for non-vegetative levels of arousal and arousal state transitions (Lin et al., 2011). However, no one region is solely accountable for sleep-wake transitions, as single region lesions do not impair arousal (Schwartz and Kilduff, 2015). For example, lesioning of substantia nigra as well as ventral tegmental area in cats induces behavioural unresponsiveness and akinesia, but cortical activation is intact. On the other hand, lesions in the large area of mediopontine tegmentum affecting the ascending noradrenergic pathway can impair waking (Jouvet, 1972), whereas electrolytic or chemical lesioning of locus coeruleus or its ascending projection to the cortex did not impair waking (del C. González et al., 1998). It is hypothesised that LH orexin and MCH activities are synchronised to modulate multiple arousal centres to increase the likelihood of arousal state transitions, tailored to homeostatic or circadian input (Saper et al., 2010).

The sleep-wake ‘flip-flop’ switch model describes the arousal state transition in mammals (Lu et al., 2006). The flip-flop system is comprised of the reciprocal inhibitory GABAergic input between sleep-promoting preoptic nuclei (VLPO) and wake-promoting cholinergic (LDT, PPT), glutamatergic (PB) and monoaminergic (DR, LC, TMN, vPAG) nuclei. Upon waking, the wake-promoting centres directly inhibit the VLPO, boosted by stimulating orexin neurons (Saper et al.,
2005). However, during homeostatic sleep (for example when jetlagged), excitatory input to VLPO can induce sleep during the day (Saper et al., 2010). At night, collective activity of the VLPO supersedes that of the wake-promoting centres, which induces transition from wake to NREM sleep (Gvilia et al., 2006). Circadian processes, including SCN input, can affect this transition by influencing both VLPO and orexin, as ablation of the SCN induced a fluctuation in orexin CSF levels (Abrahamson et al., 2001). In any case, OXA has been demonstrated to promote both homeostatic and circadian processes of sleep, in studies revealing that the ablation of SCN in rats induced fluctuations in the cerebrospinal fluid (CSF) level of orexin (Zhang et al., 2004) whereas mice SCN clock neurons were inhibited by orexin in vitro (Belle et al., 2014). The REM-NREM switch is regulated by reciprocal GABAergic inhibition between REM-off neurons in the VLPO and REM-on neurons in the peri-locus coerules (P-LC) and sublaterodorsal nucleus (SLD), where SLD neurons can disinhibit P-LC neurons and disinhibit medullary interneurons to promote REM-associated atonia (Lu et al., 2006). MCH has also been demonstrated to inhibit VLPO REM-off neurons and initiate NREM to REM transitions (Saper et al., 2010; Jego et al., 2013).

Although LH orexin neurons only discharge during wakefulness, and MCH neurons fire during sleep that is maximum during REM, other arousal centres reinforce this firing profile, such as inhibition of MCH neuron firing by histamine (Parks et al., 2014). It is established that orexin neurons prolong the duration of wakefulness and MCH neurons prolong the duration of REM within the flip-flop switch model, which underlies the narcoleptic pathology associated with impaired orexin signalling (De la Herrán-Arita et al., 2011).

### 1.6.2. Role in feeding and energy balance

Although it is well-known that both orexin and MCH systems are orexigenic, orexin promotes food seeking behaviour or so-called foraging and these neurons are activated when an animal is in contact with the food rather than the digestion of food (Gao and Horvath, 2016), whereas the MCH system can promote caloric intake consumption and both systems can be stimulated by food intake, especially palatable food (Barson et al., 2013). Many studies have demonstrated that OX1R activation by OXA contributes to increased chow intake using local microinjection of OXA into hypothalamic areas of the brain (Dube et al., 1999) and reduced palatable food intake following
peripheral administration of OX1R antagonist (SB-649868) with no changes in sucrose self-administration in rats (Choi et al., 2010).

In addition, prepro-orexin gene knockout mice exhibit no changes in chow-food intake compared to wild type, implying that this neuropeptide is likely not essential for normal food intake (Sharf et al., 2010b). From the available evidence it is suggested that OX1R, but not OX2R, is mainly responsible for the link between orexins and palatable food intake as well as chow intake, as an OX2R antagonist (JNJ-10397049) had no effect on binge eating of a high-fat, high-sucrose food in rats (Piccoli et al., 2012). Although MCH neurons have been shown to be strongly depolarized by the orexin system by administering OXA and OXB in slice preparation (Van den Pol et al., 2004), the MCH system operates in a complementary way to orexin in terms of feeding and energy balance. MCH gene expression is upregulated after 24 h food deprivation (Barson et al., 2013). Increased body weight following a standard diet was observed in mice with an over expression of the ppMCH gene (Ludwig et al., 2001), whereas MCH1R receptor knockout mice had lean body weight with hyperphagia and hyperactivity (Marsh et al., 2002), in opposite of the hypophagia reported in MCH knockout mice (Shimada et al., 1998). Genetically obese mice are reported to have elevated MCH mRNA and plasma peptide levels and chronic i.c.v injections of MCH elevated the caloric efficiency and body fat mass (Mondal et al., 2002) which was reversed following the administration of an MCH1R antagonist in rats (Shearman et al., 2003). In terms of energy homeostasis, orexin and MCH have been shown to have opposite roles. Central injection of orexin elevated energy expenditure by increasing oxygen consumption, which is believed to be due to the action of the OX2R in promoting energy expenditure. This idea was supported by observations of resistance to dietary obesity in mice lacking OX1R, but not in those lacking OX2R. The chronic central injection of OX-B, which binds to OX2R, resulted in prevention of fat-induced obesity (Funato et al., 2009). MCH, on the other hand, promotes energy conservation, reflected by a decrease in oxygen consumption after central administration of MCH in rodents (Asakawa et al., 2002; Semjonous et al., 2009).
1.6.3. Roles in stress response

Conflicting findings regarding the orexin system response to stress and anxiety have been reported. For example, rats that had undergone restraint-induced stress (Tung et al., 2016) and noxious but not conditioned fear stimuli (Zhu et al., 2002) exhibit Fos-immunoreactivity in orexin neurons. Studies have indicated that the increased Fos-immunoreactivity in orexin neurons following restraint, shock or ‘panic’ might be due to increased wake and grooming after an aversive stimulus, rather than the aversive situation itself, when compared to control (Blouin et al., 2005). MCH neurons, on the other hand, seem to be activated by the HPA stress axis and i.c.v administration of this peptide has anxiolytic effects in animals exposed to inescapable footshock (Carlini et al., 2006). In addition, footshock decreases the level of MCH mRNA (Presse et al., 1992; Presse et al., 2014) and chronic or repeated stress in mice elevated MCH1R receptor and MCH in rodents (Roy et al., 2007; Kim and Han, 2016). MCH signalling also interacts with the HPA axis, as application of corticosterone onto mouse hippocampal neurons in vitro increased MCH expression (Kim and Han, 2016). On the other hand, orexin release is associated with stress, anxiety, PTSD and panic attack responses, which are outcomes associated with various excitatory orexin projections to arousal areas. For example, orexin projections to LC excite noradrenergic neurons, increasing arousal (Horvath et al., 1999). Orexin also excites serotonergic neurons in the DRN (Brown et al., 2002) and adrenergic neurons in the RVLM (Antunes et al., 2001) (Antunes et al., 2001). This also suggests orexin involvement in anxiety since many anxiolytic drugs target monoaminergic systems including tricyclic antidepressants (Rifkin et al., 1981) and monoamine oxidase inhibitors (Kelly et al., 1971) or drugs that alter activity of specific monoaminergic systems [e.g., serotonergic (SSRI) or norepinephrine (NRI) reuptake inhibitors, see review (Johnson et al., 2012)].

1.6.4. Roles in reward behaviour

Importantly, orexin signalling is principally activated under conditions of high motivational relevance, such as the states of physiological need, exposure to threats or reward opportunities. It is hypothesized that many behaviours arising from orexin signalling reflect an essentially integrated function for orexins in translating motivational activation into organized collections
of psychological and physiological processes supplementing adaptive behaviours (Johnson et al., 2012; Mahler et al., 2014). In contrast, lower levels of orexin signalling in areas such as hippocampus with lower expression of orexin-A can result in depressive-like behaviour, as found in the FST in mice (Arendt et al., 2013).

In terms of rewarding behaviour, orexin and MCH have opposing effects via modulation of limbic dopamine levels. Orexin increases the reinforcing effect of abused substances, whereas MCH suppresses reward behaviour and promotes depressive and anxious behaviour (Barson et al., 2013). OXA injected into the VTA promoted conditioning for morphine in a conditioned place preference paradigm in a dose-dependent manner in rodents (Taslimi et al., 2012), whereas peripheral administration of the OX1R antagonist SB-334867 or OX2R antagonist TCS-OX2R-29 suppressed place preference acquisition and expression (Sharf et al., 2010a; Tabaeizadeh et al., 2013). In contrast, ethanol conditioned place preference appears to be mainly influenced by OX2R, since acquisition, expression, and reinstatement for ethanol were attenuated by systemic treatment of the OX2R antagonist JNJ-10397049, but not the OX1R antagonist SB-408124 (Shoblock et al., 2011) or SB-334867 (Voorhees and Cunningham, 2011).

The reinforcing effects of naturally rewarding stimuli may also involve the orexin system. In male rats, conditioned cues which were associated with sexual behaviour in a conditioned place preference paradigm induced Fos-immunoreactivity in orexin neurons, whereas orexin neuron lesions blocked the formation of place preference for a chamber paired with sexual behaviour (Di Sebastiano et al., 2011). Therefore, orexin signalling may be important for reward processing of both drugs of abuse as well as natural rewards, such as sexual activity or palatable food intake.

In contrast, the MCH system appears more associated with anxiety and depression rather than rewarding reinforcement. For example, there was no difference between MCH1R knockout mice and their wild-type counterparts in cocaine- or amphetamine-induced conditioned place preference (Tyhon et al., 2008) and knockout mice exhibited hypersensitivity to the locomotor activating effects of the psychostimulant, d-amphetamine (Smith et al., 2008), which suggest that these mice have enhanced drug-induced reward processing. Furthermore, MCH signalling is associated with anhedonic behaviour in the forced swim test, where the immobility time during the test positively
correlated with prepro-MCH mRNA expression (Garcia-Fuster et al., 2012). Consistent with this, systemic or local (NAc shell) administration of an MCH1R antagonist produced an antidepressant effect in the forced swim test (Borowsky et al., 2002; Gehlert et al., 2009), whereas injection of MCH in NAc shell produced an opposite effect (Georgescu et al., 2005). Taken together, these findings indicate that MCH signalling operates in opposition to orexin, and is anti-rewarding in its effects.

1.6.5. Roles in autonomic reflexes and glucose sensing

Many studies have established the glucose responsiveness and sensitivity of both orexin and MCH neurons. Burdakov and colleagues, using patch-clamp recording techniques in vitro, demonstrated that orexin neurons are inhibited by glucose, whereas MCH neurons are excited by glucose in mice (Burdakov et al., 2005). These studies also revealed adaptive glucose-sensing responses of orexin and MCH neurons, which allow them to sense changes in blood glucose levels rather than its absolute concentration, in a similar manner to neuropeptide-Y, proopiomelanocortin, ventromedial hypothalamic steroidogenic factor-1 neurons; all of which are important for glucose homeostasis (Karnani and Burdakov, 2011). These regions, in turn, are responsible for stimulation of the sympathetic outflow to the adrenal gland, liver and pancreas to increase blood glucose, which produces negative feedback inhibition from these organs (Karnani and Burdakov, 2011).

On the other hand, a recent study using MCH knockout rats, observed an enhanced hypercapnic chemoreflex only in wakefulness, in addition to decreased body weight and basal glucose levels, but unchanged metabolic rate (Li et al., 2014b). This suggested a contribution of MCH to the regulation of the central hypercapnic chemoreflex that is dependent on vigilance state, and a considerable role in respiratory control (Li et al., 2014b).

1.7. Nucleus incertus (NI) and the relaxin-3 system

1.7.1. NI neuroanatomy and chemoarchitecture

The nucleus incertus (Latin for uncertain nucleus) is a region in the ventromedial central grey ventral to the fourth ventricle of the pons, first described in the human by George Streeter (Streeter, 1903). In the rat, the NI extends for around 0.7 mm rostrocaudally, and is comprised of two clusters
of neurons that are densely packed in the midline (pars compacta or NiC) or loosely arranged laterally from NiC (pars dissipata or NiD) (Goto et al., 2001). The Ni has also been referred to as ‘nucleus O’ (Paxinos and Watson, 1986; Olucha-Bordonau et al., 2003), and lies ventromedial to the posterodorsal tegmental nucleus, dorsal to medial longitudinal fasciculus, and ventral part of the caudal central grey of the rat and mouse (Olucha-Bordonau et al., 2003). For further anatomical details of the Ni, see Figure 4.1. This nucleus was initially described as expressing high levels of GABA-related enzyme activity in rat, and more recent studies have revealed that GABAergic neurons of the Ni are the primary source the neuropeptide, relaxin-3 (Ma et al., 2007) (Figure 1.7). Ni neurons also exhibit immunoreactivity for the calcium-binding proteins, calbindin, in both relaxin-3 negative and positive neurons, as well as calretinin, which is often associated with GABAergic neurons (Ma et al., 2007). There is limited information on the expression of excitatory neurotransmitters within the Ni, but markers for these neurons such as vGlut1-3 mRNA are present in the region in rat and mouse brain. A recent study reported low numbers of vGlut2-immunoreactive neurons in Ni that were back-filled from the MS (Nunez et al., 2006). Dense glutamatergic terminations have also been reported in both Ni subdivisions (Cervera-Ferri et al., 2012). A glutamatergic/excitatory influence on the activity of Ni-to-septum neurons seems to parallel GABAergic inhibition, which may contribute to the hippocampal theta rhythmicity (Cervera-Ferri et al., 2012). There are also other neuropeptides expressed by neurons in this region such as calbindin (Figure 1.7), neuromedin B and cholecystokinin (CCK) (Ryan et al., 2011), whereas minimal 5-HT-immunoreactivity is detected in the Ni relative to the more anterior DR, although one report described the existence of 5HT3 receptors in Ni (Kumar et al., 2016). In addition to peptides, various other receptors are also detected in Ni, including CRF receptor-1 (CRF1) (Walker et al., 2017), 5HT1A receptors expressed by relaxin-3 neurons (Miyamoto et al., 2008), mGluR3 (Ohishi et al., 1993), OX1R and OX2R (Blasiak et al., 2015), MCH1R (Saito et al., 2001), cholinergic muscarinic (M3 and M4) and nicotinic receptors (Vilaro et al., 1994; Ryan et al., 2011).

The Ni receives projections from medial prefrontal cortex, MS and nucleus of the diagonal band, septofimbrial nucleus, medial part of lateral habenula, rostral zona incerta, lateral and posterior hypothalamus, supramammillary nucleus, lateral preoptic nucleus, PAG, interpeduncular nucleus,
caudal pole of the parafascicular nucleus, MR, caudal DR, nucleus prepositus and from contralateral NI neurons (Goto et al., 2001). In turn, NI neurons project broadly throughout the brain, with the largest projections targeting the hippocampal formation, medial septal nucleus and nucleus of the diagonal band; and moderate projections to the amygdala, bed nucleus of stria terminalis, mediodorsal thalamic nucleus, several midline and intralaminar thalamic nuclei, lateral habenula, suprachiasmatic nucleus, lateral preoptic area, lateral and posterior hypothalamus, lateral mammillary nucleus supramammillary nucleus, VTA, superior colliculus, interpeduncular nucleus, MR, LDT, capsule surrounding dorsal and ventral tegmental nuclei, DR, nucleus prepositus, and medial accessory inferior olive nucleus (Goto et al., 2001; Olucha-Bordonau et al., 2003) (Figure 1.8). In this regard, the distribution of relaxin-3-immunoreactive fibers and RXFP3 mRNA and binding sites generally correspond with the distribution of NI efferents (Ma and Gundlach, 2007), suggesting that a major component of relaxin-3 transmission originates from NI neurons. In addition, the distribution of relaxin-3/RXFP3 system in rats (Ma and Gundlach, 2007) and mice (Smith et al., 2010) are consistent with that in macaque (Ma et al., 2009b), suggesting that the systems are conserved, although a thorough characterisation of the human relaxin-3/RXFP3 system is yet to be reported.
Figure 1.7. The chemoarchitectonic appearance of the nucleus incertus (NI).

Double label immunofluorescence for RLX3 in the NI. (A, B, C) Confocal photomicrographs illustrating localization of RLX3 and (A', B', C') GAD in neuronal cell bodies of the NI. (A", B", C") Merged immunofluorescent images reveal that most, if not all, RLX3 cells colocalize with GAD (white arrows). In some sections, GAD-positive only cells were detected (black arrows). (D, E, F) Confocal photomicrographs illustrating localization of RLX3 and (D', E', F') calbindin (CB) in neuronal cell bodies of the NI. (D", E", F") Merged immunofluorescent images reveal approximately 30% of cells show colocalization of RLX3 with calbindin (white arrows). Numerous RLX3-only and CB-only cells were observed (hollow arrows). Scale bars, 30 μm (A, A', A"), 85 μm (D, D', D") and 20 μm (B–C, E–F) (Ma et al., 2007).
Figure 1.8. Key pathways related to the NI brainstem network.

The interconnected NI-IPN-MR projections to and inputs from different brain regions including hypothalamus influence a number of functions important for behavioural output. For abbreviations refer to (Goto et al., 2001).
1.7.2. NI functions in modulating arousal and behaviour

To date, NI function has been investigated using rodent models, through lesioning, *in vivo* and *in vitro* electrophysiology, and pharmacological studies. The activity of NI neurons in response to acute and chronic stress has been tested following the observation of high levels of CRF1 mRNA -but not CRF2 receptors- co-expressed with immunoreactive Fos protein in the NI, after i.c.v. injection of CRF in rats (Bittencourt and Sawchenko, 2000). In addition, NI neurons exhibit long-lasting and non-desensitising depolarisation in response to (Ma et al., 2013), which is different from the dose-dependent characteristics of DR neurons in response to CRF, 5-HT and other neuromodulators (Kirby et al., 2008). Physical stress such as different types of restraint (Senba et al., 1993; Cullinan et al., 1995; Tanaka et al., 2005) increased Fos protein immunoreactivity in NI. In addition, 10 min forced-swim test, water immersion and immobilisation, footshock, or paw pinch, also significantly increased NI Fos-immunoreactivity whereas pairing with a previously-exposed predator environment decreased NI Fos immunoreactivity (Ribeiro-Barbosa et al., 2005; Ryan et al., 2011).

In contrast, physiological stressors such as infection, hypoxia or hypercapnia do not increase NI neural activity (Elmquist et al., 1996; Teppema et al., 1997). It has been hypothesised that NI activation occurs when a behavioural or locomotor response is required. In fact, rats that display freezing behaviour in a conditioned fear paradigm or in response to cat (predator) odour, exhibit decreased NI Fos-immunoreactivity compared to control rats (Ribeiro-Barbosa et al., 2005). In this regard, the active locomotor component or ‘flight’ response, but not the active immobilisation component or freezing, of the behavioural stress response appears to be mediated by CRF1-expressing NI neurons (Ryan et al., 2011). Consistent with this, saporin lesions of CRF1-expressing NI neurons increased immobilisation/freezing in a fear conditioned paradigm (Lee et al., 2014). Furthermore, NI Fos-immunoreactivity also increased with food anticipatory activity in rats with restricted food access (Poulin and Timofeeva, 2008). Poulin and Timofeeva (2008) demonstrated that the septohippocampal system together with PVN and DMH act in concert as a food-entrainable oscillator (Goto et al., 2001; Poulin and Timofeeva, 2008).
GABAergic neurons in NI (a population which expresses relaxin-3) have been shown to influence hippocampal theta rhythm by direct projections to ‘pacemaker’ regions, such as the supramamillary nucleus and medial septum/diagonal band (MS/DB) and directly to the hippocampus (Olucha-Bordonau et al., 2012). *In vivo* electrophysiological studies revealed that hippocampal theta rhythm was evoked by electrical stimulation of the NI, and by *nucleus reticularis pontis oralis* or sensory stimulation, and was significantly attenuated by electrical lesion or muscimol injection into the NI (Nunez et al., 2006). Studies by my laboratory have revealed that relaxin-3 neurons (which all express CRF1) exhibit a phase-locked firing pattern with hippocampal theta oscillations, compared to non-relaxin-3 neurons, which suggests a functional connection between NI GABA/relaxin-3 neurons and hippocampus (Ma et al., 2013). Since NI neuronal firing frequency is greater (13-25 Hz) than theta frequency (Olucha-Bordonau et al., 2012), it is unlikely that the NI would convert tonic RPO input to phasic theta firing directly. It is more probable that NI GABA/relaxin-3 neurons influence principle hippocampal neurons indirectly through actions on septohippocampal GABA/PV interneurons and/or hippocampal interneurons. Consistent with this, infusion of RXFP3 agonist or antagonist into MS of awake rats induced or impaired hippocampal theta rhythm, respectively (Ma et al., 2009a). In addition, inactivation of NI with lidocaine infusion impaired the acquisition and retrieval of spatial reference memory (Nategh et al., 2015). Taken together, these findings highlight the role of NI in promoting hippocampal function and theta rhythm, which is important for spatial memory (Smith et al., 2014b).

Anatomical studies have identified a reciprocal neural connection between NI, interpeduncular nucleus (IPN) and median raphe (MR) that forms a brainstem system modulating behavioural activities (Goto et al., 2001) *(Figure 1.8)*. These nuclei have common projection targets, including the hippocampus, MS/DB, mediodorsal thalamic nucleus, subfornical region of LH, posterior hypothalamus, ventral tegmental area, dorsoventral tegmental nucleus, and supramammillary nucleus (Goto et al., 2001). It is hypothesised that MR and IPN mainly inhibit behavioural activation, whereas NI has an excitatory role by disinhibiting MR and IPN. However, experimental evidence supporting this model is still lacking. Previous studies using electrical or chemical lesions of MR resulted in increased locomotor activity in rats (Jacobs et al., 1974). Administration of
nicotine into IPN induced hypoactivity, whereas electrolytic lesions of IPN blunted the hypoactivity induced by nicotine (Ren and Sagar, 1992; Hentall and Gollapudi, 1995). Moreover, untrained rats that display immobility when placed in a Morris water maze exhibited less Fos immunoreactivity in the NI than trained rats that swim in search of an escape platform (Goto et al., 2001). This is again consistent with at least a contribution of NI to general levels of activity in animals in a stressful context.

These common projection areas are also interconnected to each other and have crucial contributions to certain behaviours (Goto et al., 2001). For example, the mediodorsal thalamic nucleus is involved in temporally orchestrating behaviour via projections to the prefrontal cortex (O'Leary, 2014), as well as locomotor activity through nucleus accumbens and ventral tegmental area (Guan et al., 2002). The two latter areas are also receive direct and indirect inputs from MR, IPN and NI (Goto et al., 2001). Like MR and NI, the laterodorsal tegmental nucleus also integrates prefrontal cortex and lateral habenula inputs (Bianco and Wilson, 2009), and lesion reduced scopolamine-induced locomotion in rats (Laviolette et al., 2000).

The hippocampal formation, which is essential for mnemonic processing, spatial learning and navigation, as well as modulatory role in adoptive behaviour in specific sensory events, also receives inputs from the MR-NI-IPN circuit. Hippocampal lesions can induce hyperactivity, which is blunted by nucleus accumbens lesion, suggesting a subiculo-accumbal pathway mediating hippocampal related locomotor activity (Laviolette et al., 2000). In addition, the hippocampus influences the function of the medial hypothalamic area in goal-orienting behaviours (Fanselow and Dong, 2010) and via the lateral septal nucleus affects the lateral preoptic area and LH in relation to foraging and defensive behaviour (Staiger and Nürnberg, 1991; Risold and Swanson, 1997). Moreover, the subfornical region of the ventromedial LH, which regulates fight-flight behaviour, receives direct inputs from the NI-IPN-MR system (Vertes et al., 1999; Goto et al., 2005).

In addition to neural inputs, the NI-MR-IPN circuit is influenced by CRF, which could be from neural sources or circulating in cerebrospinal fluid (Bittencourt and Sawchenko, 2000), via CRF1 and CRF2 in the IPN and MR, and CRF1 in NI (Chalmers et al., 1995; Korosi et al., 2006; Ma et
al., 2013). In the NI, the actions of CRF via CRF1 are long-lasting and non-desensitising, suggesting that this nucleus is highly reactive to stress (Ma et al., 2013). Furthermore, it has been shown that CRF, via CRF1, directly depolarised relaxin-3 positive (which are all CRF1 positive) neurons, in addition to hyperpolarised relaxin-3 negative neurons (Ma et al., 2013). Taken together, the NI appears to be important for the ‘active locomotor component’ of behavioural responses to stress and its inactivity is associated with ‘active immobility’ or freezing behaviour (Ma and Gundlach, 2015).

1.7.3. Relaxin-3/RXFP3 system functions

Relaxin-3 is a highly conserved neuropeptide in mammalian brain (Ma et al., 2017b), which binds and activates the $G_{i/o}$-protein coupled receptor, relaxin-family peptide receptor-3 (RXFP3) (Liu and Lovenberg, 2008). Neurons expressing relaxin-3 mRNA are found in four distinct hindbrain populations - the NI, pontine raphe, PAG, and a dorsal area of substantia nigra. Although relaxin-3 has a lower affinity for RXFP1 and can bind to RXFP4 (which is a pseudogene in rats), it binds with highest affinity to RXFP3, which has a distribution that is complementary to that of relaxin-3 immunoreactive fibers (Bathgate et al., 2013). There is co-expression of both RXFP1 and RXFP3 in some brain regions, such as the PVN and supraoptic nucleus (Ganella et al., 2013b). In addition, different downstream pathways are associated with RXFP3 activation, depending on the cell type investigated (Ganella et al., 2012). There is growing evidence for a role for the relaxin-3/RXFP3 system in arousal and stress (McGowan et al., 2008; McGowan et al., 2009; Smith et al., 2010; Ryan et al., 2011; McGowan et al., 2014), spatial learning/memory (Ma et al., 2009a), innate and social anxiety (Ryan et al., 2013a; Zhang et al., 2015), reward behaviour (Ryan et al., 2013b; Ryan et al., 2014), and feeding (McGowan et al., 2005; McGowan et al., 2006; McGowan et al., 2007; Farooq et al., 2016). However, as relaxin-3 is usually upregulated in response to stressors and released under high neural tone, it has been suggested that the neuropeptide is present in pathological stressful conditions, which in turn suggest its receptors antagonist as a putative therapeutic target to correct related disorders (Kumar et al., 2017).
Figure 1.9. Nucleus incertus and relaxin-3 location and projection in the rodent brain.

(A) Low and high magnification micrographs of a coronal section through the mice NI, displaying neurons positive for relaxin-3-like fluorescent immunoreactivity. The region displayed in (B) is outlined in (A). The location of the midline (m/l) is indicated with a dotted line. Scale bars A, 100 μm; B, 250 μm. (C) Schematic parasagittal representation of the rodent brain, illustrating the ascending relaxin-3 system and the distribution of RXFP3 in regions grouped by function. For abbreviations refer to (Smith et al., 2014b).
1.7.3.1. Role in stress

Stress conditions, like other aversive external stimuli, promote a complex counter-adaptive mechanism and many studies indicate that the dysregulation of these adaptive mechanisms underlies pathological conditions, such as depression and anxiety (Stephens and Wand, 2012). The relaxin-3 system is potentially an adaptive neural circuit, since relaxin-3 NI neurons in the rat co-express CRF1 and are activated by CRF (Ma et al., 2013). Several studies have investigated the functional interactions between these two systems. Various stressor paradigms, such as swim stress in rodents or central injection of CRF, increased the expression of c-fos in many regions including NI which was blocked if rats were pre-treated with a CRF1 antagonist (Banerjee et al., 2010). In addition, infusion of CRF or electrical stimulation of NI affected hippocampal long-term potentiation (LTP), which is an indicator of neural plasticity in the hippocampal-medial prefrontal cortical pathway (Rajkumar et al., 2016). Similarly, infusion of CRF1 antagonist reversed stress-induced suppression of LTP (Rajkumar et al., 2016). Inactivation of NI by lidocaine infusion prior to stimulation of the hippocampal perforant path also blocked the LTP induction in the dentate gyrus (Nategh et al., 2015). Taken together, this suggests CRF1 signalling in the NI appears to mediate stress-related plasticity through an NI-hippocampus-medial prefrontal circuit that could be important for stress-related cognitive dysfunction (Rajkumar et al., 2016). On the other hand, central relaxin-3 administration has a stimulatory effect on CRF neuron activity and increases the release of corticosterone, ACTH and prolactin via HPA axis activation, as well as increases in c-fos and CRF mRNA expression in CRF neurons in PVN, but there is no data available that indicates whether or not these effects are direct or indirect, despite the high density of RXFP3 mRNA in the PVN (Ma and Gundlach, 2007; McGowan et al., 2014).

1.7.3.2. Role in behavioural activation, cognition, memory and arousal

Anatomical evidence demonstrates that there are mutual projections between NI, RPO, MRN, IPN and LPO (Goto et al., 2001), which implies that there is a contribution of NI to behavioural activity, such as food anticipatory activity or the behavioural inhibitory system via the septohippocampal formation (McNaughton and Gray, 2000). Hippocampal theta activity is related to memory consolidation, anxiety, sleep states, exploration as well as arousal (McNaughton, 2006).
Hippocampal theta rhythm can be anatomically influenced by various pathway originating from NI which pass though: (1) medial septum/vertical limb of diagonal band (GABAergic and glutamatergic projections), (2) RPO (as a brainstem ‘generator’ of hippocampal theta waves, (3) habenula and interpeduncular nucleus (as a ‘controller’ of hippocampus), (4) MRN (which desynchronises theta rhythm), and (5) posterior hypothalamus (Kumar et al., 2017). It has been shown that NI relaxin-3 neurons contribute in the induction of, display coherence with, and discharge in a phase-locked manner with hippocampal theta oscillations (Ma et al., 2009a; Albert-Gascó et al., 2017).

It has been shown that anxiolytic drugs increase the threshold of septal-induced, and reduce the threshold level of RPO-induced hippocampal theta waves (McNaughton et al., 2007), in a similar manner to the effects following septohippocampal lesion (Laviolette et al., 2000). Based on the hypothesis that the hippocampus functions as a comparator between expected and actual behavioural outcomes and a modulator of attention, arousal and behavioural inhibition, particularly at times of stress, the NI, along with septal pacemaker neurons, pedunculopontine tegmental nucleus, amygdala, superior colliculus and substantia nigra, could provide important inputs to hippocampus. In contrast, the NI is thought to mediate the effects of the RPO on the hippocampus during exploratory tasks, as inhibition of NI attenuates RPO-stimulation-induced hippocampal theta waves (Nunez et al., 2006). It is therefore, suggested that NI might modulate anxiety by altering the inputs to the septohippocampal system and contribute to this stress-responsive circuit (Ryan et al., 2013a).

In addition, there is other evidence for a contribution of the NI to behavioural activation. For example, dopamine D2 receptors are co-expressed by relaxin-3 and CRF1 positive NI neurons, and infusion of D2 receptor agonists into the NI decreased locomotion (Kumar et al., 2015). The activation of D2/D3 receptors in the NAc shell by stress also reduced feeding behaviour, indicating the contribution of the mesolimbic dopaminergic system during chronic stress or motivated behaviour (Yau and Potenza, 2013). In addition, chronic electrical microstimulation of NI induced a robust forward locomotion as well as rotational behaviour, however the latency of the movement suggests these effects are via activation of premotor areas rather than direct activation of motor
Interestingly, hippocampal theta waves are highly related to locomotion and processing of spatially-related sensory input in behaving animals (Vanderwolf, 1969). Recent studies in my host laboratory revealed that selective activation of NI using chemogenetics (designer receptors exclusively activated by designer drugs (DREADDs)) resulted in cortical desynchronisation, heightened arousal, and increased locomotion and vigilance, all suggesting that the NI is important for stress-related modulation of behavioural activity (Ma et al., 2017a).

1.7.3.2.1. Role in anxiety and depression

Anxiety is defined as a sustained state of vigilance, which is evident by arousal and behavioural inhibition (Davis and Whalen, 2001). The roles of the amygdala, bed nucleus of stria terminalis (BNST), ventral hippocampus and prefrontal cortex (all of which are anatomically connected with the NI) in mediating anxiety has been extensively characterised (Gold et al., 2015; Lebow and Chen, 2016; Padilla-Coreano et al., 2016). It has been shown that the relaxin-3 system is positioned to suppress anxiety, as central i.c.v infusion of an RXFP3 agonist is associated with an anxiolytic effect in rats (Ryan et al., 2013a), which may be related to effects on oxytocin signalling. Oxytocin is an important mediator in relation to anxiety, which has been investigated using fear conditioning in preclinical studies (Zoicas et al., 2014), and there is a high level of oxytocin mRNA expressed in PVN and supraoptic nucleus neurons (Viero et al., 2010). On the other hand, selective ablation of NI by CRF-saporin injection or electrolytic lesion, resulted in a deficit in ‘extinction’ of conditioned fear, which is a preclinical model for PTSD, panic disorder and phobias, but had no effect on fear acquisition or retrieval of extinction memory (Pereira et al., 2013). Interestingly, RXFP3 agonist administration had no effect on basal anxiety, but attenuated ‘elevated’ anxiety in mice suggesting that relaxin-3 signalling is heightened during elevated anxiety (Zhang et al., 2015).

Depression is suggested to be a result of a dysfunctional HPA axis, based on observations in depressed patients (Varghese and Brown, 2001). Central infusion of an RXFP3 agonist also activates the HPA axis in rats (McGowan et al., 2014). In a behavioural paradigm, such as a forced swim test, which is a preclinical test for depressive-like behaviour, it has been shown that RXFP3
agonist i.c.v infusion reduced immobility time, whereas immobility in relaxin-3 knockout mice was increased, implying that relaxin-3 promotes an antidepressant effect (Smith et al., 2009a). However, further characterisation of the behavioural phenotypes of knockout mice using other preclinical paradigms is still necessary.

Human positron emission tomography (PET) studies revealed that serotonin binding to its receptors is reduced in depressed patients, particularly in the raphe, limbic and cortical regions (Sargent et al., 2000). Interestingly, the depletion of serotonin in a preclinical rat model increased relaxin-3 mRNA in NI, along with an increase in 5-HT$_{1A}$ receptor immunoreactivity (Miyamoto et al., 2008). Taken together, these findings suggest that relaxin-3 signalling is regulated by serotonin, which might be related to effects on sleep, appetite, and mood. If depression is considered as a state triggered by an uncontrollable stress, it is plausible to think that the same stress/rewarding pathway is activated between NI relaxin-3 and BNST (as a rewarding centre mainly activated in addiction) in reinstatement of alcohol addiction following stress (Kastman et al., 2016).

1.7.3.2.2. Role in appetite and feeding regulation

Appetite is a physiological behaviour mediated by the orchestration between many neurotransmitters and neuromodulators, such as dopamine, serotonin, insulin, ghrelin, AgRP, beta-endorphin, orexin and NPY and its dysregulation is considered a robust symptom in various mental disorders (Gao and Horvath, 2016). Acute and chronic i.c.v infusion of relaxin-3 or RXFP3 agonist induced hyperphagia and body weight gain, with related metabolic changes in rats (Liu et al., 2005b). It is believed that these effects are mediated by actions on feeding centres in the hypothalamus, such as PVN, supraoptic and arcuate nuclei, anterior preoptic area (Ganella et al., 2012).

Several studies have observed hyperphagia following i.c.v or local hypothalamic infusion of relaxin-3 in rats (Ganella et al., 2013b). However, in KO mice there are reports on mixed effects on feeding following relaxin-3, which depends on the palatability or availability of the food (Smith et al., 2014a). I.c.v injection of human relaxin-3 at doses of 54 and 180 pmol significantly increased 1 h food intake in satiated rats in both the light and dark phase (McGowan et al., 2005). In addition,
i.c.v injection of RXFP3 agonist produced the same effect when injected acutely and chronically and its effect was blocked by an RXFP3 antagonist (Kuei et al., 2007). Local injection of relaxin-3 in PVN, supraoptic and arcuate nuclei, as well as anterior preoptic area increased food intake without any significant increase in expression of local orexigenic neuropeptides such as NPY, proopiomelanocortin (POMC) or agouti-related peptide (AgRP) (McGowan et al., 2009). In addition, chronic local injection of human relaxin-3 (180 pmol for 7 days) increased food intake and leptin plasma level with body weight and epididymal fat mass gain and no significant change in BAT weight, energy expenditure and physical activity (McGowan et al., 2006). However, 14 days injection of human relaxin-3 (600 pmol) or RXFP3 agonist increased food intake, body weight, as well as plasma leptin and insulin (Sutton et al., 2009; Smith et al., 2011).

1.8. Gaps in research and aims of this project

Orexin was discovered >20 years ago, but little is known about its role in controlling blood glucose during hypoglycaemia. The first part of this thesis describes an investigation of the involvement of the neurotransmitter glutamate that is co-expressed in orexinergic neurons in the LH, in the activation of adrenal sympathetic activity. The goal was to determine whether glutamate has a similar crucial role to that described earlier for orexin in relaying hypothalamic activation to adrenaline release in rats (Korim et al., 2014; Korim et al., 2016), as this is an important pathway/mecchanism for the restoration of blood glucose in type 1 diabetic patients.

Orexin and MCH systems act in concert to fine-tune arousal and motivated behaviours. The brainstem NI has been demonstrated to modulate arousal and stress-related behaviour. The second part of this thesis describes investigations of the impact of the hypothalamic neuromodulators, orexin and MCH on the NI and on arousal and feeding behaviour. Recent data from in vitro electrophysiological studies of the NI confirmed an excitatory orexin input to NI via different orexin/LH populations (Blasiak et al., 2015). Studies of these neuropeptide circuits and reciprocal interactions should eventually inform better treatments for various diseases associated with blood glucose dysregulation, and arousal and mental disorders, such as anxiety and depression.
Chapter 2- GENERAL MATERIALS AND METHODS
2.1. Ethical approval

These studies were approved by either the Animal Ethics Committee of Austin Health (electrophysiological studies) or The Florey Institute of Neuroscience and Mental Health (anatomical and behavioural studies). All experiments complied with the principles outlined in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2. Animals

For electrophysiological studies (Chapter 3), male Sprague-Dawley rats from the Animal Resources Centre (Perth, WA), weighing between 250-300g were acclimatised to the Austin Health Bioresources Facility for a week under constant room temperature, 40-50% humidity, and access to food and water *ad libitum* prior to experimentation. For behavioural (Chapter 4) and immunohistochemical studies (Chapter 5), male Sprague-Dawley rats from the Animal Resources Centre, weighing between 250-350g were acclimatised to the Florey Bioresources Facility for a week under similar conditions prior to experimentation.

2.3. Drugs, antibodies and reagents

Table 2-1. Name and supplier of the materials used in this thesis studies

<table>
<thead>
<tr>
<th>Drug, Antibody or Reagent</th>
<th>Supplier (Cat Number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoflurane</td>
<td>Delvet Pty Ltd, Seven Hills, NSW Australia</td>
</tr>
<tr>
<td>Urethane (ethyl carbamate)</td>
<td>Ajax Chemicals, Sydney, NSW, Australia</td>
</tr>
<tr>
<td>2-deoxyglucose (2-DG)</td>
<td>Sigma-Aldrich Co, St Louis, MO, USA</td>
</tr>
<tr>
<td>Artificial cerebrospinal fluid</td>
<td>Prepared in laboratory from standard reagents</td>
</tr>
<tr>
<td>Orexin-A</td>
<td>Mimotopes, Melbourne, VIC, Australia</td>
</tr>
<tr>
<td>Melanin-concentrating hormone</td>
<td>Tocris Bioscience, Bristol, UK</td>
</tr>
<tr>
<td>Relaxin-3</td>
<td>Florey Institute for Neuroscience and Mental Health</td>
</tr>
<tr>
<td>Paraformaldehyde (PFA)</td>
<td>Sigma-Aldrich Co, St Louis, MO, USA</td>
</tr>
<tr>
<td>Goat pro-MCH antibody</td>
<td>Santa Cruz Biotechnology, Dallas, TX, USA (14509)</td>
</tr>
<tr>
<td>Goat polyclonal orexin A antibody</td>
<td>Santa Cruz Biotechnology, Dallas, TX, USA (sc-8070)</td>
</tr>
</tbody>
</table>
2.4. General surgical procedures for in vivo electrophysiology studies

Rats were anaesthetised in a chamber saturated with isoflurane vapour and transferred to the stereotaxic frame once breathing was deep and slow, and there was a lack of righting response. An anaesthetic nose-cone was placed over the rat’s snout. Adequacy of the depth of anaesthesia was confirmed by an absence of a response to firm toe-pinch and gentle eye probing. An electronic thermometer covered with paraffin grease was inserted 2-3 cm into the rectum to measure core temperature. Body temperature and anaesthetic level were recorded every 15 min throughout each experiment. An incision was made in the ventral mid-cervical region and the connective tissue, sublingual and submaxillary gland were pushed aside. The sternohyoideus was divided so that the trachea was exposed. A small incision was made in the trachea, below the thyroid, and a tracheal tube was inserted, which was connected to a ventilator. The supply of isoflurane was then changed from nose cone to tracheal cannula. The tracheal cannula was sutured in place using a silk thread.

2.5. Blood vessel cannulation

The left carotid artery was identified beside the trachea, under the sternohyoideus muscle, and was isolated for about 2 cm using blunt forceps and a stereomicroscope. At the cranial end of the isolated artery, a tight ligature was made and was used to gently retract the artery. At the cardiac

<table>
<thead>
<tr>
<th>OX1R antibody</th>
<th>Abcam, Cambridge, UK</th>
</tr>
</thead>
<tbody>
<tr>
<td>OX2R antibody</td>
<td>Alpha Diagnostic, San Antonio, TX (USA): gift from Dr Anna Blasiak, Jagiellonian University, Krakow, Poland</td>
</tr>
<tr>
<td>Donkey anti-goat antibody Alexa 594</td>
<td>ThermoFisher Scientific, Waltham, MA, USA</td>
</tr>
<tr>
<td>Donkey anti-rabbit antibody Alexa 594</td>
<td>ThermoFisher Scientific, Waltham, MA, USA</td>
</tr>
<tr>
<td>Donkey anti-mouse antibody Alexa 488</td>
<td>ThermoFisher Scientific, Waltham, MA, USA</td>
</tr>
<tr>
<td>Biotinylated rabbit anti-goat IgG antibody</td>
<td>Vector Laboratories, Burlingame, CA, USA</td>
</tr>
<tr>
<td>Biotinylated rabbit anti-mouse IgG antibody</td>
<td>Vector Laboratories, Burlingame, CA, USA</td>
</tr>
</tbody>
</table>
end, a loose ligature was made and an artery clip was used to stop the circulation in the isolated artery segment. An incision was made using iris scissors near the distal ligature and a PE-8 cannula was inserted ~1 cm, which was connected to a syringe filled with heparinised saline (50 U/ml) and a blood pressure transducer. The artery clip was then removed to allow the cannula to be inserted a further ~1 cm towards the heart. The loose ligature was tightened with two surgical knots and this was repeated at the distal end. The blood pressure was then checked using Spike2 software (Version 7.09, Cambridge Electronic Design Limited, Cambridge, UK).

The left jugular vein in the superficial part of the cervical region adjacent to the sternohyoideus muscle was identified and isolated with blunt forceps. The same procedure used for cannulation of the carotid artery was performed. Placement of the catheter into the jugular vein was confirmed by withdrawing the plunger of a saline-filled syringe and noting the presence of venous blood in the cannula.

After blood vessel cannulation, the rat’s head was positioned in the stereotaxic apparatus (David Kopf, Tujunga, CA, USA) using ear bars. The incisor bar was set at 3.3 mm below the interaural line so that the skull was horizontal between bregma and lambda.

2.6. Intrathecal cannulation

A 20 cm length of PE-8 tubing was inserted into the lumen of an 18 cm length of PE10 polyethylene tubing to deter occlusion. The distal (spinal) end of the PE-8 cannula was marked to 8.5 cm, which was approximately the distance from the occipital bone to the lumbar enlargement in the adult rat. The head was shaved and a 5 cm incision was made in the midline of the scalp. The fascia under the incision was cleared by blunt dissection. The incisor bar was moved so that the head was now at 45° using the incisor bar. Respiration of the rat in this position was carefully monitored.

A 1 cm bilateral incision was made from the occipital crest to detach the related muscles and reveal an insertion point in the capitus muscles on the interparietal bone. By careful blunt dissection of the capitus muscles, the atlanto-occipital (AO) membrane was observed. A sterile 27-gauge needle was used to make a lateral incision around 1 mm to side of AO midline. Extra care was taken not
to damage the underlying vasculature of the dorsal medulla while penetrating the dura (AO) with the needle. Cerebrospinal fluid (CSF) was observed from the incision, and the end of the cannula was inserted as shallow as possible, to avoid damage to the spinal cord and medulla. When the cannula mark was reached, the tip of the cannula was roughly near the lumbar enlargement or the end of the T12 thoracic vertebra. Kynurenic acid (KYN) (0.5 μmol in 10 μl) was administered in a 10 μl volume followed by a 10 μl flush with artificial cerebrospinal fluid (aCSF) using a 50 μl glass barrelled Hamilton syringe. Xanthurenic, a metabolite of KYN without glutamate receptor antagonist properties, was injected followed by an aCSF flush in the control group.

2.7. Peripheral sympathetic nerve recording technique

2.7.1. Adrenal sympathetic nerve recording

After shaving the left lumbar area starting from the last rib toward the hips, a longitudinal incision in the left flank was made to reveal the underlying muscles. Using blunt scissors, a retroperitoneal dissection was made in the muscle underneath until the kidney was observed. Using cotton buds, the abdominal content was pushed down, being careful not to the damage the peritoneum. The adrenal nerve was identified below the suprarenal ganglion, which was at the junction between the coeliac ganglion and the greater splanchnic nerve. A retractor was used to pull the last rib rostrally for a better exposure. Using cotton buds, the connective tissue on top of the nerve bundle was gently moved aside. To avoid stretching the nerve, the adrenal nerve bundle (going parallel to or covered by the adrenal artery/vein) just below the ganglion was dissected free. Using a fine-tipped probe, any fat deposit below the nerve bundle was cleared. A double ligature was applied to the caudal end of the nerve bundle using 5.0 thread and the nerve bundle between the knot and adrenal gland was cut using iris scissors. The nerve bundle was placed onto a bipolar Teflon-coated silver recording electrode. After blotting the nerve-electrode junction to remove excess moisture, the nerve was covered with paraffin or a silicone–based resin (Kwik-cast, WPI International, Sarasota, FL, USA) to prevent drying. The nerve signal was amplified and filtered (30-3000 Hz band pass). The filtered nerve signal was directed to an oscilloscope and a nerve traffic analyser and nerve action potentials are rectified and integrated (5 ms time constant) which produced a smooth discharge of the activity and was recorded as waveform averaging (microvolts-seconds).
Hexamethonium chloride (40 mg/kg), a ganglionic blocker, was injected i.v. to block any postganglionic nerve activity and to determine the ratio of pre- to post-ganglionic fibres of the adrenal nerve bundle. At the end of the experiment, clonidine (200 µg/kg, i.v.), a centrally-acting α₂-adrenoceptor agonist, was injected to suppress adrenal sympathetic nerve activity. Clonidine operates through two mechanisms: it causes peripheral vasoconstriction and it inhibits the activity of RVLM adrenergic (C1) neurons through activation of α₂-adrenoceptors. The activity recorded after clonidine injection revealed the noise present during the experiment and was deducted from the experimental activity for further normalization of the data. In an appropriate recording, the signal-noise ratio was around 4 for the adrenal nerve.

2.7.2. Lumbar sympathetic nerve recording

After shaving the abdominal area, a longitudinal incision was made in the midline to reveal the underlying muscles. The abdominal contents were pushed away using cotton buds, covered in moist gauze and fixed to the abdominal wall using a retractor. The iliolumbar vessels (artery and vein pair) were identified just below the kidney, dissected gently, and ligated using a pair of 5.0 surgical thread sutures. The vessels were bisected using iris scissors and by retracting the ligature, the genitofemoral nerve close to the abdominal aorta was observed. Care was taken to ligate and cut any other small vessels while retracting the iliolumbar vessels. The genitofemoral nerve was dissected carefully about 2 cm with fine forceps and the muscle beneath was retracted proximally in order to identify the lumbar sympathetic chain (L3-L5) lying just below the abdominal aorta. The nerve was then carefully isolated for ~2 cm rostrally and the rostral section was placed onto a bipolar recording electrode made of Teflon-coated silver wires. After applying Kwikcast or paraffin to prevent drying, the abdomen was closed. Lumbar SNA was amplified (5,000-10,000 times) and filtered (30-3000 Hz bandpass). The amplified and filtered signal was routed to an oscilloscope and an amplified loudspeaker. Nerve activity was obtained after rectifying and integrating the action potentials (5 ms intervals) using a data acquisition system as described above. Hexamethonium chloride (40 mg/kg) was injected to determine the contribution of pre- and post-ganglionic fibres of the chain although about 95% of the lumbar sympathetic activity consists of postganglionic activity. Finally, the rat was injected with clonidine (200 µg/kg, i.v.) to determine the noise level which was subtracted from the recording to calculate lumbar SNA.
2.8. RVLM single unit recording

A short (0.5 cm) incision starting near the ear and along the lower jaw line was made to reveal the mandibular branch of the facial nerve ipsilateral to the side of the brain from which recordings were made. The tip of a concentric bipolar stimulating electrode was placed onto the nerve and fixed into position. Stimulation of the nerve was applied using 1-2 mA pulses with 0.1 ms pulse width at 0.5 Hz. This caused twitching of the vibrissae via orthodromic propagation of the stimulation-induced depolarisation. The depolarisation was also conducted antidromically into the brain manifesting as field potentials in the motor neurons of the facial nucleus. Since the facial nucleus lies just rostral and dorsal to the presympathetic neurons of the RVLM, it provided an extremely robust method of locating the RVLM.

After an incision on the scalp starting between the eyes to the occipital crest was made, the underlying connective tissue was removed until the skull and related landmark sutures (lambda and bregma) were noted. Using a dental drill, an interparietal craniotomy was carefully performed in the area between the lambdoidal suture and the occipital crest. The thinned bone was finally removed using fine-tip forceps and rongeurs. The dura was then penetrated using a 27-gauge needle and iris scissors were used to expose the dorsal cerebellum. A glass microelectrode filled with 1% Pontamine Sky Blue in 0.5 M sodium acetate was fitted to a micropositioner attached to a micromanipulator (David Kopf Instruments, Tujunga, CA, USA). The glass microelectrode was connected to an amplifier and electrode tip was positioned over the sagittal suture using a high-power stereomicroscope. After reading the mediolateral coordinates, the electrode was moved 2 mm laterally from midline. The same procedure was repeated for the Y-arm of the micromanipulator using the lambdoidal suture of the skull and the electrode was then moved 3 mm caudally. The microelectrode was then advanced into the brain tissue. The signals from the recording electrode are amplified (×1000), filtered (50-8000 Hz bandpass) and stimulation of the facial nerve (0.5 Hz, 0.1 ms pulse duration, 1-2 mA) were monitored. When the electrode entered the region containing the motor neurons of the facial nerve a field potential was recorded at a depth of 8.5-8.8 mm below the dorsal surface of the cerebellum. The contour of the facial nucleus was mapped by systematically recording the magnitude of the facial field potentials. After completion of facial field mapping, the region just caudal, medial and ventral was explored using a narrow
band pass (400-4000 Hz). Usually respiratory neurons with a rhythmic (on-off) firing pattern were found. Around 100-300 µm ventral to the respiratory area, spontaneously discharging, barosensitive neurons with fast- or slow-firing rates were found. These neurons were tested for their sensitivity to elevation of arterial blood pressure (barosensitivity) by injection of the vasopressor agent phenylephrine (5-10 µg/kg, i.v.). RVLM presympathetic neurons are powerfully inhibited by elevation of arterial blood pressure (Brown and Guyenet, 1985). After identification of an RVLM presympathetic neuron, the recording electrode was replaced with a microstimulation electrode.

2.9. Hypothalamic stimulation

After a partial frontal craniotomy, a stimulating electrode was placed into the PeH using the coordinates: 2.9-3.4 mm caudal to bregma, 1.1-1.3 mm lateral to midline and 8.6-8.7 mm ventral to surface of the brain. A laser Doppler blood flow probe was placed on top of one of the adductors muscles in the medial part of the hind limb, to record the effects of PeH stimulation on muscle blood flow.

2.10. Brain microinjection

In studies, to localise the site of drug action, microinjection of small volumes of different drugs, e.g. agonists or antagonists, were applied to different brain regions. Microinjection can be used as a single event in anesthetised animals to study neural pathways. A 1 µl Hamilton syringe needle was attached to a pulled glass pipette tip using super glue. The microinjector was affixed to a cannula holder.

2.11. Stereotaxic implantation of cannula targeting the NI

A guide cannula was implanted into the target brain region and microinjection was performed using microinjectors connected to 1 µl Hamilton syringes by PE-10 polyethylene tubing. The guide cannula consisted of two-26-gauge stainless steel tubes with 0.5 mm distance from each other (Plastics One, Roanoke, VA, USA). Microinjectors consisted of two 33-gauge stainless steel tubes which project 1-1.5 mm from the end of the guide cannulae (Plastics One, Roanoke, VA, USA). All guide cannulae, dummies and microinjectors were autoclaved 24 h before surgery. PE-10
tubing was used to connect the Hamilton syringes to the microinjectors. Dummy cannulae consisted of a two 33-gauge stainless steel tubes which were inserted into the full length of the guide cannula but not beyond. Caps were sections of 22-gauge polyethylene tubing which kept the dummies in their place by sliding into guided cannula. Placement of dummies and caps on guide cannula was done after complete hardening of the dental cement used to fix the guide cannulae to the top of the skull.

2.12. Microinjection of drugs into the NI in awake rats

For microinjections, Hamilton syringes and PE tubing were filled with distilled water. A 0.4 µl air bubble separated the distilled water from the drug solution. Rats were gently restrained in a towel, which was well tolerated if they have been familiarised with the procedure during the surgical recovery period. Infusion of drugs bilaterally (600 pmol/each side) into the NI were made at 500 µl/min/ per side. Microinjectors were cleaned with 70% ethanol between rats.

2.13. Behavioural testing

2.13.1. Open field test

The open field activity monitoring system broadly assesses locomotor and behavioural activity levels of the rats, which can be correlated with locomotor function. The arena was a square box (43.2 × 43.2 × 30.5 cm; Med Associates, Fairfax, VT, USA) in which rat movement was tracked by 3 × 16 beam infrared arrays spaced 2.5 cm apart, located on the X and Y axes for horizontal movement, and Z axis for detection of rearing. Parameters assessed were: ambulatory distance travelled, number of ambulatory movements, average ambulatory velocity, and rearing frequency. Arenas were cleaned with 70% ethanol between rats. For these studies, rats were placed in arenas immediately after intracranial infusion of drugs, for a testing trial of 4 h and the automated software (Med Associates) monitored the parameters for further analysis.

2.13.2. Feeding behaviour

Pellets of food were pre-weighed (10 g). Immediately after intra-NI injection, each rat was placed in new cage to avoid any food intake resulting from food in the bedding, and pre-weighed food was placed on empty lids above new cages. The food intake for each rat was measured 0.5, 1, 2,
and 4 h after injection. At the end of the experiment, the cumulative food intake for each rat was calculated and the mean of the treated group was compared to the control group.

2.13.3. Brain collection

Rats were deeply anaesthetised with pentobarbitone sodium (100 mg/kg, i.p.) and transcardially perfused with 300 ml ice-cold 0.1 M phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 11.2 mM Na$_2$HPO$_4$, pH 7.4) followed by 400 ml ice-cold 4% formaldehyde fixative solution in 0.1 M PBS, pH 7.4. Following perfusion, the rats were decapitated and the brains were rapidly removed and post-fixed for 1 h in 25 ml fixative. Brains were then transferred to a 30% sucrose in 0.1 M PBS for cryoprotection for ~48 h at 4°C.

2.14. Immunohistochemistry and immunofluorescence staining

2.14.1. Tissue preparation

Deeply anaesthetised with pentobarbital sodium (100 mg/kg, i.p.), rats were transcardially perfused with 300 ml ice-cold 0.1M phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 11.2 mM Na$_2$HPO$_4$, pH 7.4) followed by 400 ml ice-cold 4% formaldehyde fixative solution in 0.1M PBS, pH 7.4. Following completion of the perfusion, rats were decapitated and brains were rapidly removed, kept in fixative for 24 h and then cryoprotected (dehydrated) in 30% sucrose in 0.1M PBS until they sunk, which usually took 48 h being kept at 4°C. Brains were then blocked, frozen over dry-ice and stored at -80°C for later processing or taken for immediate cryostat sectioning. For sectioning, frozen brain blocks were attached to a microtome chuck using Tissue-Tek® OCT (Optimum Cutting Temperature) compound (Sakura Finetek USA Inc., Torrance, United States) and cut at 30-40 µm in the coronal plane at -18°C (Cryocut 1800, Leica Microsystems, Heerbrugg, Switzerland). Sections were collected free-floating and stored in a 4-well series in cryoprotectant (20% glycerol, 2% DMSO in 0.1 M PBS) at -20°C.

2.14.2. Immunohistochemistry

All brain sections were incubated in blocking agents and the incubation and washing steps (with 0.1 M PBS) were performed on an orbital shaker protected from light at room temperature:
1. Sections were blocked using 10% NHS in PBT, by incubation for 1 h, at room temp, on a orbital shaker.

2. Primary antibody, orexin-A, MCH, relaxin-3, at different concentrations, was applied to each well containing blocked sections. A single well was used as a reference (-1°Ab) control for each experiment. Positive control brain structures were selected, including LC for the orexin antibody (with staining of soma and/or fibres predicted) and LH for orexin-A/MCH antibody (with staining of soma). Optimization for observing OXA/MCH fibres in NI was performed by using optimal concentrations for the respective soma in LH (1:400 for MCH, 1:1000 for OXA) but a longer incubation time (3 nights) was applied to the sections at a lower temperature (4°C). The relaxin-3 antibody dilution was previously optimized in my laboratory at 1:5 with an overnight incubation at room temperature.

3. Sections were washed 3 times for 10 min with 0.1 M PBS.

4. Secondary antibody e.g. donkey anti-goat Alexa 594 (1:500) for OXA, MCH, as well as donkey anti-mouse Alexa 488 (1:2) were applied to sections in each well under foil cover and left for 1 hour in room temperature on slow speed shaker.

5. Sections were washed 3 times for 5 min with 0.1 M PBS.

6. Free floating sections in 0.1 M PBS were mounted onto gelatin-coated glass-slides.

7. Before the sections were completely dried, they were coverslipped with Fluoro-mount and observed under confocal microscope.

2.14.3. 3, 3’-Diaminobenzidine (DAB) immunohistochemistry

For the purpose of obtaining a specific and durable staining for recording using a brightfield microscope, 3,3'-diaminobenzidine (DAB) immunohistochemistry was used. Free-floating sections were washed (3 × 5 min) to remove cryoprotectant, then quenched by incubation in 50% methanol and 1% H$_2$O$_2$ in 0.1 M PBS for 15 min and then the endogenous-peroxidase free sections were washed (3 × 5 min). Sections were then incubated for 1 h in blocking solution (10% NHS in PBT) as mentioned. The remaining procedure was as follows:

1. Primary antibodies, orexin-A/MCH goat IgG (1:1000, 1:2000, 1:4000) or relaxin-3 mouse IgG (1:5) were incubated on sections overnight at room temperature on a slow-speed shaker.
2. Sections were washed 3 times for 10 min with 0.1M PBS.
3. Biotinylated rabbit anti-goat IgG antibody (1:500) or biotinylated rabbit anti-mouse IgG (1:500) were added to sections in incubation wells and left for 1 h on a slow-speed shaker.
4. Sections were washed 3 times for 5 min with 0.1M PBS.
5. Sections were incubated with 1:100 dilution of avidin-biotin complex (ABC Elite PK-6100 Standard; Vector Laboratories, Burlingame, CA, USA) in 0.1M PBS. Mixing ABC kit took placed 30 min before adding to the sections.
6. Sections were washed 3 times for 5 min with 0.1M PBS.
   A pre-prepared DAB solution (Sigma-Aldrich, 0.25 mg/ml DAB w/v and 0.005% H₂O₂ in 0.1M PBS, mixed with NiCl₂) was added to the sections. For better contrast and visibility of stained fibres in NI sections, the RLN3 antisera staining was processed with DAB only, to produce a brown reaction; while in LHA sections, the RLN3 antisera was processed with DAB + nickel chloride to produce a black reaction.
7. H₂O₂ was immediately added to the sections wells and each well left for exactly 2 min.
8. After 2 min sections were transferred to PBS to stop the DAB reaction.
9. Free floating sections in 0.1M PBS were mounted on gelatin-coated glass-slides and dried overnight. Slides were then sequentially dehydrated in a series of alcohol (50% EtOH, 70% EtOH, 90% EtOH, 2 × 100% EtOH, 2 × xylene) for 2 min in each solution and coverslipped (Menzel-Gläser, Braunschweig, Germany) with safety mount (Fronine Pty Ltd, Scoresby, VIC, Australia).

2.14.4. Nissl staining - verification of cannula location

For verification of the cannula implant and correctly targeted injections, sections through the area of interest (NI) were mounted onto gelatin-coated microscope slides directly after sectioning and left to dry overnight. Slides were gradually rehydrated, using a reverse order of alcohol incubations (see Section 1.8.3.), and incubated for 90 s in a thionin stain (0.01% v/w) before dehydration in 50-100% ethanol and coverslipping as described (Section 1.8.4.).
2.15. Microscopy and image acquisition

Fluorescence immunohistochemistry was imaged on a Leica DM LB2 epifluorescence microscope (Leica Microsystems Pty Ltd, Wetzlar, Germany) using a mercury lamp for detection of m-Cherry and GFP fluorophores; or a Zeiss LSM 780 confocal microscope (Carl Zeiss AG, Oberkochen, Germany) with 405/488/594/647 diode lasers. The Leica DM LB2 microscope was also used to obtain bright field images of DAB and Nissl staining. For qualitative assessments and illustrations of the distribution of immunostaining, images were manipulated (cropped, adjustments of brightness and contrast, addition of scale bars) using FIJI (Schindelin et al. 2012) and processed (reconstructed) using Imaris (Ausdenmoore, 2011).

2.16. Infrared spectrometry

A minimal sample volume (2 μl) of the vehicle and prepared solution (of orexin or MCH) was applied on a card with hydrophilic polytetrafluoroethylene (PTFE) membrane which was readable by the infrared spectrometer (Direct Detect, Merck Millipore, VIC, Australia) and the final concentrations of the prepared solution ranging from 0.2 to 5 mg/ml was accurately measured for calculating further intra-NI injection.
Chapter 3- OREXIN REGULATION OF AUTONOMIC FUNCTION: EFFECTS ON THE SYMPATHO-ADRENAL AXIS
Perifoncal hypothalamic pathway to the adrenal gland: Role for glutamatergic transmission in the glucose counter-regulatory response

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ABSTRACT

Adrenaline is an important counter-regulatory hormone that helps restore glucose homeostasis during hypoglycaemia. However, the neuroanatomy that controls the hypothalamic regulation of the adrenal sympathetic outflow to the chromaffin cells is poorly understood. We used electrical microstimulation of the perifoncal hypothalamus (PVH) and the rostral ventromedial medulla (RVM) combined with adrenal sympathetic nerve activity (ASNA) recording to examine the relationship between the RVM, the PVH and ASNA. In urethane-anesthetised male Sprague-Dawley rats, intravenous single pulse electrical stimulation of the rostral ventromedial medulla (RVM) elicited an evoked ASNA response that consisted of early (60±3 ms) and late peaks (125±4 ms) of preganglionic and postganglionic activity. In contrast, RVM stimulation elicited responses in spinal sympathetic nerve activity that were most entirely postganglionic. PVH stimulation also produced an evoked excitatory response consisting of both preganglionic and postganglionic excitatory peaks in ASNA. Both peaks in ASNA following RVM stimulation were reduced by intrathecal hyaluronidase (YIN) injection. In addition, the ASNA response to systemic noradrenaline injection induced by 2-deoxy-g-p-glucose was abolished by bilateral microinjection of YIN into the RVM. This suggests that a glutamatergic pathway from the perifoncal hypothalamus (PVH) stays in the RVM to activate the adrenal SN and to modulate ASNA. The main findings of this study are that (i) adrenal premotor neurons in the RVM may, at least in part, glutaurenergic and (ii) that the input to these neurons that is activated during noradrenergic injection is also glutamatergic.

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1. Introduction

Adrenaline is a hormone that is critically involved in the glucose counter-regulatory response especially in Type 1- and advanced Type 2 diabetes (Geyer, 1993; Verberne et al., 2016; Verberne et al., 2014). Electrical stimulation of the adrenal sympathetic nerves (Edwards and Jones, 1993), systemic administration of the glucoprivic agents 2-deoxyglucose (2-DG) or insulin and haemorrhage (Mundinger et al., 1997; Vollmer et al., 2000), increase circulating catecholamines, while desensitization of the adrenal medulla (Abbott et al., 2005) decreases catecholamine levels. Adrenal sympathetic preganglionic neurons (SPNs) have been characterized anatomically (Edwards et al., 1996; Keske et al., 1988) and physiologically (Gao and Morrison, 2000; Morrison and Gao, 2000). The cell bodies of rat adrenal SPNs project to the adrenal and noradrenaline-synthesising chromaffin cells and are located in the intermediolateral cell column of the spinal cord between T1-T5 with the highest density at T5 (Strack et al., 1989). Individual adrenal SPNs target either adrenaline-synthesising or noradrenaline-synthesising chromaffin cells (Edwards et al., 1996; Keske and Morrison, 2000; Morrison and Gao, 2000). However, adrenaline responses to stimulation of the PVH were most entirely postganglionic (Edwards et al., 1996) and excitatory responses were elicited by intrathecal injection of 2-DG (Edwards et al., 1996). Electrical stimulation of the PVH resulted in an increase in firing rate of both adrenaline and noradrenaline SPNs at different latencies (Gao and Morrison, 2000). This finding is consistent with single unit recordings made from the RVM medullar neurons which had distinct ascending conduction velocities (Brown and Guyenet, 1985; Schreiber and Guyenet, 1997).
Chemical stimulation of the RVM in the cat using excitatory amino acid injections elicits robust pressor responses accompanied by increases in plasma catecholamines (McAllen, 1986). Our laboratory identified slow-conducting (conduction velocity <1 m/s), slow-firing, barosensitive, medullocervical neurons in the RVM that were activated by glomus stimulation suggesting that the RVM may be involved in the arterial response to glomus excitation (Verberne and Sator, 2010).

Adrenal catecholamine release can also be elicited by hypothalamic stimulation. Thus, electrical or chemical stimulation in the hypothalamic nuclei altered the blood glucose level or elicited adrenal secretion (Folko and Von Euler, 1954; Matsuo, 1972; Verberne et al., 2014). Recently, a role for paraventricular hypothalamic (PVH) neurons in the arterial response to systemic glucagon has been identified (Korin et al., 2014; Korin et al., 2016; O'Donnell et al., 2016). In these studies it was proposed that PVH neurons couple the responses of hypothalamic glucose-sensing neurons to RVM presymptomatic neurons that control adrenal secretion (Verberne et al., 2016). However, a neuroanatomical analysis of the pathway that connects the RVM and adrenal sympathetic outflow has not been reported. The present study was designed to characterize the neural pathways between brain glucose-sensing neurons and the adrenal gland by electrical stimulation of the RVM and PVH and recording ASNA.

In order to explore the connection between the RVM and ASNA, we used intermittent electrical stimulation of the RVM to construct post-stimulus microprobes in which we could identify sympathetic components with different latencies. In addition, the relative contribution of pre- and post-ganglionic activity was assessed by using the ganglion blocker hexamethonium. RVM presymptomatic neurons send projections to adrenal SPN (Morrison and Gao, 2000) and PVH neurons provide polysynaptic input to the adrenal sympathetic outflow (Korin et al., 2007). Therefore, we examined the effect on the adrenal sympathetic outflow to electrical stimulation of the PVH as well as the RVM. Some PVH neurons express vGlut2 mRNA, a marker for glutamatergic neurons (Kosinetz et al., 2003). The increase in ASNA that occurs in response to systemic glucagonization is blocked by inhibition of neuronal function in the PVH (Korin et al., 2014). Therefore, we tested the hypothesis that iontophoretic glutamate injections in the RVM are involved in the excitatory responses in ASNA elicited by glucagonization using systems 2.1G. This was achieved by perfusing bilateral microinjections of kynurenic acid or vehicle into the RVM. Several previous studies have also suggested that glutamate is the major excitatory neurotransmitter of RVM neurons that project to SPN (Huang et al., 1994; Morrison, 2008) although this has not been established for the premotor pathway to the adrenal SPN. Therefore, the involvement of glutamate receptors at the level of the SPN was tested by administration of kynurenic acid intrathecally while recording evoked ASNA responses following RVM stimulation.

2. Materials and methods

Experiments were performed according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by the Animal Ethics Committee of Austin Health (Heidelberg, Victoria, Australia; Approval numbers 12/4/64 and 14/5/191).

2.1. General preparation

Male Sprague-Dawley rats (250-300g) were anaesthetised with 2% isoflurane, tracheotomised and connected to a rodent ventilator (50-90 breaths/min; 10 ml/kg; supplying isoflurane (1.5-3.0% in O2). The depth of anaesthesia was monitored regularly and confirmed by the absence of response to toe pinch and corneal probing. Rectal temperature was maintained at 35.5-37.5°C by a thermocouple-controlled heating pad. The jugular vein and carotid artery were cannulated for intravenous drug injection and measurement of blood pressure, respectively. After completion of all surgery, the isoflurane anaesthesia was discontinued and urethane (1.4g/kg, i.v.) was administered. Urethane was chosen for its minimal effects on blood glucose (Verberne and Sator, 2010), and supplemented when necessary. The animal was paralysed with pancuronium bromide (2 mg/kg, i.v. with 0.2 mg/kg supplement if required) before performing electrical stimulation. After neuromuscular blockade, anaesthesia was maintained at a level in which paw pinch produced minimal changes in blood pressure (<10 mm Hg).

2.2. Intravascular cannulation

The animal was placed into a stereotaxic apparatus with the incisor bar at -3.3 mm below the intracranial line. The atlanto-occipital membrane was exposed, a fine polyethylene cannula (SP-8, C.D 0.5 mm, ID 0.2 mm) filled with artificial cerebrospinal fluid (aCSF) was inserted through a small opening in the dura mater, and its tip was positioned at the end of the T4-5 thoracic vertebra. Rynynuric acid (KYN) (0.5 μmol in 1 μl) or aCSF mixed with fluorescent latex microspheres was slowly administered followed by a 10 μl flush with aCSF (Verberne et al., 1996). The effect of intravascular RVM or vehicle on the ASNA responses in RVM stimulation was tested.

2.3. Extracellular single-unit recording of RVM barosensitive neurons

A partial parietal craniotomy was performed to access the ventromedial thalamic nuclei (RVM) using a transcrullate approach. The procedure was performed as described previously (Brown and Gayer, 1985) using transcranial insertion of a glass microelectrode (4-8 MΩ impedance at 30 Hz), filled with a solution of 0.1% dextran blue (2%,0.3 M sodium acetate), into the ventral medulla at the level of the facial motor nucleus (coordinates 3.6 mm caudal to lambda, 2.0 mm lateral to midline, 8.0-9.0 mm ventral to the surface of cerebellum). A band pass amplifier (Model WDR 420, Intracranial Inc., Orange, Connecticut, USA) was used to filter and amplify the field potentials of facial motor neurons during facial nucleus mapping (50 Hz-8 kHz) and a bandpass of 400 Hz-4 kHz was used for recording RVM presynaptic neurons.

Facial field potentials were recorded in response to electrical stimulation of the mandibular branch of the facial nerve (0.5 Hz, 0.1 ms, 1.0-2.0 mA). RVM presymptomatic neurons are found immediately caudal and ventral to the caudal pole of the facial nucleus (Verberne et al., 1996) extending caudally for 500 μm. RVM presymptomatic neurons are barosensitive and have spatiotopographically projecting axons (Brown and Gayer, 1985; Verberne et al., 1996) or are barosensitive, spatiotopographically projecting and are activated by neuroglucopenia (Verberne and Sator, 2010). Since these two populations are intermingled, we used the location of a barosensitive neuron to guide placement of the stimulation electrode. Spontaneously active RVM neurons were tested for barosensitivity by increasing arterial blood pressure using the selective α2-adrenoceptor agonist phenylephrine (10 μg/kg, i.v.), as well as phenylephrine (10 μg/kg, i.v.), a 5-HT agonist and activator of the von Bezold-Jarisch reflex (Verberne and Gayer, 1992a). Once an RVM barosensitive neuron was found, the recording electrode was replaced with a monopolar, stainless steel stimulation electrode at identical stereotactic coordinates.

2.4. Adrenal and lumbar sympathetic nerve activity

Levels of circulating adrenaline, but not norepinephrine levels, correlate with adrenal preganglionic sympathetic nerve activity (ASNA) during systemic neuroglucopenia (Korin et al., 2014). Therefore, ASNA seems to be a reliable index for studying adrenal gland catecholamine release in experimental hypoglycemia or neuroglucopenia.

The left or right adrenal gland was exposed using a retroperitoneal approach. The transient nerve bundle in the between the aortic ganglia (Celler and Schramm, 1981) and adrenal gland were carefully
dissected free from the surrounding connective tissue and fat. The nerve bundle was tied together using surgical silk, cut distally, and mounted onto a silver wire bipolar hook recording electrode. The nerve was covered with paraffin oil to avoid shunting of the nerve signal (Mueller et al., 2011). In another group of animals, lumbar sympathetic nerve activity was recorded in order to compare a vagal tone outflow to ASNA. The lumbar sympathetic chain was exposed after retracting the abdominal viscera laterally at the level of the 12th lumbar vessels. The 5-6 mm segment of the lumbar sympathetic chain was dissected free from surrounding tissue and placed onto the recording electrode (Verberne and Guyenet, 1992a). Adrenal or lumbar sympathetic nerve activity (ASNA and LSNA) was amplified x 10,000 (719B; Grass Instruments, Quincy, MA), filtered (300 Hz-3 kHz), and sampled at 6 kHz using aCED Power1401 (Cambridge Electronic Design Ltd., Cambridge, UK) with Spike2 v7.02 software. ASNA or LSNA was rectified and averaged over 0.005-s intervals and recorded as baseline activity. All neurograms were normalized with reference to the resting level before application of any stimulus (100%) after subtraction of the noise (0%), determined after saline administration (200 µg/kg, iv: Sigma-Aldrich) (Verberne and Guyenet, 1992b). Hexamethionum (40 mg/kg, iv) was used to identify pre- or post-ganglionic components of the ASNA or LSNA after the stimulation protocol. SNA was considered preganglionic if 75% remained after hexamethionum. Conversely, SNA was considered postganglionic if 50% remained after hexamethionum.

25. Stimulation of the RVLM and the PeH

A stainless steel monopolar stimulating electrode (2-3 MD impedance at 1 kHz; Frederick Harr, Bowdoinham, Maine, USA) was inserted into the RVLM and replaced the glass microelectrode used for field potential and neuronal recording. To confirm the positioning of the microelectrode in the RVLM, train stimulation (30 Hz, 50 µA, 16 s) was initially applied to the microelectrode to observe a presynaptic response (40-60 mV, Hg) (Fig. 1C). Once the RVLM stimulation electrode was in place the preparation was allowed to stabilize for 30 min and the electrode was replaced with a microinjector.

In a separate group of 5 animals, the response to intermittent electrical stimulation of the RVLM on LSNA was tested for comparison with the ASNA response to RVLM stimulation. In another group of animals, the stimulating electrode was placed into the PeH (Paxinos and Watson, 1986) according to the following stereotaxic coordinates: 3.8-3.7 mm caudal to bregma, 1.1-1.4 mm lateral to midline and 1.3-1.8 mm ventral to the surface of dura. Therewith, intermittent stimulation of the RVLM or PeH areas was performed using single or twin pulses (0.5 Hz, 100-400 µA, 0.5 ms; twin pulse, 3 ms interpulse interval) (Verberne et al., 1993a) and sympathetic nerve activity was recorded following 50-100 repeats of stimulation. Spike 2 software (Cambridge Electronic Design, Cambridge, UK) was used to perform waveform averaging using a 400 ms post-stimulus sampling time and 100 ms prestimulus sampling time. All stimulation pulses were driven by a Grass S88 stimulator (Grass Instruments, Quincy, Massachusetts, USA) and a laboratory built constant current unit and stimulus isolation unit.

26. Microinjection into the RVLM

Rats received bilateral microinjections of KYN (5 nmol/100 nl) or the equivalent amount of aCSF (as vehicle) into the RVLM after locating a spontaneous firing baroreflex neuron subsequent to facial field mapping (Verberne and Guyenet, 1992b). These agents were injected slowly using glass micropipettes cemented to the tip of a 1 µl Hamilton syringe (Verberne and Guyenet, 1992b). Fluorescent latex microspheres (2%, Invitrogen, California, USA) were incorporated into the KYN and vehicle injectate. In these experiments, rats received a bilateral microinjection of either aCSF or KYN prior to injection of the glucoseotropic agent 2-deoxyglucose (2DG, 200 µg/kg, iv) while monitoring ASNA.

![Fig. 1. Average evoked adrenal sympathetic nerve activity (ASNA) following electrical stimulation of the RVLM (0.5 Hz, 100-400 µA, 0.5 ms; twin pulse, 3 ms interpulse interval).](image)

2.7. Histology

While deeply anaesthetised each rat was perfused with saline (NaCl: 0.9% w/v) followed by 1% formalin. Brains were removed, fixed in formalin overnight, and cut with a vibratome in 100-μm coronal sections. Sections were mounted onto gelatin-subbed slides for identification of lesion or injection sites. Sections containing deposits of fluorescent microspheres were examined under epifluorescence. The centers of the injection sites were photographed (Infinity 2 digital camera, Lumenera, Canada) and plotted on plates from a standard rat brain atlas (Paxinos and Watson, 1996).

2.8. Drugs

2-DC (Sigma-Aldrich; 250 mg/kg, i.v.), clonidine (Sigma-Aldrich; 200 μg/kg, i.v.), hexamethonium (Sigma-Aldrich; 20-40 mg/kg, i.v.), hyoscine (Sigma-Aldrich), phenytoin (Novo-Nordisk; 100 μg/kg, i.v.), and phenylephrine (Sigma-Aldrich; 10 μg/kg, i.v.) were used for this study. KYN was suspended in ACSF and dissolved with addition of a molar equivalent of NaOH solution. The pH was then adjusted back neutrality by addition of HCl solution. KYN had the following composition (in mM): NaO, 125; KCl, 2.6; NaH2PO4, 1.3; NaHCO3, 2; CaCl2, 1.3; MgSO4, 0.9.

2.9. Statistical analysis

Averaged stimulation-evoked newgrams of adrenal or lumbar SNA following intermittent stimulation of the RVM or the PeH were generated using the waveform average function in Spike2 before and after injection of hexamethonium to identify preganglionic and postganglionic components of the evoked response. Peaks were identified as positive components of the evoked newgram that were 20% above the baseline activity. Peak latencies were calculated by measuring the time (in ms) between the beginning of the stimulation artefact and the maximal responses and peak latencies (as mean ± SEM) before and after hexamethonium were compared using Student’s paired t-test. The effect of KYN microinjection into the RVM on the ASNA response to systemic glucoprivation (2-DC) was analysed using repeated measures two-way ANOVA followed by Sidak’s post-hoc test for comparing KYN4- and control-treated group. The level of statistical significance was set as P < 0.05.

3. Results

3.1. RVM stimulation-evoked ASNA responses

Stimulation of RVM evoked a response in ASNA that consisted of two peaks with latencies of 60 ± 3 ms and 135 ± 4 ms (N = 8). In four of the eight experiments, ganglionic blockade (hexamethonium) markedly reduced the amplitude (~75%) of the peaks in the ASNA newgrams suggesting the activity was predominantly preganglionic with peak latencies of 56 ± 6 ms and 138 ± 5 ms (N = 4) (Fig. 1A). In the remaining experiments, the reduction in amplitude was less (~75%) indicating the response was mostly preganglionic with peak latencies of 63 ± 3 ms and 132 ± 7 ms (N = 4) (Fig. 1B). Hexamethonium reduced the magnitude of the first peak from 98 ± 18 to 45 ± 9% (P < 0.01, N = 8) and that of the second peak from 100 ± 18 to 58 ± 12% (P < 0.05, N = 8) (Fig. 1C).

We also compared the ASNA responses to the LSNA responses (Fig. 2). Stimulation of RVM evoked a response in LSNA that consisted of two peaks with latencies of 86 ± 4 ms and 166 ± 8 ms (Fig. 2A, N = 5). Hexamethonium treatment almost abolished all evoked activity in the early (100 ± 19 vs 7 ± 4%, P < 0.05, N = 5) and late (100 ± 16 vs 20 ± 15%, P < 0.05, N = 5) peaks (Fig. 2A, B).

3.2. Blockade of spinal glutamate receptors

As shown in Fig. 3A and B, intrathecal administration of KYN (0.5 μmol in 10 μl) reduced the amplitudes of both the early peak (98 ± 18 vs 76 ± 6%, P < 0.05, N = 5) and the late peak (100 ± 13 vs 71 ± 7%, P < 0.05, N = 5) in the stimulation-evoked ASNA responses. However, KYN treatment did not change any of the peak latencies (61 ± 5 ms and 147 ± 7 ms) (P > 0.05, N = 5 for all comparisons) (Fig. 3C).

3.3. Pre-H stimulation-evoked ASNA responses

Consistent with the findings noted in the RVM stimulation experiments, excitatory responses following electrical stimulation of the PeH were either mixed preganglionic and postganglionic activity (Fig. 4A and B) or predominantly preganglionic activity (Fig. 4C and D). Electrical stimulation of the PeH elicited responses that consisted of a broad peak (~50%) of evoked ASNA with a latency of 23 ± 2 ms in combination with an additional peak with a latency of 152 ± 4 ms in preganglionic activity resulting in a dominant peak at 153 ± 5 ms in the postganglionic activity group (Fig. 4C). Following administration of hexamethonium in the postganglionic activity group, the magnitude of the first peak decreased (100 ± 42% vs 30 ± 13%, P < 0.05, N = 5; Fig. 4E) whereas the second small peak was not altered significantly (100 ± 24% vs 57 ± 25%, P > 0.05, N = 5; Fig. 4E). In contrast, the latency of the first peak in the preganglionic group was shifted to 152 ± 5 ms (P < 0.05). The magnitudes of the first (100 ± 42% vs 75 ± 6%, P > 0.05, N = 8) and second peaks (100 ± 30% vs 79 ± 64%, P > 0.05, N = 8) in the preganglionic group were not changed following administration of hexamethonium (Fig. 4B). The locations of the PeH stimulation sites are depicted in Fig. 4D and are all located within 200 μm of the fornix.

3.4. Blockade of RVL glutamate receptors and the ASNA response to 2-deoxyglucose

Systemic injection of 2DG (250 mg/kg, iv) increased ASNA which reached a maximum compared to baseline 25 min after injection (100 ± 0.2% vs 230 ± 20%, P < 0.01, N = 6; Fig. 5B) in the control group that received bilateral microinjections of vehicle (aCSF) into the RVM (Fig. 5A). In contrast, ASNA did not increase over the 25 min recording period after 2-DG administration in animals pretreated with bilateral microinjections of KYN (50 µmol/100 µl) into the RVM (96 ± 1% vs 119 ± 25, P > 0.05, N = 6, Fig. 5B, Aii). Thus, compared to aCSF treatment, KYN microinjection blocked the increase in ASNA after systemic...
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Fig. 5. Aii, A1: Measurement depicting changes in adrenal sympathetic activity (ASNA) following systemic injection of 2-DG (250 mg/kg, i.e., arrow heads) in rats pretreated with artificial cerebrospinal fluid (aCSF; 180 ml, AL) or hyperosmotic acid (KYN, 50 ml/180 ml, AL) microinjected bilaterally into the RVLM (N = 6 for each group). B, C: Group data for time-course changes in ASNA in rats pretreated with 2-DG or aCSF. Data are mean ± SEM. KYN compared to aCSF, two-way ANOVA followed by the Sidak multiple comparison test. *P < 0.05; **P < 0.01. C1: An example of a microinjection site in the RVLM. C2: Reconstruction of the microinjection sites in the RVLM following KYN (N = 6) and aCSF (N = 6). Each dot represents a bilateral site of injection.

4. Discussion

The main findings of this study are that (i) adrenal preterminal neurons in the RVLM may be, at least in part, glutamatergic and (ii) the input to these neurons that is activated during neurogulation is also glutamatergic. The first conclusion cannot be stated with any certainty and will require further studies to clarify this equivocal result.

Several pieces of evidence support the hypothesis that a pre-RVLM-glutamatergic pathway controls adrenal catecholamine secretion during glucoprivation. First, pioneering studies by Follow and von Euler showed that hypothalamic stimulation in cats increased catecholamine secretion in the circulation (Follow and von Euler, 1964). Importantly, the adrenal/noradrenaline secretion ratio was dependent on the precise location of the stimulation electrode in the hypothalamus. Second, tracing studies using neurotropic viruses injected into the adrenal medulla results in labeling of Peh neurons (Kerrin et al., 2007). Third, our laboratory has shown that local glucoprivation of the Peh increases ASNA and catecholamine secretion (Kerrin et al., 2014). Finally, Peh neurons are activated by muscin-induced hypoglycemia (Briski and Sylwester, 2007; Ciarcia et al., 2001; Li et al., 2013; Morisuchi et al., 1999; Thacs et al., 2007) and these project to the RVLM (Kerrin et al., 2016). In turn, the RVLM contains neurons that are responsible for controlling adrenal secretion and these are intermingled with those that control the sympathetic vasomotor outflow (Verberne and Sabehghadian, 2010). Previously, we showed that stimulation of the RVLM neurons using local microinjections of the GABA antagonist bicuculline produced an increase in arterial blood pressure and an increase in blood glucose that was abolished by adrenalectomy (Verberne and Sabehghadian, 2010). This suggests that a source of adrenal preterminal sympathetic drive arises from the RVLM as has been previously suggested by others (McAllen, 1986; Morrison and Cao, 2000).

RVLM stimulation elicited a pattern of evoked response in ASNA that consisted of two distinct peaks with different latencies. This result is consistent with the studies performed by Morrison and colleagues that indicate that the adrenomedullary sympathetic outflow can be controlled differentially and by medial-lateral pathways that have differing conduction velocities (Morrison and Cao, 2000). In the present study, we recorded a relatively modest preganglionic ASNA response evoked by RVLM stimulation and it was evident that most adrenomedullary neurons consist of both preganglionic and postganglionic activity as judged by responses obtained after ganglionic blockade. A comparison of RVLM stimulation-evoked ASNA responses was made to stimulation-evoked responses in the lumbar sympathetic outflow. The dual peak pattern of response has also been noted previously for RVLM and midbrain stimulation-induced responses in LSNA and spinohypothalamic (Huangfu et al., 1994; Lam and Verberne, 1997) and is best explained by the presence of C1- and non-C1 sympathetic preganglionic neurons in the RVLM which have different spinal afferent conduction velocities (Schreiber and Guyenet, 1997). In the present study, the ASNA response to RVLM stimulation was almost abolished by hexamethonium and this is consistent with a vasomotor role for LSNA. In contrast, the adrenal nerve has both vasomotor and non-vasomotor (cholinergic cell preganglionic) components (Verberne et al., 2016). We have also shown previously that systemic 2-DG treatment increases ASNA but not LSNA (Kerrin et al., 2014).

On average the peak latencies of the RVLM stimulation-evoked ASNA response (60 ms and 125 ms, respectively) were comparable to the time of the latency of the RVLM stimulation-evoked response in adrenal SPN (20 and 125 ms, respectively) and the average antidromic conduction latency of the axons of the adrenal SPN (37 and 35 ms) resulting in a total of 66 ms and 364 ms (Morrison and Cao, 2000). This suggests that the RVLM-ASNA pathway is a direct pathway that does not involve any additional synapses.

We noted partial blockade of ASNA responses by intracarotid KYN following electrical stimulation of the RVLM suggesting that glutamate is at least one of the neurotransmitters involved in the RVLM-ADRENAL SPN pathway. Although KYN has the advantage that it is a broad spectrum (excitotoxins) receptor antagonist, it is not very potent (pA2 = 4) (Burton et al., 1984). The dose chosen was identical to the dose used in a previous study which led to a significant reduction in blood pressure and heart rate but which led to a partial blockade of sympathetic postganglionic responses elicited by stimulation of the midbrain noradrenergic nucleus (Lam and Verberne, 1997; Verberne et al., 1990). It may be concluded that some component of the sympathetic postganglionic response is mediated by a glutamatergic input to adrenal SPN although it cannot be stated with any certainty whether this is the preganglionic component of the evoked response. While Morrison reported a more substantial block of glutamatergic excitatory responses in SPN elicited by RVLM stimulation, the antagonist was applied iontophoretically rather than into the cerebrospinal fluid (Morisuchi et al., 2003). On the other hand, blockade of RVLM glutamate receptors completely inhibited the ASNA response to systemic glucoprivation. This suggests that the Peh pathway to the RVLM adrenal preterminal sympathetic neurons recruited during glucoprivation is critically dependent on glutamate receptors in the RVLM.

PeH stimulation also elicited an evoked ASNA response that had both preganglionic and postganglionic components. As expected, the peak latencies of the PeH stimulation-evoked responses (86 ± 4 ms and 177 ± 5 ms) were longer than the RVLM stimulation-evoked responses (50 and 135 ms). The difference corresponds approximately...
to the average conduction velocity (0.5 m/s) of the majority of RVL-
projecting PeH neurons (Korim et al., 2016). It was also shown that
hexamethonium did not block the entire evoked ASNA response and
led to the appearance of a longer latency peak. This observation
may suggest that an additional preganglionic pathway may be involved in
the connection between the PeH and the adrenal sympathetic outflow
that increased activity in response to baroreceptor unloading after
hexamethonium.

5. Conclusion

In conclusion, the modulatory action of peripherally hypothalamic
neurons on adrenal sympathetic drive is dependent on glutamatergic
transmission. Glutamate acts at the level of the RVL preganglionic
neurons to help restore blood glucose levels back to normal during
hypoglycemia. Some RVL premotor sympathetic neurons that control
ASNA (and therefore adrenaline secretion) appear to use glutamate as
an excitatory transmitter. These neurons give an excitatory input
from premotor glutamatergic neurons in the PeH.

Conflict of interest

The authors have no conflicts of interest to declare.

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References


Chapter 4- MODULATORY EFFECTS OF HYPOTHALAMIC OREXIN AND MCH SYSTEMS ON MOTIVATED BEHAVIOUR: FOCUS ON ACTIONS WITHIN THE NUCLEUS INCERTUS
4.1. Introduction

Hypothalamic-originating orexin and MCH systems are well known for their contribution in motivated behaviour. There is a fundamental contribution of orexin-induced wakefulness and vigilance, as well as MCH-induced energy balance, required for feeding, drinking, locomotor activity in search of food, emotional behaviour and reward seeking as well as mating and maternal and defensive behaviours (Sakurai, 2014; Diniz and Bittencourt, 2017). The recently discovered relaxin-3 neuropeptide located in the brainstem, and which forms an N1 GABAergic system, potentially has similar arousal-related function to orexin and MCH, based on recent studies (Ma and Gundlach, 2015; Ma et al., 2017b). This chapter is designed to investigate the interactions between these three arousal systems, focusing on established motivated behaviours, such as food intake and locomotor activity in an experimental rat model.

4.1.1. Motivated behaviours and the orexin system

Wakefulness and a certain level of vigilance are always required for the execution of motivated behaviours. The reward system is faithfully associated with arousal, as both the cues that predict reward and reward-seeking behaviour are arousal-dependent. Reward-related behaviour is based on the activation of the circuit between NAc-LHA-VTA (via dopaminergic neurons) and -DR (via serotonergic neurons) (Sakurai, 2014). In emotional negative-motivated behaviour, the limbic system, especially the amygdala, is involved and is in close reciprocal contact with the LH area previously known as the “defence area” due to its capacity to produce defensive, aggressive behaviours following electrical stimulation (Hess and Akert, 1955). The LH is, in turn, in contact with the sympathoadrenal axis and drives the neuroendocrine and autonomic systems.

In terms of feeding behaviour, orexin was first shown to be involved with this behaviour as orexin neurons projected to the LH ‘feeding centre’ (Moriguchi et al., 1999). Central administration of OXA/B or i.c.v. or i.p. administration of orexin or OX1R antagonist increased food intake or reduction of feeding, respectively (Sakurai et al., 1998). Knockdown of orexin gene expression by RNAi reduced food intake and orexin-deficient mice consumed less food than littermate, wildtype mice (Sharf et al., 2010b). Interestingly, orexin neurons increase food intake by sensing metabolic
and sleep-wake states, and increase locomotor activity and wakefulness during periods of starvation (Tsujino and Sakurai, 2009). Prepro-orexin mRNA levels also increase in fasting rats and mice (Tsujino and Sakurai, 2013).

In terms of low energy balance, orexin neurons can sense metabolites such as glucose, triglycerides and amino acids and changes in CO₂ and pH levels directly, as well as leptin and ghrelin signalling indirectly, via their ARC neuron inputs (Williams et al., 2007; Goforth and Myers, 2017). Other neurons involved in the feeding circuit include those glucose sensitive neurons in VMH, which can be inhibited by orexin neurons, while NPY and MCH neurons in ARC and LHA, respectively, can be stimulated by orexin (Brunetti et al., 2005). Neurons in the PVN and DMH in the hypothalamus, as well as AP and NTS neurons in brainstem, contribute in this sensing behaviour, as induction of hypoglycaemia to these areas stimulates feeding and consequently activation of sympathetic outflow (Jordan et al., 2010).

On the other hand, food-related environmental cues or contexts can affect orexin neurons via inputs from the limbic system (e.g. BNST, CeA and NAc). At the same time, perception of cues which stimulate reward might activate the pathway between NAc and orexin neurons and their output to dopaminergic neurons in VTA (Johnson and North, 1992). This fact has also been confirmed in a study demonstrating that hypothalamic orexin neurons, but not MCH neurons, display Fos immunoreactivity after a learned food-cue, which stimulates feeding in satiated rats (Petrovich et al., 2012). On the other hand, orexin neurons also modulate NAc neurons via OX1R signalling (Tsujino and Sakurai, 2009) and, in relation to the hedonic aspect of feeding, injection of an opioid agonist into the NAc can increase palatable food intake in rats (de Macedo et al., 2016). Thus, it is suggested that the NAc to orexin neuron pathway might be involved in driving the ‘seeking’ for food, especially when motivation is high, e.g. for palatable food, under food restricted conditions or in the presence of condition cues which can, in turn, maintain motor activity and wakefulness until the reward (i.e. food) is obtained.

Furthermore, arousal linked to motivated feeding behaviour might be activated by the projections of activated orexin neurons to the monoaminergic systems located in DR, LC and TMN (Sakurai, 2014). The incidence of obesity in orexin deficient animals (Hara et al., 2005) and humans (Nixon
et al., 2015) with narcolepsy is twice that in the normal population and this can be related to a reduced energy expenditure baseline, decreased peripheral metabolism (muscles) and/or hypoactivity even if these subjects eat less food. In contrast, overexpression of orexin receptors can result in mice that are resistant to the weight gain associated with a high fat diet and the insulin insensitivity (Funato et al., 2009). These effects are mainly mediated by OX2R rather than OX1R, via its effects on leptin signalling mediating an increase in the anabolic to catabolic ratio, where leptin-deficient mice are totally resistant to the metabolic changes following OX2R overexpression (Funato et al., 2009). Diet-induced thermogenesis driven by increased sympathetic outflow can occur in the presence of orexin, and is triggered by VMH input stimulation, which can partially prevent obesity, especially that seen after a high-fat diet exposure (Gaur et al., 2014). Thus, the net effect of orexin signalling is body weight loss via increased energy expenditure even though food intake is increased.

Psychological stressors such as social stress, novelty and contextual fear, rather than physical stressors such as cold, restraint, and tail pinch, activate the orexin system. Cardiovascular responses were blocked in rats by orally-administered DORA or almorexant (a non-selective orexin receptor antagonist) (Furlong et al., 2009), or 1-SORA ACT-335827 (an OX1R-selective antagonist) (Steiner et al., 2013) or in orexin-knockout mice (Kayaba et al., 2003); but the origin of inputs that triggers the orexin system following these stressors is not clear. Furthermore, orexin is involved in relaying salient emotional cues or contexts that lead to activation of the limbic system to maintain wakefulness during emotional arousal, which, in turn, can produce self-regulatory effects on the limbic system by the orexin system (Yoshida et al., 2006). For example, in fMRI studies, there is impaired amygdala activation in narcoleptic human subjects (Khatami et al., 2007). In addition, projections of orexin neurons to the NA-ergic system in the LC, which projects to the lateral amygdala, form a circuit responsible for the appearance of emotional/fear memory (Samuels and Szabadi, 2008). Humans and mice lacking OX1R also display reduced freezing or amygdala activation in response to contextual/cued fear stimuli (Ponz et al., 2010; Soya et al., 2013). This behaviour is corrected in the mice after re-expression of OX1R by viral gene transfer (Soya et al., 2013).
Although, the function of OX1R and OX2R in terms of responses to contextual versus cued fear is different, the learning of fear memory is mainly via OX1R. Furthermore, it is known that OX2R is mainly responsible for maintaining arousal, while OX1R is mostly involved in processing emotive/rewarding memory formation, as OX2R antagonist treatment diminished amphetamine-induced activation of frontal cortex and thalamus, areas responsible for regulating arousal; whereas OX1R antagonist treatment attenuated the activation of the extended amygdala, BNST, and NAc, regions that are key for emotional memory formation (Stamatakis et al., 2014).

In regard to the reward system, an increasing body of evidence indicates that orexin is part of the reward aspects of addiction, not by affecting the reinforcing or priming effects of reward, but by supporting the motivated behaviour to obtain the reward such as drugs, food or sex; and this effect is apparently mediated by CRF1 located on orexin neurons in LHA (Ryan et al., 2013b) or via the NA-ergic system. CRF levels in the PVN are increased after i.c.v. injection of orexin which, in turn, results in HPA axis activation and ACTH level increases (Al-Barazanji et al., 2001). This reciprocal connection is relevant to the psychological but not physical stress-induced activation of the orexin system, which is increased to provide the required vigilance and attention. Dynorphin released from orexin neurons can bind to κ-opioid receptors and is known to mediate negative emotional states such as depression, and it might also be involved in the stress-induced activation of the orexin system and the occurrence of depressive changes in animals under psychological stress (Sakurai, 2014).

4.1.2. Adaptive behaviours and MCH system modulation

Anatomical studies reveal that orexin and MCH systems are targets of ascending arousal systems. Reciprocal/complementary actions of MCH and orexin systems, as well as coupling of their receptors to other transmembrane proteins in their target areas, can imply multimodal fine tuning of activated signalling cascades, especially in terms of arousal and motivated (goal-oriented) behaviours (Adamantidis and de Lecea, 2008). MCH receptors and MCH-containing axons are abundant in regions of the brain that display the highest metabolic activity produced by electrical stimuli of the LH rewarding system, which suggests a possible role for MCH in reward and motivation (Valenstein and Campbell, 1966).
In terms of the sleep-wakefulness cycle, it has been shown that during active wakefulness with strong locomotor activity and during REM sleep-to-wake transitions, orexin neuron firing rates increase. Orexin neurons activate MCH neurons, which in turn diminishes the waking state by inhibiting the activation of the adjacent orexin neurons, and thereby decreases orexin drive of downstream arousal neurons. Sleep begins when MCH neurons are activated and REM sleep begins when MCH neurons inhibit GABA neurons in the pons, and disinhibited GABAergic neurons in the pons ensure REM sleep (Pelluru et al., 2013). Non-REM sleep ends when MCH neurons auto-inhibit themselves, but notably, sleep can still occur if MCH neurons are ablated, as pre-optic ‘sleep-active’ neurons can still generate sleep (Konadhode et al., 2015). These neurons are mainly responsible for the timing of sleeping, as they receive direct input from the retina, whereas MCH neurons might be involved under special conditions such as post-prandial sleep, as they are in a region sensing glucose and can be activated following increases in blood glucose, whereas orexin neurons are activated by low blood glucose, which in turn activates descending arousal system, such as LC (Burdakov et al., 2005; Kong et al., 2010; Konadhode et al., 2015).

In terms of motivated behaviours, the MCH system acts differently to the orexin system. In feeding, MCH is orexigenic and similar to orexin, and in rats, i.c.v. administration of MCH or injection of MCH into hypothalamic nuclei that express MCHR1, induces hyperphagia with increased body weight (Abbott et al., 2003). However, chronic i.c.v. administration of MCH increased food intake without increasing food digestion over 24 h, which resulted in no overall change in body weight (MacNeil, 2013). In mice, i.c.v. injection of MCH increased their intake of a high fat-diet, but not standard chow, which may be due to the increased desire for reward caused by the neuropeptide (Gomori et al., 2003). However, both normal chow and high fat-diet fed groups displayed a significant increase in body weight and the latter group had significant increases in liver weights, plasma glucose, insulin, and leptin levels, which implies a major role for MCH in energy homeostasis (Gomori et al., 2003).

In another study, it was shown that rats injected i.c.v. with orexin tended to choose a high-fat diet over standard food, in contrast to a lack of selective behaviour in MCH treated rats (Borgland et al., 2009). On the other hand, several studies showed that i.c.v injection of MCH promotes the
intake of a high- or medium-fat diet, more than a standard chow, and i.c.v or peripheral injection of an MCH antagonist decreased intake of, and operant responding, for these diets (Shearman et al., 2003). Transgenic mice that overexpress ppMCH exhibit increased consumption of a high-fat diet compared to wild-type mice (Ludwig et al., 2001). However, it seems that the relationship of MCH with palatable food does not relate to sweet, noncaloric saccharin (Karlsson et al., 2012), indicating that MCH signalling may be related more to energy conservation than to the intake of palatable food per se. It was also reported that the expression of MCH is more responsive to fat content than to general reward, and MCH level are also similarly increased in the PeF of rats fed laboratory chow, rather than those which were prone to over-consuming a high-fat diet (Morganstern et al., 2010b). Similar results was observed in mice, as mice that overexpressed MCH consumed greater quantities of a high-fat diet than wild-type mice (Ludwig et al., 2001). In conclusion, orexin signalling increases short-term food intake (Sakurai et al., 1998) by increasing the motivation to eat and increasing energy expenditure- possibly via opioid receptors (Clegg et al., 2002) but most likely via glutamate/orexin receptors, whereas MCH signalling increases the sustainability of feeding and conserves energy. To further support this hypothesis, it was comprehensively shown that acute or chronic administration of MCHR1-specific antagonists decreased body weight and spontaneous or induced feeding either by fasting MCH in rodent (MacNeil, 2013). In mice, MCH mRNA is elevated in obese (ob/ob) mice and MCH knockout mice exhibit decreased body weight, associated with hypophagia associated with reduced meal size and increased energy expenditure and this effect was not dependent on hindbrain circuits as the microinjection of MCH in 4th ventricle did not reduce these effects (Mul et al., 2011). MCHR1-deficient mice were lean, despite consuming more food than control littermates. However, decreasing MCH neuron activity, in the presence of low glucose levels, would suppress sleepiness and promote activity, while conserving energy required for food seeking (Burdakov et al., 2005; Barson et al., 2013).

In terms of the contribution of MCH system in stress, i.c.v. administration of MCH in rats both had no effect on anxiety in the elevated-plus maze (Duncan et al., 2005). However, recent studies reported that MCH acted as pro-depressive agent following administration of this neuropeptide in local nuclei such as DR (Urbanavicius et al., 2013).
4.1.3. Role of *nucleus incertus* in motivated behaviour

A growing body of evidence suggests a contributing role of GABAergic neurons of *nucleus incertus* in motivated behaviours, of which a significant proportion are relaxin-3-positive. Relaxin-3 and its GPCR, RXFP3, likely act synergically with GABA signalling in target neurons, based on *in vivo* and *in vitro* studies to investigate the modulatory effects of RXFP3 activation in behavioural arousal and motivated behaviours, including sleep/wakefulness, and feeding and drinking (Smith et al., 2014b). The role of relaxin-3 signalling in feeding behaviour, as an example of motivated behaviour, has been widely investigated. Central injection of human relaxin-3 (54 and 180 pmol) into satiated adult male Wistar rats, stimulated feeding in the first hour after injection - either in the dark or light phase - (McGowan et al., 2005). This effect was reproduced by central injection of relaxin-3 analogues that are more selective for RXFP3 than the native peptide (Liu et al., 2005a; Haugaard-Jönsson et al., 2008), and RXFP3 related feeding is blocked by RXFP3 antagonists (Haugaard-Kedstrom et al., 2011; Shabanpoor et al., 2012). Central injection of human relaxin-3 (50-150 ng) also increased water drinking in male rats (Bathgate et al., 2006), mimicking the effect of the related peptide-hormone, relaxin (Thornton and Fitzsimons, 1995).

Local injection of relaxin-3 into hypothalamic nuclei such as PVN (18, 180 pmol), ARC, supraoptic nucleus and anterior preoptic area (180 pmol) also increased feeding in rats, with no increase after injection into the LH, suggesting putative sites of action for endogenous relaxin-3 (McGowan et al., 2007). Although acute central administration of relaxin-3 increased food intake for no more than one hour, sub-chronic (180 pmol/day for 7 days) or chronic (600 pmol/day for 14 days) administration of the neuropeptide or its analogues in PVN or i.c.v., increased food intake, leptin and/or insulin levels in blood and bodyweight, with no indication of an increase in energy expenditure or locomotor activity (Hida et al., 2006; McGowan et al., 2006; Sutton et al., 2009). Studies of the orexigenic effect of relaxin-3 in mice produced divergent results. Relaxin-3 knockout mice, back-crossed on a C57BL6/J background, did not have metabolic differences or lower body weights *c.f.* wildtype littersmates (Smith et al., 2009a). In contrast, i.c.v. injection of an RXFP3 antagonist in C57BL/6J mice reduced the food anticipatory activity before meal time,
generated by prior food restriction and regular feeding for a fixed period of 4 h/day (Smith et al., 2014a). In addition, consumption of highly palatable food, as well as regular chow was reduced at the beginning of the dark phase or after food deprivation (4 h). Food intake was not increased after i.c.v. or intra-PVN injection of RXFP3 agonist in this strain of mice, which highlights an experimental difference in feeding behaviour related to relaxin-3 signalling in mice and rats (Smith et al., 2014a).

In regard to other motivated behaviours, such as arousal and wakefulness, decreased in voluntary running wheel activity was observed in both relaxin-3 knockout mice (Smith et al., 2012) and RXFP3 knockout mice (Hosken et al., 2015), as well as differing effects on locomotor activity in rats after central relaxin-3 injection (McGowan et al., 2005; Hida et al., 2006; Sutton et al., 2009). These findings implicate relaxin-3 signalling in arousal and reward pathways (Smith et al., 2012). As discussed in Chapter 1, the activity of NI neurons in response to acute and chronic stress has been reported, following the observation of high levels of CRF1 receptor expression in the NI (Bittencourt and Sawchenko, 2000). In this regard, long-lasting and non-desensitising depolarisation was observed in response to CRF in in vivo single unit recordings in rats (Ma et al., 2013). Physical stress, such as different types of restraint, forced-swim test, water immersion and immobilisation, foot shock, or paw pinch, also significantly increased NI Fos-immunoreactivity; whereas freezing behaviour with exposure to a previously-exposed predator environment decreased NI Fos immunoreactivity (Ribeiro-Barbosa et al., 2005; Ryan et al., 2011).

In contrast, physiological stressors such as infection, hypoxia or hypercapnia did not increase NI neural activity (Elmquist et al., 1996; Teppema et al., 1997) suggesting that NI activation occurs when an active locomotor component or ‘flight’ response, but not the active immobilisation component or freezing response is required. This was displayed when rats with freezing behaviour in a conditioned fear paradigm or in response to cat (predator) odour exhibited decreased NI Fos-immunoreactivity (Ribeiro-Barbosa et al., 2005).

In terms of contribution to arousal, in vivo electrophysiological studies revealed that hippocampal theta rhythm was evoked by electrical stimulation of the NI and was significantly attenuated by electrical lesion or muscimol injection into the NI (Nunez et al., 2006). Studies by my host
laboratory have revealed that relaxin-3 neurons (which all express CRF1) exhibit a phase-locked firing pattern with hippocampal theta oscillations, compared to non-relaxin-3 neurons, which suggests a functional connection between NI GABA/relaxin-3 neurons and hippocampus (Kizawa et al., 2003; Ma et al., 2013). Consistent with this, infusion of RXFP3 agonist or antagonist into MS of awake rats induced or impaired hippocampal theta rhythm, respectively (Ma et al., 2009a). In addition, inactivation of NI with lidocaine infusion impaired the acquisition and retrieval of spatial reference memory (Nategh et al., 2015). Taken together, these findings highlight the role of NI in promoting hippocampal function and theta rhythm, which is important for spatial memory (Smith et al., 2014b).

4.2. Gaps in knowledge and effects of the orexin system on the nucleus incertus

Recent electrophysiological and anatomical studies in anesthetized rats confirm the existence of an excitatory orexinergic projection to the NI (Blasiak et al., 2015). However, the interaction between orexin and NI different systems including relaxin-3 system in conscious, freely-moving rats has not been exhaustively investigated. On the other hand, there are limited studies regarding the anatomical, behavioural, and functional interactions of MCH with other arousal systems mainly focusing on sleep-wakefulness and to date there is no study showing any reciprocal interaction of this system with NI and relaxin-3 system in terms of other motivated behaviours. In this study, experiments focused on determining the effect of orexin and MCH signalling within the NI and the potential behavioural output of this interaction was measured in the context of feeding and exploratory locomotor activity. The behavioural effects of these hypothalamic systems are supported by further study on the pattern of anatomical projections and the diurnal changes of these neuropeptides trafficking to NI. Supplemental results conducted by an external collaborator that complement this data are discussed in the discussion section.
4.3. Materials and Methods

4.3.1. Materials

The inhalational anaesthetic, isoflurane (Pharmachem, QLD, Australia) was used for induction and maintenance of anaesthesia. Orexin A was supplied by Mimotopes (Notting Hill, VIC, Australia; with content of 78%, as described in certificate of analysis). MCH (Tocris Bioscience, Bristol, United Kingdom; with the content of 88.5% as described in certificate of analysis) was dissolved in artificial CSF (aCSF). Supplies of aCSF previously prepared were kept at 4°C. Different doses of orexin A and MCH (200, 600, 1000 pmol) were prepared by serial dilution immediately before starting each experiment, with the concentration measured using Direct Detect and transported to the experimental room on ice.

Bupivacaine hydrochloride solution 0.25% (Pfizer, New York, NY, USA) and Meloxicam (Randlab, NSW, Australia) were provided by the Florey Animal Resource Facility. Silk surgical suture thread with USP: 4.0 was used to suture the skin after the surgery. Guide cannula (5.0 mm, C235G-SPC, 26 gauge), dummy (C235D-SPC, 33 gauge), and internal cannulas (C235I-SPC, 33-gauge, 7.3 mm fit with 1.5 mm projection from the tip of guide cannula) and 10-PE tubes were purchased from Plastics One Bioscientific (Roanoke, VA, USA). Syringe pump (11-Plus) was from Harvard Apparatus (Holliston, MA, USA). Hamilton syringes (1 µl) were purchased from SGE Analytical Science (Melrose Park, NSW, Australia). Dental cement and its solvent was purchased from Stoelting Co (Wood Dale, IL, USA).

4.3.2. Methods

4.3.2.1. Brain cannulation and injection

Rats underwent stereotaxic surgery for implantation of a permanent guide cannula directed to the nucleus incertus using the following coordinates: anterior/posterior -2.2 mm, medial/lateral +0.3 mm, dorsal/ventral -6.3 mm (from the surface of the skull); with the incisor bar set at -12.5 mm. All rats were placed on a heat pad during surgery. Following adjustment of the ear bars and fixing the head at +3.3 mm on the incisor bar, the top of the head was shaved and scrubbed with iodine solution. Bupivacaine (0.1 ml; 1:10 in saline) was injected subcutaneously on top of the skull to
Figure 4.1. Sagittal and coronal views of NI for cannulation.

(A) Sagittal and coronal view for the nucleus incertus which is located between Bregma − 9.12 mm to Bregma − 9.84 mm. Green bars show the cannula position. (B) Nissl stained section through the mid-rostrocaudal extent of the nucleus incertus, located adjacent to the posterior dorsal tegmental nucleus (PDTg) and dorsal to the medial longitudinal fasciculus (mlf). Approximate borders of the pars compacta (NIC) and the pars dissipata (NID) are outlined. Abbreviations: Me5, trigeminal nerve nucleus, 4 V, fourth ventricle (Ryan et al., 2011)
reduce the pain of the incision. Meloxicam (0.05 ml/each rat) was administered intraperitoneally.
A midline incision was made from between the eyes caudally to where the occipital bone joins the
neck muscles, and the connective tissue underneath the skin was held back by forceps from both
sides. Following cessation of local bleeding, 3 holes were drilled into the left frontal and both
parietal bones and three screws were inserted using a small screwdriver. The incisor bar was then
lowered to -12.5 mm. The guide cannula was placed in a cannula holder and adjusted on the
midline using a microscope (Figure 4.1).

Using the left cannula bar as a reference, it was moved +0.3 mm from midline and then -2.2 mm
from lambda. The place the tip of the cannula bars touched the skull was marked and drilled to
make the hole for the cannula to be inserted to a depth of +6.3 mm from the surface of the skull.
Following the cessation of bleeding caused by the cannula insertion, the edge of the cannula plastic
base/holder was glued to the skull with superglue. In addition, the barrel/thread of the screws was
also covered in glue. For better protection of the cannula and to prevent their movement, dental
cement was applied on top of the skull around the cannula and after drying, the overlying skin was
sutured. Dummy pins were inserted into the cannula and a cap was screwed on top. Rats received
meloxicam i.p. injection for another 2 days following the surgery and their body weight was
recorded to confirm recovery after the surgery (e.g. no consistent decrease in body weight). During
the 7-day recovery period, rats were handled daily and underwent daily mock injections to be
habituated to the injection procedure.

On the experimental day, injections were performed over one minute using a perfusion pump, and
the microinjector was left in the place for another 2 min afterwards to complete drug extrusion.
Dummies were placed back into the guide cannula to avoid blocking, and the caps were screwed
back on to the guide cannula. The injector was washed with isopropyl alcohol between injections
in different rats. In addition, the injector was soaked in 70% ethanol between sessions, with 48 h
intervals between, to avoid blocking or microfilm layer production around the tips. For verification
of the cannula placement, rats were anesthetised in a chamber using isoflurane at the end of the
experiments and an ink injection was performed using the same procedure as used for peptide
injections. The rats were then deeply anesthetised with sodium pentobarbital (100 mg/kg) and were
decapitated following respiratory arrest. Brains were frozen on dry ice, wrapped in Parafilm and stored at -20°C. Cryostat sections (40 µm) were cut and the location of the tip of the cannula was determined by standard histological procedures using thionin staining.

4.3.2.2. Injection protocols

The protocols used for these peptide injection studies were counter-balanced or a balanced Latin square design (used when the number of conditions is even: aCSF, 200 pmol, 600 pmol, 1000 pmol), with a 48 h interval between each session. The principle is as follows:

Where the treatment factor levels are the letters in the Latin square design. The number of rows and columns correspond to the number of treatments (e.g. aCSF, 200 pmol, 600 pmol, 1000 pmol). Each condition appears once in each row and column. Furthermore, each condition appears before and after each other condition an equal number of times. Thus, imbalance is eliminated.

Despite the advantages of a within-subjects design (as represented by counterbalance design), a between-subjects (represented as balanced Latin square) design is sometimes preferred in order to avoid interference between the conditions. For this study, as the “sensitivity” of the injection site (NI) after repeated injections was important and a within-subject design might have not been valid enough, considering the small number of rats used (n = 8), using the Latin square design eliminated this bias. Therefore, the protocol adopted for this study was as follows:
Table 4.1. The design of microinjection to NI of the animals used for food intake and locomotor activity studies using counter-balanced Latin square design. Each dose is shown as pmol/0.5 µl/each side. A = 200 pmol, B = 600 pmol, C = 1000 pmol, D = aCSF

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In a pilot study, three different doses of OXA (200, 600, 1000 pmol/0.5 µl) were injected using infusion pump (over 3 min with 1 min waiting period in the injecting site) into rats during the light phase (n=8, indicated by Latin number as in above table, n=2 cannula was blocked at time of injection and used as sham replicates), using a counter-balanced Latin square design. With this design all 4 treatments were presented during each experimental session including open field with food accessible in each locomotor cell 30 min after each injection and each rat received all the possible treatments with at least 48 h between treatments.

For the main experiments, the selected dose of OXA (600 pmol/0.5 µl) was injected into two different groups of rats assigned for open field test without food and home cage food intake using counterbalanced design with the interval of 48 h between injections.

4.3.2.3. Open field test

**Orexin studies.** For acclimatisation, rats were kept overnight in their home cage in the testing room and on the experimental day, rats were placed in chambers with the lid on and left for 30 min to acclimatise. The chamber timers were stopped and each rat was infused with OXA (200, 600 or 1000 pmol/each side in the pilot study, or 600 pmol/each side in the main study, 500 µl/min) or aCSF and placed back into the chamber. The ambulatory distance, ambulatory count and vertical count were recorded over 4 h. After finishing the experiment, rats were returned to their homecage and the locomotor parameters for all rats were downloaded to an Excel file for processing. Horizontal and vertical counts were binned into 5 min samples for 270 min after drug administration.

**MCH studies.** For acclimatisation, rats were kept overnight in their homecage in the testing room. On the experimental day, the software was set for a 240 min recording for two different groups of rats with 30 min habituation for one group and no habituation for the other group. Each rat was injected with MCH (600 pmol) or aCSF and immediately placed in chambers with the lid on and the recording started as soon as the rat touched the floor of the chamber. After finishing the experiment, and returning the rats to their home cage, the locomotor parameters were downloaded and binned into 5 min samples. In a separate group, locomotor activity was measured after MCH
infusion in unhabituated rats, during the dark phase (4 h) where rats are more active, and compared to activity in the control group.

4.3.2.4. Home cage food intake

Pellets of standard chow (~10 g) were pre-weighed. Immediately after injection, each rat was placed in new cage to avoid any food intake from the bedding and pre-weighed food was placed on empty lids above the new cage. The food intake for each rat was measured 0.5, 1, 2, and 4 h after injection. At the end of the experiment, the cumulative food intake for each rat was calculated and the mean of the treated group was compared with that of the control group. In the first cohort, rats were fed ad lib and MCH (600 pmol) solution or aCSF were infused (flow rate: 500 µl/min, concentration: 2.14 µg/µl) at 12 pm and intake of pre-weighed food (~10 g) was recorded at 0.5, 1, 2, and 4 h after injection during the light phase, similar to the orexin protocol. As the intake levels were not significantly different, the experiment was repeated under different conditions.

Different group of rats were restricted from access to food for 6 h during light phase (9 am-3 pm) and intake of pre-weighed food (~10 g) was measured 1, 2 and 4 h after introduction of food to each cage. The same protocol was applied to these rats when they were not fasted and the food intake under these two conditions were compared. As there was no significant difference between the total food intake within 1 h by the fasted and non-fasted rats during the light phase, in which rats are mostly sleeping and inactive, another study was conducted during the dark phase, when rats are active and actively feeding. Thus, baseline food intake was measured using pre-weighed food (30 g) introduced at 7 pm, when the dark phase began, and the consumption was measured every hour for 4 hours (7-11 pm). The time at which the highest amount of food was consumed by rats during this period was around 8 pm. Therefore, 24 h later, MCH (600 pmol) or aCSF was infused into the NI at 7 pm (flow rate: 400 µl/min, concentration 4.037 µg/µl) and pre-weighed food was presented to each rat with food consumption recorded every hour for 4 h for each cage. (Rats were assessed in a new cage to ensure there was no left-over food in the bedding). At the end of the experiment, the cumulative food intake during 4 h was calculated for each rat and the means of each group were compared. Baselines of locomotor activity and food intake in naïve rats were also recorded to highlight the handling/injection stress effects in both assays.
4.3.2.5. Immunohistochemistry and reconstruction of fibres distribution in the NI

Naive rats (n = 4 per diurnal phase) were transcardially perfused with fixative at 13:00 h (during the light phase) or 01:00 h (dark phase). Rats were deeply anesthetised with sodium pentobarbital (100 mg/kg, i.p.) prior to transcardial perfusion with 300 ml ice-cold 0.1 M phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 11.2 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) followed by 400 ml 4% formaldehyde in 0.1 M PBS. Brains were isolated and post-fixed for 1 h, then transferred to 30% sucrose-PBS solution at 4°C for 48 h. Blocks containing the NI of each brain were sectioned (30 µm) and collected free floating in PBS.

Brain sections were firstly blocked using 10% normal horse serum (NHS) in PBT (PBS with 0.1% Triton-X100) for 1 h at room temperature. Sections were then incubated in primary antibody solution orexin-A (1:1000 dilution) overnight and 4-hour secondary antibody for goat anti-orexin A (Abcam, Cambridge, United Kingdom) or 1:400 dilution goat anti-MCH primary antibody (Abcam, Cambridge, United Kingdom) with 1:5 mouse anti-relaxin-3 (The Florey Institute of Neuroscience and Mental Health; (Kizawa et al., 2003; Ma et al., 2017b) containing 2% NHS in PBT, overnight at 4°C. Sections incubated without primary antibody were used as a negative control. Sections were washed 3 × 10 min in PBS prior to incubation in secondary antibody solution (1:500 dilution donkey anti-goat AlexaFluor-594 and donkey anti-mouse AlexaFluor-488; Thermo Fisher Scientific, North Ryde, NSW, Australia) for 1 h at room temperature. Sections were washed 3 × 5 min in PBS, mounted on glass slides and coverslipped with Fluoromount-G (Southern Biotech, Birmingham, AL, USA).

Reconstruction of the distribution of MCH and OXA fibres and relaxin-3 positive soma was based on a series of 8 projections made from z-stacks (1024 × 1024, 20×/0.8) combined into one image using ImageJ (NIH, Bethesda, MD, USA). Distance from bregma was assessed by matching the tissue to the corresponding plate of a rat brain stereotaxic atlas (Paxinos and Watson, 2007).

4.3.2.6. Confocal imaging and analysis

Imaging was performed using an LSM 780 Zeiss Axio Imager 2 confocal laser scanning microscope (Carl Zeiss AG, Jena, Germany). The system is equipped with a stitching stage, and
Zen software (Carl Zeiss AG) was used to stitch tiled images. Each fluorescence channel was imaged sequentially using a 20× or 63× objective. Stitched mosaic images were collected and analysed.

In studies to assess diurnal fluctuations in immunofluorescence, imaging parameters were optimised and set across all samples. In each section, two axonal segments in the vicinity of either a relaxin-3 immunopositive soma located in the NI were sampled. Images were quantified using FIJI (NIH). Quantified particles indicative of axonal varicosities of each segment were pooled for analysis. Images were also analysed using Imaris (v8.3; Bitplane, Zurich, Switzerland) for surface rendering of relaxin-3 soma (surface) and spot rendering of orexin/MCH axons/vesicles reconstructed as ‘spots close to surface’ (<0.5 µm from the surface) and ‘spots far from surface’ (>0.5 µm from the surface).

**4.3.2.7. Statistical analysis**

Data were analysed for statistical significance using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). For preliminary study, repeated measure two-way ANOVA (different doses 200, 600, 1000 pmol over time 1, 2, 4 h after injection) was applied to test the significant interaction of treatment doses over time of recording. One-way ANOVA followed by Bonferroni post-hoc was also performed to test the significant treatment dose compared to control group. After identifying the effective dose (600pmol), in the main study two-way ANOVA (with repeated measures where appropriate) with Holmes-Sidak’s post-hoc was used to determine the significance of the interaction between time and treatment on locomotor activity parameters or food intake and the P value (P<0.05) for this interaction was considered as significant. Students t-test at each time point of recording was also performed followed by the Bonferroni post-hoc test to examine the significance of the effect of treatment compared to control group at a specific time point.

For rejecting the hypothesis that multiple injections reduced the effectiveness of each injection, a one-way ANOVA (dependent variable: day, independent variable: food intake at 4 h after 1000 pmol dose on different days) was completed. Although this analysis revealed no significant differences, as this analysis was limited in power to detect differences, in another analysis
including all doses of OXA across days (corrected for the baseline food), n was equal to 7-9 rats per day with the power of 0.78. This analysis with the enhanced power also revealed no significant difference in response to OXA due to day. Rats might have been conditioned to eat or not eat during different days, so the effect of day on food-intake of all saline-treated rats was analysed by one-way ANOVA at each injection time (day, independent variable, 4 h food intake, dependent variable) which revealed no significant differences in 4 h intake in control rats across treatment days (P>0.05).

For data related to the diurnal frequency of the intensities of presynaptic vesicles and varicosities data were illustrated as mean ± SEM. For analysis of diurnal changes in immunofluorescence intensity, frequency histograms that passed the normality test (D’Agostino and Pearson normality test; α = 0.5, P > 0.05), were analysed for differences by unpaired t-test. Frequency histograms that did not pass the normality test (D’Agostino and Pearson normality test; α = 0.5, P < 0.05) were considered non-Gaussian in nature and were analysed using a non-parametric, two-tailed t-test followed by a Kolmogorov-Smirnov post-hoc test.
4.4. Results

4.4.1. Differential effects of intra-NI infusion of orexin A and MCH on locomotor activity and food intake

In the studies of locomotor activity, following inspection of the data, the initial 30 min of activity was excluded from the main analysis, as the high levels of activity observed during this period were likely influenced by/attributed to handling and infusion stress (Table 4.1). Before analysing the data, rats with off-target injections were also excluded from further analysis (Figure 4.2). Intra-NI infusion of OXA (600 pmol) during the light phase when rats are usually inactive, resulted in significantly increased total ambulatory distance travelled compared to aCSF-infusion. Cumulative distance travelled was significantly increased from 75 min post-infusion through the end of the experiment in 240min post injection ($F_{(17,85)} = 2.86; P<0.001$) indicating prolonged activity beyond initial handling/stress phase while preventing the normal slow down to sleep/wake pattern of the animals in the locomotor cells. In contrast to the sustained activity of the OXA-treated rats, aCSF-infused rats exhibited bouts of low level activity (4-5 min in length characterised by elevated distance travelled and elevated rearing) between periods of inactivity (25-30 min in length), likely related to rest and/or sleep (Figure 4.3A).

In contrast to the effect of OXA, intra-NI infusion of MCH (600 pmol) during the light phase resulted in a trend for decreased total distance travelled compared to aCSF-infusion (Figure 4.3B). Notably, the (4-5 min) bouts of low level activity between periods of inactivity observed in the control group were not observed in the MCH-infusion group. In light of a likely ‘floor-effect’ in tests of reduced locomotor activity in habituated rats during the light phase, the effect of intra-NI MCH infusion was assessed in rats that did not receive prior habituation to the arena during the light phase, and infusion during the early dark phase, when rats are more active (Figure 4.3C). Intra-NI MCH infusion in rats for which the arena was a novel environment did not produce any difference in cumulative distance travelled or cumulative vertical counts (rearing) (data not shown). In the study conducted in the early dark phase, both aCSF- and MCH-infused rats exhibited greater locomotor activity than those tested during the light-phase (Figure 4.3C), but intra-NI MCH infusion did not significantly alter locomotor activity ($F_{(46, 322)} = 0.73; P > 0.05$).
Consistent with the activation of locomotor activity, intra-NI infusion of OXA (600 pmol) during the light phase, significantly increased cumulative food intake of satiated rats compared to aCSF infusions over a 4 h test period (**Figure 4.4A**; $F_{(3, 27)} = 4.906; P < 0.01, n = 12$). In contrast, intra-NI MCH infusion (600 pmol, $n = 7$) during the light phase, did not significantly alter food intake (**Figure 4.4B**). Again, in light of a likely ‘floor-effect’ in tests of reduced feeding in satiated rats during the light phase, the effect of intra-NI MCH infusion was assessed in rats that were food deprived for 6 h (9:00-15:00 h) prior to infusion during the light phase, or during the early dark phase (16:00-22:00 h). Food intake was higher in rats that underwent a 6 h food deprivation during the light phase prior to infusion, but intra-NI MCH infusion did not significantly alter this intake (**Figure 4.4C**). Similarly, food intake was higher in rats studied during the early dark phase than during the light phase, and interestingly intra-NI MCH infusion resulted in significantly increased food intake in the first 2 h compared to aCSF-infusion ($F_{(3, 18)} = 8.57; P < 0.05$ at 60 min and $P < 0.01$ at 120 min post-infusion).
Table 4-1. Locomotor activity in the first 30 min after handling and intra-NI infusion of aCSF or peptide.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cumulative Ambulatory distance (0-30 min)</th>
<th>Cumulative Vertical Counts (0-30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Light Phase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aCSF</td>
<td>133.7 ± 60 (n=6)</td>
<td>5.5 ± 2.7 (n=6)</td>
</tr>
<tr>
<td>OXA</td>
<td>150.1 ± 85.3 (n=6)</td>
<td>4.1 ± 2.1 (n=6)</td>
</tr>
<tr>
<td>aCSF</td>
<td>187.7 ± 110.4 (n=6)</td>
<td>5.2 ± 1.9 (n=6)</td>
</tr>
<tr>
<td>MCH</td>
<td>160.2 ± 86.7 (n=8)</td>
<td>5.2 ± 2.8 (n=8)</td>
</tr>
<tr>
<td><strong>Dark Phase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aCSF</td>
<td>413.8 ± 138.1 (n=7)</td>
<td>9.60 ± 3.3 (n=7)</td>
</tr>
<tr>
<td>MCH</td>
<td>328.1 ± 120.1 (n=7)</td>
<td>7.1 ± 3.0 (n=7)</td>
</tr>
</tbody>
</table>

Data is represented as mean ± SEM of the distance (cm) travelled or the vertical count in open field during the 30 min after injection.
Figure 4.2. Cannulation sites and targeting of the nucleus incertus.

Sites within the NI or surrounding areas targeted by cannulae and infusions in studies of (A) locomotor activity and (B) food consumption. Sites assessed as ‘hits’ (blue circles) and ‘misses’ (red circles) are indicated in the different cohorts of rats used to study the effects of peptide and aCSF infusions during the light or dark phases.
Figure 4.3. Effect of intra-NI orexin-A (OXA) or melanin-concentrating hormone (MCH) infusion on locomotor activity of rats during the light or dark phase.

Time-course for cumulative ambulatory distance travelled (cm) during the 30-240 min after: (A) intra-NI OXA (600 pmol/min-filled circles) or aCSF infusion (n = 6/group, open circles) during the light phase; and (B, C) intra-NI MCH (600 pmol/min, filled square) or aCSF(open square)infusion during (B) the light phase (n = 8/6 per group) and (C) dark phase (n = 7 per group). Data is shown as mean ± SEM and analysed using 2-way rmANOVA followed by Holmes-Sidak’s post-hoc with $P<0.05$ set as significant (**$P<0.01$). Blue arrows indicate the phases of waking/activity following bouts of resting (30-35 min) in aCSF treated rats, which were largely absent in MCH treated rats.
Figure 4.4. Effects of intra-NI orexin-A (OXA) or melanin-concentrating hormone (MCH) infusion on cumulative food intake in satiated and fasted rats during the light or dark phase.

Time-course of cumulative food intake following (A) intra-NI OXA (600 pmol/min) or aCSF infusion (n = 12/group); and (B) intra-NI MCH (600 pmol/min, n = 7) or aCSF infusion (n = 6), during the light phase. (C,D) Cumulative food intake following intra-NI MCH (600 pmol/min) or aCSF infusion (n = 6/group) in prior, 6 h-fasted rats, during the (C) light and (D) dark phase. (E) Food consumption of 6 h-fasted rats after intra-NI MCH or aCSF infusions (n = 10/group) during the dark phase (solid lines) compared to consumption by the same rats without fasting or by sham-treated rats (dashed line). Data is shown as mean ± SEM and analysed using 2-way rmANOVA followed by Holmes-Sidak’s post-hoc with *P<0.05 set as significant (**P<0.01, ****P<0.001).
4.4.2. Diurnal differences in orexin and MCH projections to NI and putative synaptic vesicle content

Characteristics of OXA and MCH innervation of the NI in immunostained coronal sections of the rat brains collected during the mid-light or mid-dark phase were examined. Surface/spot rendering and automated analysis of fluorescence intensity was undertaken to examine OXA- and MCH-positive elements >0.5 µm from relaxin-3 soma (indicative of axonal segments) and elements <0.5 µm from relaxin-3 soma (indicative of terminations). The distribution and mean frequency of fluorescence intensity of OXA-containing axonal segments was unchanged ($P = 0.1714$, unpaired two-tailed t-test) between light (64.11 ± 0.61, 8 sections/rat, $n = 4$ rats) and dark phase (65.12 ± 0.42, 7 sections/rat, $n = 4$ rats). However, the mean frequency of fluorescence intensity of MCH-containing axonal segments was significantly lower ($P < 0.0001$, Kolmogorov-Smirnov test) in the light phase (77.21 ± 0.62, 8-9 sections/rat, $n = 4$ rats) than in the dark phase (80.60 ± 0.42, 7 sections/rat, $n = 4$ rats) (Figure 4.5A-D). Analysis of light-phase OXA-immunofluorescence revealed a positive skewness in the histogram distribution compared to the dark-phase, suggesting the presence of high intensity vesicles in the orexin axonal segments during the light, but not the dark, phase. In contrast, the mean intensity of dark-phase MCH immunofluorescence was significantly higher than that in the light phase (Figure 4.5E-F), suggestive of increased trafficking of MCH in varicosities of axons projecting to the NI during the dark phase.

Three-dimensional reconstruction of surfaces (relaxin-3 immunoreactive soma) and spots (OXA and MCH immunoreactive boutons) were analysed (Figure 4.6A-D) to determine the frequency of fluorescence intensity of spots located close to the surface (<0.05 µm), which are reflective of synaptic vesicles (Ausdenmoore, 2011). Fluorescence intensity for orexin during the light phase (52.69 ± 2.76, 5-6 sections/rat, $n = 4$ rats) was not significantly different ($P = 0.52$) to that observed during the dark-phase (52.17 ± 2.59, 5-6 sections/rat, $n = 4$ rats) (Fig. 5). In contrast, fluorescence intensity for MCH during the light phase (57.06 ± 9.85, 5-6 sections/rat, $n = 4$ rats) was significantly lower ($P < 0.01$) than that observed during the dark-phase (81.90 ± 9.80, 4 sections/rat, $n = 4$ rats) (Figure 4.6E-F), suggesting a higher level of MCH in presynaptic vesicles close to relaxin-3 soma (surface) during the dark phase.
Figure 4.5. Orexin-A (OXA) and melanin-concentrating hormone (MCH)-positive segmental axons projecting to the nucleus incertus during the light phase, and the diurnal shift in neuropeptide trafficking in fibres projecting to the NI.

Illustrative high magnification (63×) confocal images during the light phase of (A) OXA-positive varicosities (magenta) with a large XY dimension (around 2.0 µm), illustrating a constant trafficking of neuropeptide throughout the segmental axons (inverted greyscale image); and (B) MCH-positive varicosities (blue) with a smaller XY dimension (1.0 µm) illustrating an intermittent trafficking of the neuropeptide (inverted greyscale image). (C, D) The diurnal histograms related to the distribution of neuropeptide intensities in the NI-projecting segmental axons. (C) There was a significant shift in the median value of histograms related to the expression of orexin-A in segmental axons in the dark and light phases (**, $P<0.01$, unpaired t-test, followed by Kruskal-Wallis post-hoc, data from 157 segmental axons from 4 rats, with 2× sampling) and (D) a significant shift in the median value of histograms related to MCH in segmental axons in the dark and light phases (****, $P<0.001$, unpaired t-test, followed by Kruskal-Wallis post-hoc, data from 163 segmental axons from 4 rats with 2× sampling). Scale bars, 5 µm.
Figure 4.6. Distribution pattern of OXA and MCH-positive nerve fibres in NI during the light or dark phase.

Illustrative images of reconstructed ‘close-to-spot’ orexin/MCH vesicles during the light phase, and the diurnal shift of orexin/MCH (‘release’) in presynaptic vesicles during the light and dark phases. (A, B) NI relaxin-3 (RLN3) immunofluorescent soma/elements (Green, 20×) with OXA (A row, red) and MCH immunoreactive fibres (B row, red) at different AP (anterior, mid and posterior) levels. (C, D) Examples of reconstructed relaxin-3-positive neurons (green surfaces) with reconstructed putative OXA-positive presynaptic vesicles (C) and MCH-positive presynaptic vesicles (D) within 0.5 µm of relaxin-3 neurons. Insets illustrate high magnification images of the orexinergic/MCH projections to relaxin-3-IR neurons in NI (E, F) Histograms illustrating the diurnal-related distribution of neuropeptide-positive intensities in putative presynaptic vesicles. (D) There was no significant shift in the median value of the histograms related to the ‘release’ of orexin varicosities close to relaxin-3 neurons during the dark and light phases ($P$>0.05, unpaired t-test, followed by Kruskal-Wallis post-hoc, data from 36 sections from 4 rats); whereas (E) a significant shift occurred in the mean value of the histograms related to the ‘release’ of MCH varicosities close to relaxin-3 neurons during the dark and light phases (*, $P$<0.05, unpaired t-test, followed by Kruskal-Wallis post-hoc, data from 38 sections from 4 rats). Scale bar, 100 µm.
4.5. Discussion

In the current studies and related concurrent studies by other laboratories, several novel aspects of the interaction between the lateral hypothalamic MCH/MCH1R signalling system and the nucleus incertus (NI) GABA/relaxin-3 system in rats were identified. In collaboration with other groups, this study results were completed and the presence of NI-projecting, MCH-synthetising neurons in the hypothalamus as well as the topographic distribution of orexin-A and MCH-positive nerve fibres and their receptors, OX2R and MCH1R, respectively, in the NI were identified. The effect of MCH and orexin-A on the neurophysiological activity of NI neurons in vitro has been explored (Blasiak A et al., unpublished data), along with the functional effects of local infusion of orexin-A and MCH into the NI on behavioural activity and food consumption. In addition, the effect of diurnal phase on the dynamics of orexin-A and MCH in axonal segments and synaptic vesicles within the vicinity of NI relaxin-3 neurons was also assessed.

These findings complement and advance existing knowledge of the neuronal mechanisms involved in MCH and orexin system actions in brainstem areas involved in arousal and food intake control (see e.g. (Sakurai, 2014; Konadhode et al., 2015; Diniz and Bittencourt, 2017) for review). For the first time, the NI innervation by MCH neurons, with MCH-positive fibres present throughout the nucleus has been mapped, relative to the adjacent, parallel innervation by orexin-A-containing axons/terminals (Blasiak et al., 2015).

As anticipated, the presence of MCH and orexin processes/terminals in the NI region was associated with the presence of mRNA encoding MCH1R and OX2R receptors in NI neurons. It has been observed previously that the rat NI is enriched in OX2R relative to OX1R mRNA (Blasiak et al., 2015), and the present study revealed that neurons expressing OX2R mRNA can express relaxin-3 mRNA. The NI was shown to express MCH1R mRNA (see (Saito et al., 2001)), and similar to OX2R mRNA, it was expressed by both relaxin-3 mRNA positive and negative neurons (Blasiak A et al., unpublished data). Furthermore, the type I and II NI neurons classified electrophysiologically as expressing different ion channels (Blasiak et al., 2015), were not clearly differentiated by their receptor expression profile. Thus, based on the neurons tested by scRT-
PCR, orexin and MCH1R receptor mRNA was expressed by different cells, suggesting the existence of separate populations of NI neurons, influenced by either orexin or MCH signalling.

The likely existence of separate populations of NI neurons, was further supported by results of RNAscope *in situ* hybridisation studies in my laboratory (Ma S et al., unpublished data). GABAergic (vGAT mRNA-positive), MCH1R mRNA-positive neurons were distributed throughout the Nlc and NId, whereas OX2R mRNA-positive neurons were more abundant in the NId, where OX2R mRNA was present in vGAT mRNA (GABA)/MCH1R mRNA-positive neurons or vGAT mRNA only neurons, as well as vGAT mRNA-negative (non-GABA) neurons. The density of OX2R mRNA expressing neurons detected in the Nlc was somewhat lower than in the NId and these transcripts were more frequently present in non-GABAergic neurons in the Nlc.

Further studies aimed at identifying neurochemical or other factors that clearly differentiate specific NI neuron populations, are still required to complement existing models of how orexin- and MCH-related signalling mechanisms orchestrate vital behaviours via actions in other brain areas (see e.g. (Konadhode et al., 2015; Diniz and Bittencourt, 2017) for review). The current data indicate that populations of NI neurons that differentially express OX2R and MCH1R receptors share electrophysiological (type I and II) characteristics and some neurochemical features (relaxin-3 positive/negative), therefore other differentiating factors, such as possible differences in inputs from, or outputs to, specific target areas, need to be examined.

Recent concurrent studies in coronal brain slices demonstrated that NI neurons are hyperpolarized in response to bath application of MCH, and many of these neurons were insensitive to subsequent orexin-A application (Blasiak A et al., unpublished data). These electrophysiological data are consistent with the expression pattern of MCH1R and OX2R mRNA detected and the frequent lack of MCH1R and OX2R co-expression by NI neurons. Electrophysiological recordings in the presence of TTX demonstrated a sustained sensitivity of NI neurons to MCH, and together with the scRT-PCR results, indicate the postsynaptic localisation of functional MCH1R receptors on NI neurons. Similarly, previous electrophysiological studies have shown direct postsynaptic, inhibitory effects of MCH on different types of neurons involved in energy metabolism and arousal.
control in the LH (Gao and van den Pol, 2001; Gao and van den Pol, 2002) or GnRH neurons in the rostral hypothalamus (Wu et al., 2009).

Notably, the postsynaptic localisation of MCH1R receptors, and the demonstrated postsynaptic expression and actions of OX2R receptors in the NI (Blasiak et al., 2015; current study), do not exclude possible effects on presynaptic terminals within the region, but do demonstrate direct actions of MCH and orexin signalling systems on NI neurons. The possible direct inhibitory action of MCH and direct excitatory action of orexin-A on different NI neurons, suggests a high level of complexity in neuropeptide modulation of the NI neuronal network and consequent associated effects on the behaviours controlled by this network (see e.g. (Ma and Gundlach, 2015) and further discussion below).

In this respect, immunohistochemical studies have revealed that >90% of NeuN-positive neurons in the NI are GABAergic, reflected by GABA and GAD65 immunostaining (Ma et al., 2007; Singleton CE, Gundlach AL, Ma S, unpublished data). The NI is also the primary source of the neuropeptide, relaxin-3, which co-localises with GAD65 (Ma et al., 2007), consistent with the high levels of vGAT mRNA detected in the region in the current study, and a large population of CRF1 receptor-positive neurons (Ma et al., 2013), activation of which may contribute to behaviour in response to CRF signalling and neurogenic stress. Indeed, NI neuron activation reflected by Fos-immunoreactivity, is increased following exploration (Ma et al., 2009a) and various types of neurogenic stressors in rats (Tanaka et al., 2005; Banerjee et al., 2010; Ryan et al., 2013b). These findings suggest that NI activity is important to the active, locomotor components of stress responses (i.e. fight and flight). Future studies will examine the expression profile of OX2R and MCH1R receptors with other neuropeptides expressed by NI neurons, besides relaxin-3, including cholecystokinin, also present in GABA neurons (Olucha-Bordonau et al., 2003); Singleton CE, Gundlach AL, Ma S, unpublished data) and CRF, present in smaller neurons, which may be non-GABAergic in nature (Walker et al., 2017).

The general contribution of orexin signalling to promoting arousal and locomotion has been demonstrated in orexin gene-knockout mice following intracerebroventricular infusion of orexin-A, which increased wake and locomotion (Anaclet et al., 2009). The excitatory effect of orexin-A
on NI neurons in vitro (Blasiak et al., 2015) is consistent with this data. Recently, it was demonstrated that chemogenetic activation of rat NI neurons resulted in increased locomotor activity in both the home cage and a locomotor cell open field, and hypervigilant behaviours in response to a fear-conditioned cue, which was associated with enhanced cortical desynchronization during periods of immobility and rest (Ma et al., 2017a). As such, the NI is emerging as a key node of a brainstem arousal network, which includes the neighbouring ventral tegmental nucleus of Gudden (Brown and McKenna, 2015), laterodorsal tegmental nucleus (Schwartz and Roth, 2008) and parallel locus coeruleus system (Carter et al., 2010), which does not receive any meaningful input from the NI/relaxin-3 system (Ma et al., 2017a).

In these functional studies in adult rats, intra-NI infusion of orexin-A had a stimulatory effect on locomotor activity and food intake when administered to satiated rats during the light phase, consistent with activation of NI networks and increased arousal, locomotion and potential motivation to consume available food. In contrast, intra-NI infusion of MCH appeared to suppress locomotor to some small degree or had little or no effect on these parameters. Furthermore, the effects of intra-NI infusion of MCH were influenced by the diurnal phase during testing. Thus, MCH infusion into the NI appeared to suppress exploratory behaviour in the light, but not the dark phase. The lack of effect during the active dark phase may be due to an increased tonic activity of NI neurons during the dark phase and wakefulness, as reflected by increased c-fos and relaxin-3 mRNA levels in the NI under conditions of stimulated locomotion (Banerjee et al., 2010; Ma et al., 2017a), thus rendering the neurons more resistant to inhibition by MCH. Although optogenetic excitation of MCH neurons can induce sleep, it has been demonstrated that microinjection of this neuropeptide into the DR altered REM and non-REM sleep states, but it did not induce sleep (Benedetto et al., 2013). Thus, the effects of MCH are not necessarily related just to sleep induction, as it also has a role in the control of sleep stage duration/transitions. Furthermore, the magnitude of the impact of the MCH projection from the LH to the NI may be less than that to other key nodes of MCH action.

There are several putative circuits downstream from the NI that may mediate its impact on arousal and locomotor behaviour, as the NI is known to project widely to brain regions important for
controlling these parameters as well as behavioural state and planning (Goto et al., 2001; Oluchabordonau et al., 2003). One such NI projection is to the lateral preoptic/basal forebrain area, which contains GABAergic and cholinergic neurons that regulate cortical activity and other arousal-related behaviours (Li et al., 2015; Zant et al., 2016).

In addition, there are established anatomical connections between NI and medial septum (Oluchabordonau et al., 2012; Sánchez-Pérez et al., 2015) which, in turn, is known to have a significant role in entrainment and pacing of theta oscillations, spatial navigation, learning and memory, as well as initiation and velocity of locomotion (Hangya et al., 2009; Robinson et al., 2016). Electrophysiological data suggests the NI also contributes to the generation of hippocampal theta rhythm (Nunez et al., 2006; Martinez-Bellver et al., 2015; Ma et al., 2017a; Martinez-Bellver et al., 2017). In this regard, NI (relaxin-3) and MCH (and orexin) circuits may have common downstream targets (Kilduff and de Lecea, 2001; Ma et al., 2007), and recent evidence indicates that MCH projections to the septum and changes in MCH neuron activity regulate hippocampal theta activity and aspects of REM sleep (Jego et al., 2013). Similarly, orexin circuits provide a robust innervation of different cell types in the MS and hippocampus, orexins have neurophysiological actions on these neurons, and these peptides have been shown to have direct effects on hippocampal theta activity in vitro and in vivo (Wu et al., 2002; Selbach et al., 2004; Wu et al., 2004; Bocian et al., 2015). Thus, given the importance of NI activity to the optimal generation of hippocampal theta activity (Nunez et al., 2006; Martinez-Bellver et al., 2015; Martinez-Bellver et al., 2017), it is possible that MCH and orexin signalling within this area, might modulate theta via influences on different populations of NI neurons, such as those identified in the current study.

In terms of feeding and metabolism, pharmacological studies have established the likely role of the relaxin-3 signalling system in promoting feeding (e.g. (McGowan et al., 2005; Lenglos et al., 2015; de Ávila et al., 2017; Ma et al., 2017a), but studies assessing NI neuron involvement or specifically whether feeding is attributable to relaxin-3 NI neurons have not been completed. In this regard, in preliminary studies, relaxin-3 and c-fos gene and peptide/protein expression in the
NI exhibited diurnal regulation, whereby levels of both peak during the active, dark phase and trough during the inactive, light phase in rats (Banerjee et al., 2006; Banerjee et al., 2010). Therefore, future studies are needed to assess the functional relevance of the colocalisation of MCH1R and OX2R on relaxin-3 neurons, the downstream projection targets of these NI neurons, and the behavioural effects attributed to these specific neural circuits, perhaps using a combination of viral, optogenetic, neurophysiological and pharmacological techniques in rats or normal and relevant transgenic mice, if the current data is verified in this species.

Orexin and MCH neurons of the LH have multi-transcriptional profiles and can release various neurotransmitters, dependent upon input signals (Harthoorn et al., 2005). This feature is key to the regulation of targets innervated by these two LH populations. Although it was initially believed that the orexin system was influenced by energy homeostasis, more recent findings suggest its primary role is to modulate arousal and motivation rather than specifically affect food intake (Olszewski et al., 2009; Morganstern et al., 2010b; Sakurai, 2014).

Orexin neurons are highly responsive to morphine and their activation is associated with preference for cues related to drugs and food reward (Harris et al., 2005). In this regard, an excitatory effect of orexin-A on relaxin-3 and non-relaxin-3 NI neurons was observed (Blasiak et al., 2015). Although both orexin receptor transcripts were expressed in NI, OX1R mRNA was considerably less abundant than OX2R mRNA (Blasiak et al., 2015). Indeed, OX2R signalling in the NI was shown to contribute to alcohol seeking and stress-related relapse in alcohol-preferring (iP) rats. The current study identified a potential distinct distribution of OX2R mRNA expression in the NI, with higher levels detected in the NId, and varied colocalisation with MCH1R and vGAT mRNA, as well as expression in vGAT mRNA-negative neurons. Notably, the presence of OX2R mRNA in the NI is consistent with the earlier description of putative OX2R-immunostaining in the NI (Blasiak et al., 2015; Kastman et al., 2016).

The current study also identified putative neural contacts between orexin and MCH immunoreactive fibres and relaxin-3 neurons, and since neural processes under peptidergic control exert circadian fluctuations (see (Blasiak et al., 2017) for review), their potential diurnal regulation
was assessed. Previous studies have optimized the use of Imaris to analyse putative presynaptic terminals (spots <0.5 μm from soma surface) and axonal segments (spots >0.5 μm from soma surface) from immunohistochemically stained sections (Ausdenmoore, 2011). In this study, overall MCH immunofluorescent elements within the rat NI were more abundant than the equivalent orexin elements. Using similar analysis methods (Ausdenmoore, 2011), the fluorescence intensity associated with MCH in axonal segments and putative presynaptic terminals close to relaxin-3 soma were found to be significantly higher during the dark phase than during the light phase. In contrast, orexin immunofluorescence was unchanged. These data suggest a diurnal rhythm in MCH signalling onto relaxin-3 neurons and its upstream modulation, whereas low level orexin inputs appear relatively consistent, based on this type of analysis. These data can be compared to other descriptions of changes in MCH system components under different arousal and sleep conditions (Dias Abdo Agamme et al., 2015). Relaxin-3/RXFP3 signalling has been shown to influence circadian activity and rhythmicity of arousal-related behaviours (Smith et al., 2012; Blasiak et al., 2013; Hosken et al., 2015); therefore, further studies are required to assess how diurnal rhythm might influence the independent effects of orexin and MCH neuropeptide systems on arousal and feeding, as well as their interactions.

Furthermore, the synaptic location of these peptides is suggestive of distinct functions. For example, synapses onto distal dendrites are involved in synaptic integration, whereas axo-dendritic synaptic sites, may favour information relay, and synapses onto soma (axo-somatic) suggest faster, direct information relay, which, as a result, have a higher probability of modulating the postsynaptic neuron activity than those on distal dendrites (Schneider Gasser et al., 2006). Therefore, further anatomical studies to assess the precise subcellular location of orexin- and MCH-positive synapses on NI GABA/relaxin-3 neurons are warranted.

4.6. Conclusions

The current studies and concurrent studies in other laboratories described the anatomical distribution and modulation of key aspects of orexin-OX2R and MCH-MCH1R signalling systems in the rat, along with the neurophysiological and behavioural effects of orexin-A and MCH signalling in the NI. Together these physiological and behavioural findings, along with diurnal
changes in the nature of MCH inputs to the NI, reflect a neuromodulatory role for these peptide signalling systems in activation and inhibition of heterogeneous GABA neuron populations in the NI, and are consistent with the NI being a key downstream target for LH orexin and MCH signalling in arousal and feeding processes.
Chapter 5- NEUROANATOMICAL INTERACTION BETWEEN OREXIN/MCH AND NI/RELAXIN-3 SYSTEMS
5.1. Introduction

Studies of the trafficking of neuropeptides within neuronal axons and terminals, as well as the pattern of their release into the synaptic cleft (Figure 5.1), is an emerging research field that requires sophisticated techniques to explore the regulatory mechanisms involved (Bean, 2007). Research methods developed so far for study of neuropeptides and neurotransmitters trafficking range from real-time video recording of fluorescent core vesicle movement through axons (Kwinter and Silverman, 2009), to tagged presynaptic vesicles that when released into synapses can be quantified for their expression of neurotransmitters from confocal images (Kavalali and Jorgensen, 2014).

There are many software programs and techniques to analyse and quantify neurotransmitter levels at the synapse, such as the freeware ImageJ and Fiji (National Institutes of Health, Bethesda, USA) (Nijhof et al., 2016). In addition, there are other cutting-edge, software programs, such as Imaris (Bitplane AG, Zurich, Switzerland) that can perform 3D rendering of high-resolution confocal images and associated analysis (Handschuh et al., 2010).

5.1.1. Neuronal inputs to and outputs from the NI

Early comprehensive anterograde and retrograde neural tract-tracing studies of the NI described the major telencephalic inputs to NI arising from prelimbic, anterior cingulate and orbital cortices, and the nucleus of the diagonal band (Goto et al., 2001; Olucha-Bordonau et al., 2003). In addition, diencephalic NI inputs were observed from the medial region of lateral habenula, rostral zona incerta, lateral preoptic and LH (Goto et al., 2001). At the brainstem level, inputs from the suprachiasmatic nucleus of the PAG, superior central nucleus (aka median raphe nucleus), interpeduncular nucleus, pontine periventricular grey, and DR were observed, which mainly innervated the midline Nlc (Goto et al., 2001).

In general, NI projections are present in two bundles of ascending fibres, one that passes through the pons, midbrain, diencephalon and telencephalon, and a second that descends to innervate brainstem areas (Olucha-Bordonau et al., 2003). The primary telencephalic projection target is the septohippocampal system. In the septum, NI projections innervate the lateral border of medial
septal nucleus, but not the central core. NI projections also reach the hippocampus in the temporal pole of the dentate gyrus, the medial and lateral parts of entorhinal area, and temporal regions of parasubiculum, presubiculum and subiculum (Olucha-Bordonau et al., 2012). On the other hand, hypothalamic projection of NI neurons innervate the rostral level of peri-supraoptic LH and the lateral preoptic area (Goto et al., 2001; Smith et al., 2013a). In addition, relaxin-3 terminals have been visualised in the lateral part of lateral hypothalamus in mice and rats (Tanaka et al., 2005; Smith et al., 2013a). Furthermore, recent studies of the distribution of RXFP3 mRNA revealed clusters of expression in the areas related to reproductive neuroendocrinology within the LH such as SON, preoptic area and PVN (Ma et al., 2007; Ganella et al., 2013b).

Figure 5.1. Schematic illustration of peptide trafficking within neurons from biosynthesis to release.

Neuropeptides are produced from their precursor peptide in the rough ER and are transferred to the Golgi apparatus to be transformed to an active peptide transmitter. By repeated anterograde trafficking (and retrograde trafficking, after recycling) through axons, secretory granules are matured and turn into dense-core vesicles, which are visible as varicosities along axons. When the core vesicles reach the presynaptic terminals they become synaptic vesicles, which are ready to be fused to the membrane and released into the synaptic cleft. Adapted from www.motofolio.com (Motifolio, 2017).
5.1.2. Orexin and MCH systems inputs to the NI

Two early immunohistochemistry labelling studies reported the existence of MCH-containing nerve fibres in what was termed an ‘extended part of the DR’ (Bittencourt et al., 1992; Bittencourt et al., 1998), which in fact, appears to be a clearly delineated region of the NiC. Consistent with this possibility, MCH1R mRNA was observed within the NI, at weak to moderate intensity, compared to the dense expression in the adjacent LC (Saito et al., 2001).

Similarly, recent studies in my host laboratory revealed orexin-immunoreactive fibres in close apposition to NI neurons, with relaxin-3 immunoreactivity colocalised with the presynaptic marker protein, synaptophysin. Furthermore, NI neurons were found to express high levels of OX2R mRNA, but low levels of OX1R mRNA (Blasiak et al., 2015; Kastman et al., 2016).

5.2. Gaps in understanding of orexin and MCH neuron interactions with the NI

The hypothalamus is known as the main integrating hub via different neuropeptides for the regulation of motivated and arousal related- behaviours. These hypothalamic neuropeptides include thyrotropin-releasing hormone (TRH), growth hormone-releasing hormone (GHRH), somatostatin, orexins, MCH, agouti-related peptide (AgRP), pro-opiomelanocortin (POMC), α-melanocyte-stimulating hormone (α-MSH), neuropeptide Y (NPY), corticotropin-releasing factor (CRF) and urocortins, gonadotropin-releasing hormone (GnRH), arginine vasopressin (AVP) and oxytocin, all acting via GPCRs. However, among these neuropeptides orexin and MCH are the main peptides contributing directly to arousal-related behaviours. Interestingly, relaxin-3 acting via its GPCR, RXFP3, has been shown to influence motivated and arousal-related behaviour. However, until now, few studies have investigated if this impact could be via a hypothalamic neuropeptides hub. Earlier anatomical studies reported the presence of relaxin-3 and NI projections within the hypothalamus (Tanaka et al., 2005; Smith et al., 2013a), their target neurons within LH and specifically whether it is a bidirectional reciprocal interaction with orexin and/or MCH neurons were not identified. The studies described in this Chapter aimed to further characterise the nature of the orexin and MCH innervation of the NI, and investigate its possible diurnal regulation; and to further characterise the relaxin-3-containing and NI neuron inputs to the LH, using a
combination of immunohistochemistry to identify neuronal populations and peptidergic inputs to
the LH and anterograde neural tract-tracing to identify the existence of NI projections and
terminals within the LH and any contacts with MCH and/or orexin soma.

5.3.Materials and methods

5.3.1. Brain collection and processing

Rats were transcardially perfused with fixative as described in Chapter 2. In studies of
immunofluorescence and DAB immunostaining, rats were perfused during the light phase (1100
h). For the diurnal immunofluorescence study, rats (n=4/phase per neuropeptide) were perfused
during the light phase (1300 h, 1 pm) or dark phase (0100 h, 1 am). Coronal sections (30 µm) were
cut using a cryostat, as described in Chapter 2.

5.3.2. Immunofluorescence studies

Brain sections were incubated in primary antibodies for 48 h at 4°C, followed by secondary
antibodies for 2 h at room temperature (see Chapter 2 for full detailed protocols). Primary IgG
antibodies used were: goat anti-OXA antibody (1:1000; Cat# sc-8070, Santa Cruz Biotechnology),
goat anti-MCH antibody (1:1000; Cat# 14509, Santa Cruz Biotechnology), rabbit anti-calretinin
antibody (1:1000; Abcam), mouse anti-calbindin antibody (1:5000; Abcam), and mouse anti-
relaxin-3 antibody (1:5, cell culture media generated in-house; (Kizawa et al., 2003). Secondary
antibodies used were: donkey anti-goat conjugated Alexa Fluor-594 (1:500; ThermoFisher
Scientific, Waltham, MA, USA), and donkey anti-mouse conjugated Alexa Fluor-488 (1:500;
ThermoFisher Scientific).

5.3.3. Confocal imaging and quantification using Imaris

Digital images of LH and NI sections processed from brains collected during the light- and dark-
phase (6-8 sections/brain) were obtained using a confocal microscope (Zeiss 780, Carl Zeiss AG,
Oberkochen, Germany). The immunofluorescent-stained NI sections were examined at low (20×)
and high (63×) magnification and the confocal microscope (20× objective) was adjusted according
a standard protocol of settings (Sharf et al., 2010a) (Table 5.1). The images were saved for later quantitative analysis using Imaris v6.0 (Bitplane AG).

Images were analysed by surface rendering of the relaxin-3 neurons (reconstructed as ‘surface’) and spot rendering of orexin/MCH axons/vesicles (reconstructed as ‘spots’; Imaris, 6.0) The labelled elements were then further categorised as “spots close to surface” (< 0.5 µm) and “spots far from surface” (> 0.5 µm, Figure 5.2). Analysis was performed within a fixed frame of 1948 × 817 µm, including all z-stacks available in the Nlc area or an area containing a maximum number of relaxin-3 positive neurons. Smoothing of the surface (0.493) and the intensity threshold of the surface (30 ± 2) were optimized and consistent during each batch experiment. The threshold was chosen based on “absolute intensity”. Spots within the same frame for surface were identified based on background subtraction to obtain a local contrast and within the XYZ dimension of 1.5 µm for orexin and 1.0 µm for MCH. A quality threshold, which is a combination of intensity and size of the particles, was optimized (6.30 ± 0.5 for orexin, 11.0 ± 0.5 for MCH) and was held constant for all images. The “close-to-surface” macro was used to filter spots <0.5 µm of the surface (Figure 5.2). The maximum intensity of “close-to-surface” spots were then selected and exported to Excel, for statistical analysis in Prism 6 (GraphPad Software Inc., La Jolla, CA, United States).

Table 5.1. Settings for confocal microscopy at 20× magnification.

<table>
<thead>
<tr>
<th>Setting</th>
<th>Value</th>
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<tbody>
<tr>
<td>Power</td>
<td>2-5% of total saturation</td>
</tr>
<tr>
<td>Main gain</td>
<td>500-800 V</td>
</tr>
<tr>
<td>Pin hole</td>
<td>1 AU</td>
</tr>
<tr>
<td>Zoom</td>
<td>1.2</td>
</tr>
<tr>
<td>Frame size</td>
<td>1024 × 1024 pixels</td>
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<tr>
<td>Pixel size</td>
<td>0.10 µm</td>
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<tr>
<td>Pixel dwell (Speed)</td>
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</table>
Figure 5.2. An example of 3D reconstruction for “surface” layer of relaxin-3 neurons vs “close to surface” spots layer of putative presynaptic neurons using Imaris software.

Surface layer was adjusted with absolute background intensity, smoothed and set with consistent intensity threshold. Spot layer was adjusted using local contrast background (background subtraction), and the XY dimension for spots was measured. The reconstructed close spots to the surface (distance <0.5 µm, pink dots indicated by red arrowhead) were defined as “putative synaptic vesicles”. Spots farther than this distance (distance >0.5 µm, blue spots indicated by blue arrow) were excluded from the experiment. Scale bar, 50 µm.
5.3.4. Confocal imaging and quantification using Image J

The NI slides were also imaged using 63× magnification. Confocal imaging settings were kept constant across all selected segments to allow comparable analysis (Table 5.2). The XY pixel size was adjusted to 0.07 µm according to the size of axonal varicosities (200 nm) and based on the Nyquist sampling theorem. The Z-stack interval was set at 0.15 µm, which was 2× XY pixel size of 0.07 µm. Over-saturated images were excluded from further quantification. The 63× images collected were saved for further analysis and quantification, using Image J software (National Institutes of Health, Bethesda, USA). The maximum intensity of Z-stacks was quantified using the red channel dynamic range threshold set at 45 (for orexin-related images) for all images. The quantified ‘particles’, which are indicative of axonal varicosities, of each segment were exported and pooled in an Excel spreadsheet. The averages of the pooled data obtained from brains collected during the light and dark phases were calculated in GraphPad Prism 3.0 (Graphpad Software Inc., La Jolla, CA, USA).
Table 5.2. Settings for confocal microscopy at 63× magnification.

<table>
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<tr>
<th></th>
<th>1024 × 256 pixels</th>
<th>1.27 µsec (max)</th>
<th>1.8</th>
<th>256 (8-bit colour)</th>
<th>0.07 µm</th>
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<tr>
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<td><strong>Red channel specification (orexin staining)</strong></td>
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<td><strong>Red channel specification (MCH staining)</strong></td>
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<tr>
<td><strong>Power</strong></td>
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<td><strong>Pinhole</strong></td>
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<td><strong>Offset</strong></td>
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5.3.5. DAB immunostaining studies

Sections were incubated in primary antibody for 16 h at room temperature, followed by secondary antibodies and horseradish peroxidase conjugated avidin-biotin solution (Vectastain Elite, Vector Laboratories, Burlingame, CA, USA) for 1 h respectively at room temperature (see Chapter 2 for full detailed protocols). Primary antibodies used were: goat anti-OXA antibody (1:1000-4000), goat anti-MCH antibody (1:1000-4000), and mouse anti-relaxin-3 (1:5). Secondary antibodies
used were: biotinylated rabbit anti-goat IgG antibody (1:500; Vector Laboratories, Burlingame, CA, USA) and biotinylated rabbit anti-mouse IgG (1:500; Vector Laboratories).

5.3.6. Statistical analysis

For analysis of immunofluorescence intensity, frequency histograms that passed the normality test (D’Agostino and Pearson normality test; $\alpha = 0.5$, $P > 0.05$), were analysed for differences by unpaired t-test. Frequency histograms that did not pass the normality test (D’Agostino and Pearson normality test; $\alpha = 0.5$, $P < 0.05$) were considered non-Gaussian in nature and were analysed using a non-parametric, two-tailed t-test followed by a Kolmogorov-Smirnov post-hoc test. $P < 0.05$ was considered as significant.
5.4. Results

5.4.1. Orexin and MCH neuronal inputs to NI neurons

5.4.1.1. Putative contacts between orexin/MCH-positive nerve fibres and relaxin-3-positive neurons in NI

DAB immunostaining of orexin-containing nerve fibres revealed their close apposition with relaxin-3 neuronal soma in the NI, consistent with an earlier report (Blasiak et al., 2015). Similarly, MCH-positive nerve fibres were also detected in close contact with relaxin-3 positive neuronal soma (Figure 5.3).

5.4.2. NI neuron projections to the lateral hypothalamus

5.4.2.1. Putative contacts between relaxin-3 nerve fibres and orexin/MCH neurons in NI

DAB immunostaining of relaxin-3 nerve fibres revealed their close apposition with orexin and MCH neuronal somas in the lateral hypothalamic area (Figure 5.4).
Figure 5.3. Putative appositions of OXA and MCH axons with relaxin-3 neurons in NI.

DAB-stained immunohistochemistry for OXA and MCH (black), imaged at low (left images) and high (right images) magnifications in comparison to that of relaxin-3 soma (brown). Scale bars for left and right images are 100 µm and 5 µm, respectively.
Figure 5.4. Putative appositions of relaxin-3 axons/terminals with orexin and MCH neurons in LH.

DAB-stained immunohistochemistry for relaxin-3 terminals (black) (A) orexin and (B) MCH neurons (brown) in the lateral hypothalamus, imaged at low (left) and high (right) magnifications. Scale bars for left and right images are 200 µm and 5 µm, respectively. 3V, third ventricle; f, fornix; mt, mammillothalamic tract.
5.4.2.2. Distribution of calretinin and calbindin neurons within orexin and MCH neurons

This pilot study was designed to obtain a general view of the types of interneurons within LH among the key neurons of the study (orexin and MCH neurons). Within the rostrocaudal extent of the hypothalamus, MCH neurons were first observed at AP -1.60 mm from bregma, within the ZI. MCH neurons were located throughout the ZI and within the lateral part of LH above the optic tract, extending to AP -2.80 mm, and then towards the PeF at the level of AP -3.30 mm from bregma. MCH neurons were also observed in DMH and appeared less dense at AP -3.80 mm, whereas no MCH cells were observed at the level of AP -4.16 mm from bregma (data not shown).

In addition, within the rostrocaudal extent of the hypothalamus, sparse orexin neurons were first observed around the fornix from AP -2.12 mm (data not shown). From AP -2.80 mm, orexin neurons were observed clustered around the medial PeF and caudal to this, the cluster spread towards DMH. At ~AP -3.80 mm, the orexin neuron population appeared to diminish and at AP -4.16 mm there were no orexin-positive neurons detected.

Calretinin and calbindin neurons were observed in dense populations at the level of AP -1.60 to -3.30 mm, in the lateral part of LH close to the optic tract, within the ZI, and in the MTu and VMH. Calretinin neurons had very little or sparse co-distribution with MCH and orexin neurons, whereas CB-immunoreactive neurons, appeared to overlap the distribution of orexin-positive neurons in perifornical areas and with MCH-positive neurons in ZI at the level of AP -3.30 mm (Figure 5.5). Therefore, calretinin was chosen as a marker for targeting interneurons in the lateral hypothalamus with a low probability of overlapping with orexin and MCH populations.
Figure 5.5. An example of the distribution of calretinin-IR, calbindin-IR, and MCH-IR or OX-IR in neurons of the LH.

(A) Immunofluorescent images (×20) of the distribution of (A) orexin-IR neurons (red) or (B) MCH-IR neurons (red) neurons and calretinin-IR (blue) and calbindin-IR (green) in the lateral hypothalamus area (AP -2.30 to -4.15 mm). The possibility of overlapping between each population is shown in merge image as an example. Scale bars 150 µm. 3V, third ventricle; f, fornix; mt, mammillothalamic tract, opt; optic tract.
5.4.2.3. Distribution of NI and relaxin-3-positive neuronal projections to the LH and their possible target neurons

DAB immunostaining revealed close contacts between relaxin-3-positive nerve fibres and orexin- and MCH-positive neuronal soma in LH. However, the pattern of distribution of relaxin-3 fibres within LH suggested that the close contact of relaxin-3 elements with orexin and MCH neurons might be sparse, as in low magnification microscopic fields the distribution of relaxin-3 terminals was mainly in areas other than where orexin and MCH neuron populations were located (Figure 5.6). Further staining of these sections revealed that one of these other areas was populated with calretinin-positive neurons (Figure 5.6). Similarly, anterograde neural tract-tracing of NI projections in rats after injections of AAV$^{1/2}$-hM3Dq-mCherry into the NI (in sections provided by Dr S Ma and Dr E Ong-Pålsson, The Florey Institute of Neuroscience and Mental Health; hereafter referred to as NI-mCherry sections), revealed mCherry-containing terminals in the LH (Figure 5.7). Staining of these sections for calretinin (CR) and high magnification imaging revealed that these neurons also receive some innervation from mCherry-containing NI fibres, in addition to their sparse innervation of orexin and MCH neurons (Figure 5.7).

NI-mCherry and relaxin-3-containing projections largely innervated separate areas to those containing orexin neurons (PeF, LH, DMH) and MCH neurons (LH, PeF, ZI), and were denser in the region containing CR-positive neurons, the ventral LH (Figure 5.8A). In order to assess whether the relaxin-3 terminals in the caudoventral LH has functional synaptic actions or is mainly fibres en passage, using darkfield microscopy, the distribution of RXFP3 mRNA in the LH was examined (using LH sections kindly provided by Elena Timofeeva, Laval University, Quebec, Canada, Figure 5.8B). Comparisons of the images of RXFP3 mRNA and relaxin-3 fibres revealed a dense population of relaxin-3 fibres and neurons expressing RXFP3 in the caudoventral LH (AP -3.1 mm from bregma).
Figure 5.6. Overview of relaxin-3 projections to different lateral hypothalamic neuronal population.

The pattern of relaxin-3 projections (green) throughout the lateral hypthalamus illustrating the concentrated fibres in the ventral part of the LH at caudal levels, and only sparse putative contacts (indicated by white arrowheads) with (A) orexin and (B) MCH neurons (red). These projections also made contacts with calretinin neurons (blue) spread across the LH area (yellow arrowheads). Scale bars 200 µm (large), 20 µm (small). 3V, third ventricle; f, fornix; mt, mammilothalamic tract, opt; optic tract.
Figure 5.7. General overview of NI projections to different lateral hypothalamic neuron populations.

NI-mCherry projections (red) clustered mainly in the ventral part of lateral hypothalamus and in zona incerta (ZI), separate from the areas containing (A) orexin and (B) MCH neurons (green). However, higher magnification images from additional sections stained with calretinin (blue) revealed some putative contacts between these terminals and orexin, MCH and calretinin-positive soma or related terminals. Scale bars, 200 µm and 20 µm (inset).
Figure 5.8. The distribution of relaxin-3 fibres in ventral LH overlap with RXFP3 mRNA positive neurons.

(A) Relaxin-3 terminals (green) are spread within the regions containing orexin (red) and calretinin (blue) neurons, but are mainly clustered in the ventral lateral hypothalamic area at AP -2.80 mm. (B) *in situ* hybridisation detection of RXFP3 mRNA (E. Timofeeva, unpublished data) at the same level of lateral hypothalamus also revealed a cluster of receptor mRNA positive neurons in the ventral part. Scale bar 100 µm. 3V, third ventricle; f, fornix; opt; optic tract.
5.4.2.4. Assessment of putative relaxin-3 contacts within different neuron populations in LH

Levels of relaxin-3 immunoreactivity in nerve fibres within the LH regions containing orexin and MCH neurons, were examined in brains collected during the light phase (Figure 5.9). In terms of whole area analysis, dense relaxin-3 immunoreactive fibres were observed in the proximity of CR neurons and these levels were significantly higher than those innervating orexin (P<0.0001) and MCH (P<0.001) neurons (Figure 5.9G-H). In addition, the relaxin-3 innervation of orexin neurons was in turn significantly lower than that of MCH neurons (P<0.0001), with the exception of some sparse fibers located in the lateral LH (Figure 5.9G-H). In terms of regional differences between the relaxin-3 innervation of different hypothalamic populations, there was no significant regional difference between the relaxin-3 innervation of MCH and CR neurons in the LH, PeF and DMH regions (P>0.05) (Figure 5.9A-D). Therefore, the relaxin-3 immunofluorescence intensity in putative presynaptic vesicles close to CR and MCH soma was not statistically different to the median of the data range resulting in an equal distribution throughout the different hypothalamic areas (LH, PeF and DMH) (Figure 5.9A,C). However, the relaxin-3 innervation of DMH orexin neurons was significantly greater than that of the other orexinergic populations in the LH (P<0.01) (Figure 5.9E-F), with the high intensity sparse vesicles observed in the histograms of the orexin neurons in DMH suggesting the existence of rare, but high-trafficking, relaxin-3 projections to this area (Figure 5.9F).
Figure 5.9. Comparison of the histograms and group data for the intensity (level) of relaxin-3 in putative presynaptic vesicles in the vicinity of different hypothalamic populations at distinct regions.

(A, C, E, G) Histogram distribution of intensities and (B, D, F, H) median with interquartile range of the intensities for relaxin-3 presynaptic vesicles close to (A, B) calretinin-IR neurons, (C, D) MCH-IR neurons (E, F) orexin-IR neurons and (G, H) the whole population in the lateral hypothalamic area. Data are shown as median ± quarter percentile (Kruskal-Wallis non-parametric one-way ANOVA followed by Dunn’s post-hoc test). ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; provided by 10-12 brain slices from whole hypothalamic area/rat, n = 3). Distinct region of lateral hypothalamic area includes lateral hypothalamus (LH), perifornical area (PeF) and dorsomedial hypothalamus (DMH) between AP -2.80 mm to -3.80 mm.
5.5. Discussion

Recent studies have demonstrated an excitatory effect of orexin-A on relaxin-3 and non-relaxin-3 NI neurons (Blasiak et al., 2015), and both OX1R and OX2R receptors are expressed in NI (Greco and Shiromani, 2001), although OX1R expression is much lower than OX2R (Blasiak et al., 2015). Furthermore, the OX2R population in the NI has been shown to contribute to the stress-induced relapse to alcohol-seeking in alcohol-preferring rats in studies of the effects of OX2R antagonist infusion into the NI (Kastman et al., 2016).

These data were extended in the studies described in this thesis, whereby putative contacts between orexin fibres and relaxin-3 neurons in NI, and the influence of diurnal state on these interactions were assessed. In addition, earlier studies have demonstrated that MCH neurons project to the NI, which at the time was incorrectly described as the ‘extended part of dorsal raphe’ (Bittencourt et al., 1992; Goto et al., 2001). Thus, the current studies are the first to identify an MCH innervation of the NI, and examine putative contacts between MCH fibres and NI relaxin-3 neurons. Furthermore, the influence of diurnal state on MCH-relaxin-3 elements was examined. Previous studies have described the existence of MCH1R in the NI (Saito et al., 2001), which could mediate functional effects of MCH on NI neuron activity.

The circadian cycle is mainly regulated by a SCN-to-SPZ-to-DMH pathway, which in turn has inhibitory (GABA) projections to VLPO and excitatory (glutamate and TRH) projections to orexin/MCH neurons in LH (Saper et al., 2005). In the current studies, relaxin-3 neurons were found to send a significant projection to DMH, despite low levels of RXFP3 mRNA expression, implying that relaxin-3 fibres in DMH represent fibres en passage in this region. However, it was previously shown that DMH orexin neurons have a somewhat more prominent projection to the NI region than those in the PeF and LH areas (Blasiak et al., 2015). Therefore, it is possible that the relaxin-3 positive fibres in DMH may release other neuromodulators in the region, such as GABA, which have been shown to be colocalised with relaxin-3 in NI neurons (Ma et al., 2007). This possibility could be further investigated using NI-mCherry anterograde tracing to DMH combined with electron microscopic detection of inhibitory synapses on orexin neurons and/or identification of RXFP3 expression by orexin neurons. In this regard, distinct regions of dense
RXFP3 mRNA detected in LH in this study, were consistent with the level of relaxin-3 ‘spots close to surface’ detected in the LH region containing a high density of orexin neurons. Several studies describe the contribution of LH to several physiological and motivated behaviours, such as feeding (see (Sakurai, 2014) for review) and its activation associated with hunger, which in turn, is a physiological condition in which relaxin-3 signalling is thought to play a role (Ganella et al., 2012; Ganella et al., 2013a).

MCH neurons appeared to have significantly more close contacts than orexin neurons with relaxin-3 soma. Although the interaction between the MCH system and other arousal systems is not fully understood, an interaction between relaxin-3 and MCH systems may contribute to stress responses through direct or indirect activation of the hypothalamic-pituitary-adrenal axis (Smith et al., 2009b), which has been demonstrated for relaxin-3 signalling (Ganella et al., 2013b). There is a glutamatergic projection from DMH to LH that contributes to circadian rhythm (Saper et al., 2010), and the DMH plays other roles in circadian responses, including corticosterone secretion and circadian feeding cycles (Marcheva et al., 2013). DMH output to the medial PVN regulates CRF neurons, which in turn control pituitary gland effects on corticosteroid secretion (Leal and Moreira, 1997). In the current study, relaxin-3 fibres appeared to make close contacts with MCH neurons, but this potential synaptic interaction requires confirmation using electron microscopy. Nonetheless, it can be speculated that the relaxin-3/GABA innervation from NI might have an inhibitory effect on MCH neurons resulting in increased arousal and wakefulness. The electrophysiological effects of the NI innervation transmitters (GABA and peptide) on MCH neurons requires investigation.

In the current studies, the strongest relaxin-3-containing projection innervated CR-positive neurons in the LH (Kelsom and Lu, 2013). Recent studies have demonstrated that GABA (inter)neurons contribute to mediating the effects of relaxin-3 in dorsal (Haidar et al., 2017) and ventral hippocampus (Rytova V et al., manuscript under review), which include regulation of theta rhythm, and fear and anxiety behaviour (Sun et al., 2001; Tovote et al., 2015). In a recent anatomical study, it was hypothesised that the NI has an inhibitory projection to parvalbumin
neurons of the LH and VLPO in mice, although no direct evidence was available (Smith et al., 2013a).

Although there was no significant difference in the density of relaxin-3 projections across different hypothalamic areas, the LH exhibited brighter relaxin-3 ‘close spots’ (Figure 5.10). A recent study demonstrated that injection of the RXFP3 antagonist, R3(B1-22)R, into the PeH and LH increased alcohol-seeking in rats (Walker et al., 2015), whereas decreased alcohol-seeking behaviour was associated with injections into other hypothalamic areas (Walker et al., 2015). Furthermore, YFP-positive neurons in RXFP3-Cre×YFP reporter mice, which are assumed to express RXFP3 in the adult brain, did not co-express orexin or MCH, and were mainly observed close to the borders of the fornix (Perry C et al., unpublished data). Apart from the sporadically brighter ‘close spots’ in LH observed to contain relaxin-3 immunoreactivity, separate in situ hybridisation studies of RXFP3 mRNA expression demonstrated moderate densities of RXFP3 mRNA expression around the fornix, consistent with the observed YFP staining pattern in the RXFP3-Cre-YFP mice. Therefore, it is possible that non-orexin neurons located in the medial part of LH, close to the fornix, could be CR positive. A population of these neurons project to the VTA, which has a dominant role in reward and goal-directed behaviours (Morales and Margolis, 2017), suggesting that the relaxin-3 system may regulate these behaviours through modulation of an NI-LH-VTA circuit. This possibility warrants further investigation.

Close ‘synaptic’ contacts between relaxin-3 immunofluorescent elements and CR-, orexin-, and MCH-negative neurons observed in the lateral LH, is consistent with the distribution of RXFP3 mRNA observed in the rat lateral LH using in situ hybridisation. Furthermore, a recent study identified a population of vGAT-positive neurons in the LH of vGAT-ires-Cre mice, which are distinct from orexin and MCH neurons, and when activated chemogenetically caused wakefulness and increased exploratory behaviour; and when inhibited, induced sleep (Konadhode et al., 2013). Furthermore, this population of neurons projected to important arousal centres, visualised using anterograde tracing with AAV-FLEX-ChR2-eYFP (Jennings et al., 2015). Therefore, it is possible that a relaxin-3 innervation of LH might target this population of GABA neurons, which may
contribute to the effects of this neuropeptide system on arousal. Such interactions can be investigated in future studies.

Studies of synaptic anatomy can be used to investigate likely function. For example, synapses on distal dendrites suggest a primary input pathway for synaptic integration (Gulledge et al., 2005), whereas other synaptic sites can imply a role in relaying information (Stiles and Jernigan, 2010). Synapses with soma imply more robust information relay and is more likely to activate the postsynaptic neuron than those with distal dendrites (Stiles and Jernigan, 2010). Synaptic inputs, particularly those that are inhibitory and close to the axon hillock, where the action potential initiates, gate the output of neurons by disrupting transmission of action potentials (Kole and Stuart, 2012). In contrary, synapses with axon terminals generally gate transmitter release, based on inhibitory or excitatory input, resulting in increased or decreased transmitter release at the synapse (Burt et al., 2011).

5.6. Conclusions and future directions

The data presented demonstrated that there is a bidirectional neural pathway between the lateral hypothalamus and NI/relaxin-3 neurons. It also demonstrated that there are diurnal shifts in neuropeptide expression levels in presynaptic terminals and axonal varicosities. It was revealed that there is a significant diurnal-dependent ‘release’ of MCH and consistent release of orexin from ‘putative’ terminals projecting to relaxin-3 neurons in NI. On the other hand, NI-mediated functions activated by orexin trafficking and release are shown to be circadian-independent. Therefore, it is suggested that circadian-dependent trafficking and release of MCH to NI might be involved in the actions mediated by relaxin-3. The observed changes in diurnal levels of orexin/MCH-IR NI-projecting axons ‘putative’ synaptic vesicles might be associated with (i) different speed of trafficking and/or depletion of orexin vs MCH in synaptic terminals, (ii) circadian independent orexin/MCH mRNA expression and neural content in rodents (Harthoorn et al., 2005; Stütz et al., 2007), and (iii) circadian regulation of synaptic plasticity (Appelbaum et al., 2010).
Knowing that the established orexin/MCH hypothalamic arousal systems have direct projections to NI, the current findings demonstrated that NI is an emerging brainstem arousal centre with a likely indirect reciprocal feedback to hypothalamic arousal circuits via local interneurons. It is possible that it acts similarly to orexin as a ‘stabiliser’ signal in the flip-flop arousal circuit (Saper et al., 2005). However, anatomical studies probing the possible overlap between vGAT- and CR-positive interneurons in LH and their functional inputs from relaxin-3/NI neurons could further support this theory. It is possible that these LH neurons project to other arousal areas, in addition to orexin and MCH neurons, and these other circuits could be studied using viral neural tract-tracing, electrophysiological and optogenetic methods.
Chapter 6- GENERAL DISCUSSION
6.1. Introduction

Motivated behaviour is designed to maintain energy balance of a living system and requires increased arousal obtained through neural processing within the hypothalamic-brainstem ascending arousal system and autonomic nervous system (Pfaff, 2006). In the following sections, the contribution of orexin, MCH and relaxin-3 in regulation of motivated behaviour will be discussed and the impact of the internal and external factors such as hormones as well as diurnal changes will be highlighted for these regulations. The key findings of this thesis study will be then summarised and the future directions would be discussed based on the findings.

6.2. Orexin system contribution to motivated behaviour and arousal

There is a functional and physiological difference between wakefulness and alertness. Wakefulness is a binary state either being awake or asleep (Prerau et al., 2014), whereas alertness or arousal (active awake) is an analog state that is continuous and variable (Oken et al., 2006). Orexin contributes to stabilising the state of alertness, and its promotion of feeding and locomotor activity is not the by-product of increased wakefulness (Kotz et al., 2012; Sakurai, 2014), which was confirmed in studies that demonstrated a dose-dependent behavioural response to orexin that is graded and not merely ‘on and off’ (Kotz, 2006). Interestingly, animals with narcolepsy and narcolepsy patients are still capable of being awake in spite of fragmented sleep/wake patterns (Nishino, 2007), suggesting the role of the orexin system in arousal is not exclusive and is, in fact, a system that orchestrates the control of arousal and vigilance in response to external stimuli (Tsujino and Sakurai, 2013).

Changes in level of Fos-immunoreactivity of neurons located in the lateral and PeF area of the hypothalamus and the dorsal premammillary nucleus revealed that this descending circuit may be mainly involved in food anticipatory activity or foraging behaviour in rats (Goto et al., 2005), although orexin neuron firing discharge levels are less during eating, as this requires a lower arousal level (Mileykovskiy et al., 2005). This is consistent with a study in which foraging behaviour was reduced in orexin knock-out mice (Akiyama et al., 2004). Furthermore, loss of physical activity along with altered feeding and drinking behaviours following lesioning or genetic ablation of orexin neurons suggest that this neuropeptide is important in integrating these
behaviours (McGregor et al., 2011; Tabuchi et al., 2014; Brown et al., 2015). Importantly, spontaneous activity is more affected than feeding behaviour in the absence of orexin (Brown et al., 2015), hence weight gain in narcoleptic patients or orexin knockout mice is possible (Chemelli et al., 1999; Chabas et al., 2007). The orexin-driven pathway involved in food intake might be different from that controlling spontaneous activity, and both of these pathways might differ from that controlling enhanced wakefulness. Taken together, the food intake pathway activated by orexin signalling may contribute to stress-induced food intake and might also involve dopaminergic projection targets (Johnson et al., 2012; Yau and Potenza, 2013).

6.3. MCH system contribution to motivated behaviour and arousal

Studies have reported that MCH microinjections into the rat dorsal raphe promoted a depressive-like state (López Hill et al., 2013). It was shown that MCH fibres were widely distributed throughout the rostrocaudal extent of the dorsal raphe and mainly in close apposition to 5-HT and GABA-containing neurons (Bittencourt, 2011) and a reduced density of these fibres were observed at the most caudal level, where the dorsal raphe neighbours with NI. Microdialysis studies have also shown a biphasic effect of MCH on 5-HT release in rats with a long lasting decrease in 5-HT after a low dose of MCH and a slight increase after a higher dose of MCH (Urbanavicius et al., 2013), suggesting a modulatory effect of MCH on serotoninergic neurons. On the other hand, i.c.v. administration of MCH unexpectedly increased both alcohol and sucrose intake with no effect on anxiety tested in the elevated plus maze in rats (Duncan et al., 2005). Taken together, these findings indicate MCH acts as a pro-depressive agent depending on the dose and region of administration.

6.4. Other factors affecting orexin and MCH systems in regulating motivated behaviour

Orexin and MCH neurons of the LH have multi-transcriptional profiles and can release various neurotransmitters, depending upon the nature of their input signals (Harthoorn et al., 2005). This feature is important for the regulation of targets innervated by these two LH populations. Although it was initially believed that the orexin system was influenced by energy homeostasis, more recent findings suggest that its primary role is in modulating arousal and motivation rather than effects specifically on food intake (Olszewski et al., 2009; Morganstern et al., 2010b; Sakurai, 2014).
Although the glucose-sensing capabilities of orexin and MCH neurons have been shown to contribute to feeding behaviour following activation by hypo- or hyper-glycaemia (Ouedraogo et al., 2003; Karnani and Burdakov, 2011; Gonzalez et al., 2016), there has been little investigation to date of the metabolic parameters that activate orexin neurons to induce locomotor activity, suggesting it is a complex circuit that underlies effects of orexin on locomotor activity. Temporary satiety after exercise (or purposeful activity) via gut satiety hormones such as CCK, ghrelin, and GLP-1 (Ueda et al., 2013), CRF and leptin (Bi et al., 2005) and adrenaline (Verberne et al., 2016) indicate that spontaneous locomotor activity is not a direct result of orexin activation or MCH suppression by negative energy balance or glucose fluctuation sensing, but interestingly, might be a product of activation of the third subset of LH neurons which are GABAergic (GAD65-immunoreactive) and in turn are activated by orexin neurons and are implicated in locomotor promotion (Kosse et al., 2017). In addition, other specific neural circuits are influenced by orexin and MCH in response to autonomic, physiological and endocrine regulation, such as monoaminergic, cholinergic (Inutsuka and Yamanaka, 2013; Li et al., 2014b; Parks et al., 2014) and GABAergic neurons in the brainstem (Blasiak et al., 2015), which indicates specific contributions of orexin and MCH to ‘arousal-related’ behaviours.

Rodent models of stress, such as physical immobilisation or cold exposure in rats, activate orexin neurons, leading to increased orexin mRNA expression (Ida et al., 2000). Similarly, central i.c.v. injection of orexin-A activated CRF neurons in the PVN and amygdala (Sakamoto et al., 2004). In addition, i.c.v administration of orexin-A activated the HPA axis dose-dependently, by increasing CRF mRNA levels in the PVN and levels of corticosterone and ACTH, which could be blocked by systemic pretreatment with a CRF antagonist in rats (Kuru et al., 2000). Reciprocally, it was shown that application of CRF onto orexin neurons ex vivo dose-dependently increased the membrane potential of these neurons and their firing rate. Orexin neuron activity was decreased following acute stress in CRFR1 knockout mice, wherein only 50% of orexin neurons could be activated following acute stress (Winsky-Sommerer et al., 2004). This suggests orexin signalling mediates some of the CRF-mediated behaviours induced by stressful situations and CRF receptor knockout mice have an impaired HPA axis, partly because of impaired orexin activation (Winsky-Sommerer et al., 2004). Although older studies identified populations of CRF-containing neurons

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innervating orexin neurons as located in limbic areas such as the BNST, DRN and Barrington’s nucleus, as well as PVN; my laboratory recently demonstrated that the rat NI contained neurons influenced by CRF and orexin, but the direct or indirect pathways mediating these effects are still unclear. It has been suggested that the orexin system acts as a link between stress and feeding, and the effect of orexin to increase food intake is an outcome of a balance between an orexin-CRF circuit for inhibiting food intake and orexin-NPY for stimulating food intake (Domingos et al., 2013). Chronic fasting has been demonstrated to upregulate MCH mRNA expression in LH of rats (Presse et al., 1992).

Unlike orexin neurons, MCH neurons are not sensitive to glucoprivic CSF, but are stimulated by high extracellular glucose concentrations, and MCH mRNA levels increased following the glucose counter-regulatory response to the injection of the glucoprivic agent, 2-deoxyglucose (Yamanaka et al., 2003). Similar to orexin, MCH has been shown to stimulate the HPA axis in rats, associated with increased CRF mRNA expression in the PVN, and increased blood levels of corticosterone and ACTH, and these effects are proposed to be via projections to the median eminence and pituitary (Cvetkovic et al., 2003; Hassani et al., 2016). In addition, a recent study investigated the role of MCH in motivated behaviours for rewarding substances, such as alcohol, and reported that acute consumption of ethanol increased MCH mRNA levels in the LH, whereas chronic intake of ethanol decreased levels (Morganstern et al., 2010a). Local injection of MCH into various brain regions of rats has been demonstrated to result in a selective increase in ethanol intake, without affecting food/water intake, when injected into the PVN and NAc, but resulted in an opposite effect when injected into the LH (Morganstern et al., 2010a). Taken together, these findings highlight the contributing role of other factors such as stress on MCH system and on motivated behaviour and its malfunction which alters feeding and reward behaviour centres such as the PVN and NAc and finally resulted into diseases such as obesity and addiction (Barson et al., 2012). Based on the optogenetic studies the following circuit was proposed recently for motivated behaviours such as feeding (Stuber and Wise, 2016) (Figure 6.1).
Figure 6.1. Proposed neurocircuit in lateral hypothalamic area based on optogenetic studies.

LHA GABAergic neurons inhibit VTA GABAergic neurons to disinhibit VTA dopamine neurons. Dopamine is released within the NAc, where it excites D1R-expressing MSNs and induces plasticity. These inhibitory signals then feedback to inhibit LHA GABAergic neurons to terminate feeding bouts. BNST GABAergic neurons preferentially inhibit LHA glutamate neurons, some of which may project to the lateral habenula (Stuber and Wise, 2016).
6.5. Circadian influences on orexin and MCH systems

A recent study demonstrated that orexin and MCH levels in human CSF samples exhibited a similar pattern and were highest during active wake and low during inactive sleep (Blouin et al., 2013), leading to a model for the tonic and phasic patterns of these neuromodulators. According to this model, orexin and MCH might induce specific behavioural transitions depending on circadian time, and the net function of these two systems will be either tonic (slow release) or phasic (fast or burst of release) (Adamantidis and de Lecea, 2008). Tonic release during the active period might be a direct result of increased neuronal activity or an indirect effect of prolonged presence of these neuropeptides in the extracellular space, whereas phasic release might be a consequence of a transient release into the synaptic cleft after a burst mode (Adamantidis and de Lecea, 2008). The tonic release of orexin during the active period or ‘super-arousal’ occurs during sustained, active, motivated behaviour (de Lecea et al., 2012), and the tonic release of MCH might counteract this effect to dampen overactivity of arousal centres (Adamantidis and de Lecea, 2008).

On the other hand, MCH has been shown to reduce food intake in rats when injected i.c.v and intra hypothalamic in low doses (1-100 ng) at the end of the light phase as early as 2 h after injection and for the next 24 h (Presse et al., 1996). In contrast, bilateral injection of 1 ng MCH into the lateral hypothalamus or 0.5 µg PVN in the early light phase but not at the onset of the dark phase increased the food intake (Rossi et al., 1999). In another study, i.c.v injection of MCH (0.15-15 µg) at the beginning of the light phase dose dependently increased the food intake for 2 h only with the higher dose and MCH i.c.v injection in the beinning of dark phase increased food intake only in at the highest dose of 5µg MCH. 24 hrs feedig as well as body weight remained unchanged and no dose inhibited the food intake (Rossi et al., 1997). This non-consistent orexigenic and anorectic effects highlight the food intake as a consequent behaviour mediated by different coordinated variety of neural pathways in which many neuropeptides interact and are influenced by the diurnal changes. In addition, other studies indicated that fasting and food deprivation by 2-deoxy-D-glucose and insulin can activate MCH gene expression through likely distinct regulatory pathways, therefore genetic or environmental factors may alter the response of MCH gene activity after food deprivation (Presse et al., 1996).
6.6. Relaxin-3 systems contribution to motivated behaviour and arousal

Similarly, the relaxin-3/RXFP3 signalling system, which was first identified a few years after the orexins/OX1/2 receptor system (Sakurai et al., 1998; Bathgate et al., 2001; Bathgate et al., 2003), has since been reported to display a strong association with the regulation of homeostatic systems. It was first shown that acute human relaxin-3 (H3) injected i.c.v. (54, 180 pmol), or into the PVN (18, 180 pmol), arcuate nucleus, supraoptic nucleus, or anterior preoptic area (180 pmol) resulted in increased food intake in rats (McGowan et al., 2006). However intra-LH injection of relaxin-3 had no effect on food intake 1 h post-injection in both early light/dark phases in satiated male Wistar rats, and this was consistent with unaltered mRNA levels for various feeding-related neuropeptides (McGowan et al., 2005; McGowan et al., 2007). Similarly, i.c.v injection of relaxin-3 (54-540 pmol) increased water intake in rats (McGowan et al., 2008), which likely reflected the involvement of subfornical organ neurons expressing the relaxin receptor, RXFP1 (McGowan et al., 2007; de Ávila et al., 2017; Ma et al., 2017b) and the key role of the SFO in drinking (Leinninger et al., 2011; Beier et al., 2015). Thus, it is known that human relaxin-3 can bind/activate RXFP1 and RXFP4 as well as RXFP3 (Liu et al., 2003), although RXFP4 is a pseudogene in rats (Kocan et al., 2017). The differences between the effects of intra-PVN injection of orexin-A or human relaxin-3, are the increased locomotor activity and arousal along with increased food seeking induced by orexin (Kiwaki et al., 2004) versus the increased food intake only with relaxin-3 (McGowan et al., 2005). Moreover, chronic i.c.v. injection of human relaxin-3 (600 pmol/day) for 14 days in rats significantly increased food consumption, body weight, and plasma levels of insulin and leptin, with no changes in locomotor activity during the 7 day treatment (Hida et al., 2006), which suggests the increased body weight is associated with higher energy saving, rather than lower expenditure. In addition, 7-days intra-PVN administration of relaxin-3 (180 pmol) increased cumulative food intake and body weight, in addition to epididymal fat mass and blood leptin levels, although intrascapular brown adipose tissue was unaltered, which is again indicative of more effect on energy preservation rather than energy expenditure (McGowan et al., 2006). However, in all these studies, effects of relaxin-3 via receptors other than RXFP3 could not be discounted.
To address this confound, later studies used a synthetic single chain RXFP3-specific agonist and antagonist, and demonstrated that acute i.c.v. administration of the RXFP3 agonist, R3/I5 (1 nmol), increased food intake in the first hour post-infusion in rats, and this effect was significantly blocked by pretreatment with the RXFP3 antagonist, R3(B1-22)R (4 nmol, i.c.v.) (Hojo et al., 2016). Taken together, these studies illustrate that relaxin-3 is primarily acting via RXFP3 to produce its biological effects, despite its ability to activate RXFP1 when used in a pharmacological fashion.

In contrast, studies in mice have revealed somewhat different roles for relaxin-3 in feeding control compared to those described in rats. Sutton et al. (2009) reported a reduction in body weight and metabolism in 129S5:B6 relaxin-3 gene knockout mice compared to wildtype mice. This, however, was not replicated in C57BL6/J relaxin-3 gene knockout mice, which suggests that differences in metabolic phenotype were instead associated with the mixed genetic background of the strain used and not relaxin-3 (Smith et al., 2009a; Sutton et al., 2009). Furthermore, 129S5:B6 relaxin-3 gene knockout mice also revealed effects on coordinated responses to feeding as a reward after overnight fasting (Shoblock et al., 2010). However, a caveat for using whole-of-life transgenic knockout mice is that these inconsistent findings may be related to compensatory mechanisms during development. More recently, a study using naive C57BL/6J mice revealed that acute administration of the RXFP3-specific agonist, R3/I5 (0.5 nmol), i.c.v. (Smith et al., 2013b) or intra-PVN (110 pmol) (Smith et al., 2014a), had no significant effect on food consumption in satiated mice during the first hour post-injection during the light phase, when compared to that of vehicle-injected controls. However, the consumption of palatable food during the light phase was decreased by i.c.v administration of an RXFP3 antagonist. The reduction of normal food consumption in satiated rats in early dark or mildly fasted mice in light phase indicates a robust involvement of this system in modulating behavioural state linked into food intake in mice. These findings also suggest there is a variable function for RXFP3 in the hypothalamus of rats and mice (Smith et al., 2013b).

Although studies in rats have shown that RXFP3 agonist-induced food intake was blocked by pretreatment with an RXFP3 antagonist, infusion of the antagonist alone was without effect on light phase or dark phase behaviour (Kuei et al., 2007; Sutton et al., 2009). In contrast, mice
injected i.c.v. with RXFP3 antagonist (1.1 nmol) exhibited reduced food anticipatory activity after mild food deprivation, and consumption of highly palatable food (Smith et al., 2014a) suggesting a triggering of motivated food seeking by relaxin-3/RXFP3 signalling in mice and a species difference in this system and/or circuits downstream influenced by relaxin-3/RXFP3 signalling. The functional significance of the relaxin-3/RXFP3 system in behavioural arousal is also consistent with circadian-dependent c-fos expression observed in the NI of rats (Banerjee et al., 2010).

6.7. Other factors affecting relaxin-3 system in regulating motivated behaviour and arousal

Recent studies also revealed a contribution of relaxin-3/RXFP3 signalling to alcohol-seeking behaviour by identifying the association between NI relaxin-3 mRNA expression levels and alcohol intake levels in rats (Ryan et al., 2014). It was also shown that alcohol self-administration and stress-induced relapse in alcohol-preferring (iP) rats was attenuated by administering RXFP3 antagonist i.c.v, and the circuit involved involves the bed nucleus of the stria terminalis (Ryan et al., 2013b). Consistent with these rat data, RXFP3 knockout mice (C57/B6jRXFP3TM1/DGen) displayed comparable baseline alcohol preference to wildtype littermates, whereas repeated exposure to acute stressors (repeated restraint, followed by swim stress) decreased alcohol preference of RXFP3 KO mice suggesting that RXFP3 signalling preserves high levels of alcohol preference after stress exposure, but does not appear to regulate the baseline reinforcing effects of alcohol (Walker et al., 2015). Notably, alcohol consumption in male, but not female, relaxin-3 knockout mice (on a C57BL/6J background) was significantly higher than wild-type control mice in the two-bottle choice test, although there was no difference in sucrose or quinine preference (Shirahase et al., 2015).

In line with these studies, i.c.v. administration of RXFP3 agonist did not alter anxiety-like behaviour under basal conditions, whereas the RXFP3 antagonist, R3(B1-22)R, decreased an ‘exogenously elevated’ anxiety level that followed systemic administration of an anxiogenic substance. This finding suggests that endogenous relaxin-3/RXFP3 signalling weakly contributes in regulating ‘basal’ anxiety-like behaviour but this system reduce elevated levels of anxiety in mice (Zhang et al., 2015). Although there is a weak and unexplored NI projection to areas
important for reward behaviour, such as the mesolimbic dopamine pathway from the ventral tegmental area to nucleus accumbens, NI neurons have been observed to strongly project to other reward areas, such as the LH and amygdala, which are responsible for feeding (Goto et al., 2001; Olucha-Bordonau et al., 2003; Ganella et al., 2012).

This is consistent with recent findings regarding the contribution of relaxin-3/RXFP3 signalling to binge-eating. Relaxin-3 mRNA levels in the NI were reported to be increased in ‘binge-eating prone’ rats and sucrose/chow intake was considerably higher under stressful conditions (after foot shock). These rats had higher RXFP3 mRNA levels in the paraventricular and supraoptic nuclei of the hypothalamus and i.c.v administration of RXFP3 antagonist in these rats resulted into blockade of a stress-induced increase in sucrose intake which was more prominent in female rats (Calvez et al., 2016).

In more recent studies from the Timofeeva laboratory, food intake was increased following i.c.v. administration of relaxin-3 or RXFP3 agonist, but the orexigenic effects of RXFP3 agonist was significantly stronger than relaxin-3 from 30 min after injection. The levels of water intake, plasma corticosterone and testosterone as well as c-fos mRNA levels in the parvocellular (PVNp) and magnocellular (PVNm) paraventricular and supraoptic (SON) hypothalamic nuclei, in the ventral medial preoptic area (MPAv), and in the organum vasculosum of the lamina terminalis (OVLT) were significantly amplified following central injection of relaxin-3 which was all absent following RXFP3 agonist treatment. However, the level of c-fos mRNA in the perifornical lateral hypothalamic area was increased after injection of both relaxin-3 and RXFP3 agonists.

These findings suggest that RXFP1 is involved in the relaxin-3 stimulation of water intake and activation of the HPA and HPG axes (de Ávila et al., 2017); and these data could be further confirmed by studies to visualise the relative distribution of RXFP3 and RXFP1 mRNAs (and proteins, if possible), along with functional data confirming their activity in specific brain areas such as PVN. The lower level of food intake stimulation by relaxin-3 compared to RXFP3 agonist implies the activation of both orexigenic and anorexigenic circuits by relaxin-3, which might represent dual actions at RXFP1 and RXFP3 on orexin and MCH neurons as well as oxytocin and vasopressin neurons (Blasiak et al., 2017; Ma et al., 2017b).
6.8. Summary of key findings from these studies

The studies described in this thesis have demonstrated the anatomical, behavioural and functional interaction between hypothalamic and brainstem arousal systems (Figure 6.2). An increase in food intake and locomotor activity was observed following orexin injection into the NI in satiated rats during the light phase, in which rats were resting and inactive. In the current studies in adult male rats, several aspects of the interaction between the lateral hypothalamic MCH/MCH1R signalling system and the *nucleus incertus* (NI) GABA/relaxin-3 system was also identified. NI-projecting, MCH-synthetising neurons were identified in the hypothalamus and the topographic distribution of orexin-A- and MCH-positive nerve fibres, and their receptors, OX2R and MCH1R, respectively, were mapped in the NI. In parallel studies, the effect of MCH and orexin-A on the neurophysiological activity of NI neurons *in vitro* was determined (Blasiak A and others, unpublished observations). Furthermore, the functional effects of local infusion of orexin-A and MCH into the NI on behavioural activity and food consumption was examined. Finally, the effect of diurnal phase on the dynamics of orexin-A and MCH immunoreactivity was assessed in axonal segments and synaptic vesicles within the vicinity of NI relaxin-3 neurons.

These findings complement and advance existing knowledge of the neuronal mechanisms by which MCH and orexin signalling systems act in brainstem areas involved in arousal and food intake control (see e.g. (Sakurai, 2014; Konadhode et al., 2015; Diniz and Bittencourt, 2017) for review).
Figure 6.2. Summary of thesis findings on the interactions between hypothalamus and NI in brainstem, with a proposed functional pathway.

Orexin and MCH have complementary functions via direct projections to brainstem arousal centres, including the NI, along with direct/indirect projections to activate the sympathetic nervous system via SPN. Similar to other brainstem arousal centres, there is a minor direct NI projection to orexin and MCH neurons, along with a major indirect NI projection, which might engage calretinin-positive GABA (inter)neurons, which might contact other GABA neuron populations (e.g. VLPO).
6.8.1. Orexin and MCH systems - anatomical interactions with NI

For the first time, the *nucleus incertus* innervation by MCH neurons was detailed, with MCH-positive fibres present throughout the nucleus, relative to the adjacent, parallel innervation by orexin-A-containing axons/terminals (Blasiak et al., 2015). Moreover, in line with earlier similar reports in the cat (Torterolo et al., 2008), MCH-immunoreactive tanycytes were observed, with stained processes extending from the lining of the 4th ventricle into the NI (Blasiak and others, unpublished data). In the cat brain, MCH immunoreactive tanycytes innervate the midbrain raphe nuclei and adjacent subependymal regions (Torterolo et al., 2008). Therefore, hypothalamic MCH neurons innervating the NI, may not be the only source of MCH in this nucleus, as tanycytes, which transport various molecules including neuropeptides, from cerebrospinal fluid to neural tissue (Fernandez-Galaz et al., 1996), may constitute another source of MCH for neuronal activation in the NI region, depending on the peptide release characteristics of these cell processes.

As anticipated, the presence of MCH and orexin processes/terminals in the NI region was associated with the presence of mRNA encoding MCH1R and OX2R receptors in NI neurons. Previous studies had shown that the rat NI is enriched in OX2R relative to OX1R mRNA (Blasiak et al., 2015), and the present study revealed that neurons expressing OX2R mRNA can express RLN3 mRNA. The presence of MCH1R mRNA in the *nucleus incertus* was confirmed (see (Saito et al., 2001)), and similar to OX2R mRNA, it was expressed by both relaxin-3 mRNA positive and negative neurons. The likely existence of separate populations of NI neurons, was further supported by results from RNAscope *in situ* hybridisation studies. GABAergic (vGAT mRNA-positive), MCH1R mRNA-positive neurons were found distributed throughout the Nlc and NId, whereas OX2R mRNA-positive neurons were more abundant in the NId, where OX2R mRNA was present in vGAT mRNA (GABA)/MCH1R mRNA-positive neurons or vGAT mRNA only neurons, as well as vGAT mRNA-negative (non-GABA) neurons. The density of OX2R mRNA-expressing neurons detected in the Nlc was somewhat lower than in the NId and these transcripts were more frequently present in non-GABAergic neurons in the Nlc. Further studies aimed at identifying neurochemical or other factors that clearly differentiate specific NI neuron populations, are still required to complement existing models of how orexin- and MCH-related signalling
mechanisms orchestrate vital behaviours via actions in other brain areas (see e.g. (Konadhode et al., 2015; Diniz and Bittencourt, 2017) for review).

Notably, the postsynaptic localisation of MCH1R and the demonstrated postsynaptic expression and actions of OX2R in the NI (Blasiak et al., 2015), do not exclude possible effects on presynaptic terminals within the region, but do demonstrate direct actions of MCH and orexin signalling systems on NI neurons. The possible direct inhibitory action of MCH and direct excitatory action of orexin-A on different NI neurons, suggests a high level of complexity in neuropeptide modulation of the NI neuronal network and consequent associated effects on the behaviours controlled by this network (see e.g. (Ma and Gundlach, 2015) and further discussion below).

In this respect, immunohistochemical studies have revealed that >90% of NeuN-positive neurons in the NI are GABAergic, reflected by GABA and GAD65 immunostaining (Ma et al., 2007; Singleton CE, Gundlach AL, Ma S, unpublished data). The NI relaxin-3 co-localises with GAD65 (Ma et al., 2007), consistent with the high levels of vGAT mRNA detected in the region in the current study, and a large population of CRF1 receptor-positive neurons (Ma et al., 2013), activation of which may contribute to behaviour in response to CRF signalling and neurogenic stress. Indeed, NI neuron activation reflected by Fos-immunoreactivity, is increased following exploration (Ma et al., 2009a) and various types of neurogenic stressors in rats (Tanaka et al., 2005; Banerjee et al., 2010; Ryan et al., 2013b). These findings suggest that NI activity is important for the active, locomotor components of stress responses (i.e. fight and flight).

Future studies will examine the expression profile of OX2R and MCH1R with other neuropeptides expressed by NI neurons, besides relaxin-3, including cholecystokinin, also present in GABA neurons (Olucha-Bordonau et al., 2003; Singleton CE, Gundlach AL, Ma S, unpublished data) and CRF, present in smaller neurons, which may be non-GABAergic in nature (Walker et al., 2017). Furthermore, an excitatory effect of orexin-A on relaxin-3 and non-relaxin-3 NI neurons has been observed (Blasiak et al., 2015). Although both orexin receptor transcripts were expressed in NI, OX1R mRNA was considerably less abundant than OX2R mRNA. Indeed, OX2R signalling in the NI was shown to contribute to alcohol-seeking and stress-related relapse in alcohol-preferring (iP) rats. The current study identified a distinct distribution of OX2R mRNA expression
in the NI, with higher levels detected in the NId, and varied colocalisation with MCH1R and vGAT mRNA, as well as expression in vGAT mRNA-negative neurons. Notably, the presence of OX2R mRNA in the NI is consistent with earlier description of putative OX2R-immunostaining in the NI (Blasiak et al., 2015; Kastman et al., 2016).

6.8.2. Orexin and MCH systems and interactions with NI in motivational behaviour

Recently, it was demonstrated that chemogenetic activation of rat NI neurons increased locomotor activity in both the home cage and a locomotor cell open-field, and induced hypervigilant behaviours in response to a fear-conditioned cue, which was associated with enhanced cortical desynchronization during periods of immobility and rest (Ma et al., 2017a). As such, the NI is emerging as a key node of a brainstem arousal network, which includes the neighbouring ventral tegmental nucleus of Gudden (Brown and McKenna, 2015), laterodorsal tegmental nucleus (Schwartz and Roth, 2008) and the apparently parallel locus coeruleus system (Carter et al., 2010), which does not receive any meaningful input from the NI (relaxin-3) system (Ma et al., 2017a) or provide any strong input to the NI (Goto et al., 2001; Olucha-Bordonau et al., 2003).

In functional studies in adult rats, intra-NI infusion of orexin-A had a stimulatory effect on locomotor activity and food intake when administered to satiated rats during the light phase, consistent with activation of NI networks and increased arousal, locomotion and potential motivation to consume available food. In contrast, intra-NI infusion of MCH appeared to suppress locomotor activity to some small degree or had little or no effect on these parameters. Furthermore, the effects of intra-NI infusion of MCH were influenced by the diurnal phase in which testing was undertaken. Thus, MCH infusion into the NI appeared to suppress exploratory behaviour in the light, but not the dark phase. The lack of effect during the active dark phase may be due to an increased tonic activity of NI neurons during the dark phase and wakefulness, as reflected by increased c-fos and relaxin-3 mRNA levels in the NI under conditions of stimulated locomotion (Banerjee et al., 2010; Ma et al., 2017a), thus rendering the neurons more resistant to inhibition by MCH. Although optogenetic excitation of MCH neurons can induce sleep, it has been demonstrated that microinjection of this neuropeptide into the DR altered REM and non-REM sleep states, but it did not induce sleep (Benedetto et al., 2013). Thus, the effects of MCH are not
necessarily related just to sleep induction, as it also has a role in the control of sleep stage duration/transitions. Furthermore, the magnitude of the impact of the MCH projection from the LH to the NI may be less than that to other key nodes of MCH action.

Along with feeding circuits, such as NPY, POMC/AgRP neurons projecting from ARC to PVN (Jennings et al., 2013; Jennings et al., 2015; Venner et al., 2016), another anorexic circuit involving PVN neurons may include those co-expressing oxytocin and RXFP3 in rats (Ma et al., 2007) (Kania et al., 2017). Therefore, it is possible that a GABAergic projection from the NI activated by orexin or MCH systems could inhibit the anorexic effect of oxytocin (Nieh et al., 2015), leading to increased food intake. Taken together, relaxin-3 projections target neural centres involved in arousal, which consist of thalamus, septum, hippocampus, and limbic and hypothalamic circuits. MCH and orexin neurons from the hypothalamus project to, and modulate, GABAergic NI neurons projecting to these areas to influence alertness and motivated behaviours depending on circadian phase.

6.8.3. NI system and its functional interaction with orexin and MCH systems

There are several putative circuits downstream from the NI that may mediate its impact on arousal and locomotor behaviour, as the NI is known to project widely to brain regions important for controlling these parameters as well as behavioural state and planning (Goto et al., 2001; Olucha-Bordonau et al., 2003). One such NI projection is to the lateral preoptic/basal forebrain area, which contains GABAergic and cholinergic neurons that regulate cortical activity and other arousal-related behaviours (Li et al., 2015; Zant et al., 2016). In addition, there are established anatomical connections between NI and medial septum (Olucha-Bordonau et al., 2012; Sánchez-Pérez et al., 2015) which, in turn, are known to have a significant role in entrainment and pacing of theta oscillations, spatial navigation, learning and memory, as well as initiation and velocity of locomotion (Hangya et al., 2009; Robinson et al., 2016). Electrophysiological data suggests the NI also contributes to the generation of hippocampal theta rhythm (Nunez et al., 2006; Martinez-Bellver et al., 2015; Ma et al., 2017a; Martinez-Bellver et al., 2017). In this regard, NI (relaxin-3) and MCH (and orexin) circuits may have common downstream targets (Kilduff and de Lecea, 2001; Ma et al., 2007), and recent evidence indicates that MCH projections to the septum and
changes in MCH neuron activity regulate hippocampal theta activity and aspects of REM sleep (Jego et al., 2013). Similarly, orexin circuits provide a robust innervation of different cell types in the MS and hippocampus, orexins have neurophysiological actions on these neurons, and these peptides have been shown to have direct effects on hippocampal theta activity in vitro and in vivo (Wu et al., 2002; Selbach et al., 2004; Wu et al., 2004; Bocian et al., 2015). Thus, given the importance of NI activity to the optimal generation of hippocampal theta activity (Nunez et al., 2006; Martinez-Bellver et al., 2015; Martinez-Bellver et al., 2017), it is possible that MCH and orexin signalling within this area, might modulate theta via influences on different populations of NI neurons, such as those identified in the current study.

In terms of feeding and metabolism, pharmacological studies have established the likely role of the relaxin-3 signalling system in promoting feeding (e.g. (McGowan et al., 2005; Lenglos et al., 2015; de Ávila et al., 2017; Ma et al., 2017a), but studies assessing NI neuron involvement or specifically whether feeding is attributable to relaxin-3 NI neurons have not been completed. In this regard, relaxin-3 and c-fos gene and peptide/protein expression in the NI exhibits diurnal regulation, whereby expression of both peak during the active, dark phase and trough during the inactive, light phase in rats (Banerjee et al., 2006; Banerjee et al., 2010).

Therefore, future studies are needed to assess the functional relevance of the colocalisation of MCH1R and OX2R on relaxin-3 neurons, the downstream projection targets of these NI neurons, and the behavioural effects attributed to these specific neural circuits, perhaps using a combination of viral, optogenetic, neurophysiological and pharmacological techniques in rats or normal and relevant transgenic mice, if the current data is verified in this species.

6.8.4. Diurnal pattern of orexin and MCH trafficking on regulation of NI

In the current study putative neural contacts between orexin and MCH immunoreactive fibres and relaxin-3 neurons were identified and since processes under peptidergic control exert circadian fluctuations (see (Blasiak et al., 2017) for review), their potential diurnal regulation was assessed. Previous studies have optimized the use of Imaris to analyse putative presynaptic terminals (spots <0.5 μm from soma surface) and axonal segments (spots >0.5 μm from soma surface) from
immunohistochemically stained sections (Ausdenmoore, 2011). In this study, overall MCH immunofluorescent elements within the rat NI were more abundant than the equivalent orexin elements. Using similar analysis methods (Ausdenmoore, 2011), the fluorescence intensity associated with MCH in axonal segments and putative presynaptic terminals close to relaxin-3 soma were found to be significantly higher during the dark phase than during the light phase. In contrast, orexin immunofluorescence was unchanged. These data suggest a diurnal rhythm in MCH signalling onto relaxin-3 neurons and its upstream modulation, whereas low level orexin inputs appear relatively consistent. These data can be compared to other descriptions of changes in MCH system components under different arousal and sleep conditions (Dias Abdo Agamme et al., 2015). Relaxin-3/RXFP3 signalling has been shown to influence circadian activity and rhythmicity of arousal-related behaviours (Smith et al., 2012; Blasiak et al., 2013; Hosken et al., 2015); therefore further studies are required to assess how diurnal rhythm might influence the independent effects of orexin and MCH neuropeptide systems on arousal and feeding, as well as their interactions.

Furthermore, the synaptic location of these peptides is suggestive of distinct functions. For example, synapses onto distal dendrites are important for synaptic integration, whereas axo-dendritic synaptic sites, may favour information relay, and synapses onto soma (axo-somatic) suggest faster, direct information relay, which, as a result, have a higher probability of modulating the postsynaptic neuron activity than those on distal dendrites (Schneider Gasser et al., 2006). Therefore, further anatomical studies to assess the precise subcellular location of orexin- and MCH-positive synapses on NI GABA/relaxin-3 neurons are warranted.

### 6.9. Concluding remarks and future directions

Much is known about the functional role of the orexin system in arousal-based behaviour and state control, yet new knowledge is still being discovered, which highlights the complexity of arousal neural processes in general. Among the influences of other neuromodulatory systems on motivated behaviours and stress responses, the contribution of the hindbrain NI and the relaxin-3 neuropeptide system is being revealed. The interactions between orexin and MCH hypothalamic systems and the relaxin-3 system in normal physiological conditions, such as regulating circadian
rhythm, is a new field of research. Specifically, the baseline physiological interactions between these systems were unknown prior to the current thesis studies, which have investigated the interactions between orexin/MCH and relaxin-3 systems in the context of normal physiological conditions, specifically circadian-related behaviours and the interactions between these hypothalamic and brainstem arousal systems.

Further studies and understanding different hypothalamic population and their neural projections and interactions with other arousal centres and motivated behaviour neural networks along with the recently discovered NI/relaxin-3 system, will provide greater insight into how brain circuits influence arousal and its related outcomes such as feeding and locomotor activity (Figure 6.3). The current studies described the relative anatomical distribution of the orexin-OX2R and MCH-MCH1R signalling systems in the rat, along with the neurophysiological and behavioural effects of orexin-A and MCH signalling in the NI. Together these physiological and behavioural findings, along with diurnal changes in the nature of MCH inputs to the NI, reflect a neuromodulatory role for these peptide signalling systems in activation and inhibition of heterogeneous GABA neuron populations in the NI, and are consistent with the NI being a key downstream target for LH orexin and MCH signalling in arousal and feeding processes. Better understanding of how these systems interact would allow us to better understand pathophysiology of the related disorders and to design more accurate pharmaceutical treatments for the ever-increasing rates of these related disorders, such as obesity, type 1 and 2 diabetes, or stress-induced eating disorders. Future studies extending electrophysiological and behavioural findings, will further identify the functional importance of this arousal network, which might underpin normal circadian behaviour and pathological conditions of impaired arousal or hyperarousal.
Figure 6.3. Actual and putative circuits and pathways between hypothalmaus and arousal centres, including NI, responsible for motivated behaviors.

(A) Feeding behaviour, overlapping with reward-related behaviour. Note a putative subpopulation of LHA\textsuperscript{GABA} neurons defined by Pdx1. (B) Sleep and wake behaviour. Note a putative subpopulation of LHA\textsuperscript{GABA} neurons defined by LepRb. (C) Stress-related behaviour, including LepRb neurons. Abbreviations: BNST\textsuperscript{GABA} (GABAergic neurons in the bed nucleus of the stria terminalis); LC, locus coeruleus; LHb, lateral habenula; NAc\textsuperscript{D1R/GABA} (GABAergic D1R-expressing medium spiny neurons in the nucleus accumbens); POA\textsuperscript{GABA} (GABAergic neurons in the hypothalamic preoptic area); PVN, paraventricular nucleus of the hypothalamus; TMN\textsuperscript{HA} (histaminergic neurons in the tuberomammillary nucleus); TRN\textsuperscript{GABA} (GABAergic neurons in thalamic reticular nucleus); VTA, ventral tegmental area. Adapted from (Bonnavion et al., 2016).
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