Functional properties of GAD65 neurons in the lateral hypothalamus

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"Imagination is the Discovering Faculty, pre-eminently. It is that which penetrates into the unseen worlds around us, the worlds of Science. It is that which feels & discovers what is, the real which we see not, which exists not for our senses."

Ada Lovelace | Letter to Charles Babbage | January 1841
Declaration

I, Christin Lena Kosse, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
Publications

Results presented in this thesis might have been presented and published previously in the following publications:


Abstract

The brain’s ability to sense energy levels and adjust behaviour accordingly is vital for survival in mammals. The lateral hypothalamus (LH), which contains energy-spending (orexin) and energy-conserving melanin-concentrating hormone (MCH) neurons, is thought to be the brain's master energy sensor and generator of motivated behaviour. Recently, other classes of non-MCH, non-orexin neurons, such as vesicular GABA transporter (VGAT) and glutamic acid decarboxylase 65 (GAD65) expressing neurons, have been discovered in the LH. VGAT\textsubscript{LH} neurons have been shown to be essential for appetitive and consummatory behaviour. However, the properties and behavioural roles of GAD65\textsubscript{LH} neurons remain unclear, and are the focus of this thesis. The thesis’ three parts examine cellular, circuit, and behavioural roles of GAD65\textsubscript{LH} neurons.

Firstly, whole cell patch clamping was used to determine firing responses of GAD65\textsubscript{LH} neurons to injections of oscillatory input currents. GAD65\textsubscript{LH} neurons were found to have similar frequency-preferences of firing resonance to those of VGAT and MCH neurons, whilst orexin neurons showed a different, “high frequency inhibited” frequency-preference profile. Moreover, histochemistry was employed to characterise GAD65\textsubscript{LH} neurons further by quantifying their overlap with other GABAergic LH cell types. It was found that GAD65\textsubscript{LH} neurons overlapped only partially with VGAT\textsubscript{LH} neurons, and that neuropeptide Y (NPY) and leptin receptor (LepRb) expressing neurons were largely distinct from GAD65\textsubscript{LH} cells.

Secondly, cell-type specific channelrhodopsin-assisted circuit mapping was used to probe up- and downstream functional targets of GAD65\textsubscript{LH} neurons. It was found that GAD65\textsubscript{LH} neurons were excited by orexin neurons and inhibited by VGAT\textsubscript{LH} neurons, and that they preferentially inhibited MCH\textsubscript{LH} and NPY\textsubscript{LH} neurons.

Finally, chemogenetic excitation or inhibition of GAD65\textsubscript{LH} cell activity was used to investigate the role of these cells in behaviour. It was found that GAD65\textsubscript{LH} neurons were weight-loss-promoting, and essential and sufficient for normal locomotor activity.
Overall, these results define and characterise a new cellular network component in LH function.
Impact Statement

Considering the existing and still growing prevalence of obesity, especially in the developed world, and its role in the causation of diabetes (Minamino et al. 2009), cardiovascular diseases (Van Gaal et al. 2006) and even cancer (Calle and Kaaks 2004), it is paramount to understand the control of energy expenditure and body weight. Since it has been suggested that obesity is a brain based disease (Locke et al. 2015), it is of essential value to understand the complex interaction of neuronal circuits that control and produce the fine balance of keeping an appropriate body weight by producing adaptive food intake and energy expenditure.

This work aims to provide a novel insight into how behaviour emerges from specific neuronal signals in the LH, and how these signals can be controlled by the local circuitry. Thereby providing new knowledge about how basic functions like energy balance and body weight might be controlled by the brain, but also adding to the understanding of how neuropeptidergic circuitries might differ in form and function from other brain areas. This work will hopefully not only help basic science progress by opening further avenues for dissecting neurophysiological mechanisms of body weight control, but also provide a basis for clinical research and application to build upon this knowledge.
Acknowledgement

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>aDREADD</td>
<td>activating DREADDs-hM3Dq</td>
</tr>
<tr>
<td>alpha-MSH</td>
<td>alpha-melanocyte-stimulating hormone</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AOB</td>
<td>accessory olfactory bulb</td>
</tr>
<tr>
<td>AP</td>
<td>Action potential</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine tri-phosphate</td>
</tr>
<tr>
<td>Agrp</td>
<td>agouti-related peptide</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>CNO</td>
<td>clozapine-n-oxide</td>
</tr>
<tr>
<td>CRACM</td>
<td>channelrhodopsin-assisted circuit mapping</td>
</tr>
<tr>
<td>ChR2</td>
<td>channelrhodopsin-2</td>
</tr>
<tr>
<td>DREADD</td>
<td>designer receptors exclusively activated by designer drugs</td>
</tr>
<tr>
<td>DTR</td>
<td>diphtheria toxin receptors</td>
</tr>
<tr>
<td>EEG</td>
<td>electroencephalographic</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric-acid</td>
</tr>
<tr>
<td>GAD65</td>
<td>glutamic acid decarboxylase 65</td>
</tr>
</tbody>
</table>
GAD67  glutamic acid decarboxylase 67
GCaMP  genetically encoded calcium indicator
GFP    green fluorescent protein
GPCR   G-protein coupled receptors
icv    intracerebroventricular
iDREADD inhibitory DREADD-hM4Di
i.p.    intraperitoneal
ING    interneuron network gamma
JAK2   Janus-kinase 2
K-ATP  ATP-inhibited K+ channel
K2P    Tandem-pore potassium channel
KO     knockout
LH     lateral hypothalamus
LepRb  leptin receptor
MB     midbrain
MCH    melanin-concentrating hormone
MCH1R  melanin concentrating hormone receptor 1
NARP   neuronal activity-regulated pentraxin
NEAT   non-exercise activity thermogenesis
NEI    neuropeptide EI
NGE    neuropeptide GE
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMDA</td>
<td>N-methyl-d-aspartate</td>
</tr>
<tr>
<td>NP1</td>
<td>neuronal pentraxin-1</td>
</tr>
<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
</tr>
<tr>
<td>ns</td>
<td>non-significant</td>
</tr>
<tr>
<td>OXR2</td>
<td>orexin receptor type 2</td>
</tr>
<tr>
<td>PING</td>
<td>pyramidal interneuron network gamma</td>
</tr>
<tr>
<td>pSTAT3</td>
<td>phosphorylated form of Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>REM</td>
<td>rapid-eye-movement</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>TH</td>
<td>thalamus</td>
</tr>
<tr>
<td>TRAP-seq</td>
<td>Translating Ribosome Affinity Purification analyzed by RNA-seq</td>
</tr>
<tr>
<td>VGAT</td>
<td>vesicular GABA transporter</td>
</tr>
<tr>
<td>VGLUT1</td>
<td>vesicular glutamate transporters 1</td>
</tr>
<tr>
<td>VGLUT2</td>
<td>vesicular glutamate transporters 2</td>
</tr>
<tr>
<td>VMH</td>
<td>ventro-medial hypothalamus</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
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Chapter 1. Introduction

1.1 A historic and evolutionary view of the hypothalamus

The origin of hypothalamic cells can be traced back to protochordates (Candiani et al. 2008), annelids (Tessmar-Raible et al. 2007) and insects (Hartenstein 2006). Moreover, it has been theorised that some endocrine cells of the vertebrate pituitary and some neurons of the hypothalamus and the olfactory system evolved by functional segregation of sister cell types, which emerged from a chemosensory–neurosecretory organ that was similar to the Hatschek’s pit (a structure that secretes mucus to entrap food particles in the water, see Figure 1.1) in lancelets. These fish-like marine chordates, which are also known as amphioxus, are representatives of the subphylum Cephalochordata (Figure 1.1) (Arendt 2008). Intriguingly, this theory sees the evolutionary progenitor of hypothalamic cells already involved in the function of feeding, and furthermore suggests that an olfactory-hypothalamus-pituitary circuitry has a long-standing evolutionary history.

**Figure 1.1: Phylogenetic tree of Chordata and anterior anatomy of the lancelet**

Left, phylogenetic tree of Chordata. Right bottom, example of Branchiostoma lanceolatum, a species of the subphylum Cephalochordata. Right top, zoom of the anterior anatomy of Branchiostoma depicting the location of the Hatschek’s pit. Pictures modified from Bertrand and Escriva 2011.

Despite the postulated evolutionary ancient history, scientific interest only started to majorly concern itself with the hypothalamus during the 20th century (reviewed in Brooks 1988). After William His introduced the term hypothalamus as an anatomical, neurological and physiological entity for the first time at the end of the 19th century
(His 1895), and Ramon y Cajal started to identify hypothalamic tracts and nuclei with his histological advances in 1904, scientists started to investigate the function of the hypothalamus. Interestingly, one of the first functions proposed was control of somatic actions. That the hypothalamus is essential for complex motor behaviour by integrating somatic and visceral processes, was first indicated by experiments by Goltz and his famous de-cerebrated dogs (where cutting across the brainstem removed the cerebrum), showing that sparing “thalamic areas”, which included the hypothalamus, would preserve locomotion including eating (Goltz 1892). This was later refined by Hinsey and Ranson (1928), who showed that locomotor activity was much higher in hypothalamic cats (where the cut to remove the cerebrum across the brain stem spared the hypothalamus) compared to fully de-cerebrated cats. Moreover, Bard who lesioned the hypothalamus noticed increased lethargy in the five cats, that survived after surgery for long enough (Bard 1940). Loss of motor initiative was also noted in lesion studies in monkeys by Ranson, who destroyed the lateral part of the hypothalamus (Ranson 1939), suggesting that hypothalamus-dependent changes in somatic actions were not limited to cats and dogs. In addition to this, early electrical stimulation experiments of cat hypothalami by Nobel laureate Hess, produced somatic and autonomic effects that could differ depending on how anterior/lateral or posterior the electrodes were placed. Whilst anterior/lateral stimulation lowered blood pressure and respiration rate, and increased hunger and thirst behaviour, more posterior stimulations led to aggressive and defensive behaviour. Thereby giving the first indication that the hypothalamus contains different components that are responsible for a variety of somatic and behavioural effects.

1.2 The lateral hypothalamus: classic studies

1.2.1 The lateral hypothalamus in the context of wakefulness

Von Economo, in the 1920s, investigated post-mortem brains of patients with “sleepy sickness” (Encephalitis lethargica), a viral infection causing lesions in different brain tissues (Economo 1930). He found that major neuronal damage in areas like the lateral hypothalamus was often accompanied by a behavioural impairment that prevented his patients to be awake for sustained periods of time. Patients suffering from the disease seemed often to be frozen or asleep and could only be woken up
with sufficient external stimuli. For example, patients could catch a ball when one was thrown at them, but they could not self-initiate movements to throw a ball. His concluding hypothesis was that the LH is a wakefulness centre of the brain (Saper et al. 2001). In addition to this, these observational conclusions from human patients have been extended onto monkeys and rats, where experimental lesions in the LH produced similar somnolence (Nauta 1946; Ranson 1939).

It is noteworthy, that even though Encephalitis lethargica was named “sleepy sickness”, Von Economo never used electroencephalographic (EEG) recordings to measure or quantify sleep. Considering that it wasn’t until 1953, when Aserinsky and Kleitman used EEG for the first time to characterise and describe different stages of sleep, any earlier studies would not have had the same criteria and definition of sleep that we use nowadays. Therefore, a preferred interpretation of these classic studies is to see the LH as wakefulness centre in the sense that the LH is necessary for somatic control and movement initiation instead of implying any relation to sleep.

1.2.2 The lateral hypothalamus in the context of energy and eating

Another function of the LH was proposed later during the 1950s and 1960s, when electrolytic lesions in rats showed that destruction of the LH led to an aphagic phenotype with animals suffering radical weight loss up to the point where they died of starvation (Anand and Brobeck 1951b, 1951a). These results were in stark contrast to earlier lesion studies ablating mainly the ventro-medial hypothalamus (VMH), which led to an obese phenotype (Hetherington and Ranson 1940). Consequently, it was postulated that the LH is the hunger centre and the VMH is the satiety centre of the brain.

Of interest is also one lesion study suggesting an alternative hypothesis for the role of the LH. Instead of seeing the LH as a hunger or feeding centre, they suggest it represents a body weight set point (Powley and Keesey 1970). Starvation prior to lesioning resulted in a much shorter period of aphagia and anorexia and sometimes even hyperphagia. Reduction in body weight was chronic and inversely correlated
with the size of damage to the LH, suggesting that the LH does not produce a uniform behaviour but instead its activity represents a set point for body weight.

The caveat with many electrolytic lesion studies like these is that they are not specific to cell bodies but also ablate fibres of passage going through the hypothalamus, such as the medial forebrain bundle (Morgane 1961b; Nieuwenhuys et al. 1982) and fornix (Swanson and Cowan 1975). One study sought to differentiate these effects and observed that stimulation of the “far” LH caused rats to cross an electrified barrier to press a lever for food (a sign of increased motivation or appetitive behaviour). However, when the medial forebrain bundle was lesioned, LH stimulations only caused the animals to eat but did not motivate them to cross the same electrified barrier (Morgane 1961a). This suggests that appetitive behaviour is actually not mediated by cells in the LH but instead by fibres going through the LH, whilst the LH drives consummatory behaviour only.

Furthermore, most of the LH lesioned animals showing aphagia recovered or could be rescued. In the case of mild lesions, the inability to eat was often only temporarily, whilst more severe cases needed force feeding. However, for both cases, animals recovered their feeding abilities over time. Rats with LH lesions would go through defined stages of what is called the “hypothalamic syndrome”, a four stage process: aphagia and adipsia; anorexia and adipsia; adipsia with a secondary dehydration-aphagia; and recovery (Teitelbaum and Epstein 1962). Therefore, showing that the LH as a hunger and feeding centre can be replaced by other brain areas representing the same drives and keeping the animals alive. Of note is that high-fat diets seemed to elicit most of the recovering hunger in LH lesioned rats, meaning that rats ate foods rich in fat, but refused to eat normal, low fat foods and preferred to starve. Even after recovery, a higher hunger for fat-rich diets was observed (Teitelbaum and Stellar 1954), indicating that there is maybe a fat-hunger centre, that lies outside the LH.

The idea of the LH as a source of feeding and reward signals was further supported by experiments using electrical stimulating in the LH of rats. This stimulation led to voracious food intake (Delgado and Anand 1952), but also reward-seeking behaviour when rats were given the opportunity to self-stimulate by lever pressing (Olds and
Interestingly, self-stimulation was inhibited after excessive feeding (Hoebel and Teitelbaum 1962) and *vice versa* food restriction would cause an increment in self-stimulation (Margules and Olds 1962).

Overall, manipulations of the LH by lesioning or electrical stimulation cause decreases or increases respectively in feeding that suggest that the LH is essential and sufficient for feeding behaviour. However, even though feeding impairments caused by LH lesions can be so severe that animals die of starvation, mice can recover their feeding abilities over time, which shows that other hunger drives exist in the brain, and limits the necessity of the LH for feeding. In addition to this, there are further limitations to the LH as hunger centre, if one considers that some effects of LH lesions might be accounted for by damage to fibres on passage which seem to have an effect on appetitive behaviour.

### 1.2.3 The lateral hypothalamus in the context of locomotion and other behaviours

Besides the obvious link between feeding and reward properties, other more subtle phenotypes were also observed during electrolytic lesion and stimulation studies of the LH. Lesioned monkeys would fail to regulate their own body temperature (Ranson and Ingram 1935) and contralateral sensory neglect was often observed in unilateral LH lesions in rats (Marshall 1978; Marshall et al. 1971).

One study investigated potential cognitive deficits, in the form of learning and memory, resulting from electrolytic lesions in rats (Schwartz and Teitelbaum 1974). They found that, after recovery of feeding behaviour, rats were still able to remember a previously learned taste aversion, but they were not able to acquire new taste aversions with different flavours. Thus, they concluded that the LH is not essential for memory storage or retrieval but for learning and memory acquisition.

In addition to this, scientists were able to use LH specific lesions to recapitulate some phenotypes of the broad hypothalamic lesions and de-cerebrations, that resulted in impairments of somatic actions. Monkey studies using lesions in the LH reported loss
of motor initiative (Ranson 1939). Moreover, a behavioural studies using LH lesioned rats, that had been force-fed, showed that rats suffered from an extreme form of akinesia (Levitt and Teitelbaum 1975). Rats would try to avoid movements at all cost, but were physically able to perform them. For example, rats would spontaneously freeze for a long time, either during movements like grooming (Figure 1.2B) or in awkward positions with their head resting on surfaces between their limbs (Figure 1.2C) or when put into an awkward position, such as on a block of wood (Figure 1.2D). However, most striking is their lack of movement when put into a beaker of warm water. A situation in which normal rats would happily swim to the surface, lesioned rats sank to the bottom and remained there until they were running out of oxygen and were forced to briefly swim to the surface to breath before sinking back to the bottom (Figure 1.2A). This illustrates that lesioned rats were physically able to perform swimming movements, but would avoid them if possible.

Figure 1.2: Movement deficits of rats with bilateral LH lesions

Posture and movement in rats with bilateral lateral hypothalamic lesions. (A) In warm water, rat swims little or not at all. Cycle of exhalation under water as in photograph, followed by thrust against bottom of tank with rear leg propelling rat to surface for inhalation may be observed. (B) "Frozen" stance following short period of grooming. (C) Gradual subsiding follows "freezing" and sometimes leads to top of head resting on table tucked between forelegs. (D) Rat remains in cataleptic
awkward posture for indefinite period. (Figure and description reproduced from Levitt and Teitelbaum 1975).

A point to note is that the most extreme akinesia was observed after rats were force fed, and whilst the authors conclude that therefore any spontaneous locomotion is dependent on a signal from the empty stomach, another possible explanation may be that the LH is essential for non-hunger motivated locomotion.

1.3 Genetically defined neuronal populations in the LH

Considering that the LH consist of diverse heterogeneous cell populations (Mickelsen et al. 2017), lesions and electrical stimulations are methods too crude to assign behavioural outputs to specific LH cell types. Therefore, methods to manipulate genetically identifiable populations of the LH open a new avenue to try to assign some of the striking phenotypes from broad LH lesions to specific Cre-populations.

Two major cell populations found in the lateral hypothalamus are genetically distinct and named after neuropeptides that they express. These are MCH (Nahon et al. 1989; Vaughan et al. 1989) and orexin/hypocretin (Sakurai et al. 1998; de Lecea et al. 1998) neurons, which have cell bodies exclusively in the hypothalamus, but project throughout most of the brain (Bittencourt et al. 1992; Peyron et al. 1998). In addition to their slow-acting neuropeptides, some of these neurons are also able to release fast-acting neurotransmitters. MCH neurons express glutamic acid decarboxylase 67 (GAD67) for the synthesis of gamma-aminobutyric-acid (GABA) (Jego et al. 2013), and orexin neurons express the vesicular glutamate transporters 1 and 2 (VGLUT1) and (VGLUT2) for the transport of glutamate into vesicles (Rosin et al. 2003). Moreover, recent single cell quantitative polymerase chain reaction (qPCR) studies found that nearly all MCH and roughly half of all orexin neurons expressed markers for glutamatergic and GABAergic neurotransmitter machinery but were lacking the vesicular GABA transporter (VGAT) (Mickelsen et al. 2017). This shows an unexpected diversity of markers for fast neurotransmitter being transcribed in MCH and orexin neurons. However, single cell qPCR does not prove neurotransmitter release but functional studies have reported GABA release from
MCH neurons and glutamate release from orexin neurons (see section 1.4.1 and 1.5.1).

Furthermore, there are other types of neurons found in the lateral hypothalamus that are not exclusive to this area. These are GAD65 neurons (Karnani et al. 2013), neuropeptide Y (NPY) neurons (Marston et al. 2011), and leptin receptor (LepRb) neurons which express GAD67 like MCH neurons (Elias et al. 2008). All of them are distinct from MCH and orexin neurons (Leinninger et al. 2009). However, it is still unclear whether GAD65LH neurons are leptin responsive. LepRb neurons have been reported to express GAD67 and since most GABAergic neurons in the CNS express both GAD67 and GAD65 (Erlander et al. 1991; Soghomonian and Martin 1998), it raises the question of what is the relationship between GAD65 and LepRb neurons.

Thanks to this distinct protein expression, it is possible to target these LH neurons separately via their gene expression for various techniques like chemogenetic and photogenic activation and inactivation (Packer et al. 2013).

### 1.4 Orexin neurons

#### 1.4.1 Intrinsic properties of orexin neurons

Orexin, as a neuropeptide, can be released as orexin-A or orexin-B, which result from cleavage of a single precursor protein. There are two specific orexin G-protein coupled receptors (GPCR) that they can bind to, with OXR1 being Gq coupled and preferably binding orexin A, and OXR2 being Gi/o or Gq coupled and binding to both orexins equally well (Sakurai et al. 1998; Zhu et al. 2003). In addition to this, orexin neurons also express other neuropeptides such as dynorphin (Chou et al. 2001) and neuronal activity-regulated pentraxin (NARP), a peptide important for AMPA receptor clustering and regulation of glutamatergic signalling (Reti et al. 2002). Besides slow-acting neuropeptides, orexin neurons can also release glutamate which can act upon ionotropic AMPA-receptors, as it has been shown at the orexin cell to tuberomammillary histamine cell synapse (Schöne et al. 2012, 2014).
From a biophysical perspective, there seem to be at least two distinct subclasses of orexin neurons, namely H and D-type orexin neurons depending on the existence of a hyperpolarizing or depolarizing post-inhibitory rebound potential. These two classes also differ in dendritic architecture, synaptic inputs and expression of a low-threshold A-type K+ channel (Schöne et al. 2011). Therefore, orexin neurons do not represent a homogenous class.

Projections of orexin neurons target most brain areas including the cerebellum (Peyron et al. 1998; Nisimaru et al. 2013) and thus are suspected to have an impact on many different neuronal processes. Dense projections are found to target regions regulating behavioural states (España et al. 2005), like the locus coeruleus, dorsal raphe, periaqueductal gray and paraventricular nucleus of hypothalamus, which may be important for the arousal-promoting effect of orexin neurons and are described in detail in section 1.2.1 below. Furthermore, projections to the posterior nervous system such as the hindbrain and spinal cord are thought to implement control over the sympathetic and autonomic nervous system, which can influence metabolism, thermogenesis and locomotor activity (Kerman et al. 2006; Kerman et al. 2007; Oldfield et al. 2007). Dense projections are also found to the mesolimbic dopamine system including the amygdala, septal area, medial preoptical area, ventral tegmental area and nucleus accumbens (Vittoz et al. 2008; Korotkova et al. 2003). Interactions of orexin neurons and reward circuits could play an important role in hedonic food intake, but also in stress (Narita et al. 2006; Baldo et al. 2003).

### 1.4.2 Orexin neurons as internal sensors

Orexin neurons respond to a huge variety of indicators of the state of the body and the body’s needs. Glucose inhibits orexin neurons in vivo (Cai et al. 2001) and in vitro (González et al. 2008) due to a background potassium current likely involving a tandem-pore channel (K2P) (Burdakov et al. 2006). However, glucose sensing in orexin neurons is rather complex, since orexin neurons are a heterogeneous group with dichotomous electrophysiological properties (Schöne et al. 2011). Some orexin neurons respond to glucose with sustained hyperpolarisation and thus track absolute glucose levels, whilst other orexin cells adapt to constant glucose elevation,
comparable to adaptive changes in the sensory systems (Williams et al. 2008). In addition to this, orexin neurons seem to be conditional glucose sensors that reduce their glucose-sensing ability when energy availability, through other compounds, is high. This was shown by dose-dependent blockage of glucose evoked hyperpolarisation by other energy substrates such as lactate, pyruvate and adenosine tri-phosphate (ATP) (Venner et al. 2011), and might indicate that orexin neurons are only glucose sensors when general energy levels are low. In addition to this, leptin, which is produced by fat tissue, inhibits orexin neurons whilst ghrelin, a hunger hormone produced by an empty stomach, excites orexin neurons (Yamanaka et al. 2003). Considering all these points, it was suggested that orexin neurons are essential for connecting energy balance with wakefulness. This theory is based on studies showing that wild type mice increase their arousal and activity in response to fasting, but orexin neuron ablated mice do not show this increase (Yamanaka et al. 2003).

Orexin neurons seem also to be part of the brain network involved in sensing of amino-acids that can either be taken up through diet or result from tissue break-down to gain energy during starvation (L’Heureux-Bouron et al. 2003). It has been shown that orexin neurons detect these non-essential amino acids *in vivo* and *in vitro* (Karnani et al. 2011). Mixtures of non-essential amino acids excite orexin neurons via closure of K-ATP channels and electrogenic system-A amino acid transporters. Additionally, the range of physiologically-critical substances sensed by orexin neurons is completed by H+ and CO₂, which are regulated by breathing (Nattie and Li 2010). Orexin neurons seem to have chemoreceptors for these substances, since H+ and CO₂ increased the activity of orexin neurons *in vivo* (Sunanaga et al. 2009) and *in vitro* (Williams et al. 2007).

Besides sensing nutrients and compounds, that signals the state of the body, orexin neurons also respond to applications of neurotransmitters from potential neuronal inputs. For example, corticotropin-releasing factor excites a subgroup of orexin neurons (Winsky-Sommerer et al. 2004), which could explain how orexin neuron activity plays a role in stress responses and anxiety (Suzuki et al. 2005; Johnson et al. 2010). In addition to this, noradrenalin and serotonin, neuromodulators implicated in the regulation of arousal, hyperpolarise orexin neurons (Yamanaka et al. 2003;
Muraki et al. 2004). However, the relationship between orexin cells and cells expressing these monoamines seems to be complex, since orexin neurons also excite some of the nuclei they originate from, and thus form reciprocal connections (Yamanaka et al. 2003). Another reciprocal connection was found between orexin neurons and local glutamatergic neurons excited by orexin. Specifically, orexin indirectly excites orexin neurons (Li et al. 2002). This orexin-evoked glutamate input seems to be negatively regulated by MCH, because the glutamate-orexin synapse is potentiated in melanin concentrating hormone receptor 1 (MCH1R) knockout (KO) mice (Rao et al. 2008). Antagonistically, there also seems to be a GABAergic input to orexin neurons during sleep that can be blocked with GABA_A receptor antagonists resulting in higher c-fos expression in orexin neurons (Alam et al. 2005). Additionally, LepRb neurons in the LH are also GABAergic and seem to project onto orexin neurons (Leinninger et al. 2011; Louis et al. 2010), however, it is unclear whether LepRb neurons inhibit orexin neurons during sleep. As orexin neurons are not constantly active during wakefulness, but rather peak during exploration and sensory stimuli, orexin neurons may receive neuronal input about the environment and possibly novelty (Mileykovskiy et al. 2005). The neuronal identity and nature of these synaptic inputs has not been identified yet.

Overall, orexin neurons appear to act like master sensors of many humeral and neuronal inputs. Integration of these signals potentially allows orexin neurons to generate appropriate arousal according to the current physiological state of body and environment.

### 1.4.3 Orexin neurons and feeding, body weight and metabolism

That orexin neurons can also affect body weight and metabolism is known from the pathology of orexin-deficient human patients (Dauvilliers et al. 2007), as well as mouse models with orexin neuron ablation (Hara et al. 2001). Both orexin-deficient humans and mice develop obesity, and for mice it has been shown that food intake was lowered. Reduction in feeding was also confirmed by intracisternal injection of orexin antibodies (Yamada et al. 2000). Considering obesity and a decrease in food intake as a result of orexin neuron loss, this implies that loss of orexin neurons
causes either a change in metabolism or a reduction in locomotor activity accounting for the higher body weight despite the decrease in food intake. In contrast, a recent study (González et al. 2016) using orexin-DTR mice, allowing for an ablation of orexin neurons in the fully developed mouse, showed how loss of orexin neurons caused overweight but also overeating with an altered feeding pattern. This paper also demonstrated for the first time that orexin neurons are inhibited during feeding, using whole population fibre photometry.

The effect of orexin neurons on metabolism was investigated by studies on brown adipose tissue (BAT), suggesting that orexin activity is necessary for the development and function of BAT. Orexin KO mice show impaired BAT thermogenesis due to preadipocytes failing to differentiate properly (Sellayah et al. 2011). Recent studies used ataxin-3 induced orexin neuron-ablated rats to monitor BAT thermogenesis and body temperature over the basic rest-activity cycle (Mohammed et al. 2014). Their findings show a reduced amplitude and slope of body temperature and BAT thermogenesis during the active phases, which occur every 1-2 hours, and during exposure to salient environmental stimuli such as an intruder. If BAT thermogenesis fails due to a loss of orexin neurons, that could account for a higher preservation of energy seen in orexin neuron ablations.

### 1.4.4 Orexin neurons and sleep

Narcolepsy is a rare neurological disorder that occurs in 0.05% of the general population (Ohayon et al. 2005) and is characterised by daytime sleepiness with abnormally frequent rapid-eye-movement (REM) sleep phases, and cataplexy, the sudden loss of muscle tone (Nishino and Kanbayashi 2005). This suspected autoimmune disease (Partinen et al. 2014) is due to a loss of orexin neurons. Pathophysiological studies in humans show a loss of up to 85-95% of these neurons (Dauvilliers et a. 2007; Thannickal et al. 2000). Animal models with knockouts of the orexin receptor type 2 (OXR2), the preproorexin gene or the orexin neurons show the same phenotype as the narcoleptic patients (Nishino and Kanbayashi 2005; Chemelli et al. 1999), suggesting that orexin neuron activity and OXR2 are essential for stable wakefulness. Moreover, it does not seem like orexin neuron activity is
necessary for wakefulness per se, but rather that it is needed to maintain a stable and appropriate level of arousal, since the overall amount of sleep is not changed in narcolepsy, only more fragmented (Hara et al. 2001).

Expression of the immediate early gene c-fos, a measure of neuronal activity (Chung 2015; Dragunow and Faull 1989), was used to measure the activity of orexin neurons during the active and sleep period (Estabrooke et al. 2001). A positive correlation of c-fos staining with the amount of wakefulness was found, indicating that orexin neurons are mainly active during the dark phase when mice are active. This result was confirmed by measurements of orexin-A levels in cerebrospinal fluid, which found high levels during the active phase and falling levels in the sleeping phase (Yoshida et al. 2001). However, because of the placements of the taps, which were in close proximity to the lumbar spine, a time delay of changes in orexin concentration could have been, inadvertently, introduced.

To gain a better understanding of the exact role of orexin neuron activity in sleep and arousal, it is necessary though to record activity directly from the cells to be able to correlate neuronal activity with behavioural state. Recordings from hypothalamic neurons *in vivo* are rarely done, as the area is difficult to access. Single unit recordings of 6 orexin neurons of head-fixed rats over their sleep-wake cycle (neurons were juxtacelullarly labelled with neurobiotin to confirm their identity) show that orexin neurons are not constantly firing during wakefulness, but are rather peaking during active waking when postural muscle tone is high (Lee et al. 2005). During quiet waking there is still some activity, but during sleep orexin neurons nearly cease to fire at all. These findings were complemented by experiments where orexin neurons were optogenetically activated via channelrhodopsin-2 (ChR2). Sleeping mice woke up faster upon light stimulation of ChR2 expressing orexin neurons, indicating that orexin neuron activity is sufficient for this arousal state transition (Adamantidis et al. 2007). Similar experiments, using chemogenetic activators like designer receptors exclusively activated by designer drugs (DREADD) showed a decrease of REM and non-REM sleep when orexin neurons were activated (Sasaki et al. 2011). This again shows how orexin neuron activity can alter the behavioural state of the animal from sleep to wakefulness.
1.4.5 Orexin neurons and other awake behaviour

Another study of 9 orexin neurons, recorded in freely moving rats with micro wires, suggested that there is low firing activity during quiet awaking and high firing activity during active awakening (Mileykovskiy et al. 2005). Furthermore, they also recorded activity transients during sensory stimulation and behaviour. Grooming and eating evoked equally high activity, but peak activity was observed during exploratory behaviour. These findings indicate that orexin neurons are not just involved in the transition from sleep to wakefulness, but maybe also in transitions to different arousal states, such as during sensory stimulation and exploration. This supports the theory of orexin neurons communicating an arousal error signal, that corresponds to a desired change in arousal level (Kosse and Burdakov 2014).

In addition to this, locomotor activity also seems to be affected by orexin. Intracerebroventricular (icv) administration of orexin caused increased arousal and locomotor activity (Hagan et al. 1999). Moreover, chemogenetic activation of an excitatory Cre-dependent DREADD construct in orexin neurons caused an increase in locomotion and respiratory exchange rate (Inutsuka et al. 2014). However, an optogenetics study in rats (Heydendael et al. 2014), found that activation of orexin neurons only increased locomotion in the presence of a novel stimulus, for instance an unfamiliar rat, and there was no change in locomotion in their home cages.

Orexin neurons also seem to influence general, non-food-related, reward behaviour and anxiety. Orexin, when icv injected into rats, seems to negatively regulate brain reward circuitry, since it increased the threshold for self-stimulation and reinstated cocaine seeking (Boutrel et al. 2005). Panic disorder in humans is accompanied by elevated orexin levels in cerebrospinal fluid, and in rats the panic-prone state can be avoided by orexin gene silencing or administration of orexin antagonists (Johnson et al. 2010). This could indicate that high orexin activity goes hand in hand with high arousal that might lead to a stress like state. Furthermore, fibre photometry recordings have shown that orexin neurons are quickly and strongly activated by stressors like air puffs and immobilisation (González et al. 2016), supporting the idea of orexin neuron activity responds to external stress factors.
1.5 MCH neurons

1.5.1 Intrinsic properties of MCH neurons

The prepro-MCH precursor peptide yields not only MCH but also neuropeptide EI (NEI) and neuropeptide GE (NGE) (Nahon et al. 1989). NGE and NEI might mimic alpha-melanocyte-stimulating hormone (alpha-MSH) actions on melanocortin receptors at high concentrations (Hintermann et al. 2001), but their physiological function is still elusive. So far, NEI seems to be able to affect binding at the dopamine D1 receptor (Sanchez et al. 2001) and has an effect specifically on cholinergic afferents to dopaminergic cells (Berberian et al. 2002).

MCH has two different GPCRs, with MCHR1 occurring in all mammals and MCHR2 occurring in humans, primates, dogs and ferrets, but not rodents (Tan et al. 2002). MCH1R binds to Gi/q whereas MCH2R only binds Gq (Chung et al. 2009). MCH neurons are thought of as mainly having an inhibitory effect, the opposite of the neuroexcitatory effect of orexin neurons. Inhibition can occur either via MCH1R (Wu et al. 2009) or the fast transmitter GABA (Cid-Pellicer and Jones 2012; Jego et al. 2013). GABA release by MCH neurons has been shown in vitro at the tuberomammillary MCH neuron to histamine neuron synapse, where MCH is speculated to have only a presynaptic effect to facilitate GABAergic transmission. Furthermore, this MCH neuron projection to histaminergic neurons has been shown to prolong and maintain REM sleep in vivo when MCH neurons are optogenetically activated (Jego et al. 2013). The same effect has been observed in MCH gene ablated mice, indicating that non-MCH transmitters like GABA are responsible for the effect on REM sleep. MCH neurons express also other transmitters like nestafin, cocaine- and amphetamine-regulated transcript (CART) and neuronal pentraxin-1 (NP1) (Reti et al. 2002; Elias et al. 2001) which might play important roles in glucose tolerance. A comparison of MCH neuron KO mice with MCH gene ablated mice showed that a loss of the MCH neurons but not the MCH neuropeptide only led to an improved glucose tolerance (Whidden and Palmier 2013), implying that the loss of transmitters not transcribed by the MCH gene, such as nestafin and CART, is the underlying cause of the improved glucose tolerance. In addition to this, it still has to be shown that MCH neurons actually release MCH with a postsynaptic effect.
MCH neurons, as orexin neurons, can be divided into two biophysical classes (Hausen et al. 2016). They are silent (not firing at resting potential) or spontaneously active, expressing a hyperpolarisation activated outward rectifier. Most of the MCH neurons (62%), however, are silent.

The projections of MCH neurons mostly overlap with those of orexin neurons, and include most of the brain areas besides the cerebellum (Bittencourt et al. 1992). The overlap of orexin and MCH projections to reward areas like the nucleus accumbens (Georgescu et al. 2005) and the autonomic nervous system via the nucleus solitarius (Kerman et al. 2007; Oldfield et al. 2007), could enable them to have physiological antagonistic roles in most aspects considered. Interestingly, there is also a vast symmetry between inputs going to MCH and orexin neurons (Gonzávez, et al. 2016), with the highest number of inputs coming from the hypothalamus itself. MCH neurons also receive strong direct GABAergic inputs from VGAT neurons in the amygdala and bed nucleus of the stria terminalis, both areas are implicated in stress and anxiety.

1.5.2 MCH neurons as internal sensors

A physiological increase in extracellular glucose concentration excites MCH neurons \textit{in vitro}, showing the opposite response of orexin cells (Burdakov et a. 2005). The glucose response of MCH neurons is mediated via a β-cell like mechanism of a glucose dependent closure of Kir6.2/SUR1 containing ATP-inhibited K+ (K-ATP) channel (Kong et al. 2010). It was suggested that MCH neuronal activity was necessary for evaluation of different glucose-containing food choices (Domingos et al. 2013), since preferences of sucrose over sucralse are lost when MCH neurons are ablated. Based on increased dopamine release upon optogenetic stimulation of MCH neurons during ingestion of sucralse, it was suggested that MCH neuron activity communicates the nutrient value of sugar. However, this connection to dopamine neurons might also have a reciprocal effect. Dopamine can depress MCH neurons directly via α2-noradrenergic receptors and a complex dose-dependent indirect mechanism (Conductier et al. 2011). Low dopamine seems to act via
dopamine 1-like receptors activating GABAergic inputs and high dopamine acts via dopamine 2-like receptors inhibiting GABAergic inputs. As a result, feedback loops may exist between MCH neurons and dopamine neurons in reward centres.

Vasopressin and oxytocin selectively excite MCH neurons, but not other GAD65/67 neurons in the hypothalamus (Yao et al. 2012). Consequently, it has been postulated that vasopressin and oxytocin dependent effects on energy homeostasis, water intake, anxiety and stress might be exerted in part via MCH neurons. In addition to this, MCH neurons have also been shown to be directly excited by insulin, the body’s most prominent anabolic hormone, and to downregulate locomotion and insulin sensitivity in response (Hausen et al. 2016).

Other neuromodulators, that have been shown to have an inhibitory effect on MCH neurons (when bath applied), are monoamines of the arousal systems such as serotonin, noradrenalin and the neuropeptide NPY (van den Pol et al. 2004). This could indicate that there are projections from arousal centres, which have subsequently been confirmed with monosynaptic rabies tracing (González et al. 2016), that inhibit the sleep-promoting MCH neurons when arousal is increased. However, an arousal promoting local neurotransmitter, namely orexin, excites MCH neurons in vitro when bath applied, via a direct inward current that causes the otherwise silent MCH neurons to spike frequently. Since the two neuronal types seem to have opposite physiological functions, it does not seem to make sense for one to excite the other. Opposing the finding that orexin excites MCH neurons, a recent study (Apergis-Schoute et al. 2015) re-examined the effect of orexin on MCH neurons, and found that orexin peptide-mediated excitation only occurs in a minority of MCH neurons when bath applied. Moreover, in vitro optogenetic activation of orexin neurons led to a GABA\textsubscript{A} dependent inhibition of MCH neurons. This could mean that a minority of MCH neurons can be excited directly by orexin but the majority is inhibited in an indirect way via GABA. Nevertheless, it is not clear what the identity of these GABAergic neurons is, that inhibit MCH neurons.
1.5.3 MCH neurons and feeding, body weight and metabolism

Besides being sleep promoting (see section 1.5.4 below), MCH neuron activity also affects body weight and feeding. Again, a rather opposite effect to that of orexin is observed. MCH gene KO mice are reported to be lean and hypophagic with a decrease in bodyweight and fat mass, due to an increase in energy expenditure and decrease in food intake (Shimada et al. 1998). However, after this first report there seem to be some controversies in reported effects of MCH neurons function. If the MCHR1, the only MCH receptor in rodents, was deleted instead of the MCH gene, mice were lean, hyperactive and hypermetabolic as reported for MCH KO mice, but hyperphagic (Marsh et al. 2002; Chen et al. 2002). An ablation of 98% of MCH neurons via diphtheria toxin receptors (DTR) targeted to MCH neurons, confirmed the lean and hyperactive phenotype, but found normal food intake (Whiddon and Palmiter 2013). The advantage of using diphtheria toxin to ablate neurons is that it can be used in adult mice allowing normal development of brain circuitry without compensatory mechanisms that might occur in KOs. A recent meta-analysis tried to make sense of these different findings, and came to a conclusion (Takase et al. 2014): loss or disruption of MCH neuron signalling leads to higher food intake, lower body weight and fat mass and elevated body temperature with higher oxygen consumption and heart rate. Alternatively, comparing the different MCH neuron signalling manipulations and their differences in feeding behaviour might also help to speculate about the different roles of transmitters expressed. Comparing the phenotype of the MCH gene KO with the MCHR1 KO, the only difference lies in the hyperphagia in the MCHR1 KO which could potentially be due to NGE and NEI still being expressed and promoting feeding. Consequently, hypophagia in MCH gene KOs could be caused by the lack of the mixture of feeding inhibiting MCH and strongly feeding promoting NGE and NEI.

Overall, MCH neuron activity seems to play an important role in appetite, metabolism, obesity and production of fat mass, especially white adipose tissue (Imbernon et al. 2013). Due to its ability to communicate the nutrient value of sugar (see section 1.5.2), it was suggested that MCH neurons are involved in predictive control of stable blood glucose levels by influencing learning associations between
fast sensory information about food and their nutritious value (Kosse and Burdakov 2014).

1.5.4 MCH neurons and sleep

The function of MCH neuron activity is often described as physiologically antagonistic to orexin neurons (see Figure 1.3). Based on c-fos expression experiments and single unit recordings (Hassani et al. 2009), it was found that MCH neurons are indeed mainly active during sleep. icv administration of MCH also increased the amount of REM and non-REM sleep in a dose-dependent manner (Verret et al. 2003). Moreover, single unit recordings in head-fixed rats (Hassani et al. 2009) provide information that MCH neurons fire occasionally during non-REM sleep, but maximally during REM sleep. Therefore, MCH neurons fire in a reciprocal activity pattern to orexin cells. However, one of the caveats of this study is that cells for recordings were mainly chosen based on their firing during sleep, thus potentially biasing against sleep-silent neurons.

A causal relationship between MCH neuron activity and non-REM sleep was attempted to be demonstrated with selective optogenetic stimulation of MCH neurons. Light stimulation every 5 min for 24 hours hastened onset of sleep and increased non-REM and REM sleep length (Konadhode et al. 2013). A different study focused on the role of MCH neurons for REM sleep (Jego et al. 2013). Stimulation at the onset of REM sleep exclusively extended the length of REM, but not non-REM sleep. In addition to this, the use of archaerhodopsin as a silencer of MCH neurons had no effect on REM sleep length. Consequently, MCH neuron activity seems sufficient to increase sleep, REM and non-REM, but it is not essential for REM sleep.

1.5.5 MCH neurons and other awake behaviours

Interestingly, there seems to be one kind of behaviour where orexin and MCH neurons may have similar effects (Figure 1.3). Disruption of MCH neuron signalling through administration of MCHR1 antagonists (Borowsky et al. 2002) or MCHR1 ablation (Roy et al. 2006) seems to be anxiolytic in animal models. This seems to be
the same effect that orexin cell silencing and orexin antagonists produce to avoid the panic prone state (Johnson et al. 2010). Whereas, MCH administration (icv) increased anxiety-like behaviour in the elevated-plus maze (Smith et al. 2006).

Figure 1.3: Summary of the properties of MCH and orexin neurons

Blue arrows indicate inputs and modulators that orexin/MCH neurons respond to, orange arrows indicate projections and neurotransmitters that are expressed, beige arrows indicate somatic effects of orexin/MCH neuron activity.

Still, there is one persistent problem with manipulation of MCH or its receptor: it does not include fast neurotransmitters and other neuropeptides that might, under physiological circumstances, be co-released with MCH. Whilst MCH administration in the ventromedial nucleus stimulates sexual activity in female rats, this could be partially antagonised by NEI (Gonzalez et al. 1998). However, the interactions between different neuropeptides expressed by MCH neurons seem to be complex, as NEI increases grooming, rearing and locomotion when administered icv (Sanchez et al. 1997) or into the ventral tegmental area (Sánchez et al. 2001), but MCH has no effect on these behaviours and even antagonises and annihils the effects of NEI when co-administered. Interestingly, chemogenetic activation of MCH neurons with activating DREADDs (aDREADDs) decreases locomotion (Hausen et al. 2016), which is contrary to the increase of locomotion seen with NEI administration. There are two possible interpretations of this. First, fast neurotransmitters or neuropeptides
other than NEI have an inhibitory effect on locomotion which outweighs any locomotion-promoting effect that NEI might have. Second, there are slight differences in the behaviours that are measured between the different studies. Whilst, NEI induced activity was measured in a novel environment and has more the characteristics of investigative movements towards the environment (rearing and physical activity), the decrease of locomotion after chemogenetic activation of MCH neurons was measured in a familiar home cage lacking incentives for investigation. Therefore, a decrease in locomotion does not have to be equivalent to a decrease in investigative behaviour (Leyland et al. 1976).

MCH neurons have also been implicated in learning, especially in respect to food choices, and memory. Feeding can be seen as a process which requires predictive control and predictive control requires learning. An association has to be formed between a neutral but easily measurable parameter, such as location, shape or smell (‘conditioned stimulus’ in psychology) and the actual nutrient value of the food (‘unconditioned stimulus’) which reaches the brain with a long delay as nutrients first need to be extracted by the gut. Subsequently, detection of a conditioned stimulus, sight or taste, can be used for efficient (i.e. rapid) action selection and for preparing the body for subsequent arrival of glucose or other nutrients to blood. Recent experimental evidence suggests that MCH neurones are a key part of such associative learning. For example, Sherwood et al. found that genetic or pharmacological disruption of the MCH-1R reduced the ability of a previously learnt conditioned stimulus to serve as a reinforcer in a new instrumental behaviour task (Sherwood et al. 2012). This raises the possibility that MCH signalling may be important for the response transfer in dopamine neurones (Schultz 2006), which shift their activity from occurrence of the reward to the earliest conditioned stimulus, while the stimuli closer to the reward (e.g. taste) serve as conditioned reinforcers for earlier occurring stimuli (e.g. colour, shape of food). This would enable formation of associative memories, which is a prerequisite for good predictive control. Among projection targets of MCH neurones are also several memory-related structures, including the hippocampus and septum (Adamantidis and de Lecea 2009; Jego et al. 2013), where activation of MCH receptors may promote synaptic plasticity. Ablation of MCHR1 leads to postsynaptic down regulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-d-aspartate (NMDA) receptors in the
hippocampus and impaired long-term synaptic plasticity (Pachoud et al. 2010). Thus, providing a mechanism how MCH neurons could be implicated in the formation of memory. Moreover, mice with MCHR1 ablation also showed an impairment at the passive avoidance test, which is a hippocampus dependent memory task (Adamantidis et al. 2005).

A recent study using optogenetic stimulation of MCH neurons, which has the advantage of manipulating the neuron with all of its neurotransmitters, suggested that MCH neurons might signal the nutrient value of sugars (Domingos et al. 2013). However, there are some issues with this study that prevent this to be the only possible conclusion. Whilst increased MCH neuron activity was sufficient to shift the mouse’s preference form nutrient-containing sucrose to a sucralose solution without any nutrients, it has neither been shown that MCH neurons are essential for the signalling of nutrients, nor was increased MCH neuron activity sufficient to shift the preference to flavourless water. Considering that sweet taste was still required to result in a consumption preference when paired with MCH neuron stimulation, this might mean that MCH neurons play a role in the process of learning the association of taste with nutrient content. In the case of consumption of a sucrose solution, nutrients are sensed by the brain with a delay from experiencing its taste, thus future decisions about its consumption will involve a degree of learned associative nutrient value.

### 1.6 GABAergic non-MCH non-orexin neurons

Juxtacellular recordings in head-fixed rats have found GABAergic non-MCH non-orexin neurons in the lateral hypothalamus are maximally active during sleep, and especially REM sleep (Hassani et al. 2010). However, these ‘sleep-max’ neurons only accounted for 53% of the non-MCH non-orexin neurons. What the activity pattern of the remaining 47% is unclear. Furthermore, they could only confirm the GABAergic nature of two ‘non-REM max’ neurons and eight ‘REM max’ neurons, which is overall a small sample size, considering that they recorded from 104 cells. Nonetheless, GABA in the LH seems to be important for sleep. GABA-A receptor antagonists, administered into the perifornical area, decrease the amount of sleep during the light phase and increase c-fos expression in orexin neurons. This
suggests, that there is an increased endogenous GABAergic inhibition on orexin neurons during sleep (Alam et al. 2005). However, as MCH neurons can be GABAergic as well, it is not clear if the source of inhibition are separate, non-MCH GABAergic neurons or MCH neurons.

As there are several classes of GABAergic non-MCH non-orexin neuron in the LH with different additional markers and physiological roles, this section serves to give a brief overview.

1.6.1 NPY neurons

NPY neurons in the LH are activated by hunger \textit{in vivo} and inhibited by increased concentrations of glucose \textit{in vitro} (Marston et al. 2011). Even though other NPY neurons, like those co-expressing agouti-related peptide (Agrp) neurons in the arcuate nucleus (Hahn et al. 1998), have well described phenotypes, in the LH the function of NPY cells is not further described.

1.6.2 LepRb neurons

Whilst LepRb neurons are not restricted to the LH, they seem to exert much of their anorectic effect via the LH (Leinninger 2011). LepRb neurons in the LH, which are GABAergic and express GAD67, seem to influence body weight and feeding as intra-LH administration of leptin leads to a decrease in both (Leinninger et al. 2009). Since leptin can have inhibitory and excitatory effects, it is not clear what the underlying mechanism of leptin action in the LH is. However, it is thought that LepRb neurons have direct and indirect effects on orexin neurons, resulting in orexin neuron inhibition (Leinninger 2011; Louis et al. 2010; Leinninger et al. 2009).

A fraction of LepRb neurons in the LH also express neurotensin, which made it possible to create a neurotensin dependent LepRb knockout mouse that has LepRb loss restricted to the LH. These mice showed early onset obesity and a modestly increased feeding behaviour with decreased locomotor activity, which supports the idea of leptin as hunger-suppressing (Leinninger et al. 2011). Interestingly though,
Translating Ribosome Affinity Purification analyzed by RNA-seq (TRAP-seq) data (Allison et al. 2015) has shown that LepRb neurons in the hypothalamus express many markers for neuropeptides and that neurotensin is only expressed in a fraction of them.

### 1.6.3 VGAT neurons in the LH

VGAT neurons in the LH have been investigated intensely in the recent years. Surprisingly, there is no overlap between VGAT neuron and MCH or orexin neurons in the LH (Jennings et al. 2015) but it is not known what or if there is any connectivity between those neurons. Furthermore, it is possible that VGAT$_{LH}$ neurons are overlapping with some other markers like NPY or LepRb.

VGAT$_{LH}$ neurons drive appetitive and consummatory behaviour when optogenetically activated (Jennings et al. 2015). Furthermore, ablation of VGAT$_{LH}$ neurons shows a decrease in those behaviours, suggesting that VGAT$_{LH}$ neurons are essential for feeding and appetitive behaviours. Interestingly, *in vivo* calcium imaging of these neurons shows that neurons are either active during appetitive or consummatory behaviour but not both, indicating that this group of neurons is not homogenous in their function (Jennings et al. 2015). It is worth mentioning that VGAT$_{LH}$ neuron driven consummatory behaviour is not restricted to calorific food but can also be directed towards non calorific objects (Navarro et al. 2016).

Studies investigating the projections of VGAT$_{LH}$ neurons to the ventral tegmental area (VTA) found, that these projections can drive feeding behaviour but not compulsive sucrose-seeking (Nieh et al. 2015), indicating that there must be other projections from the LH to the VTA responsible for compulsive sucrose seeking. In addition to this, optogenetic activation of VGAT$_{LH}$ projections to the VTA increased dopamine release in the nucleus accumbens and promoted ‘behavioural activation’ (Nieh et al. 2016). Thus, stimulation of VTA projecting VGAT$_{LH}$ neurons can also drive behaviours outside the feeding context, such as novel object and conspecies investigation, and supports positive reinforcement as was shown in a real time place preference test.
One caveat with targeting VGAT neurons in the LH is that it is technically challenging to restrict injection sites to the LH without having any spill-over to the zona incerta where VGAT neurons show a very similar and strong feeding-promoting phenotype (Zhang and van den Pol 2017).

1.6.4 GAD65 neurons in the LH

GAD65 neurons in the LH have been extensively characterised in vitro (Karnani et al. 2011). They are partially (40%) inhibited by increases in external glucose concentrations and can be clustered into 4 different classes depending on their electrophysiological profiles (fast spiking, late spiking, low threshold spiking and regular spiking). Compared to cortical GABAergic neurons these classes are very similar with the exception that in the LH GAD65 neurons are intrinsically active. However, not much is known about the role of GAD65<sub>LH</sub> neuron activity in behaviour or their overlap with above described cell types. Moreover, it is also still unclear what the effect of GAD65 neuron activity is on other LH neurons and if there are any intra-LH circuitries.

1.7 Can silencing of genetically identifiable populations recapitulate the results of electrolytic LH lesions?

Considering the drastic anorectic and apathetic phenotype seen with electrolytic lesion of the LH, one might wonder if this effect can be accounted for by one of the genetically identifiable population of the LH. The reduction in feeding in MCH ablated mice (via DTR) is rather mild and cannot compare to the change seen with electrolytic lesion (Whiddon and Palmiter 2013). Orexin neurons, in contrast, suppress feeding and thus their ablation with DTR leads to an increase in feeding by mainly changing the temporal feeding pattern (González et al. 2016). VGAT cells in the LH, however, might account for the feeding drive of the LH as their activation leads to consummatory behaviour and their ablation with caspase decreases food intake (Jennings et al. 2015). Still, even though weight gain is attenuated, mice lacking VGAT<sub>LH</sub> neurons do not lose body weight at any point. Consequently, genetically
identifiable cell populations, in particular VGAT<sub>LH</sub> neurons, might be able to account for the lack of feeding seen in LH lesioned animals, but no cell type can account for the drastic weight loss yet.

Considering the locomotor impairments that animals with LH electrolytic lesions display, it is even more complicated to compare this phenotype to effects of ablations of genetically identifiable populations, as some of their activation seems to actually inhibit movement, namely MCH neurons. Mice with DTR ablation of MCH neurons (Whiddon and Palmiter 2013) are hyperactive, especially during a fast, which has also been shown with MCH gene deletion (Shimada et al. 1998). In contrast, orexin neuron ablation (ataxin-3) impairs locomotion but only slightly and not to the extent seen with electrolytic lesion (Hara et al. 2001). VGAT neuron manipulations in the LH do not cause any changes in locomotor activity (Navarro et al. 2016; Jennings et al. 2015). Overall, the small effect of these individual neuron population ablations on locomotion leads to the question if there is another LH neuron population that could account for the impairment of locomotor activity in LH lesioned animals.

1.8 Aims of this thesis

This thesis aims to answer and elucidate the currently-unknown functional and molecular properties of GAD65 neurons in the LH. Even though some initial in vitro characterisation previously described the biophysical properties of GAD65<sub>LH</sub> neurons, it is still unclear what their firing responses to injections of oscillatory current inputs are, and how these responses compare to other LH neurons. This is an especially interesting aspect, if one considers the effect of gamma oscillations in the LH in vivo, where they cause food approach behaviour. Furthermore, it is still unknown how GAD65<sub>LH</sub> neurons relate to other GABAergic non-MCH non-orexin LH neurons, and if there are any overlaps with them. This leads to the general question of how neurons in the LH are organised, and if they are synaptically connected via GAD65<sub>LH</sub> neurons. In addition to this, it is not known if and how GAD65<sub>LH</sub> neurons affect behaviour, and if GAD65<sub>LH</sub> neuron silencing could recapitulate any of the LH lesion effects. In order to answer these questions, this thesis will pursue the following specific aims:
First, intrinsic properties of GAD65\textsubscript{LH} neurons including projection areas, overlap with other known GABAergic non-MCH non-orexin cell types, and firing responses to injections of oscillatory input currents, will be investigated. This will enable a comparison of GAD65\textsubscript{LH} neurons to known LH cell types, and the use of GAD65 as a marker for a previously uncharacterised distinct cell population.

Second, LH network functional connectivity involving GAD65\textsubscript{LH} neurons will be investigated to probe what kind of neurotransmitters are released by GAD65\textsubscript{LH} neurons and onto what neurons, and in addition to this, potential neuronal inputs to GAD65\textsubscript{LH} cells and their responsiveness to neuropeptides will be investigated. These results will give new information about a potential local LH network, and if different LH cell types are interconnected via GAD65\textsubscript{LH} neurons.

Third, it will be investigated what the behavioural impact of GAD65\textsubscript{LH} neuron activity is with respect to: mouse body weight, food, water intake and locomotor activity. This will show any potential necessity or sufficiency of GAD65\textsubscript{LH} neuron activity for vital behaviours, and allow to compare and contrast them with known LH cell types.

Altogether, these experiments should result in a comprehensive study into the function and role of GAD65\textsubscript{LH} neurons and their involvement in a local circuitry of previously characterised classes of neurons.
Chapter 2. Materials & Methods

2.1 Reagents

All chemicals were from Sigma-Aldrich, ThermoFisher Scientific Inc. or Tocris Bioscience unless stated otherwise.

2.2 Solutions and concentrations

For brain slice recordings, artificial cerebrospinal fluid (ACSF) and ice cold slicing solution were gassed with 95% O₂ and 5% CO₂, and contained the following (in mM)

ACSF: 125 NaCl, 2.5 KCl, 1 MgCl₂, 2 CaCl₂, 1.2 NaH₂PO₄, 21 NaHCO₃, 2 D-(-)-glucose, 0.1 Na+-pyruvate, and 0.4 ascorbic acid. Slicing solution: 2.5 KCl, 1.3 NaH₂PO₄.H₂O, 26.0 NaHCO₃, 213.3 Sucrose, 10.0 D-(+)-glucose, 2.0 MgCl₂, 2.0 CaCl₂ (Schöne et al. 2012).

For standard whole-cell recordings, pipettes were filled with intracellular solution containing the following (in mM): 120 K-gluconate, 10 KCl, 10 HEPES, 0.1 EGTA, 4 K₂ATP, 2 Na₂ATP, 0.3 Na₂GTP, 2 MgCl₂, pH 7.3 with KOH.

Liquid junction potential were estimated to be 10.1mV and were subtracted from voltage clamp measurements.

Concentrations used for in vitro recordings (bath applied): Orexin-A: 300 nM; Clozapine-n-oxide (CNO): 5 µM; synaptic blocker mix: D-AP5 50µM, PicROTOXIN 10 µM, CNQX 10 µM, CGP-35348 10 µM; Gabazine: 3 µM.

Concentrations for in vivo intraperitoneal (i.p.) injections: clozapine n-oxide (CNO) 5 mg/kg (experiments involving hM4Di) or at 0.5 mg/kg (experiments involving hM3Dq), SB-334867 (in vehicle, 0.9% NaCl 10% DMSO 0.3% hydroxypropyl-β-cyclodextrin). Cryoprotectant for storage of brain slices at -20°C: 30% Glycerol, 30% Ethylene Glycerol, 30% dH₂O and 10% Phosphate buffer.
2.3 Animals

2.3.1 Transgenic mice and breeding

All procedures followed United Kingdom Home Office regulations and were approved by local welfare committees. Adult male and female mice (at least 8 weeks old) were used for *in vitro* experiments. Male mice were used for behavioural experiments and single housed. All mice were kept on a standard 12 h light-dark cycle in a temperature regulated room and had free access to standard mouse chow and water. Previously characterised and validated transgenic mouse lines were used, where indicated (see Table 1 for breeding strategy and references).

### Table 1. Mouse lines and breeding strategies

<table>
<thead>
<tr>
<th>Mouse lines and breeding strategies</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAD65-Ires-Cre mice</td>
<td>bred in hom-WT pairs with C57BL/6 mice</td>
<td>(Taniguchi et al. 2011)</td>
</tr>
<tr>
<td>Orexin-eGFP mice</td>
<td>bred in het-WT pairs with C57BL/6 mice</td>
<td>(Burdakov et al. 2006)</td>
</tr>
<tr>
<td>GAD65-GFP mice</td>
<td>bred in het-WT pairs with C57BL/6 mice</td>
<td>(Karnani et al. 2013)</td>
</tr>
<tr>
<td>Orexin-Cre mice</td>
<td>bred in het-WT pairs with C57BL/6 mice</td>
<td>(Matsuki et al. 2009)</td>
</tr>
<tr>
<td>MCH-Cre mice</td>
<td>bred in het-WT pairs with C57BL/6 mice</td>
<td>(Kong et al. 2010)</td>
</tr>
<tr>
<td>NPY-hrGFP mice</td>
<td>bred in het-WT pairs with C57BL/6 mice</td>
<td>(Pol et al. 2009)</td>
</tr>
<tr>
<td>VGAT-Ires-Cre mice</td>
<td>bred in hom-WT pairs with C57BL/6 mice</td>
<td>(Vong et al. 2011)</td>
</tr>
<tr>
<td>CAG-tdTomato mice</td>
<td>bred in hom-hom pairs</td>
<td>(Madisen et al. 2010)</td>
</tr>
</tbody>
</table>

Orexin-Cre and GAD65-GFP line were crossed in Figure 4.2. GAD65-Ires-Cre and NPY-GFP line were crossed in Figure 4.6. Orexin-eGFP and GAD65-GFP lines were crossed in Figure 4.7. MCH-Cre and GAD65-GFP line were crossed in Figure 4.4. VGAT-Ires-Cre and GAD65-GFP line were crossed in Figure 4.3. To investigate the overlap of VGAT and GAD65 neurons, VGAT-Cre and GAD65-GFP lines were crossed and then injected with ChR2-mCherry or crossed with a CAG-tdTomato line (Figure 3.8). For the overlap of GAD65 and NPY neurons, GAD65-Ires-Cre and NPY-GFP lines were crossed and injected with ChR2-mCherry in Fig. 2 and 3.
2.3.2 Gene transfer and viruses

Mice were anaesthetised with isoflurane and injected with Meloxicam (2mg per kg bodyweight, subcutaneous) for analgesia. After placing into a stereotaxic frame (David Kopf Instruments), a craniotomy was performed and a boroscillate glass pipette was used to inject viral vectors (see Table 2 for details and references) into the lateral hypothalamus bilaterally with pressure (coordinates AP/DV/ML = −1.3 / −5.15 to −5.25 / 1.0, -1.0 mm; infusion speed = 75 nl/min, injection volume 75 nl), see Figure 2.1 for examples of virus spread and expression. Wound closure was done by suturing. Mice were allowed to recover for at least one week after surgery whilst single housed.

Table 2. Viruses and their used concentrations

<table>
<thead>
<tr>
<th>Description</th>
<th>Virus Type</th>
<th>Concentration (gc/ml)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cre-dependent iDREADD</td>
<td>rAAV8/hSvn-DIO-hm3D(Gq)-mCherry</td>
<td>2.2x10^12</td>
<td>UNC Vector Core (Armbruster et al. 2007)</td>
</tr>
<tr>
<td>Cre-dependent aDREADD</td>
<td>rAAV8/hSyn-DIO-hM4(Gi)-mCherry</td>
<td>5.3x10^12</td>
<td>UNC Vector Core (Krashes et al. 2011)</td>
</tr>
<tr>
<td>Cre-dependent ChR2-mCherry</td>
<td>AAV1.EF1.flox.hChR2(H134R)-mCherry.WPRE.hGH</td>
<td>8.78*10^12</td>
<td>UNC Vector Core (Atasoy et al. 2008)</td>
</tr>
<tr>
<td>Cre-dependent ChR2-YFP</td>
<td>AAV1.EF1.DIO.hChR2(H134R)-EYFP.WPRE.hGH</td>
<td>6.2*10^12</td>
<td>UNC Vector Core (González, Iordanidou, et al. 2016)</td>
</tr>
<tr>
<td>MCH-dependent mCherry</td>
<td>VSVG.HIV.MCH.mCherry(p2428)</td>
<td>3.16*10^11</td>
<td>UPenn Vector Core (Apergis-Schoute et al. 2015)</td>
</tr>
<tr>
<td>Cre-dependent GCaMP</td>
<td>AAV9.CAG.Flex.GCaMP6s.WPRE.E.SV40</td>
<td>2.74*10^13</td>
<td>UPenn Vector Core (T.-W. Chen et al. 2013)</td>
</tr>
</tbody>
</table>

Cre-dependent DREADD constructs were used to measure the effect of neuronal activation and inhibition respectively on eating, drinking, and locomotion. For channelrhodopsin-assisted circuit mapping, “FLEX switch” channelrhodopsin-2 (ChR2) constructs were used. To label MCH neurons for patch-clamp recordings, we
injected into the LH a custom made lentiviral vector specifically targeting MCH neurons. For calcium imaging, we used GCaMP6s constructs. To investigate long ranging projections the same ChR2-YFP virus was used as for circuit mapping and ChR-mCherry and ChR2-YFP were also used to characterise overlapping cell populations.

![Image of sliced tissue](image)

**Figure 2.1: Example slices of GAD65-Ires-Cre mice injected with Cre-dependent DREADD-mCherry**

Examples of coronal LH sections from four GAD65-Ires-Cre mice injected with Cre-dependent DREADD-mCherry, illustrating the typical extent of LH labelling.

### 2.4 Histochemistry

#### 2.4.1 Thick and thin slice immunohistochemistry

For fixation after patching, 250 µm thick brain slices containing the lateral hypothalamus were incubated in 4% PFA in PBS overnight. For thin sections of 30 and 50 µm, mice were transcardially perfused through the vascular system, first with PBS and then with 4% PFA in PBS. The extracted brains were incubated in 4% PFA overnight before being transferred to a 30% sucrose in PBS solution. Once the brains were saturated with sucrose (indicated by floating at the bottom), brains were frozen and embedded with an optimum cutting temperature compound. Brains were sliced with a cryostat and slices were subsequently stored at -20°C in cryoprotectant.
After washing with PBS, slices were either mounted straight away with Vectashield hard setting mount (with DAPI staining) or first stained for orexin with goat antibody to orexin-A (sc-8070, 1:2000, Santa Cruz Biotechnology) and for MCH with a rabbit antibody to MCH (H-070-47,1:2000, Phoenix Pharmaceuticals) as primary antibodies and Alexa 647–conjugated donkey antibody to rabbit IgG (A-21424, 1:500, Invitrogen) and Cy3-conjugated AffiniPure F(ab')2 Fragment donkey anti-goat IgG (705-166-147, 1:500, Jackson Immunoresearch) for secondary antibodies. As a check for antibody specificity, we extensively examined extra-hypothalamic areas, where no labelled cell-bodies were observed as expected, because neurons expressing the peptides we stained for are found exclusively in the LH. Slices were then imaged with an Olympus VS120 slide scanner or an Olympus BX61WI laser scanning confocal microscope.

### 2.4.2 Antigen retrieval and pSTAT immunohistochemistry

To identify cells responsive to leptin, a staining for phosphorylated STAT3 (pSTAT3), a transcription factor activate by leptin, was performed by antigen retrieval and immunohistochemistry. GAD65-GFP mice were injected (i.p.) with leptin (5mg/kg) prior to transcardial perfusion and 30µm cryosections were pretreated with: 3% H₂O₂ and 1% NaOH for 20min, 0.3% glycine for 10 min and 0.03% sodium dodecyl sulfate for 10min. Staining for pSTAT3 was done with rabbit anti-pSTAT3 IgG (#9131,1:500, Cell Signaling Technology) sera overnight as primary antibody and Alexa 555 conjugated-donkey to rabbit IgG (A-31572,1:500, Life Technologies) as secondary (Xu et al. 2011). GAD65-GFP littermates injected with saline were used as controls. Slices were then imaged with an Olympus VS120 slide scanner or an Olympus BX61WI laser scanning confocal microscope.

### 2.5 Brain slice optogenetics, electrophysiology and imaging

#### 2.5.1 Brain slice preparation and in vitro recordings

Standard whole-cell slice patch-clamp recordings were carried out alone or in combination with optical excitation of ChR2 containing cells and axons (Schöne et
al. 2014). LH slices were prepared at least 2 months after the virus injection to ensure expression in cell bodies and projections. After gluing a block of brain with cyanoacrylate glue to the stage of a Campden Vibroslice, coronal brain slices of 250 µm thickness containing the LH were cut whilst immersed in ice cold slicing solution. Slices were incubated for 1h in ACSF at 35°C, then transferred to a submerged-type recording chamber.

Living neurons containing fluorescent markers were visualised in acute brain slices with an upright Olympus BX61WI microscope equipped with an oblique condenser and appropriate fluorescence filters. Excitation light for ChR2 was delivered from a LAMBDA DG-5 fast beam switcher (Sutter Instruments) with a xenon lamp and ET470/40 nm bandpass filter. A 40x 0.8NA objective was used to deliver flashes of blue light (∼10 mW/mm²) onto ChR2-containing axons around the recorded cell. Whole-cell recordings were carried out at 35 °C using an EPC-10 amplifier and PatchMaster software (HEKA Elektronik, Germany). Patch pipettes were manufactured from borosilicate glass, and their tip resistances were 4-6 MΩ when filled with K-gluconate solution (see above).

### 2.5.2 Calculation of equilibrium potentials

Theoretical equilibrium potentials \( E_x \) for known ion concentrations \([X]\) (see solutions) were calculated using the Nernst equation, with the gas constant \( R = 8.314472 \text{ J} \cdot \text{K}^{-1} \), temperature \( T = 95^\circ \text{K} \), n = valence number of the ion and Faraday’s constant \( F = 9.65 \times 10^4 \text{ C} \cdot \text{mol}^{-1} \)

\[
E_x = \frac{RT}{nF} \ln \frac{[X]_{\text{out}}}{[X]_{\text{in}}}
\]

### 2.5.3 Channelrhodopsin-assisted circuit mapping

The ChR2 evoked currents of a repetition of 3 single light flash per neuron were averaged at each holding potential to give a single measurement. Linear regressions
were fitted through the averages of each cell type at the three holding potentials to estimate an equilibrium potential.

Functional ChR2 expression was confirmed by recording light-activated action potentials in the target cells as shown Figure 2.2 for GAD65\textsubscript{LH} neurons and cell-attached recordings confirmed a 2.11 ms delay from flash (1 ms) onset to peak current. Functional identity of optically evoked postsynaptic current was confirmed by pharmacological blockade with GABA or glutamate antagonists and/or by biophysical determination of reversal potentials; where no connection was observed, the connection was always probed further at a range of holding potentials.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Recordings of ChR2-expressing GAD65 neurons in the LH}
\end{figure}
2.5.4 Oscillatory input currents and calculation of membrane impedance

To determine the frequency preference for action potential firing, a protocol of 5 s long sinusoidal currents at the following fixed frequencies was applied: 0.5, 1, 2, 3, 5, 7, 10, 15, 20, 30, 50, 70, 100, 200 Hz. Membrane time constants ($\tau$) were calculated from fitting a single exponential function to the initial part of a voltage response to a small hyperpolarising current pulse. Input resistances ($R_i$) were derived from Ohm's law by fitting a linear function to the current-voltage relationship of voltage responses to hyperpolarising current pulses. From these values, the input frequency ($f$) -dependence of membrane impedance ($Z$) was calculated as follows (based on (Pike et al. 2000; Gutfreund et al. 1995)):

$$|Z| = \frac{R_i}{\sqrt{(\tau^2(2\pi f)^2 + 1)}}$$

2.5.5 In vitro calcium imaging

For calcium imaging, brain slices were placed in a recording chamber of BX61WI Olympus microscope controlled by the Olympus Fluoview software (FV10-ASW version 4.0), perfused at 35°C with ACSF. Confocal imaging was performed at 0.5 Hz frame-rate through an Olympus 20x 0.50 NA objective, with a 488 nm Argon laser excitation, and 500-545 nm spectral detector emission collection. Motion and bleach-corrections were applied if needed (StackReg plugin, Image J). A region of interest (ROI) containing each GCamp6s-positive neuron was selected via ROI manager in Image J. Mean fluorescence of each ROI was extracted for on each frame. These raw fluorescence values (Fr) were normalized to produce $\Delta F/F$ values, were F is the mean baseline before orexin application, and $\Delta F=(Fr-F)$. Cells were randomly sampled throughout the full anatomical extent of the LH, by choosing fluorescent cells using an objective that blinded the investigator to exact intra-LH location of the
cell due to its small field of view (a high-magnification x40 objective). After recording, the intra-LH locations of recorded cells were confirmed using a low-magnification large-field objective.

2.6 Measurements of movement, eating, and body weight

Locomotor activity was assessed after a single i.p. dose of CNO or saline during the dark phase in their open home cages via video tracking (Anymaze, Stoelting or Ethovision, Noldus). Mice of the same genotype were grouped according to vectors injected into their brains, and all groups had similar composition based on sex (males), age, and body weight.

For bodyweight and feeding measurements, mice were single-housed in cages (Tecniplast) fitted with solid floors or modified home cages. Whilst mice had free access to standard mouse chow and water, food and water intake and body weight of each mouse were measured manually each day before the onset of the dark phase. An adjustment period of at least a week in the metabolic cage preceded all experiments. Five days of baseline measurements (pre) were followed by five days of 58.3 µM CNO (clozapine n-oxide, Sigma) administration in the drinking water, and five more days of baseline measurements. 0.25 mM Saccharine (Sigma-Aldrich) was added to water throughout, to mask the taste of CNO. In Fig. S3C, mice were injected twice daily with 10 µl/g bodyweight of either saline (0.9% NaCl) or 0.5mg/kg body weight CNO dissolved in the saline, as indicated.

2.7 Statistical analysis

Statistical tests and descriptive statistics were performed as specified in the figure legends, with not-significant (ns) results marked as ns or with p-values given. Datasets were plotted with single values or averages and standard error of the mean (SEM). In each experimental dataset at the cellular level, each n was a different cell (no repeated trials from the same cell were used as n values) and cells from at least three mice were analysed. Before performing parametric tests, data were assessed for normality with a D’Agostino–Pearson omnibus test or Kolmogorov–Smirnov test.
for small sample sizes, and variances were assessed for homogeneity with a Brown–Forsyth test. To compare interactions within normally distributed data with repeated measurements, repeated measures ANOVA was used, with multiple comparison tests where appropriate. Analysis was performed with GraphPad Prism, MATLAB (The MathWorks, Inc.), and ImageJ.

Quantification of the preferred firing properties of LH neurons to oscillatory input currents were performed by normalising on a single cell basis by dividing by the largest value obtained per cell. Cells were deemed active if a paired t-test comparing normalized firing and impedance values was significant after controlling for the false discovery rate (which was set to 5%) by a two-stage step-up method of Benjamini, Krieger, and Yekutieli.
Chapter 3. Intrinsic properties of GAD65LH neurons

3.1 Introduction

GAD65LH neurons have been previously characterised as intrinsically active but otherwise having similar properties to GAD65 neurons in the cortex (Karnani et al. 2013). Adding to the electrophysiological characterisation of GAD65LH neurons by Karnani et al. 2013, the first aim of this chapter is to investigate the firing preferences of GAD65LH neurons in response to a wide range of frequencies of oscillatory current inputs and compare them to firing preferences of other LH neuronal types.

It is established that chemical compounds like neurotransmitters, hormones (e.g. ghrelin (Toshinai et al. 2003)), nutrients such as glucose (Williams et al. 2008; Burdakov et al. 2005), and gasses (Williams et al. 2007) can modify the activity of LH neurons in a cell-type specific manner. This chemical control is potentially essential for appropriate physiological regulation and avoidance of co-occurrence of contradictory drives. Apart from these chemical signals, many brain regions, including the LH, contain - and are controllable by - electrical oscillations (Buzsáki and Draguhn 2004; Gray and Singer 1989; Salinas and Sejnowski 2001; Carus-Cadavieco et al. 2017). Oscillations shape the synaptic inputs onto individual neurons, which collectively results in sinusoidal oscillations of current input at varying frequencies in neurons recorded intracellularly in vivo (Leung and Yim 1986; Soltesz and Deschênes 1993). Coherent network oscillations are thought to control brain states and behaviours in a frequency-dependent manner, for example, fast oscillations (gamma frequencies, ≈30–90 Hz) orchestrate arousal, memory, sensory processing, and decision-making (Buzsáki and Wang 2012; Cardin et al. 2009; Yamamoto et al. 2014; Colgin et al. 2009). In the LH, gamma oscillations, were recently found to be associated with food approach behaviour and differentially affect subthreshold membrane potentials of MCH and VGATLH cells (Carus-Cadavieco et al. 2017). Although, it remains unknown whether different oscillation frequencies differentially modulate the physiological output (action potential firing rate) of specific LH neuronal classes. Neurons control long-range targets by action potentials fired in response to input signals. Understanding how the firing rates of molecularly-defined
LH neurons respond to oscillatory input currents, may thus reveal a new dimension of LH output tuning and input-output information transfer.

A previous in vitro study, using GAD65-GFP mice, shows that GAD65 neurons are not overlapping with two major groups of LH neurons, namely orexin and MCH expressing cells (Karnani et al. 2013). However, in order to use GAD65 as a marker for further studies and to be able to compare them to previously characterised LH neuronal populations, it is necessary to investigate their neurochemical identity and potential overlap with other known groups of neurons further. Therefore, using GAD65-Ires-Cre and GAD65-GFP mice, the second aim of this chapter is to confirm this distinction with orexin and MCH expressing neurons and to extend it to other GABAergic non-MCH non-orexin classes of LH neurons.

The third aim of this chapter is to investigate if GAD65 neurons in the LH have extra-hypothalamic projections or if they are local interneurons, which is an important consideration for designing further experiments to probe the function of the LH network.
3.2 Gamma oscillations differentially control the firing of orexin and non-orexin neurons in the lateral hypothalamus

in vitro

Using experimental paradigms established for studying the effects of oscillations on neuronal firing in other brain regions (Pike et al. 2000), here we explore how the firing of individual, molecularly-defined LH neurons is modulated by the frequency of oscillatory current inputs.

To explore how different LH neurons respond to oscillatory inputs, we selectively targeted fluorescent reporters to LH orexin, VGAT, MCH, or GAD65 cells (see section 2.3), and recorded the membrane potential responses of individual genetically-defined LH cells to sinusoidal input currents at a broad range of physiological frequencies (0.5-200 Hz; Figure 3.1). To facilitate comparisons between neurons, and to previous studies of neuronal responses to oscillations in other brain areas (Pike et al. 2000), the recordings were performed at the membrane potentials close to threshold for spike generation. This was achieved by superposing an oscillatory current on the maximum step current that itself did not elicit spikes, and using a small (20 pA) peak-to-peak sinusoidal current (based on (Pike et al. 2000)).
Chapter 3. Intrinsic properties of GAD65LH neurons

Figure 3.1: Schematic and raw examples of oscillatory currents injected into LH neurons

(A) Overview of experimental strategy. Cell types were genetically tagged with a fluorophore to target patch-clamp recordings. During whole-cell recordings, 5s long oscillatory current at fixed frequencies were injected into the cells to obtain a profile of preferred input frequencies for maximal action potential firing. (B) Individual raw traces of single cells of the investigated cell types at 3 different input frequencies.

Low input frequencies (0.5–20 Hz) resulted in robust spiking activity in all LH neuronal types (Figure 3.1 and Figure 3.2). In contrast, higher frequencies selectively silenced orexin neurons (cessation of significant firing at inputs above 7 Hz, Fig. 9), while preserving significant firing in non-orexin cell types (Figure 3.2). These differences in frequency-preferences of LH neuron firing did not appear to be related to their maximal firing rates or spike-rate adaptation. Specifically, the firing of non-orexin neurons stayed relatively invariant across oscillation frequencies, irrespective of whether their maximal firing rates were fast (VGAT, GAD65 cells) or slow (MCH...
cells), and irrespective of whether their spike-rate adaptation was high, which is the case for MCH cells (Burdakov et al. 2005; van den Pol et al. 2004), or low which is the case for GAD65 cells (Karnani et al. 2013). In turn, orexin cell firing had higher frequency-dependent decay than non-orexin cell firing, even though their initial firing was faster than MCH cells but slower than VGAT or GAD65 cells (Figure 3.2), and their spike-rate adaptation was lower than that of MCH cells (Burdakov et al. 2005). Thus, there appears to be distinct frequency-bandwidths for optimal firing of orexin and non-orexin LH neurons, which cannot be accounted for by previously-studied differences in their intrinsic excitability.

Passive membrane properties differed between the cell classes (see Table 3), with VGAT neurons having the highest membrane resistance and MCH neurons having the lowest. Membrane resistance plays a role in the effect current injections have on membrane potential with high resistances leading to larger changes in membrane potential. Nevertheless, the membrane resistance would have the same effect across all injected current frequencies and thus could not account for differences of cell spiking at different frequencies. However, to adjust for different levels of spike frequency and effects that differences in membrane resistance might have spike rates were normalised in Figure 3.3. In addition to this, considering oscillatory current inputs as an analogue current instead of direct current, membrane impedances calculated from membrane resistances and time constants are more applicable and can be seen as an extended concept of membrane resistance.

### Table 3: Membrane resistances and time constants of molecularly-defined LH cell classes

<table>
<thead>
<tr>
<th></th>
<th>MCH</th>
<th>Orexin</th>
<th>GAD65</th>
<th>VGAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane resistance (MΩ)</td>
<td>474.8571± 54.2614, n=14</td>
<td>463.0385± 56.76324, n=13</td>
<td>619.2471± 49.09955, n=17</td>
<td>724.4438± 92.54686, n=16</td>
</tr>
<tr>
<td>Membrane time constant (ms)</td>
<td>40.60857± 6.530006, n=14</td>
<td>43.24915± 7.335909, n=13</td>
<td>32.42376± 4.217719, n=17</td>
<td>32.54188± 3.505608, n=16</td>
</tr>
</tbody>
</table>

Distinct frequency-dependencies of firing in orexin and non-orexin neurons could, in theory, emerge from distinct frequency-dependencies of the passive membrane
impedances (Pike et al. 2000). Higher membrane impedance would produce greater membrane potential fluctuations in response to oscillatory inputs and thus produce greater membrane excitation and firing (Pike et al. 2000). To investigate whether such passive membrane resonance could account for the differences in spike frequency preferences (Figure 3.2, red plots), we used our data to compute impedances of resistor–capacitor equivalent circuits at each input frequency for individual LH neurons (Figure 3.2, blue plots, see section 2.5.4 for calculations and Table 3 for values). Although maximum impedances differed between cell types (orexin = MCH < GAD65 < VGAT neurons, Figure 3.2), all impedances decayed similarly with input oscillation frequency, and this decay did not follow the associated frequency-tuning of firing (Figure 3.2, compare red and blue plots).

Figure 3.2: Effects of oscillations on molecularly-defined LH cell classes
Cell population average (± SEM of n= 14,13,17,16 cells) of spike rate (in red) and impedance magnitude of equivalent passive membranes averaged (± SEM of n= 14,13,17,16 cells) for each population of cells (in blue). Tuning bandwidths (in
purple) for each cell type were calculated as the frequencies at which the average spike rate was significantly different from 0 (one sample-t test) after correcting for multiple comparisons by controlling the false discovery rate (two-stage step-up method of Benjamini, Krieger and Yekutieli).

In order to compare the frequency-tuning of firing and impedances between different LH cell types, independently of differences of absolute values in these parameters, we normalized each neuron to its own maximal firing and impedance (Figure 3.3). Similar to raw data (Figure 3.2), this revealed that orexin cell firing decayed more steeply with oscillation frequency than that of non-orexin cells (Figure 3.3A; within the normalised data the decay was significantly different between orexin and VGAT or GAD65 cells, but not between orexin and MCH cells: Figure 3.3B). This difference between orexin and VGAT/GAD65 cells emerged sharply at >7 Hz and persisted at higher frequencies (Figure 3.3B). In contrast, there was an almost perfect overlap in
the frequency-dependence of normalized membrane impedances in the four LH cell types (Figure 3.3C).

Figure 3.3: Effects of oscillations on between LH cell class differences and their passive membrane equivalents

(A) Preferred input frequencies of different LH cell types. Cell population averages (± SEM of n= 14,13,17,16 cells) of spike rate normalised to the maximum rate per
We next investigated the differences between the impedance-predicted and experimentally-observed frequency-tuning of LH cell firing at the level of individual neurons. Within each molecularly-distinct class, individual neurons displayed similar frequency-tuning of impedance (as was the case also between classes, Figure 3.3C), but differed substantially in frequency-tuning of firing (Figure 3.4A). As the input oscillation frequency increased, the firing rate decay mirrored the impedance decay in some cells (Figure 3.4A, typical examples in right column), but strikingly deviated from impedance in other cells (Figure 3.4A, typical examples in middle column). By quantifying and analysing the difference between normalized impedance and firing in each cell (see section 2.7), we estimated, within each cell type, the percentage of cells that were tuned passively (i.e. firing tuning similar to impedance tuning) or actively (firing tuning significantly deviating from impedance tuning) (Figure 3.4A, left column). This revealed that within each cell type, the majority of cells were actively tuned, but some cell classes contained more “active” cells than others (MCH>Orexin>GAD65>VGAT, Figure 3.4A).

Finally, we analysed how “cell activeness” (the difference between observed and impedance-predicted firing) varies as a function of input oscillation frequency within each cell type (Figure 3.4B). Active tuning (significant difference between observed and impedance-predicted firing) was present in all cell types at low frequencies (< 1 Hz), where firing was lower than expected from impedance (Figure 3.4B). However, as input oscillation frequency increased, the frequency-dependence of orexin population firing became indistinguishable from the frequency-dependence of orexin cell impedance, with both sharply decaying as oscillation frequency increased (Figure 3.4B). In contrast, VGAT and GAD65 populations (and to a lesser extent the MCH population) maintained substantial firing in the gamma-fast frequency range (30-200 Hz, Figure 3.4B). Thus, orexin neuron firing is subject to steep impedance-
associated decay during gamma input, but non-orexin neurons resist this decay and maintain firing during gamma input.
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A

MCH

- Actively-tuned examples
- Passively-tuned examples

Orexin

- Actively-tuned examples
- Passively-tuned examples

GAD65

- Actively-tuned examples
- Passively-tuned examples

VGAT

- Actively-tuned examples
- Passively-tuned examples

B

Activeness of cell populations

- MCH
- Orx
- GAD65
- VGAT

p-value

Oscillation frequency (Hz)
Figure 3.4: Effects of oscillations on individual neurons within LH cell classes

(A) Left, pie charts depicting the percentage of actively-tuned cells (cells whose normalised spike frequency significantly differs from its normalised impedance magnitude), and passively-tuned cells (cells whose normalised spike frequency did not significantly differ from its normalised impedance magnitude). To group neurons into these categories, firing and impedance profiles of each individual cell were compared using a paired t-test with correction for multiple comparisons by controlling the false discovery rate (two-stage step-up method of Benjamini, Krieger and Yekutieli). Middle, examples of actively-tuned cells on each. Right, examples of passively-tuned cells on each. (B) Activeness of cell populations (statistical difference between normalized spike frequency and normalized impedance of each cell type, n= 14,13,17,16 cells, across input oscillation frequencies, n numbers for each cell types are as indicated in A. The y axis shows adjusted p-values from paired t-tests with correction for multiple comparisons by controlling the false discovery rate (two-stage step-up method of Benjamini, Krieger and Yekutieli).

In summary, our study demonstrates an unexpected way of controlling the firing of orexin versus non-orexin LH neurons. Such cell-type-specific LH control was previously thought to be achievable only by cell-type-selective chemical signals, but our results now show how a single, non-selective electrical input can unexpectedly create cell-type-specific effects on hypothalamic firing.
3.3 LH GAD65-GFP neurons are not co-localised with orexin, MCH, NPY and LepRb neurons

Using GAD65-Ires-Cre mice injected with a FLEX-GCaMP6s virus into the LH, we are able to confirm the finding that GAD65 LH neurons are distinct from MCH and orexin expressing neurons (Karnani et al. 2013) while testing the expression of the GCaMP6s calcium indicator for further experiments (see Figure 3.5). Slices (30µm) of these mice, which were stained for MCH or orexin, show no overlap with GAD65-GCaMP6s neurons. Note that this finding does not contradict previous reports of GAD67 expression in MCH cells, because GAD65 and GAD67 are not always co-expressed in the hypothalamus according to single-cell RNA studies (Romanov et al. 2017; Jeong et al. 2016).

![Figure 3.5: Co-localisation of GAD65 neurons in the LH with MCH and orexin neurons](image)

Examples of confocal images of (left) MCH and GAD65 neurons in close proximity but not overlapping and (right) orexin neurons and GAD65 neurons showing that both populations are found in the same location of the LH without overlap.

As GAD65 is an enzyme that catalyses the decarboxylation of glutamic acid to GABA, it was of great interest to see if GAD65 neurons would overlap with other known GABAergic populations like NPY (Marston et al. 2011) and LepRb neurons (Vong et al. 2011; Y. Xu et al. 2012) in the LH.

To label LepRb neurons, we stained for the phosphorylated form of Signal Transducer and Activator of Transcription 3 (pSTAT3), a downstream product of the JAK2-STAT3 pathway (Banks et al. 2000; Robertson et al. 2008). GAD65-GFP mice (n=3) were injected with an i.p. dose of (5mg/kg) leptin prior to a transcardial
perfusion, in order for LepRb to activate the JAK2-STAT3 pathway and to phosphorylate STAT3, which could then be detected and visualised after antigen retrieval in slices. For each mouse, three slices, containing both hemispheres, were counted for an overlap between GAD65-GFP and pSTAT positive cells in the LH. Of the 819 GAD65-GFP cells counted, less than 7% were also pSTAT positive (Figure 3.6). Control mice, injected with an equivalent dose of saline instead of leptin, showed only a minimal level of pSTAT staining, confirming the specificity of the protocol and antibody (Scott et al. 2009; L. Xu et al. 2011).

![Image](pSTAT_GAD65.png)

**Figure 3.6: Co-localisation of LH GAD65 and LepRb neurons**

Example of pSTAT stained cells in red (right) and GAD65-GFP cell in green (middle) in close proximity in the LH but without overlap (left). A total of 11.45% of pSTAT3\textsubscript{LH} neurons contained GAD65 (analysis of 1,544 pSTAT neurons from three GAD65-GFP/pSTAT3-Alexa555 brains), and 6.89% of GAD65\textsubscript{LH} cells contained pSTAT3 (analysis of 819 GAD65 neurons from three GAD65-GFP/pS\textsuperscript{STAT3}-Alexa555 brains). The % co-localization values are averages per hemisphere.

To investigate the relationship between GAD65 and NPY neurons in the LH, we made use of a double transgenic line, a cross of NPY-hrGFP mice with GAD65-Ires-Cre mice. Three mice were injected with a Cre-dependent mCherry-ChR2 into the LH to label GAD65 cells with mCherry. For each mouse, three bilateral slices containing the LH (30µm) were counted and an overlap of 2.06% of NPY-GFP neurons that also showed GAD65-mCherry were discovered (Figure 3.7).
3.4 What is the overlap between VGAT and GAD65 neurons in the LH?

Recent studies on feeding behaviour driven by VGAT cells in the LH have received much attention (Nieh et al. 2015; Jennings et al. 2015). However, the use of VGAT as a genetic marker does not necessarily mean that GAD65 cells were targeted as well. This is because single-cell mRNA studies have shown that VGAT RNA is not always expressed in GAD65 neurons in the hypothalamus and that some hypothalamic GAD65 neurons might even contain glutamatergic markers instead (Romanov et al. 2017; Jeong et al. 2016), which has also been confirmed by in situ hybridisation (Jarvie and Hentges 2012).

To investigate the overlap between the mouse lines for these markers, we used either a triple-transgenic mouse that resulted from a cross of VGAT-Ires-Cre with CAG-tdTomato and GAD65-GFP or a double transgenic of VGAT-Ires-Cre with GAD65-GFP and injected a Cre-dependent ChR2-mCherry virus, labelling VGAT cells with tomato/mCherry and GAD65 cells with GFP.
Counting cells in the LH of three equal slices per mouse (6 hemispheres per mouse), we found that only 50% of the GAD65\textsubscript{LH} cells contain VGAT, and that most (=80%) VGAT\textsubscript{LH} cells were distinct from GAD65\textsubscript{LH} cells (Figure 3.8).

Overall, GAD65 neurons in the LH seem to be distinct from previously described neuronal types like orexin, MCH, NPY, LepRb and only partially overlapping with VGAT neurons with the majority of VGAT neurons in the LH not expressing GAD65. Thus, previous studies of VGAT\textsubscript{LH} cells likely targeted a larger and more heterogeneous cell populations compared to GAD65\textsubscript{LH} neurons studied here. Therefore, previous studies characterising these classes of neurons cannot be fully applied onto GAD65\textsubscript{LH} neurons and GAD65 can be used as a genetic marker for a distinct population of LH neurons. In addition to this, these findings also raise questions of what neurotransmitters are released by GAD65 neurons and in what way.
Co-localization of GAD65\textsubscript{LH} cells and VGAT\textsubscript{LH} cells. GAD65\textsubscript{LH} cells were labelled with GFP (GAD65-GFP transgenic mouse), and VGAT-Ires-Cre cells were labelled with tdTomato (CAG-tdTomato; VGAT-Ires-Cre transgenic mouse) or ChR2-mCherry. (A, top left) Example of LH co-localisation. (A, right and bottom) More examples of coronal slices from different anteroposterior LH locations (bregma coordinates indicated on the slides). (B) Quantification of data in A (combined cell counts from three brains).

Figure 3.8: Co-localisation of GAD65 and VGAT neurons in the LH
3.5 Are GAD65\textsubscript{LH} neurons local neurons or do extra hypothalamic projections exist?

Orexin and MCH neurons have two similar anatomical characteristics. First, their cell bodies are only found in the hypothalamus; and second, they send axons to nearly all other brain areas (Bittencourt et al. 1992; Peyron et al. 1998). GAD65 neurons, however, are not exclusive to the hypothalamus, and in regions like the cortex (López-Bendito et al. 2004) and hippocampus (Wierenga et al. 2010), they act as interneurons, projecting mainly locally instead of forming projections to other areas. This raises the question of whether GAD65 neurons in the LH are local interneurons like in cortex, or if they project outside the LH like the MCH and orexin neurons. To answer this question, GAD65-IRES-Cre mice were injected with a Cre-dependent ChR2-YFP virus into the LH. After an expression time of at least six weeks, ChR2-YFP was seen in the projections and soma of infected neurons, allowing for projection fields to be analysed in 30µm thick coronal and sagittal sections. Sagittal sections (Figure 3.9A) show that there are dense projections from GAD65\textsubscript{LH} neurons in the hypothalamic area itself, but they are not limited to that region. Projections seem to be far reaching to many subcortical areas, from as anterior as the accessory olfactory bulb, to as posterior as the dorsal raphe in the brain stem (Figure 3.9B).

It is notable that GAD65\textsubscript{LH} neuron projections from the hypothalamus seem to target many areas involved in sensory processing, especially olfaction, like the accessory olfactory bulb, that is part of the vomeronasal system and encodes conspecific and allospecific cues (Ben-Shaul et al. 2010; Luo et al. 2003), and the endopiriform nucleus where olfactory and gustatory information converge (Sugai et al. 2012). In addition, gustatory processing might also be targeted in the nucleus of the solitary tract, where sensory and tactile input from the tongue arrive among other visceral inputs (Mark et al. 1988; Halsell et al. 1993), and the nucleus raphe magnus (Yamamoto et al. 1994) which represents the hedonics and quality of taste stimuli. Besides sensory processes, areas involved in locomotor output are also among projection targets: The periaqueductal gray, an area important for the initiation (Jordan 1998) and motivation (Mota-Ortiz et al. 2012) of several locomotor programmes for instance prey pursuit (Han et al. 2017) is one of them. Additionally, other areas involved in motivation of locomotor activity such as the nucleus
accumbens (Delfs et al. 1990; Parkinson et al. 1999) are also among the projection targets. However, we could not find any projection to areas of higher cognitive functions like the cortex.

Thus, it is safe to say that GAD65\textsubscript{LH} neurons have projections to many areas outside the LH, similar to MCH and orexin neurons but not as extensive. Nevertheless, it remains unclear what the actual neuronal projection targets are and what the function of these projections are. Additional immunohistochemical stainings for synaptophysin would have given a clearer indication in which areas GAD65\textsubscript{LH} neurons form synapses and through which areas axons travel without making any connections. Further immunohistochemistry could then have been used to identify the projection targets.
Chapter 3. Intrinsic properties of GAD65LH neurons

Figure 3.9: GAD65 neurons in the LH have diverse extra hypothalamic projection targets
Axonal projections of GAD65LH cells. (A, Left) Scheme for targeting ChR2-YFP to
GAD65\textsubscript{LH} cells. (A, Right) DAPI-stained (blue) 30-\(\mu\)m sagittal section of the whole brain showing GAD65\textsubscript{LH} projections (green) to brain areas like the accessory olfactory bulb (AOB), LH, thalamus (TH), midbrain (MB), and medulla (representative example of three brains). (B) High magnification of brain areas where dense GAD65-ChR2-YFP axons are found (representative example of three brains).

### 3.6 Discussion

GAD65\textsubscript{LH} neurons are distinct from MCH, orexin, NPY and LepRb neurons. However, this finding does not contradict previous studies of MCH cells expressing GAD67 (Jego et al. 2013), because GAD65 and GAD67 are not always co-expressed in the hypothalamus which was shown in several single-cell mRNA studies (Romanov et al. 2017; Jeong et al. 2016).

That GAD65\textsubscript{LH} neurons are only partially overlapping with VGAT cells, with only half of the GAD65\textsubscript{LH} population expressing VGAT, raises some important questions for further studies. For example, what neurotransmitters are actually released by GAD65\textsubscript{LH} neurons and by what mechanism. Previous studies have found that dopaminergic neurons in the striatum can release GABA in a VGAT-independent way by using VMAT2, the vesicular monoamine transporter, instead (Tritsch et al. 2012). Moreover, POMC neurons, which have been reported to release GABA (Hentges et al. 2004), do not show any evidence of VGAT being present when staining for its mRNA with in situ hybridisation (Jarvie and Hentges 2012). These findings might start to challenge the belief that VGAT, GAD65 and GAD67 are being co-expressed and equivalent markers for the same GABAergic neurons, and furthermore that VGAT is essential for GABA release.

A more general question raised by this approach of classifying neurons according to a few genetic markers, is how homogenous these classes actually are. From a biophysical viewpoint, orexin neurons can be divided into at least two subgroups, a depolarising and hyperpolarising type that differ in their morphology, ionic currents and synaptic input organisation (Schöne et al. 2011). VGAT neurons represent a large population of neurons and it would not be surprising, if they are biophysically as diverse as the GAD65\textsubscript{LH} neurons which can be subdivided into four groups depending on their evoked firing patterns (Karnani et al. 2013). Considering this
biophysical diversity of LH cell classes, it is not unexpected that a recent qPCR single-cell gene expression study also showed that a vast neurochemical heterogeneity exists in the LH (Mickelsen et al. 2017).

Another unanswered question is what role the many projection targets of GAD65LH neurons play. Considering the large overlap with many projection targets of MCH and orexin neurons, one might wonder if they target also the same neurons and what neurotransmitters might be released. As the main projection areas seem to be either involved in locomotor control or sensory processing, especially for food and conspecifics, it is clear that GAD65LH neurons are not locally restricted interneurons but projection neurons that can influence processes that are typically associated with classical LH functions (MacDonnell and Flynn 1966; Lumb and Lovick 1993).

The frequency preference of GAD65LH neurons was very similar to that of VGAT and MCH neurons, showing a broadly tuned preference which was very different from the frequency tuning of orexin neurons (Figure 3.2). The monotonic decay in membrane impedance of all cell types, that occurs as oscillation frequency is increased, would be expected to produce concurrent monotonic decay in firing as it is seen for orexin neurons at gamma frequencies (Pike et al. 2000). However, non-orexin neurons, including GAD65LH neurons, continue to fire action potentials even at high input frequencies in the gamma range, which is significantly different from the tuning predicted by their RC-equivalent circuits. Orexin neuron firing follows the predicted decay of activity by their impedance at gamma frequencies. Therefore, it is safe to say that the differences between orexin and non-orexin neurons in frequency-dependence of firing are not a consequence of cell-type-specific variation in passive membrane impedances, which were very similar (Figure 3.3C). Instead, differences in active properties like differential expression of many different types of voltage-gated ion channels (calcium, sodium, potassium, or non-selective channels may all contribute (Puil et al. 1986; Hutcheon et al. 1996) and differences in dendritic geometry (Mainen and Sejnowski 1996) are likely to be the cause of the difference in frequency-dependent firing of orexin and non-orexin neurons at high frequencies.

If we want to speculate what the physiological purpose of this difference in frequency preference is, with orexin neurons showing a preference for low frequencies (<10
Hz), while non-orexin neurons are driven by low and high (10-200 Hz) frequencies, it might be part of the physiological control that avoids co-occurrence of contradictory LH outputs. Considering established functions of oscillations in input selection, synaptic plasticity, long-term consolidation and temporally linking neurons into assemblies (Buzsáki et al. 2004), we could see the differential frequency preference of orexin and non-orexin neurons serving as a switch between two cell assemblies: from VGAT-GAD65-MCH-orexin to VGAT-GAD65-MCH. This switch might enable, at high frequencies, a removal of orexin neuron activity which evokes the physiological hallmarks of stress and aversion (Bonnavion et al. 2015; Suzuki et al. 2005; Heydendael et al. 2014) and whose lack might be beneficial in certain situations. In some contexts, for example eating or formation of food preference driven by \( \text{VGAT}_\text{LH} \) and \( \text{MCH}_\text{LH} \) neurons respectively (Jennings et al. 2015; Domingos et al. 2013), it may be important not to associate a stress/aversion signal with food. Furthermore, this scenario of an oscillation dependent switch between cell assemblies with and without orexin neurons, would also be supported by the fact that orexin neurons are silent during feeding as was shown by in vivo imaging (González et al. 2016).

Another physiological role for this switch could be to control neuronal activity for an optimal body state of energy storage. One can view orexin neurons as an essential natural signal for weight loss, as their inactivation produces weight gain (Hara et al. 2001; González et al. 2016). In contrast, non-orexin neurons can be viewed as a natural signal for net weight gain, because MCH and VGAT cell inactivation produces weight loss (Whidden and Palmiter 2013; Jennings et al. 2015; Shimada et al. 1998). By removing the energy-expending orexin drive, gamma oscillations may shift LH output to favour weight gain. This is conceptually consistent with the recently-discovered association of LH gamma power with food approach (Carus-Cadavieco et al. 2017). An important direction for further research probing causal importance of gamma-control of LH cells would be to use some methods for controlling the influence of gamma oscillations on orexin neurons in vivo.

Overall, these insights open up new avenues for future research on how this novel control mode can be utilised physiologically via internally-occurring hypothalamic oscillations (Carus-Cadavieco et al. 2017), or – in theory – therapeutically, via a
deep-brain-stimulation paradigm promoting a particular oscillation (Sun et al. 2015; Maling et al. 2012). Considering the pivotal role of the LH in physiology and behaviour, this reveals an important new dimension of controlling the functions and malfunctions of this brain region.
Chapter 4. Local LH circuitry involving GAD65 neurons

4.1 Introduction

In order to understand what role GAD65_LH neurons play in the LH circuitry, we tried to functionally identify local inputs and outputs. Compared to other brain areas like the cortex with well characterised circuits and structures (Song et al. 2005; Brown and Hestrin 2009; Ko et al. 2013), the LH circuitry is still mostly undefined, providing many possible circuit arrangements. Attempts to elucidate the connections between the many genetically-identifiable cell populations relied often on bath applications of neuropeptides (Fu et al. 2004; Rao et al. 2008; Li et al. 2002). Even though, this approach can give useful information about putative receptor expression and neurons responding to them, it lacks physiological relevance as it cannot prove functionality of release of the transmitter and often misses out on the role of fast neurotransmitters. Therefore, this approach should only be seen as a first step to identify potential neuronal connectivity.

Tracing studies using wheat germ agglutinin, that have identified orexin cells as a potential projection target of LepRb_LH neurons (Louis et al. 2010), and channelrhodopsin-assisted circuit mapping (CRACM) studies suggesting a local microcircuitry of inhibition of MCH neurons from orexin neurons (Apergis-Schoute et al. 2015), are the only studies indicating an existence of a local LH microcircuitry. The aim of this chapter is to determine local GAD65_LH cell inputs and outputs by combining cell-type-specific presynaptic optical stimulation with postsynaptic electrical recordings. Accordingly, the light-activated excitatory actuator ChR2 was genetically targeted to a variety of hypothalamic cell types to enable optical activation of these ChR2 cells, and postsynaptic responses, evoked by ChR2 activation, in different genetically-identified surrounding networks were recorded (see section 2.5).
4.2 Orexin peptide excites GAD65<sub>LH</sub> neurons

To determine, if orexin peptide signalling can have an effect on the GAD65<sub>LH</sub> network, we first investigated the effect of applying exogenous orexin peptide. We used confocal network imaging of a Cre-dependent calcium indicator, GCaMP6s, expressed in LH slices from GAD65-Ires-Cre mice (Figure 4.1A). Exogenous orexin-A peptide robustly excited the GAD65<sub>LH</sub> network, and this excitation persisted (but was slightly reduced) in the presence of a mix of synaptic blockers (Figure 4.1B). This suggests that orexin can excite GAD65<sub>LH</sub> neurons directly instead of an indirect way via other neurons. Overall, >98% of GAD65<sub>LH</sub> cells (64 out of 65) were activated by orexin peptide in vitro. Thus, it is safe to say that GAD65<sub>LH</sub> neurons are directly activated by the orexin peptide, but it is unclear if orexin neurons also project onto GAD65<sub>LH</sub> neurons.

![Image of orexin peptide effects on GAD65<sub>LH</sub> neurons](image.png)

**Figure 4.1: Effects of orexin peptide on GAD65<sub>LH</sub> network activity**

(A) Scheme for targeting GCaMP6s (Left), example of GCaMP6s expression in GAD65<sub>LH</sub> cells (representative example of five brains) (Right), GCaMP6s response...
Chapter 4. Local LH circuitry involving GAD65 neurons

of GAD65\textsubscript{LH} cells to 300 nM orexin-A with corresponding mean ± SEM plot (n = 44) (B) (Right), and data summary without (C) and with (B) synaptic blockers (n = 44 and n = 13 cells, respectively, mean ± SEM responses of cells during 2–20 min after orexin-A infusion). P values (italics) are from sign tests of whether the response within each group is different from zero, and P-value (regular font) is from a two-tailed Mann–Whitney test comparing response amplitude between groups.

4.3 Local LH inputs to GAD65\textsubscript{LH} neurons

To examine whether orexin cells directly functionally innervate GAD65\textsubscript{LH} cells, the light-activated excitatory actuator channelrhodopsin-2 (ChR2) was targeted to orexin cells (Figure 4.2A), enabling selective optical activation of orexin-ChR2 cells. GAD65\textsubscript{LH} cells were genetically tagged with GFP throughout the brain whilst orexin-mCherry-ChR2 cells were only found at the injection site in the LH (Figure 4.2A). Subsequently, whole-cell patching in brain slices was used to examine the resulting optically induced postsynaptic responses in GAD65\textsubscript{LH} neurons. GAD65\textsubscript{LH} cells received time-locked excitatory inputs when orexin cells were stimulated with single light flashes.

These ChR2 evoked currents could be abolished (Figure 4.2B) when CNQX and D-AP5 were bath applied to block ionotropic AMPA/kainate and NMDA receptors respectively, and thus, the ChR2 evoked current in GAD65\textsubscript{LH} neurons was deemed glutamatergic. Furthermore, the current-voltage relationship (Figure 4.2C) of the ChR2 evoked current in GAD65\textsubscript{LH} neurons shows how the inward current decreases in size when the holding potential increases in positivity and the electrochemical gradient decreases. The predicted equilibrium potential of the ChR2 evoked currents, from assuming a linear relationship, is around 43mV which indicates an underlying cation channel. Based on the Nernst equation (see section 2.5.2) the equilibrium potential for potassium should be -106.46mV, whilst that of sodium is 95.7mV. As a result, an equally conductive non-selective cation channel would have a predicted equilibrium potential of -10.76mV, which is much lower than the equilibrium potential of the measured ChR2 evoked current. Considering that conductances are rarely equal, a bias towards sodium ions could explain a higher measured equilibrium potential or an additional conductance for calcium ions (with a predicted equilibrium potential of 131.43mV) could be another mechanism. An alternative possibility might be provided by the involvement of NMDA receptors which result in a non-linear
current-voltage relationship for sub-zero holding potentials due to the magnesium block.

Nonetheless, it is safe to say that orexin neurons can release fast neurotransmitters like glutamate onto GAD65_{LH} neurons, which probably act on a mix of AMPA/kainate and NMDA receptors leading to an excitatory current.

**Figure 4.2: CRACM investigating orexin\textsubscript{LH} \rightarrow GAD65\textsubscript{LH} signals**

(A) Left, targeting strategy. Centre and right, localization of Orexin-ChR2-mCherry and GAD65-GFP expression. Representative example of 5 brains. LH: lateral hypothalamus, Arc: arcuate nucleus, VMH: ventromedial hypothalamus, DMH: dorsomedial hypothalamus, 3V: third ventricle. D: dorsal, V: ventral, M: medial, L: lateral. (B) Effect of GAD65_{LH} cell photostimulation (light-blue vertical line) on GAD65 cells. Grey lines are individual trials from one cell; thick blue and yellow lines are means. Representative example of 7 cells (fraction of connected to total cells is in brackets). (C) means±s.e.m of current in 5 GAD65_{LH} cells induced by photostimulation of Oexin-ChR2 cells.
After establishing that VGAT and GAD65 neurons in the LH are not always co-expressed, CRACM was used to elucidate if VGAT\textsubscript{LH} neurons are another potential input of GAD65\textsubscript{LH} neurons. We used the same CRACM strategy as previously and targeted ChR2 to VGAT-cre neurons in the LH whilst genetically tagging GAD65 neurons with GFP (Figure 4.3A). Whole-cell patch recordings from GAD65-GFP neurons in the LH, that did not express ChR2-mCherry, showed that GAD65\textsubscript{LH} cells received time-locked inhibitory GABAergic inputs when VGAT\textsubscript{LH} cells were stimulated (Figure 4.3B). The current-voltage relation of the ChR2 evoked currents in GAD65\textsubscript{LH} neurons shows how the directionality of the current changes with a reversal potential of around -73mV which is close to the predicted equilibrium potential of chloride of -63.14mV (Figure 4.3C). At holding potentials of -20 and -40mV there is a chloride influx into the cell due to its electrochemical gradient and therefore negative charge enters the cell resulting in an outward current (Figure 4.3B). At a holding potential of -90mV chloride ions leave the cell against their concentration gradient as the negative electrical potential inside the cell repulses chloride ions, resulting in net positive charge transmission. This is characteristic for GABA\textsubscript{A} receptor-gated chloride channels which are prominent transmitter-gated chloride channels in the CNS.
To test for another potential input, this time of MCH neurons, ChR2 was expressed in a Cre-dependent manner in MCH neurons whilst genetically tagging GAD65 neurons with GFP in a MCH-Cre mouse line crossed with GAD65-GFP (Figure 4.4A,B). However, light stimulation of MCH neurons did not result in any detectable changes of current in in vitro patched GAD65LH neurons (Figure 4.4C). To ensure that ChR2 expression in MCH neurons was adequate and that the duration of the light flash was long enough to produce reliably action potentials in MCH neurons, MCH cells were patched in each slice that was recorded from. Only CRACM results from slices with MCH neurons that were responsive to light stimulation were counted.
Nevertheless, we could not record any evidence for connectivity from MCH to GAD65<sub>LH</sub> neurons with this protocol.

![Figure 4.4: CRACM investigating MCH<sub>LH</sub> → GAD65<sub>LH</sub> signals](image)

(A) Left, targeting strategy. Centre and right, localization of MCH-ChR2-mCherry and GAD65-GFP expression. Representative example of 5 brains. LH: lateral hypothalamus, Arc: arcuate nucleus, VMH: ventromedial hypothalamus, DMH: dorsomedial hypothalamus, 3V: third ventricle. D: dorsal, V: ventral, M: medial, L: lateral. (B) Schematic of CRACM strategy and fraction of connected to total cells in brackets (C) Effect of MCH<sub>LH</sub> cell photostimulation (light-blue vertical line) on GAD65 cells at different holding potentials. Representative example of 14 cells.

### 4.4 Local outputs of GAD65 neurons

To probe for signalling from GAD65<sub>LH</sub> to MCH neurons, a Cre-inducible ChR2-eYFP was injected into the LH of GAD65-ires-Cre mice, together with another viral construct coding for a mCherry label driven by a MCH promoter (Figure 4.5A). Pairing photostimulation of ChR2-containing GAD65<sub>LH</sub> neurons with recordings from mCherry-containing MCH neurons in brain slices (Figure 4.5B) revealed photostimulation-induced postsynaptic currents in nearly all MCH neurons tested (14 out of 16 cells). Postsynaptic currents are predicted to have an equilibrium potential of around -58mV (Figure 4.5C) which is close to the predicted equilibrium potential...
of chloride ions (-63.14mV) and suggests a transmitter-gated GABA$_A$ channel as underlying ionotropic channel. In addition to this, the GABA$_A$ receptor blocker gabazine abolished (Figure 4.5B) the ChR2 induced current and thus, it is safe to say that GAD65$_{LH}$ neurons can inhibit MCH neurons by GABA release which acts upon GABA$_A$ receptors in MCH neurons. The GAD65→MCH signal is unidirectional as the previous CRACM experiment of the reverse directionality (see section 4.3) found no evidence of any connectivity.

Figure 4.5: CRACM investigating GAD65$_{LH}$→MCH signals

(A) Left, targeting strategy. Centre, localization of GAD65-ChR2-eYFP and MCH-mCherry. Representative example of 5 brains. LH: lateral hypothalamus, Arc: arcuate nucleus, VMH: ventromedial hypothalamus, DMH: dorsomedial hypothalamus, 3V: third ventricle. D: dorsal, V: ventral, M: medial, L: lateral. (B) Schematic of CRACM strategy and effect of GAD65$LH$ cell photostimulation (light-blue vertical line) on MCH cell. Grey lines are individual trials from one cell; thick blue and yellow lines are means. Representative example of 14 cells (fraction of connected to total cells is in brackets). (C) means±SEM of current in 14 MCH cells induced by photostimulation of GAD65-ChR2 cells.

Another possible output target are NPY neurons which are localised in the LH as well as the arcuate nucleus, where they co-localise with Agrp neurons (Hahn et al. 1998). A Cre-inducible ChR2-mCherry virus was injected into the LH of GAD65-ires-Cre
mice which were crossed with a NPY-hrGFP line to additionally genetically tag NPY neurons with GFP (Figure 4.6A). Subsequently, slice whole-cell patching was used to examine the resulting optically induced postsynaptic responses in NPY neurons in the LH and arcuate nucleus.

Photostimulation induced postsynaptic currents were sparse (2 out of 10) in the arcuate and more frequent (10 out of 22) in the LH (Figure 4.6B), but both connectivity rates were much lower compared to the GAD65\textsubscript{LH} \textarrow{MCH} connection. The biophysical properties of the current-voltage relationship were those of a typical chloride current with an equilibrium potential of around -68mV for ChR2 evoked currents in NPY\textsubscript{LH} neurons (calculated equilibrium potential for chloride is -63.14mV) (Figure 4.6C). For post synaptic currents in NPY\textsubscript{Arc} neurons, no equilibrium potential was calculated, as only two cells received inputs but both showed a reversal potential between the holding potential of -40 and -90mV suggesting a chloride current as underlying ionic mechanism as well. Therefore, GAD65\textsubscript{LH} neurons seem to directly inhibit local NPY neurons and with a low connection rate also NPY neurons in the arcuate nucleus.
Figure 4.6: CRACM investigating GAD65\textsubscript{LH} \rightarrow NPY signals in the LH and arcuate nucleus

(A) Left, targeting strategy. Centre, localization of GAD65-ChR2-mCherry and NPY-GFP expression. Representative example of 5 brains. (B) Schematic of CRACM strategy and below, effect of GAD65\textsubscript{LH} cell photostimulation on NPY\textsubscript{LH} or NPY\textsubscript{Arc} neurons. Representative examples of 2 and 10 cells respectively (fraction of connected to total cells is in brackets). (C) means±SEM of current in 10 NPY cells induced by photostimulation of GAD65-ChR2 cells.

The last connection investigated was a potential reciprocal signal from GAD65\textsubscript{LH} to orexin neurons. A GAD65-Ires-Cre mouse line crossed with an orexin-eGFP line was use to express ChR2-mCherry in a Cre-dependent manner in GAD65 neurons, whilst tagging orexin neurons genetically with GFP (Figure 4.7A). Even though photostimulation evoked reliably action potentials in GAD65\textsubscript{LH} neurons (see Figure 2.2), slice patch-clamp recordings of orexin cells could not find any detectable photostimulation-induced postsynaptic currents (Figure 4.7C). Hence, GAD65\textsubscript{LH}
neurons receive glutamatergic inputs from orexin neurons but do not form a reciprocal connection back to them and thus form a unidirectional output of orexin neurons.

Figure 4.7: CRACM investigating GAD65\textsubscript{LH} → orexin\textsubscript{LH} signals
(A) Left, targeting strategy. Centre and right, localization of GAD65-ChR2-mCherry and orexin-GFP expression. Representative example of 5 brains. LH: lateral hypothalamus, Arc: arcuate nucleus, VMH: ventromedial hypothalamus, DMH: dorsomedial hypothalamus, 3V: third ventricle. D: dorsal, V: ventral, M: medial, L: lateral. (B) Schematic of CRACM strategy and fraction of connected to total cells in brackets (C) Effect of GAD65\textsubscript{LH} cell photostimulation (light-blue vertical line) on orexin cells at different holding potentials. Representative example of 10 cells.

4.5 Discussion

It is safe to say, that the LH circuitry around GAD65\textsubscript{LH} neurons locally connects different classes of neurons (Figure 4.8) that have different and sometimes opposing behavioural functions. Thanks to the presented CRACM experiments, we found a unidirectional tri-partite structure that allows the locomotor activity and leanness-promoting orexin neurons (Hara et al. 2001) to inhibit energy-conserving MCH neurons (Shimada et al. 1998; Gomori et al. 2003) via local GAD65 neurons. This is a connectivity that had been previously speculated and suggested to exist but until
now was not proven. It is important to point out, that there is a directionality in the connections we recorded. Orexin neurons activate GAD65\textsubscript{LH} neurons but there is no feedback projection back to orexin neurons, the same was observed for MCH neurons as the output target of GAD65\textsubscript{LH} neurons where no reciprocal inhibition by MCH neurons was observed. This type of unidirectional connectivity is very different from many connectivity patterns observed in the cortex where feedback loops and bidirectional connections are common (Song et al. 2005) and allow for continuous activation and intrinsic oscillations (Gollo et al. 2014; Womelsdorf et al. 2014).

Furthermore, GAD65\textsubscript{LH} neurons also inhibit NPY neurons in the LH, which are hunger activated and respond to increased glucose levels with hyperpolarisation (Marston et al. 2011), and to a lesser degree in the arcuate nucleus where they co-express Agrp and are one of the main hunger drives of the brain (Atasoy et al. 2012). The sparseness of the connection to NPY neurons in the arcuate nucleus might be explained by the increased likelihood of GAD65\textsubscript{LH} projection being cut due to the distance between the LH and the arcuate nucleus.

Furthermore, local VGAT neurons inhibit GAD65 neurons, which raises the question of how these two GABAergic LH population are functionally activated and coordinated. A question we could not answer with the CRACM approach is, whether the VGAT neurons that inhibit GAD65\textsubscript{LH} co-express GAD65 and VGAT or only VGAT. Moreover, the approach of using a single short light stimulus, that elicits one action potential in the ChR2 expressing cells, is prone to detect mainly fast neuronal connections based on glutamate or GABA but not on slow neuropeptides. Neuropeptides like orexin require a longer high frequency train of stimulation to be released (Schöne et al. 2014). As many peptidergic neurons can release more than one type of neuropeptide like orexin (A and B) and dynorphin (Li and Pol 2006) there might be even more neuronal activation patterns that are required for different types of neuropeptides to be released. Thus, neuropeptides that are released under physiological activation patterns by synapses onto GAD65\textsubscript{LH} or are released by GAD65 neurons themselves would remain undetected.
Figure 4.8: CRACM connection summary for GAD65LH neurons

Summary of functional connectivity. Left, connection success rate with number of cells patched indicated on each bar; Right, graphical summary, line thickness represents connection strength whereas dotted lines indicate no connection, arrows and t-bars denote excitation and inhibition respectively.
Chapter 5. What is the behavioural impact of GAD65LH neuron activity?

5.1 Introduction

The LH is thought to provide an essential drive for diverse vital behaviours, including feeding and locomotion. The human disorder encephalitis lethargica, which results in extensive damage in the peri-LH area, has been noted to make humans “sit motionless…all day” (Saper et al. 2001). Electrolytic lesions in the LH of rats cause lethal hypophagia (Anand and Brobeck 1951b) and LH lesioned animals, if force fed, show a disruption of context-appropriate physical activity and extreme akinesia (Levitt and Teitelbaum 1975).

Considering that the LH is not a homogeneous entity, but contains many molecularly distinct classes of neurons, that are thought to have different physiological roles (Stuber and Wise 2016), one might wonder if silencing of single populations of genetically-identifiable LH neurons can recapitulate parts of the phenotype observed in LH lesion studies. For example, LH neurons expressing orexin become activated in diverse stressful contexts, like acute auditory stimulation, hypoglycemia, hypercapnia, and physical capture (Yamanaka et al. 2003; Sakurai et al. 1998; Mileykovskiy et al. 2005; González et al. 2016; González et al. 2016; Williams et al. 2007). OrexinLH cell activity may thus represent an important input variable for computing context-appropriate locomotor outputs (Sutcliffe and de Lecea 2002). However, the hypoactivity phenotypes caused by cell-type specific orexinLH cell deletion (Gerashchenko et al. 2001) are much milder than the hypoactivity phenotypes observed in broader unspecific LH lesions, therefore orexin neurons alone cannot account for the locomotor drive originating from the LH.

On the contrary, MCH cell-specific deletions cause hyperactivity, implying that MCHLH cells suppress locomotion (Whiddon and Palmiter 2013). Interestingly, VGATLH neuron manipulations show the strongest feeding phenotype of all LH cell-types, with activation driving motivation to consume food and ablation leading to attenuation of weight-gain by decreased feeding (Jennings et al. 2015; Navarro et al.
2016). However, VGAT\textsubscript{LH} manipulations have no significant effect on locomotion and thus might account for the anorexia phenotype in LH lesioned animals but not for the pronounced effect on movement. Overall, these findings suggest that additional drivers of physical activity may exist in the LH.

After establishing that results of behavioural studies from VGAT\textsubscript{LH} cells cannot be extended onto GAD65\textsubscript{LH} neurons, it raises the question of what is the behavioural impact of GAD65\textsubscript{LH} neuron activity? How similar or different might the phenotype of GAD65\textsubscript{LH} neuron activation be from the robust feeding seen with VGAT\textsubscript{LH} stimulation?

We hypothesized that GAD65\textsubscript{LH} cells may be a source of natural LH signals underlying normal levels of physical activity. Furthermore, considering that GAD65\textsubscript{LH} neurons receive strong excitatory inputs from energy expenditure-promoting orexin neurons, but at the same time can inhibit energy conserving neurons like MCH and NPY, it is intriguing to suggest that GAD65\textsubscript{LH} neuron activity might also be able to have an effect on either body weight or food intake. To answer these questions, we used a chemogenetic DREADD approach as this silencer and activator bears the advantage of being suitable for long term manipulation over several days without the need of restricting or tethering the mice and interfering with their natural behaviour. A Cre-dependent virus rendered the targeted neurons active to a physiological otherwise inert compound clozapine n-oxide (CNO), whilst mice were single housed in custom made cages that allowed to measure body weight and food and water intake daily.

5.2 Chemogenetic increase of GAD65\textsubscript{LH} neuron activity can produce weight loss without affecting food intake

To selectively activate GAD65\textsubscript{LH} neurons \textit{in vivo} we targeted them with a Cre-dependent hM3Dq-mCherry virus. hM3Dq is a human muscarinic derived GPCR that is selectively activated by clozapine-n-oxide (CNO) (Roth 2016) and allows reliable long term manipulations (Krashes et al. 2011). An example section of the LH (Figure 5.1A) shows that the injected virus is exclusively expressed in the LH and when cells
expressing hM3Dq-mCherry were patched in alive slices, they were reliably and reversibly activated by bath applications of CNO (Figure 5.1B). The action potential firing of GAD65LH-hM3Dq-mCherry neurons increased significantly when exposed to CNO in the bath (Figure 5.1C) showing that DREADDs are a reliable tool to activate GAD65 neurons in the LH.

As it was intended to use CNO in vivo, administered orally via drinking water, it had to be ensured that there are no off-target effects of CNO for the behaviour we wanted to measure. CNO itself should be biologically inert but recent studies have shown that it can be metabolised to clozapine which not only has a proven effect as anti-psychotic drug (Baldessarini and Frankenburg 1991), but it also seems to be highly potent for DREADD receptors and the actual mechanism of action of CNO.

Figure 5.1: Targeting scheme and in vitro control of GAD65-hM3Dq (aDREADD)
(A) Left, targeting scheme for hM4Di-mCherry. Centre, localization of hM4Di-mCherry. (A) Right, GAD65LH-hM4Di-mCherry cells at high zoom (representative example of five brains). (B) Effect of CNO on GAD65LH-hM3Dq cell firing. Spikes are truncated at 0 mV (representative example of n = 5 cells). (C) Group data, raw values, and means (red) of cell firing of 5 GAD65LH-hM3Dq neurons during saline and CNO bath application; the on-plot P value is from a one-tailed, ratio-paired t test (t, df = 3.251, 4) (n = 5 cells). APs, action potentials.
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(MacLaren et al. 2016; Gomez et al. 2017). In order to ensure that CNO administration had no effect on food intake and body weight, we used control mice of the same GAD65-Ires-Cre strain injected with a Cre-dependent ChR2 virus and measured food intake and body weight manually in custom made metabolic cages. Control mice underwent the same procedure as experimental mice did. They were given at least one week to adjust to the metabolic cages and handling involved in daily weighting. Once mice showed stable measurements, the baseline or pre-CNO phase of days 5 began, which was followed by 5 days of CNO and 5 days post-CNO as a washout. CNO (58.3 µM) was given in drinking water that was slightly sweetened with the non-calorific sweetener sucralose to mask the slightly bitter taste of CNO.

Food and body weight of control mice given CNO did not show any significant alterations when compared to measurements of baseline days before CNO administration (Figure 5.2). Therefore, it is safe to use CNO as a chemogenetic drug to activate GAD65LH neurons in order to investigate feeding and body weight.
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Figure 5.2: Control experiments for chemogenetic manipulations

(A, B) Control experiments with LH GAD65 cells expressing ChR2 instead of hM3Dq (n = 5 mice). (A) Left, normalised body weight measurements during a 5 day long pre, CNO and post phase. Right, difference in body weight between CNO and pre measurements: p = 0.14 (ns) two-tailed one sample t test, t,df= -1.81,4. (B) Left, normalised food intake during a 5 day long pre, CNO and post phase. Right, difference in body weight between CNO and pre measurements: p= 0.36 (ns), two-tailed one sample t test, t,df= -1.022,4.

Experimental mice followed the same procedure as control mice. Surprisingly, experimental mice started losing body weight once administration of CNO began (Figure 5.3A). Weight loss at the end of the 5 day period of CNO administration was around ≈ 5%, which is comparable to the effect seen by activation of the most potently anorexigenic hypothalamic cells known, the POMC neurons (Zhan et al. 2013).

Food intake, however, was unchanged showing no significant increase or decrease in the measurements during the CNO period (Figure 5.3B). This was an unexpected finding in two regards. Firstly, VGAT_{LH} neurons, which are partially overlapping with
GAD65\textsubscript{LH} neurons, were shown to have a very prominent feeding-promoting effect (Jennings et al. 2015; Navarro et al. 2016), whilst GAD65\textsubscript{LH} neuron activity does not seem to have any effect on feeding. Secondly, if mice are losing body weight, that means their energy expenditure is higher than their intake and if intake is unchanged, there must be an GAD65\textsubscript{LH} neuron-dependent effect that increases energy expenditure.

![Figure 5.3: Effects of GAD65\textsubscript{LH} cell stimulation on eating and body weight.](image)

(A) Effect of CNO on body weight of GAD2\textsubscript{LH}-hM3Dq mice. Raw data (right, means±SEM. of 5 mice) and % change relative to baseline (left, values and means±SEM. of CNO days 8-10). *P = 0.0166, two-tailed one sample t-test, t, df = -3.96, 4, n = 5 mice. (B) Effect of CNO on food intake of GAD2\textsubscript{LH}-hM3Dq mice. Raw data (right, means±SEM. of 5 mice) and % change relative to baseline (left, values and means±SEM. of CNO days 8-10). ns, P = 0.143, two-tailed one sample t-test, t,df = -0.1816, 4, n = 5 mice.

Even though we recorded a robust change in body weight, we wanted to ensure that by administrating CNO orally with a different bioavailability compared to i.p. administration, we would get a similar result to that of injecting CNO i.p. Consequently, we injected twice daily a standard dose of CNO (0.5mg/kg body weight) and measured food intake and body weight as previously in the same
animals. The effect of i.p. administration of CNO on body weight and feeding behaviour is very similar to the previous oral administration (Figure 5.4). As the effect size of CNO administration is similar in oral and i.p treated mice, it is safe to use the oral administration of CNO. Oral administration also has the added benefit of reducing pain and stress in mice and leads to more consistent metabolic measurements. One can see an increased variance in the day-to-day single mouse measurement with i.p. injections (Figure 5.4), even though mice had training saline injections during their 5 days of baseline measurements to adjust to the procedure.

![Figure 5.4: Effect of i.p. CNO injections on feeding behaviour of GAD65-hM3Dq mice](image)

Left, body weight effect of 50 mg/g daily CNO i.p. injection on 5 mice with hM3Dq in LH GAD65 neurons. Individual faint lines are individual mice; thick blue line is the mean. One-way repeated measures ANOVA $F(14, 56) = 5.696$, $P < 0.0001$; Holm-Sidak's multiple comparison for specific days: $p$ values indicated on the graph.

Right, eating effect of 50 mg/g daily CNO i.p. injection on 5 mice with hM3Dq in LH GAD65 neurons. Individual faint lines are individual mice; thick blue line is their mean. One-way repeated measures ANOVA $F(14, 56) = 1.615$, $P = 0.1034$ (not significant, ns).

5.3 Chemogenetic inhibition of GAD65$_{LH}$ neuron activity leads to a decrease in food intake without changes to body weight

Whilst chemogenetic activation of GAD65$_{LH}$ neurons proves their neuronal activity to be sufficient to cause an increase in energy expenditure leading to weight loss, it is not proven that GAD65$_{LH}$ neuron activity is also necessary for a normal body weight.
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To investigate this, inhibitory chemogenetic DREADD with a human muscarinic derived Gi coupled receptor hM4Di was used.

*In vitro* controls showed that LH targeted virus injections, as for the Gq version, are restricted to GAD65 neurons in the LH (Figure 5.5A). Patch-clamp recordings and bath application of CNO confirmed that CNO can reliably and reversibly inhibit GAD65LH neuron activity significantly (Figure 5.3B,C).

As a further control we used C57/Bl6 mice injected with the same Cre-dependent hM4Di virus in this experiment to control for any off-target effects that the virus itself might have. Control mice went through the same experimental procedure as previously described and their food intake and body weight showed no significant changes when CNO was administered in the drinking water (Figure 5.6). Therefore,
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it can be said that the hM4Di virus does not have any unspecific effects that are not Cre-dependent and would interfere in a CNO dependent manner with feeding and body weight.

**Figure 5.6: C57/Bl6 control experiments for chemogenetic manipulations**

(A,B) Control experiments for *in vivo* use of CNO, with C57/Bl6 mice injected with a hM4Di virus (n = 5 mice). (A) Left, normalised body weight measurements during a 5 day long pre, CNO and post phase. Right, difference in body weight between CNO and pre measurements: ns = P>0.05 by two-tailed one sample t-test against 0 and (B) Left, normalised food intake during a 5 day long pre, CNO and post phase. Right, difference in body weight between CNO and pre measurements one-tailed one sample t tests against 0.

Assuming that hM4Di would result in a mirrored effect of weight loss induced by hM3Dq in GAD65LH, then a weight gain without change in feeding would be expected. However, GAD65-Ires-Cre mice that were in injected with a Cre-dependent hM4Di virus and underwent metabolic measurements at the same time as control mice in Figure 5.6, showed a different phenotype. Inhibition of GAD65LH neurons resulted in no significant change in body weight but a decrease in feeding of nearly 10% (Figure 5.7). This phenotype is different from the expected weight loss but still shows energy
conservatism. Energy intake is lowered without changes in body weight, thus suggesting a lowered net energy expenditure through ways such as physical locomotion, body temperature or metabolic rate (Novak and Levine 2007; Abreu-Vieira et al. 2015; Moruppa 1990).

An explanation for this result of unchanged body weight might be that the effect of GAD65LH neuron inhibition on body weight was too small with normal chow to show any significant difference in 5 days. High-fat diet which is often used in studies investigating differences in metabolism and food intake (Jennings et al. 2015) might have exaggerated any potential effect on body weight to a more significant level.

One interpretation of these results might be that changes in body weight are secondary and compensatory effects due to primary effects elicited by GAD65LH neuron manipulations on energy expenditure in form of locomotion. In the case of aDREADD, GAD65LH neuron activity can suppress compensatory feeding which would counteract an increase in energy expenditure (Woods et al. 2000; MacLaren et al. 2016). Whereas, inhibiting GAD65LH neurons leads to a decrease in energy expenditure, but this is met by a compensatory decrease in feeding keeping body weight stable and unchanged (Figure 5.7).
Figure 5.7: Effects of GAD65LH cell inhibition on eating and body weight.

(A) effect of CNO on body weight of LH GAD65-hM4Di mice. Raw data (right, means±SEM. of 5 mice) and % change (left, values and means±SEM. of CNO days 8-10 relative to baseline days 1-5). ns, P = 0.942, two-tailed one sample t-test, t, df = 0.0771, 4, n = 5 mice. (B) effect of CNO on food intake. Raw data (right, means±s.e.m. of 5 mice) and % change (left, calculated as in A). *P = 0.0208, one-tailed one sample t-test, t, df = -2.958, 4, n = 5 mice.

As a last contro, to ensure that the administration of CNO in drinking water did not lead to higher or lower water intake and therefore to an unequal CNO intake between control and DREADD mice or a change in body weight due to altered water consumption, water intake was measured in experimental and control mice. The water intake of inhibitory and excitatory DREADD mice when compared to controls during the CNO phase did not show any significant differences (Figure 5.8), and therefore, it is safe to say that water intake was not altered by the modulation of GAD65LH neuron activity and cannot account for changes in feeding or body weight.
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Figure 5.8: Effect of CNO on water intake in GAD65 hM4Di and hM3Dq mice and controls

(A) Left, water consumed by mice from previous figures, values are means+/−SEM. Right, CNO water consumed during the 5 days (n = 5 samples per group, each sample is average intake per mouse per day during the 5 days of CNO, error bars are SEM.), ns = P = 0.7402 by unpaired two-tailed t-test, t=0.3434 df=8. (B). Left, water consumed by mice from previous figures, values are means+/−SEM. Right, CNO water consumed during the 5 days (n = 5 samples per group, each sample is average intake per mouse per day during the 5 days of CNO, error bars are SEM), ns = P = 0.9852 by unpaired two-tailed t-test, t=0.01908, df=8.
5.4 GAD65\textsubscript{LH} neuron activity is essential and sufficient for normal locomotor activity

There are several mechanisms through which energy expenditure can be increased. Besides temperature regulation and metabolic rate, locomotor activity can be one of the factors leading to increased energy expenditure (Mayer et al. 1954). The aim of this subchapter is to investigate possible changes in locomotor activity evoked by GAD65\textsubscript{LH} neuron activity, as mice during their period of CNO administration, to activate GAD65\textsubscript{LH} neurons, looked physically more agitated than usual. In order to measure locomotor activity without any confounding factors of anxiety or novelty, the amount mice moved was measured in their open home cages after an acute i.p. injection of CNO. Mice expressing a Cre-dependent hM4Di virus in their LH robustly and significantly decreased the amount they moved by around 50% compared to their levels of physical activity when injected with saline only (Figure 5.9A). No such effect was seen in control GAD65\textsubscript{LH}-ChR2 mice that were tested at the same time, with a CNO-induced change in locomotion of 4.6 ± 8.2% ($n = 5$, $P = 0.6016$ by one-sample t test: $t, df = 0.0566, 4$). Furthermore, mice that expressed the activating hM3Dq in their GAD65\textsubscript{LH} neurons showed an even greater significant effect of more than 150% locomotion after a single acute i.p. dose of CNO, compared to their behaviour after saline (Figure 5.9B). Control mice, the same that were used in Figure 5.2 and Figure 5.6, underwent the same experimental procedure as experimental mice at the same time and showed no significant changes in their locomotor activity in response to CNO administration ($49 \pm 48.7\%$, $n = 5$, $P = 0.3684$ by one-sample t test; $t, df = 1.013, 4$). Thus, GAD65\textsubscript{LH} activity can modulate locomotor activity in a necessary and sufficient manner to up or down regulate locomotor activity depending on neuronal activity levels.
Chapter 5. What is the behavioural impact of GAD65LH neuron activity?

Figure 5.9: Effect of chemogenetic activation and inhibition of GAD65LH neurons on locomotor activity

(A) Effect of CNO on locomotion of GAD65LH-hM4Di mice. Raw data and means are shown in red (Left), and the same data are shown as % change (Right). On-plot P values are from a one-tailed paired t test (t, df = 2.767, 4; Left) and from a two-tailed one-sample t test (t, df = 3.001, 4; Right) (n = 5 mice). Corresponding negative control data are described in Results. (B) Effect of CNO on locomotion of GAD65LH-hM3Dq mice. Raw data and means are shown in red (Left), and the same data are shown as % change (Right). On-plot P values are from a one-tailed paired t test (t, df = 2.638, 4; Left) and from a two-tailed one-sample t test (t, df = 10.31, 4; Right) (n = 5 mice). Corresponding negative control data are described in Results.

Considering that orexin neurons were found in vitro to be a strong excitatory input to GAD65LH neurons and that orexin A has been shown to promote physical activity in rats (Kotz et al. 2006), a local orexin to GAD65LH neuron connectivity may be underlying the locomotor effects observed after DREADD manipulations of GAD65LH neurons. To answer the question of whether GAD65LH neurons are essential for...
orexin induced changes in locomotion, or if both neuronal populations have separate independent mechanisms of affecting activity levels, we used the established orexin receptor blocker SB-334867, alone or in combination with the chemogenetic inhibitor hM4Di in GAD65\(_{LH}\) neurons (Figure 5.10). As expected, separate i.p. injections of SB or CNO decreased locomotor activity significantly, with CNO having a slightly greater effect. However, combining CNO with the orexin receptor blockade did not add a significant effect, suggesting a shared mechanism of orexin and GAD65\(_{LH}\) neuron induced changes in locomotion.

Figure 5.10: Effects of orexin receptor inhibition combined with chemogenetic inhibition of LH GAD65 neurons

Effects of SB-334867 (30 mg/kg i.p.; Materials and Methods) on locomotion of GAD65\(_{LH}\)-hM4Di mice in the absence and presence of CNO. Repeated measures one-way ANOVA with Geisser–Greenhouse correction \[F(1.977,11.86) = 16.15; P = 0.0004, \text{on-plot values are } P \text{ values from Tukey's multiple comparison tests}\].

5.5 Discussion

Despite the long-recognized importance of the LH for maintaining normal physical activity in mammals (Hara et al. 2001), the functional relations of molecularly distinct LH subnetworks remained poorly understood. Here, a new hypothalamic circuit involved in the control of physical activity has been elucidated, that can regulate locomotion in a bidirectional manner.
Summarising, it is safe to say that GAD65LH neurons can regulate locomotor activity in a necessary and sufficient manner. Interestingly, physical activity and arousal driven by orexin neurons and orexin peptides was previously thought to be mediated mainly by projections outside the hypothalamus to areas like the locus coeruleus (Kotz et al. 2006; Hagan et al. 1999). However, our results point to local GAD65LH neurons as physiological downstream regulators of orexin neurons for the control of locomotor activity, and thus defining new key players in intra-LH information processing.

In a wider context, this orexin to GAD65LH connectivity still leaves some questions open. Even though GAD65LH neuron activity does not seem to promote feeding and therefore one might assume that GAD65LH neuron activity-evoked physical activity is not related to food seeking or foraging behaviour, it is unclear what the motivation, if there is any, behind this type of activity is. We note that in some contexts, it may be evolutionarily advantageous to develop a locomotion drive not directed toward a specific goal (e.g., food), but toward a more general goal (e.g., change of place, physical escape). It is thus tempting to speculate that the orexin-activated GAD65LH cells may provide such a general locomotion drive that serves, for example, to move the animal away from stresses that activate orexin neurons (Mileykovskiy et al. 2005; Williams et al. 2007; González et al. 2016).

These speculations about a potential purpose behind GAD65LH neuron seem in stark comparison to VGATLH mediated activity, which drives directly the motivation to work for food (progressive ratio task) (Jennings et al. 2015) and to eat even non-calorific objects (Navarro et al. 2016). The differential impact on behaviour of VGATLH and GAD65LH neurons is also reflected in the CRACM results showing that VGATLH neurons can inhibit GAD65LH neurons, and therefore a feeding and foraging behavioural drive mediated by VGATLH neurons can potentially inhibit different drives elicited by GAD65LH neurons.

The effects of GAD65LH neuron activity on feeding and bodyweight are less straightforward to interpret as the inhibitory and activating DREADDs did not produce exactly opposite phenotypes. Nevertheless, in respect of energy budgeting there seem to be opposing effects. Chemogentic activation of GAD65LH neurons led to an
increased energy expenditure without changes in feeding resulting in weight loss, which is so far surprising as body weight is usually tightly regulated and controlled to keep an optimal set-point by either increasing or decreasing feeding with changing energy demands (Woods et al. 2000; Mayer et al. 1954). However, as this approach uses a hyper-activation of GAD65LH neurons, caution is required when extrapolating the physiological role of GAD65 neurons to a more physiological activity level. When using inhibitory DREADDs, this approach reveals what the effect of a lack of physiological neural activity is and might give a better indication of the natural role of GAD65LH neurons. Therefore, the observation that with iDREADD inhibition, there is no change in body weight but a compensatory decrease in feeding to account for a decrease in energy expenditure, partly through locomotor activity effects, might hint to GAD65LH neurons having a primary effect on energy expenditure only, and that changes in feeding are secondary to keep a set point in energy balance and body weight (Keesey and Hirvonen 1997; Reddingius 1980; Mrosovsky and Powley 1977).

Approaches of neuronal activity manipulations always raise the general question of neural activity being sufficient or necessary for certain behaviours. Maybe GAD65LH cell activity is sufficient to drive weight loss by prioritising locomotor activity as this activity might be more vital than keeping to a strict body weight set point (Lima and Dill 1990). An example for such a situation might be during predator attacks or other acute stressors that require the mouse to move away from the source of danger or stress, to be in a safer, richer or otherwise favourable place and situation (Calvo–Torrent et al. 1999; Pothion et al. 2004). This interpretation would also be in line with GAD65LH neurons being a downstream mediator of stress-activated orexin neurons. That GAD65LH cell activity is only necessary for locomotor activity, but not for normal body weight, would make sense in a situation of low danger and no need for flight responses with low or inhibited GAD65LH neuron activity, where instead of focusing only on eating beyond need as a behaviour, it is more vital for the animal to focus on other behaviours like investigation of novelty (Leyland et al. 1976; Jacinto et al. 2013) or mating (Uphouse et al. 2005).

One last point to note is that there are different ways of increasing energy expenditure with locomotor activity being one of them. Much research has been done on different types of temperature control from browning of adipose tissue to changes
in body temperature, metabolic rate changes and different types of locomotor activity (exercise vs spontaneous) (Klaus 2004; Saltzman et al. 1995; Donahoo et al. 2004). However, in the end one might wonder how independent these mechanisms are or if they are causally intertwined. Evidence for this comes from studies showing that even spontaneous activity causes thermogenesis which plays a role in obesity resistance (Kotz et al. 2008; Novak and Levine 2007), additionally, this so called non-exercise activity thermogenesis (NEAT) can also play an essential role in general thermoregulation (Girardier et al. 1995). Furthermore, physical activity has been shown to also have an effect on resting metabolic rate (Speakman and Selman 2003). Thus, different mechanism of energy expenditure might be interlinked and dependent on each other.

We only measured locomotor activity without distinguishing between exercise (for example wheel running) or spontaneous activity (grooming, swaying from side to side), which could be a further interesting experiment, but to fully understand the effect of GAD65LH neuron activity on metabolism more extensive metabolic measurements including indirect calorimetry and temperature measurements would be necessary. However, a more interesting open question might also be what the purpose of GAD65LH neuron-driven physical activity is. One might wonder if this GAD65LH neuron-driven activity is pleasant and carries a positive valence or if it is aversive. Moreover, it is still unknown if GAD65LH neuron-driven activity is directed at a specific aim, such as escape, or is rather a displacement activity as seen with Agrp neuron stimulation when no food is available (Dietrich et al. 2015).
Chapter 6. General discussion

6.1 Potential caveats of using transgenic mouse lines as neuronal markers

There are two common methods of generating transgenic mouse lines that express cre recombinase or a fluorophore under the promoter of a protein such as MCH, GAD65 or orexin. The first relies upon an artificial chromosome, usually a bacterial artificial chromosome (BAC), containing cDNA of the protein specific promoter (cis-regulatory elements) and cre-recombinase/fluorophore, which is injected into the pronucleus of a mouse. This leads to random insertion of the transgene into the host genome with one or multiple copies of the BAC being inserted. Tissue and time specific expression of the transgene is achieved by taking advantage of the large size of the BAC and including cis-regulatory elements which contain binding sites for transcription factors (Heintz 2001). However, random insertion of BAC can have multiple caveats. It can alter the gene into which it is inserted and consequently have a deleterious effect on other genes than the transgene itself (insertional mutagenesis). In addition, the expression of the transgene might be affected by the level of expression of the region it is inserted into (the ‘position’ effect). Furthermore, if multiple BAC copies are inserted, their expression will also be higher and might lead to a surge depressing the expression of other genes (Chandler et al. 2007).

The second approach of targeted transgenesis or knocking-in genes uses homologues recombination in embryonic stem cells which are implanted into the mouse blastocyst. This approach ensures that the transgene is inserted into a determined genomic locus and not randomly. The transgene is then expressed under its own promoter and thus avoids any unwanted effects due to random insertion. However, the knocking-in of the transgene results in an insertional deletion affecting the transcription of the endogenous gene controlled by the used promoter (Matthaei 2007). In order to avoid this deletion an internal ribosome entry site (IRES) can be included in the DNA construct. An IRES sequence before the transgene results in a bicistronic transcription of both genes which are then expressed as separate proteins.
Both approaches of BAC transgenesis and knock-in produce several founder lines with different expression patterns of the transgene. Usually these founder lines are compared to the natural expression pattern of the endogenous gene to ensure minimal ectopic expression. Ectopic expression can occur with both methods due to transient developmental expression (Gong et al. 2007) but is more common in BAC transgenic mouse lines. One study using BAC generated mice reported 24 founder lines with different expression patterns, illustrating the effect of random insertion of the BAC transgene (Feng et al. 2000) and how one founder line rarely represents the natural expression pattern.

The main mouse lines used in this thesis, such as GAD65-cre, VGAT-cre, were IRES-cre knock-in mice and thus most of the mentioned caveats should be limited. Nevertheless, the use of the BAC transgenic lines such as NPY-hrGFP, GAD65-GFP, Orexin-GFP, Orexin-cre and MCH-cre should be used with the awareness of possible caveats as described and monitored for ectopic expression.

Considering these caveats, cre-expression and genetic fluorescent markers can be misleading and are not always equal to the expression of the marker gene. Therefore, histochemistry was used to confirm that cre-expression is restricted to their marker neurons for the mouse lines used. However, histochemistry can bare its own caveats and single cell RNA analysis would have been a more precise way to investigate cell overlap and expression and in situ hybridisation would have been a better validation for the mouse lines used.

### 6.2 Possible roles and mechanism of oscillations in the LH

The combination of the knowledge that gamma oscillations in the LH correlate and cause food approach behaviour (Carus-Cadavieco et al. 2017) with our finding of in vitro firing responses to injections of oscillatory input currents of different genetically identifiable LH neuron populations (see chapter 3.2), makes it intriguing to speculate about their function and mechanism. High frequency oscillations, including gamma, could work as an activity switch of cell assemblies, excluding orexin neuron activity. Taking into account that high frequencies such as gamma are associated with food
approach, and that orexin neuron activity is associated with stress and flight responses, such a cell assembly switch would make physiological sense. Specifically, during actions or in environment associated with feeding, it would be sensible if mice would be in a state that allows them to approach, eat and make associations about the food rather than being in a state of flight with a stress drive counteracting all these processes (Song et al. 2006; Calvez et al. 2011).

In order to understand how oscillations are generated, one must consider that there are two basic sources of oscillations: intrinsic (dynamic circuit motifs hypothesis, Womelsdorf et al. 2014) and external entrainment (Berke et al. 2004). Intrinsic oscillations are perpetuated by specific circuit motifs of intrinsically active neurons. Cortical neurons that show gamma oscillations are often organised in pyramidal interneuron network gamma (PING) or interneuron network gamma (ING) motifs (Tiesinga and Sejnowski 2009). Whilst PING motifs depend on reciprocally connected networks of excitatory and inhibitory neurons (activation of the excitatory neurons drives both excitatory and inhibitory activity and inhibitory activity dampens excitatory activity after some delay), ING motifs rely solely on recurrent inhibition coupling with mutual inhibition. If such a motif or similar arrangement would account for oscillations generated in the LH, one would expect some kind of reciprocal connectivity between the neurons involved. Oscillations coming from external neuronal inputs that entrain their targets only rely on direct feed forward connectivity from a brain region with coherent network oscillations (Sirota et al. 2008; Lakatos et al. 2008).

So far, LH neurons have not shown any local reciprocal connections even though many of them are intrinsically active. Accordingly, there is no evidence so far for an intrinsic source of gamma oscillations in the LH. However, the LH receives inputs from the septum which have been shown to inhibit feeding (Sweeney and Yang 2016) and moreover have been shown to be able to impose and entrain oscillations onto their projection neurons in the LH (when optogenetically stimulated) (Carus-Cadavieco et al. 2017). Thus, it seems likely that oscillations in the LH are entrained by feed-forward inputs from sources such as the septum and that the LH circuitry does not have any connectivity motifs that would allow them to perpetuate any oscillations without external entrainment.
6.3 Is the LH microcircuitry connecting opposing drives?

It has been a long-standing question how opposing drives, originating from the LH, are anatomically organised, and if there is a way of mutual inhibition between drives. MCH neurons as energy-conserving neurons drive a nearly opposing phenotype to the orexin neuron-driven energy expenditure. Nevertheless, some in vitro studies suggest that MCH neurons can be excited by orexin application (van den Pol et al. 2004), a finding that does not make initial sense, considering the opposing drives which MCH and orexin neurons represent. Since then orexin bath applications in vitro combined with calcium imaging of MCH neurons has shown that only a minority of MCH neurons was excited by orexin (Apergis-Schoute et al. 2015). The more physiological approach of optogenetically stimulating orexin neurons led to an increase of indirect inhibition of MCH neurons (see also section 1.5.2). The presented CRACM data (see Chapter 4) provides a unidirectional way of how orexin neuron activity can inhibit MCH neurons via local GAD65 neurons and thereby can exclude activation of an opposing drive at the same time.

With respect to the changes in locomotion caused by GAD65_{LH} neuron activity, GAD65 neurons seem to mediate the orexin neuron-driven increase in locomotion (Figure 6.1) as was shown in Kosse et al. 2017. Yet, it is still an open question if orexin neuron activity, that is often caused by stressful stimuli, recruits GAD65_{LH} neurons to inhibit MCH neurons and through this projection achieves an increase in locomotion, or if other projections targets (than GAD65→MCH neurons) are involved. Recent studies, showing that MCH neuron activity leads to a decrease in locomotion (Hausen et al. 2016), provide some support for this idea but causal proof that an orexin neuron→GAD65_{LH} neuron→MCH neuro connectivity is necessary for orexin neuron-driven locomotion is still missing.
Figure 6.1: Schematic overview of the orexin neuron to GAD65\textsubscript{LH} neuron connection and its function

Immobilisation stress is symbolised by a hand but stands for various stressors. Mouse depicted running represents a higher locomotor activity, whilst mouse with ice cream represents eating.

Besides the two classical LH cell populations of MCH and orexin neurons, VGAT neuron are also connected to GAD65 neurons in the LH by sending inhibitory projections to GAD65 neurons. It is intriguing to speculate about the behavioural role of this connection, considering that VGAT\textsubscript{LH} neurons seem to represent a hunger motivator causing appetitive behaviour (Jennings et al. 2015). If the underlying neuronal activity of this appetitive behaviour might inhibit GAD65\textsubscript{LH} neurons which drive locomotion, this would imply that GAD65\textsubscript{LH} neuron-driven locomotion is not feeding-related and maybe even opposite in intent and drive.

6.4 Are GAD65\textsubscript{LH} neurons responsible for LH lesion phenotypes?

Inhibition, via chemogenetic DREADDs, of GAD65\textsubscript{LH} neurons leads to a decrease in locomotion (Figure 5.9), which was one of the hallmark impairments observed with electrolytic lesions in the LH (Levitt and Teitelbaum 1975). This suggests that GAD65\textsubscript{LH} cell activity is essential for normal locomotion and the LH drive of movement. Yet, the locomotor impairments seen with GAD65\textsubscript{LH} silencing seem
milder than what was described in the LH lesion studies (and is depicted in Figure 1.2). This implies that another additional locomotor drive might exist in the LH.

With respect to the decrease in feeding observed with GAD65\textsubscript{LH} silencing, this effect is also in line with the anorexia caused by impairments of LH lesion animals, but it is not clear if the decrease in feeding in GAD65\textsubscript{LH} neuron-silenced mice might not be due to a secondary compensatory effect to achieve a stable bodyweight. Considering that rats with electrolytic LH lesions decrease feeding and lose weight (therefore showing a negative energy imbalance), GAD65\textsubscript{LH} neuron silencing does not produce an imbalance in energy (feeding is decreased but body weight is unchanged). Accordingly, GAD65\textsubscript{LH} neuron activity does not seem to be essential for a stable body weight and energy balance. Overall, GAD65\textsubscript{LH} neuron silencing might be able to account for some of the impairments (especially the locomotor ones) seen with electrolytic lesions in the LH, but it is far from explaining all of them. Concluding that probably several LH neuron classes are needed for the complex control of energy balance with appropriate locomotion and feeding. Nevertheless, an alternative explanation could also be that virus injections for DREADDs were not sufficient to transfect all GAD65\textsubscript{LH} neurons and that some GAD65\textsubscript{LH} neurons were not silenced.

6.5 Measuring the purpose and intent of locomotion

Understanding the nature and intent of changes in locomotion of mice will always be challenging, as we can only see changes in parameters we measure and are thereby already excluding many other possible parameters.

Real-time place preference tests can give an indication if neuronal activation or inhibition has a rewarding or repulsing effect, and open field tests can give further information about anxiety levels and exploration. However, tests using locomotion as a measurement of an emotional or cognitive state can be at risk of simply measuring changes in locomotor output without any relation to anxiety or arousal levels. How can one, with certainty, differentiate the effect of increased pure undirected locomotion from changes in locomotion caused by anxiety or any other motivation
Locomotor activity is also affected by a variety of external stimuli including novelty, stress and anxiety (Cabib et al. 1988; Hooks and Kalivas 1995). Different stressors can have an influence on the behaviour of mice that obstructs an effect that might be displayed in a familiar environment (Tuli et al. 1995; Strekalova et al. 2005). In addition to this, it seems that fearful behaviour is not simply equal to decreased locomotion. Experiments with different predator-simulating dark disks above mice found that a looming disk imitating the approach of a predator elicited a fleeing response in mice, whilst a small moving disk imitating the sweeping of a predator evoked freezing in mice (De Franceschi et al. 2016). Thus, many factors can have effects on locomotor activity, but there is no uniform fear behaviour as different stressors can elicit different responses. Recent studies seem to have struggled with problems classifying the exact intent and purpose of changes in locomotor activity during different behavioural tasks and tried to introduce descriptions like ‘general activity’ (Nieh et al. 2016) to carefully avoid this problem.

It seems more straightforward to describe behaviour once one identified its motivation. This seems especially clear cut in the case of feeding. If a mouse is more willing to work, usually by nose poking or lever pressing, for food this is interpreted as appetitive behaviour, which is dissociated from consummatory behaviour namely feeding (Jennings et al. 2015). However, often mice would consume any object independent of its calorific value, as it is the case of VGAT\textsubscript{LH} neuron activation (Navarro et al. 2016), which leads to the question if it is still valid to talk about a hunger motivator. Another interesting aspect of hunger motivation is what behaviour is displayed if there is no immediate access to food. Agrp neuron activation has been deemed as the ultimate hunger drive in the brain (Aponte et al. 2011; Betley et al. 2015), but if these neurons are activated and there is no access to food, mice display stereotypical behaviours and a decrease in anxiety (Dietrich et al. 2015). This might indicate that Agrp neuron-driven activity is not only hunger motivated, but maybe has a more general function such as to engage more with the environment and to explore which might result in obsessive-compulsive behaviour once Agrp neuron activity surpasses physiological levels. Alternatively, hunger might only recruit a subset of Agrp neurons and consummatory behaviour driven by these neurons represents only one of many behavioural drives that are controlled by Agrp neurons.
Another important point, that can be illustrated with Agrp neuron-driven hunger, is necessity and sufficiency of cell-type-specific neuronal activity for the investigated behaviour. Often only sufficiency is investigated because of technical or time restraints. An example of this is chemogenetic or optogenetic activation of Agrp neurons, where activation leads to voracious eating behaviour independent of the hunger state (Aponte et al. 2011; Krashes et al. 2011). Yet, silencing of Agrp neurons in the arcuate fails to inhibit feeding of palatable food (Denis et al. 2015), which limits the functional role of Agrp neurons in the arcuate to homeostatic feeding, and would be an easily overlooked aspect without the proof of necessity.

Therefore, it is pure speculation at this point to try to explain why mice increase their locomotion with activation (or decrease it upon inhibition) of GAD65\textsubscript{LH} neurons. GAD65\textsubscript{LH} neuron-driven increases in locomotion do not seem to be motivated by feeding, as food intake does not increase with activation of GAD65\textsubscript{LH} neurons. Yet, increased locomotion could still represent a foraging drive that is dissociated from the actual consumption. In addition to this, a fear response is equally possible, especially considering the strong excitatory input from orexin neurons which sense stress signals and drive a flight response.

### 6.6 Open questions

As already mentioned, one of the major open and challenging questions is to characterise the GAD65\textsubscript{LH} neuron-driven locomotion further. It would be interesting to extend this investigation with experiments into the behavioural role of the GAD65\textsubscript{LH} to MCH neuron projection. Considering that MCH neuron activity seems to drive a decrease in locomotion and seems to be implicated in learning and memory, it would be important to measure MCH and GAD65\textsubscript{LH} neuron activity (via GCaMP6s fibre photometry) during novel object and familiar object investigation. In addition to this, optogenetic activation and silencing should be used to investigate if GAD65\textsubscript{LH} and MCH neuron activity have a causal effect on novel object investigation and memory formation.


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