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Neurotensin and cortical arousal: an *in vitro* study

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Abstract

The information processing associated with wakefulness occurs during what is commonly referred to as a cortical desynchronized state. In contrast, deep sleep is accompanied by slow, synchronized electrical activity, perhaps best exemplified by the slow (<0.1Hz) oscillation (SO). The brain switches the cortex from the synchronized to desynchronized state through neuromodulation. Modulators are released in the cortex primarily from ascending systems, mostly signaling through biogenic amines and neuropeptides. One modulator associated with wakefulness through its effects on ascending arousal systems is the neuropeptide, neurotensin (NT). Immunohistochemical studies have revealed the presence of NT-immunoreactive fibers throughout the cortical mantle. Yet, the possibility of direct effects of NT on the cortex and cortical arousal has so far received little attention.

Multi-unit activity recordings were performed on cortical slices spontaneously exhibiting the SO. Slices were prepared from rat to investigate the role of NT in modulating cortical global network activity. Single-cell and paired recordings of and between neuronal subgroups were performed to assess NT effects on the microcircuitry in slices from rats and mice.

In this thesis a method was first developed to facilitate the investigation of cortical network activity in spontaneously oscillating rat slices (study I). Using this method, a robust SO could be recorded in combination with visually guided whole-cell recordings of single neurons. In study II, the effect of NT on spontaneous and evoked global network activity was assessed finding a depression of the spontaneous and evoked response in agreement with an arousal mediating mechanism. Through recordings of single neurons, we identified a rarely studied group of neurons residing within the cortical white matter as particularly sensitive targets for direct, excitatory actions of NT. To further investigate the role of particular neuronal subgroups transgenic mice were used in study III and IV. Neurotensin was found to excite all major classes of inhibitory interneurons, providing an explanation for the reduced global network activity.

Collectively the data presented in this thesis strongly support an arousal mediating role for NT in the cortex, and identify salient components of an arousal-promoting cortical microcircuitry.

Papers and Manuscripts

I. **Case L**, Broberger C. 2013. A method for visually guided whole-cell recordings in brain slices exhibiting spontaneous rhythmic activity. *J Neurosci Methods.* 212:64–71.

II. **Case L,** Lyons D.J, & Broberger C. 2016. Desynchronization of the rat cortical network and excitation of white matter neurons by neurotensin. *Cerebral Cortex*. *Epub ahead of print. PMID: 27095826*

III. **Case L** & Broberger C, 2016. Neurotensin recruits cortical Lhx6 positive interneurons and decorrelates their functional coupling through actions relayed via white matter neurons. *Manuscript.*

IV. **Case L** & Broberger C, 2016. Neurotensin excites 5HT3a receptor-expressing interneurons in the mouse cerebral cortex through K^+ and Ca^{2+} dependent mechanisms. *Manuscript.*

Contents

List of Abbreviations

5HT3aR	serotonin receptor 3a
ACh	acetylcholine
aCSF	artificial cerebrospinal fluid
AP	action potential
CGE	caudal ganglionic eminence
DA	dopamine
DAR	dopamine receptor
DBH	dopamine beta-hydroxylase
DIC	differential interference contrast
EEG	electroencephalogram
EPSC/P	excitatory postsynaptic current/potential
FS	fast spiking
GABA	gamma-aminobutyric acid
(E)GFP	(enhanced) green fluorescent protein
Glu	glutamate
ICV	intracerebroventricular
IPSC/P	inhibitory postsynaptic current/potential
LHA	lateral hypothalamic area
LTS	low threshold spiking
MGE	medial ganglionic eminence
NA	noradrenaline
NT	neurotensin
NTR	neurotensin receptor
pfc	prefrontal cortex
PV	parvalbumin
SO	slow oscillation
SST	somatostatin

TH	tyrosine hydroxylase
TPH	tryptophan hydroxylase
TTX	tetrodotoxin
VIP	vasoactive intestinal polypeptide
VTA	ventral tegmental area
WM	white matter

1 Introduction

The forebrain operates through vastly different modes throughout the day depending on our level of vigilance (Berger 1929). In these different modes - or states - neurons and neuronal assemblies alter their activity and inter-neuronal communication in accordance with the tasks required. How do we transition between the states of deep sleep and wakefulness? In this thesis I have investigated the potential role of the neuromodulator, neurotensin (NT), in inducing state-transitions within the cortical network and how this might be accomplished through effects on individual neurons and their connectivity schemes.

1.1 Cortical state and sensory processing

There are several theories regarding the reasons why we sleep. Sleep is known to be necessary for a healthy life as sleep deprivation results in memory impairment, psychosis and, eventually, death (Frau et al. 2008; Walker & Stickgold 2006). The association between memory consolidation and deep sleep has been extensively studied and it has been shown that *e.g.* boosting slow oscillatory activity during deep sleep enhances memory retrieval post-sleep (Marshall et al. 2006). More recently it has been discovered that sleep is necessary for clearing of debris accumulated during waking. Specifically, a decrease in noradrenergic tone associated with sleep is necessary for clearing to occur (Xie et al. 2013). The decrease in release of arousal mediating neuromodulators including noradrenaline (NA) is associated with deep sleep. The reduced cortical concentration increases interstitial space, which has been proposed to aid in the exchange between CSF and interstitial fluid, effectively “cleaning” the brain.

In the cerebral cortex, wakefulness and different stages of sleep can be distinguished through distinct electrophysiological characteristics (Berger 1929). Alert wakefulness and deep sleep are on the opposite sides of the spectrum. Electrophysiologically these two states can be identified and separated through their differences in amplitude and frequency of the EEG signal: in waking the cortical activity displays rapidly changing low amplitude fluctuations, whereas in deep sleep the activity of the entire cortical mantle is synchronized into large, slow waves (Uhlhaas et al. 2009). These radically different operating modes are thought to reflect the underlying functional connectivity which is dense and far-reaching in deep sleep but spatially restricted in waking. Differences is functional connectivity is even thought to reflect consciousness, or lack thereof, since the slow fluctuations associated with deep sleep are also seen during anesthesia and in comatose states. In the awake state sensory input is “bound” into coherent percepts through synchronization of several small functional units that, in combination with attentional mechanisms, give rise to what, in a broadly espoused definition, has been termed a conscious state (Crick & Koch 2003).

The cortex responds differently to sensory stimuli in different states of vigilance, giving rise to a relative insensitivity to sensory stimuli during sleep. During sleep electrical stimuli arriving in the cortex will elicit a temporally and spatially widespread response reminiscent of the cortex's internally generated spontaneous activity (Haider et al. 2013). Conversely, the same stimuli in wakefulness will give rise to a response that is more constrained in time and space, presumably in order to support efficient and stimulus-specific information processing. The relative relationship between cortical inhibition and excitation is thought to underlie this difference between sleeping and waking; there is a balance between these two phenomena in deep sleep, but relatively stronger inhibition is typical of the awake state (Haider et al. 2013).

The cortex transitions between states in response to neuromodulation. To a large extent, this modulation - arriving from subcortical areas such as the basal forebrain or the raphe nuclei - specifically targets inhibitory interneurons (see below), further highlighting the importance of inhibition for awake information processing (Smiley & Goldman-Rakic 1996; Beaulieu & Somogyi 1991). This has been explored in my thesis through investigation of the effects on NT on cortical state and inhibition.

1.2 Cortical neurons and microcircuitry

The cortical mantle consists of a multi-layered sheath enveloping the rest of the brain. Different cortical regions vary in their anatomical make-up such as lamination. Thus, *e.g.* the sensory cortices present a prominent layer 4 which, together with layer 6, is one of the two main sensory input layers from the thalamus (Constantinople & Bruno 2013; Brodmann 1909). Different structural makeup thus reflects the different roles of cortical regions and the underlying connectivity arising from subcortical areas as well as other cortical regions. In this thesis, I have focused on sensory cortices, especially the somatosensory cortex, since these can be utilized both to study local and global cortical activity, as well as responses to incoming sensory information from the thalamus.

The cortex's information processing is accomplished by its neurons and their interconnectivity, of which the excitatory pyramidal cells comprise 80% of the total number of cells. Inhibitory interneurons make up the remaining 20% and are more heterogeneous in their properties (Ascoli et al. 2008). A division can be made into two inhibitory subgroups based on embryonic origin; medial and caudal ganglionic eminence (MGE and CGE respectively; Lavdas et al. 1999; Miyoshi et al. 2010).

1.2.1 Interneurons

Interneurons arising from the MGE develop into **parvalbumin (PV)-expressing/fast spiking (FS)** or **somatostatin (SST)-expressing/low threshold spiking (LTS)** interneurons in the adult

animal (Lavdas et al. 1999). These two subtypes are the most studied and thus the best understood of the interneuron subtypes; they are identifiable through their expression of markers PV/SST or through their typical electrophysiological behaviour FS/LTS (Ascoli et al. 2008). I will refer to these subgroups by their electrophysiological characteristics henceforth since this has been the mode of identification throughout the presented studies. Further subdivisions can be made based on how the interneurons integrate into cortical circuits and how they appear morphologically *e.g*. (Ascoli et al. 2008). In order to directly target neurons of these two subgroups transgenic mice expressing green fluorescent protein (GFP) under the LIM-homeobox promotor (Lhx6) can be used (Lavdas et al. 1999).

1.2.1.1 FS interneurons

FS interneurons are the largest group of cortical interneurons (40% in mouse cortex; Xu et al. 2010) and are present throughout all cortical layers. They are however present in a larger proportion in layer 4 and in deeper layers while they are scarcer in more superficial layers. FS interneurons are named after their ability to maintain high frequency action potential (AP) firing without frequency or amplitude accommodation. The roles of FS interneurons in the cortex include to provide feed-forward inhibition of thalamic sensory input ensuring a temporally precise cortical response and generating and maintaining oscillatory activity in the gamma range (see below) important for attention and sensory processing (Gabernet et al. 2005; Cardin et al. 2009). FS interneurons connect to other interneurons and pyramidal cells on their cell body or the proximal axon ensuring immediate and powerful influence over AP generation (Somogyi 1977). In addition, FS interneurons are strongly interconnected via gap-junctions enabling fast synchronization of a larger population that can impact the surrounding network more efficiently (Gibson et al. 1999). The excitatory input to FS interneurons is of depressing amplitude (Beierlein et al. 2003). Modulation of FS interneurons by NT is explored in paper I and II in both rats and mice.

1.2.1.2 LTS interneurons

LTS interneurons comprise 30% of cortical interneurons in the mouse cortex and are like FS interneurons more numerous in deeper layers (Xu et al. 2010). It is their ability to fire APs from relatively hyperpolarized membrane potentials relative to their resting membrane potential that gives them their name. The roles of LTS interneurons include lateral inhibition, *i.e.* the spatial constrain of excitation ensuring precise information processing and potentially to protect from runaway excitation since excitatory synapses onto LTS interneurons facilitate (Adesnik & Scanziani 2010). Synapses from LTS interneurons onto pyramidal cells are found next to and on dendritic spines and might thus depress excitatory inputs arriving in the dendrites (Wang et al. 2004).

1.2.1.3 5HT3aR interneurons

CGE-derived interneurons have more recently been possible to identify through the discovery that they express the serotonin 3a receptor (5HT3aR; Miyoshi et al. 2010) and can

thus be targeted via genetic tools. This group is more heterogeneous than the MGE-derived interneurons and can be subdivided into several groups such as vasoactive intestinal peptide (VIP) and Reelin expressing interneurons (Rudy et al. 2011). I will hereafter refer to this group of neurons as 5HT3aR interneurons.

5HT3aR interneurons comprise the remaining 30% of cortical GABAergic neurons. In the superficial cortical layers 1 and 2/3 this subgroup is the most abundant interneuron (Rudy et al. 2011; Lee et al. 2010). The role of 5HT3aR interneurons has been proposed to depend on behavioural state as although heterogeneous they uniformly respond to cholinergic and serotonergic modulation and receive direct thalamic input. A subset of 5HT3aR interneurons have been shown to inhibit FS and LTS interneurons (Pi et al. 2013) and could disinhibit pyramidal cells during thalamo-cortical input through this mechanism. The NT modulation of 5HT3aR interneurons is explored in paper IV.

1.2.2 Pyramidal cells

Pyramidal cells comprise the bulk of cortical neurons, 80% and they are the main source of excitatory input within the cortex as well as exiting to subcortical regions such as the thalamus (Douglas et al. 1995). These neurons are usually subdivided based on their projection targets and location in the cortical mantle. In layer 6 recent genetic tools have allowed the division of pyramidal cells into cortico-cortical and cortico-thalamic due to the discovery that only cortico-thalamic pyramidal cells express the neurotensin receptor type 1 (NTR1; Gong et al. 2007). NTR1 expressing cortico-thalamic cells have been shown to influence information processing through controlling the gain of the overlaying cortical layers and are thus believed to have an important role in the early stages of sensory processing (Olsen et al. 2012). Cortico-cortical neurons on the other hand are more likely to connect to neighbouring excitatory neurons as well as to send long range axons to other cortical regions which is potentially important for information flow and binding (Bannister 2005). Pyramidal cells' direct and indirect responses to NT have been investigated in rats and mice in paper II and III, respectively.

1.2.3 White matter neurons

A group of cortical neurons that receive little attention when describing the cortical column are the white matter (WM) neurons also called; subplate, subgriseal, layer 7, layer 6b or persistent subplate neurons from which they have their origin (Chun & Shatz 1989; Clancy et al. 2009; Kostovic & Rakic 1980). It has been debated whether these neurons play a functional role in the adult cortex or if they are simply remaining at the base of layer 6 after having played their part during development (Kostovic & Rakic 1980; Viswanathan et al. 2012; Tolner et al. 2012). The WM neuron cell bodies are located within or just above the white matter below layer 6b. Their axons, however, have been shown to extend as far into the cortex as layer 1 indicative of a persisting influence over the mature network (Clancy & Cauller 1999). During development subplate neurons help guide axons from the thalamus

into the cortex as well as in the opposite direction. Their role in the adult cortex is not well understood but they strongly decrease in numbers (only 15-30% remain at postnatal day 30; Kanold et al. 2003; Kanold & Luhmann 2010; Torres-Reveron & Friedlander 2007; Clancy et al. 2001). One clue to their potential role comes from a study showing them to be the only cortical neurons found to respond to orexin (Bayer et al. 2004), a well-known mediator of arousal (Chemelli et al. 1999; Nishino et al. 2000; Hsueh et al. 2002). The WM neurons that remain in the adult cortex are primarily excitatory since GABA-ergic expression in this region is sparse and paired recordings have thus far only generated evidence of excitatory connectivity (Hoerder-Suabedissen & Molnár 2013; DeFelipe et al. 2010). White matter neurons are extensively explored in terms of their response to NT and integration into the cortical microcircuit beyond early development in papers II and III.

1.3 Cortical oscillations

The brain is always electrically active but different levels of vigilance are associated with distinct electrical patterns (Berger 1929). Functional networks vary in size from small and rapidly changing (desynchronized) in waking to large, slowly spreading (synchronized) in deep sleep. Even during waking however the cortex varies in the scope of functional connectivity; from quiet waking (synchronized) to heightened alertness (desynchronized).

Synchronization does occur in waking but it happens at a faster timescale and for a shorter time-period compared to sleep. Synchronized oscillations such as alpha (7-13Hz; Adrian & Matthews 1934; Haegens et al. 2011) and gamma (20-80Hz; Hughes 1964; Eckhorn et al. 1988; Fries 2001)are associated with information processing and attention during waking even though gamma frequency can also be detected during the slow oscillations (SO) seen in deep sleep (Compte et al. 2008).

In my thesis I have investigated the NT modulation of the cortical SO.

1.3.1 The slow oscillation

The SO was first identified as an isolated oscillation in 1993 by Steriade in anaesthetized animals where it was described as engaging almost all neurons sampled and presenting a frequency of 0.3-1Hz (Steriade, a Nuñez, et al. 1993 but see Kristiansen & Courtois 1949). The cortical SO is a hallmark of deep sleep. Alternating periods of intense synaptic activity and often APs (UP state) and quiescence (DOWN state) are present throughout the cortical mantle as travelling waves typically initiating in the frontal regions and travelling caudally towards the occipital lobes (Massimini et al. 2004; Sanchez-Vives & Mccormick 2000). During the UP states virtually all neuronal subtypes are engaged and fire APs (Steriade et al., 1993) resulting in a barrage of balanced inhibitory/excitatory synaptic input in neighboring neurons (Haider et al. 2006; Shu et al. 2003). While the SO is thus engaging the neurons the cortical

mantle is effectively shut off from the outside world and functionally unresponsive to any sensory input that might impinge on it (Watson et al. 2008). Subjective experience of sleep quality can, however, be affected and a strong sensory input can override this isolation and arouse the brain (Velluti 1997; Terzano et al. 1990). The UP states of the oscillation function as a window of opportunity for other cortical but also subcortical oscillations (*e.g.* gamma oscillation and thalamic sleep spindles, respectively) as these predominantly occur during the UP states (Compte et al. 2008; Steriade, A. Nuñez, et al. 1993).

It is debated whether the SO is an isolated cortical phenomenon emerging from intrinsic neuronal properties and/or interconnectivity or if it arises from an interplay between the cortex and thalamus (David et al. 2013). The UP state has been shown to more often initiate in layer 5 where large and highly connected pyramidal cells reside (Sanchez-Vives & McCormick 2000). *In vivo* experiments show that these neurons often receive a slowly increasing barrage of synaptic input potentially arising from spontaneous synaptic input gradually pushing the membrane potential towards threshold, thus initiating the UP state (Chauvette et al. 2010). In other experiments *in vitro* spontaneously oscillating pyramidal cells have been identified that could possibly drive and set the frequency of UP state initiation (Le Bon-Jego & Yuste 2007).

Removing the cortex and maintaining slices in isolation is a widely used method to study the oscillation itself and its modulation by *e.g.* electrical fields, temperature, inhibition and neurotransmitters (Fröhlich & McCormick 2010; Favero et al. 2012; Sanchez-Vives et al. 2010; Reig et al. 2010). Utilizing a slice that recapitulates the *in vivo* network behavior but offers easy access for electrodes and control over experimental parameters has gained many insights into the behavior of neuronal subtypes in an active network. The SO is modulated by known arousal mediators such as acetylcholine (ACh) and NA in a consistent manner; the UP state frequency is reduced/ UP states are abolished *in vitro.* The same modulators induce a persistent UP state/desynchronized state *in vivo* (Steriade, Amzica, et al. 1993; Carter et al. 2010).

1.3.2 Gamma oscillations

Gamma oscillations comprise synchronized network activity at frequencies from 20Hz up to several hundreds of Hz (Jasper & Andrews 1938; Sem-Jacobsen et al. 1956). *In vivo* studies have shown that gamma power increases locally with sensory information processing, working memory and other cognitive processes (Fries 2001; Cardin et al. 2009; Sederberg et al. 2007). Synchronization of gamma activity between sensory areas has been suggested to underlie temporal binding and thus to be necessary for perception (Engel & Singer 2001). Generation of gamma is linked to FS interneurons (Cardin et al. 2009) but there are divergent theories concerning whether pyramidal cells are involved or if gamma is a purely inhibitory oscillation (Whittington et al. 2000).

1.4 Neurotensin

Neurotensin (NT) is a 13 amino acid peptide that was first isolated and identified in the hypothalamus of oxen as a peptide that could induce hypotension in rats (Carraway & Leeman 1973). Since then it has been widely studied in the CNS, often due to its co-localization with and modulation of dopamine (DA; Beana et al. 1991; Tassin et al. 1984; Hökfelt et al. 1984; Fuxe et al. 1992). NT has been implicated in several physiological phenomena such as nociception, food intake and sleep as well as pathophysiological states such as drug abuse, autism and schizophrenia (Cape et al. 2000; Roussy et al. 2009; Sahu et al. 2001; Kinkead & Nemeroff 2004) . It is due to NT's modulation of arousal that it has been the focus of my thesis.

Neurotensin containing cell bodies have been described in a number of nuclei *e.g.* ventral tegmental area (VTA), periaqueductal gray, lateral hypothalamus (LHA) and the amygdala projecting to the prefrontal cortex (pfc), raphe nucleus, VTA and LHA/VTA respectively (Hökfelt et al. 1984; Uhl et al. 1977; Febvret et al. 1991). There is no described projection to the cortex apart from the VTA-pfc projection (Febvret et al. 1991) but NT receptors are present throughout the cortical mantle (Alexander & Leeman 1998; Sarret, Perron, et al. 2003; Morris et al. 1998; Sarret, Krzywkowski, Pascale Segal, et al. 2003).

In the cortex all known NT receptor subtypes are expressed: NTR1 (high affinity) and NTR2 (levocabastine-sensitive), which are cell membrane G-protein coupled receptors. NTR1 has been shown to couple to both stimulatory and inhibitory G-proteins, Gi, Gq and Gs whereas NTR2 is suggested to couple to Gq (Gailly et al. 2000; Labbé-Jullié et al. 1994; Yamada et al. 1993). NTR3 (also known as sortilin) is thought to be intracellular (Mazella et al. 1996; Mazella et al. 1998; Tanaka et al. 1990).

1.4.1 Neurotensin and arousal

Neurotensin has been implicated in modulation of arousal due to its awakening effects when injected intracerebroventricularly (ICV) in rats (Castel et al. 1989). When exposed to ICV NT rats spent less time in deep sleep and latency to deep sleep was increased. The exact mechanism(s) and site(s) of action of these analeptic actions is unknown but NT has since been shown to activate several subcortical ascending arousal systems.

The *Orexin/Hypocretin system* with cell bodies in the LHA is well recognized as a mediator and maintainer of arousal (Sakurai 2007). Notably, loss of orexin neurons causes narcolepsy (Chemelli et al. 1999). The LHA orexin neurons co-express and are depolarized by NT *in vitro* (Furutani et al. 2013). *In vivo* administration of a NTR1 antagonist results in an orexin dependent decrease in wakefulness indicating a significant enhancement of NT on orexin's role in arousal (Furutani et al. 2013).

The *cholinergic system* of the basal forebrain (BF) projects widely within the cortex and is activated by injection of NT *in vivo*, consequently rats that received injections spent less time in deep sleep and showed increased wakefulness in addition to increased gamma power (Cape et al. 2000).

The *serotonergic system* originating from the Raphe nuclei also responds to NT with excitation as demonstrated *in vitro* (Jolas & Aghajanian 1996).

1.4.2 Neurotensin in the cortex

In a cortical context NT has received the most attention through its co-release and modulation of DA but also in its own right through modulation of GABA and Glutamate (Glu) release in the pfc (Ferraro et al. 2000; Petrie et al. 2005; Petkova-Kirova, Rakovska, Della Corte, et al. 2008). Experiments *in vivo* and *in vitro* have shown increased release of GABA and Glu respectively (Antonelli 2004; Petkova-Kirova, Rakovska, Della Corte, et al. 2008).

Neurotensin is co-localized with DA in fibers in the pfc arising from projections from the VTA, the mesocortical projection (Hökfelt et al. 1984). It is not known under what conditions NT and/or DA would be released but according to theory a peptide like NT might be released during bursting activity while DA would be released also during tonic firing (Dutton & Dyball 1979; Gonon 1988). The NT regulation of DA activity has been proposed to be primarily stimulatory: DA release increase is probably due to NTR1 antagonistic interaction with the D2 receptor (auto-inhibitory) leading to decreased auto-inhibition and thus augmented release of DA (Petkova-Kirova, Rakovska, Zaekova, et al. 2008; Fuxe et al. 1992). Depolarization block has been observed with supra-physiological concentrations of NT leading to a decreased release of DA but whether this would occur in physiological conditions remains to be determined (Seutin et al. 1989).

Electrophysiologically, NT has been shown to depolarize pfc pyramidal cells as well as inducing an increased inhibitory tone (Audinat et al. 1989) but further studies in other cortical regions or on specific interneuronal subtypes have been lacking.

2 Aims

The overarching aim of this thesis was to investigate the mechanism of NT in mediating cortical arousal. To fully explore NTs effect on the cortical network experiments were performed from the specific single neuron whole-cell recordings to the spontaneously and evoked cortical SO. Receptor pharmacology and mechanisms of action was investigated as well as layer specificity. The first step in the thesis was, however, a method development to facilitate further studies of spontaneous network activity.

1. ***Develop a robust rodent* in vitro *SO Preparation adapted for visualized whole-cell recording.*** (Presented in study I.)

2. ***Identify (a) potential role(s) of NT in the modulation of cortical state assessed through recordings of spontaneous SO and evoked UP-states in thalamo-cortical recordings in rats.*** (Presented in study II.)

3. ***Determine the underlying microcircuitry that mediates NT actions within the cerebral cortex using paired recordings within and between neuronal subclasses in mice.*** (Presented in study III.)

4. ***Elucidate mechanisms of NT-action on different neuronal subclasses assessed through single whole cell recordings from rats and mice.*** (Presented in study II, III and IV.)

3. Methodology

The main method used to address the questions raised in this thesis is electrophysiology. Different questions can require different preparations and variants on the basic method which will be discussed here. For specific description on slice preparation see individual papers. The species chosen were rat and mouse due to the relative ease with which slices exhibiting network oscillations can be prepared and the availability of genetic tools, respectively.

3.1 Electrophysiology

When investigating the electrical behavior of a neuron or especially a neuronal network in a reduced preparation such as the slice, it is difficult to know how much of the natural behavior is lost. We know that the cortical network is never quiet *in vivo,* yet most cortical slice preparations are "silent" showing only rare occurrences of sparse activity. There are, however, alterations to the slice preparation procedure one can make to obtain a slice that exhibits a higher level of activity. Examples are; higher flow rate of perfusion media and raising the slice with the help of a grid enabling perfusion on both sides of the slice (Hájos et al. 2009). These are examples of improvements that can be made when the slice is maintained in a submerged chamber, most commonly used since it allows simultaneous visualization of neurons down to the level of axons and dendrites. Another commonly used chamber that achieves more readily induced or even spontaneous activity is the interface chamber. In this configuration only one side of the slice is exposed to aCSF and the other to humidified, oxygenated air (von Krosigk et al. 1993; Sanchez-Vives & McCormick 2000; Haas et al. 1979; Schwartzkroin 1975; Andersen & Langmoen 1980). Lack of visualization is the major drawback of this method, thus recordings need to be performed "blind".

3.1.1 The slow oscillation preparation

Historically the most common species used for *in vitro* SO experiments was the ferret (Sanchez-Vives & McCormick 2000; Compte et al. 2008). Slices from the prefrontal and visual cortex are more frequently reported in literature (Sanchez-Vives & Mccormick 2000; McCormick et al. 2003) and relatively small slices can be prepared that still exhibit oscillatory activity at a stable frequency. The ferret as a model animal is, however, not ideal due to its size, cost and relative lack of available information on its behavior, pharmacology and genetic background. Rodents (rats and especially mice) are becoming the species of choice and are increasingly common in this field of research (Fanselow & Connors 2010; Favero et al. 2012).

The term “spontaneous activity” can be debated as the aCSF needs to be slightly altered from the standard cortical aCSF in order to observe the SO (Sanchez-Vives & McCormick 2000). It should be noted, however, that different aCSFs are traditionally used for different brain regions also in slice electrophysiology where no spontaneous activity is sought (see examples in table below). Ionic concentrations that often vary are those for potassium, calcium and magnesium.

aCSF *vs* concentration in mM	Cortex	Cortex (modified)	Hippocampus	Hypothalamus
NaCl	124	124	124	127
$NaHCO_3$	26	26	30	26
Glucose	10	10	10	10
NaH_2PO_4	1.25	1.25	1.25	1.2
$MgSO_4$	2	1	1.5	1.3
CaCl2	2	1	1.5	2.4
KCl	2.5	3.5	3.5	2

Cortex, cortex modified (Sanchez-Vives & Mccormick 2000)*, hippocampus* (Andersson et al. 2012)*, hypothalamus* (Lyons et al. 2010).

It should also be noted that CSF is not the same as interstitial fluid which is what is in fact surrounding the neurons *in vivo* and it has been reported to contain ionic concentrations close to those used in the SO preparation (Ding et al. 2016; Yamaguchi 1986; Zhang et al. 1990 cf sanchez-Vives and McCormick 2000). There are, however, inherent difficulties in measuring ionic concentrations in the interstitial fluid. Mechanical disturbances with *e.g.* a probe will perturb and potentially harm neurons and astrocytes and thereby altering the concentrations one intends to measure.

The SO *in vitro* closely mimics that *in vivo* which is the most important factor for many experiments and scientific questions. Rat slices *in vitro* reliably exhibit regularly occurring UP states (Case & Broberger 2013; Favero et al. 2012)that can be used as in this thesis to evaluate methodology and neuromodulatory effects on frequency. Previously the preparation has been used to evaluate the effects of known arousal mediators such as ACh and NA. A depression in spontaneous and evoked UP state frequency is reported for these arousal mediators and is in agreement with what is described for NT in this thesis (Favero et al. 2012).

3.1.2 Whole-cell recordings

The majority of data has been collected with whole-cell recordings in submerged slices, single cell or paired recordings to analyze direct and indirect effects and cell to cell communication, respectively. Due to the submersion necessary to facilitate visualization, the neuronal networks in these experiments are predominately silent even though the modified aCSF used in SO experiments has been maintained. In paper I, whole-cell recordings were performed under visual guidance in an interface chamber with stable recordings (as monitored by access resistance) as a result. This method was, however, abandoned in subsequent studies due to focus being shifted away from the need of an active preparation and the relatively low yield compared to performing whole-cell recordings in the submerged slice.

4 Results and discussion

4.1 Establishing a robust rodent slow oscillation preparation

The aim was to develop a robust rodent *in vitro* SO preparation where individual neurons could be visualized and targeted for whole-cell recordings. Traditionally, ferret and cat preparations have been used for SO experiments without the benefit of visually guided recordings. Using rodent preparations offer genetic markers such as GFP and a large body of published information on neuronal subtypes, pharmacology and behavior for comparison. Moving from the well-established ferret slice-preparation to a rodent slice-preparation with comparable reproducibility and stability of UP state frequency involved some alterations to the established method such as recovery time and experimental yield. The submerged chamber yielded no reliable results in terms of spontaneous activity with only occasional UP states and even rarer SO (in the range of a couple in hundreds of slices), thus the classical interface chamber was employed.

Recovery time after slicing proved to be longer for rat brain slices compared to ferret brain slices where rat brain slices required at least one h in modified aCSF as opposed to half an h in order to observe rhythmic UP state activity (Sanchez-Vives & McCormick 2000; Paper I). Slices could not be cut into smaller pieces all retaining the SO (in contrast to ferret slices) somewhat reducing the possible experimental yield. Using the method developed in paper I 62% (n=77/125) of rat slices exhibited an SO with an UP state frequency of >0.1Hz.

The SO in rat slices is comparable to that in ferret slices, albeit UP states are less frequent in rats with a frequency of 0.19±0.03Hz (n=77) compared to reports from ferret where the SO exhibited an average frequency of 0.29Hz (Sanchez-Vives & McCormick 2000). The rat preparation did, however, meet our demands of a robust SO at a frequency >0.1Hz and was subsequently used to explore the effects of NT on cortical network activity in paper II.

When investigating spontaneous or induced oscillatory activity one must decide beforehand; *what constitutes an oscillation? What is an acceptable frequency? Rhythmicity? Power?* The answer must be that it depends on what questions one wants to answer using the preparation. In some cases, such as when investigating intra UP state activity, sparse and/or irregular UP states are sufficient and thus the more easily used submerged configuration can be utilized. Observing oscillation frequency or rhythmicity over time due to *e.g.* neuromodulation, however, requires a rhythmically stable oscillation necessitating the interface configuration or chemical induction. The rodent preparation developed in this thesis is suitable for such experiments.

4.1.1 Visualization while maintaining spontaneous activity

There are great benefits to enabling visualization of neurons during patch clamp experiments. Selecting the appropriate neuron increases yield and accuracy, especially while using current methods of identification such as genetically driven tagging of chemically identified neurons. In addition, active preparations are necessary for several lines of investigation such as mechanisms of oscillatory activity and the neuromodulation thereof. Finally, spontaneously active preparations have in some instances been shown to be quantitatively different to electrically or chemically induced activities *e.g*. the mechanisms underlying hippocampal gamma-oscillations (Pietersen et al. 2009) and the investigation of dendritic input (Takahashi et al. 2012), illustrating the value of spontaneously active preparations.

Due to the lack of visualization associated with the interface chamber configuration but the desire to maintain spontaneous activity a method combining the two was developed (see paper I).

The design of the chamber was based on the traditional Oslo-style interface chamber (Schwartzkroin 1975) but small enough to fit under a standard upright microscope used for patch clamp experiments. Heated oxygenated air can cause droplets to form on the objective lens at room temperature; this issue was addressed by using an objective heater. The objective used here is developed for material science (Zieringer et al. 2012) rather than biomedical use and does not lend itself to differential interference contrast (DIC) imaging but enables the experimenter to see neurons well enough to target them for patch clamp recordings. A benefit of the objective is the large working distance providing space for several electrodes for the purpose of recording, stimulation or drug application.

The versatility of the developed method was shown through recordings of spontaneous cortical SO and hippocampal gamma oscillation. Comparing the success rate of obtaining oscillating slices, frequency and duration of UP states showed that the developed method is comparable to the Oslo-style chamber. Adding visually guided patch-clamp recording to the spontaneously oscillating preparations introduced a slight depression of UP state occurrence, probably through the expulsion of high potassium concentrations through the recording electrode. In cortical slices the SO eventually returned to its previous frequency, but gamma oscillations in hippocampal slices retained a reduced power after whole-cell configuration was established.

Several studies presented in the last few years have successfully employed an oscillating mouse-preparation recorded in submersion. The spontaneously oscillating preparation is obtained by increasing the potassium concentration further, to five mM, thus increasing the excitability of the network to a level that could be required to initiate network activity in the mouse cortex (Fanselow & Connors 2010).

Another recent preparation also recorded in submersion while presenting spontaneous oscillation is the SO of the entorhinal cortex in mouse (Tahvildari et al. 2012). The entorhinal cortex is, however, structurally different to the neocortex but the preparation is valuable in terms of shedding light on cortical interneuron contributions to network activity as well as spontaneous entorhinal cortex activity in itself.

Neurotensin

The several lines of research implicating NT in the control of vigilance (see above) prompted us to design a series of experiments to determine if these effects could be explained by direct actions within the cortex and on cortical neurons by the peptide. First, we verified the presence of NT-positive fibers in the rat cortex (figure 1; paper II) and found that they are present throughout the cortical mantle but almost exclusively in deep layers and quite sparse. In addition the fibers co-express tyrosine hydroxylase (TH) but not dopamine beta-hydroxylase (DBH), tryptophan hydroxylase (TPH) or orexin.

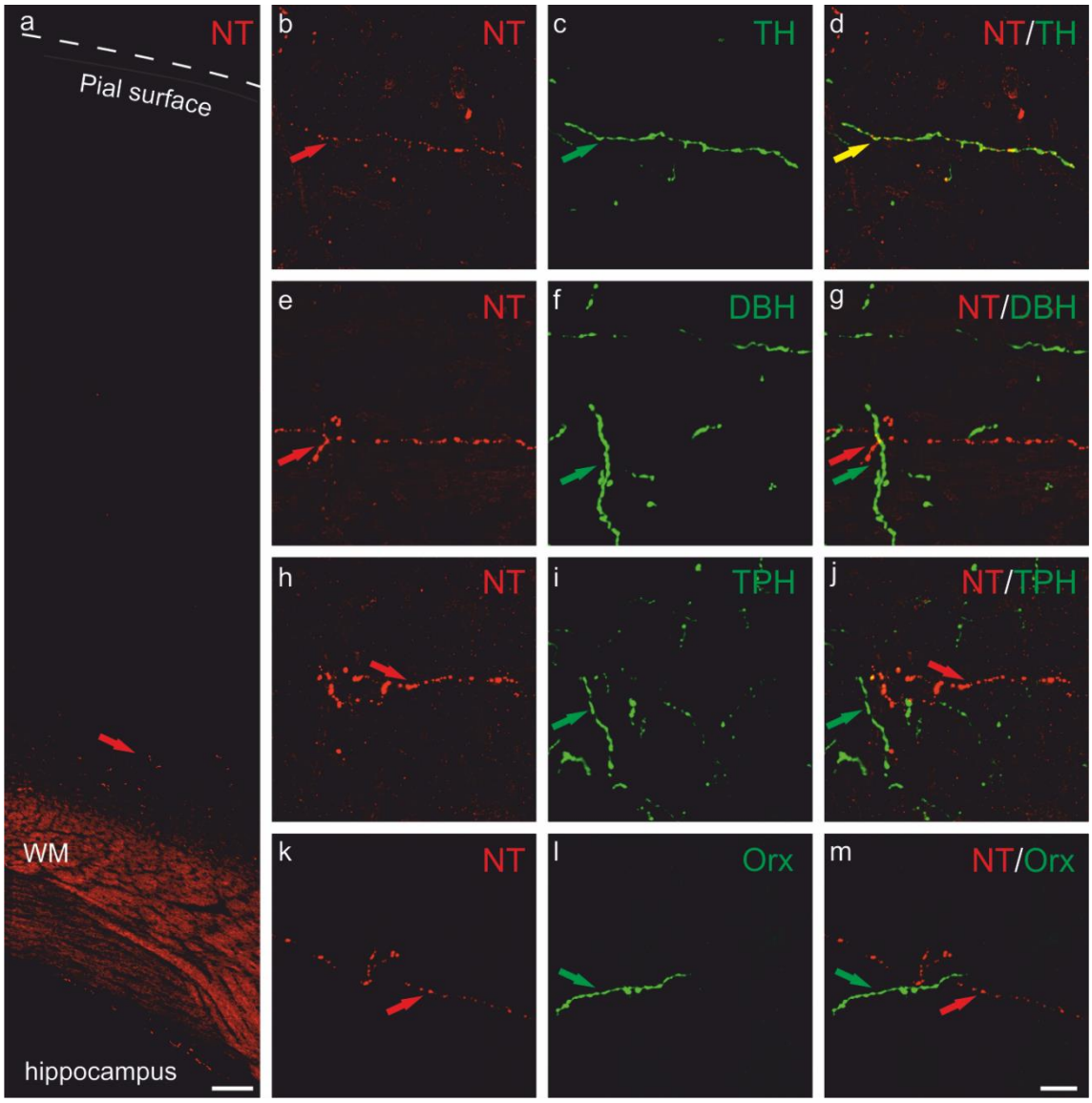

Figure 1. Confocal micrographs from coronal sections of rat somatosensory cortex double-labelled by immunofluorescence for: NT (red; a, b, e, h), TH (green; c), DBH (green, f), TPH (green, i) and orexin (Orx; green, k). Merged images are shown in (d, g, j, and m). Note sparse but discrete distribution of NT-ir fibers in the deepest cortical layers adjacent to the WM, and coexistence with TH-immunoreactivity but not with other markers.

The electrophysiological data obtained investigating the role of NT in the cortical network will be presented from the single neuron up to global network activity with data from rats and mice together but clearly indicated and contrasted when applicable.

4.2 Elucidating NT-action on single neurons

4.2.1 White matter neurons

The neurons residing in and just above the white matter below the cortical column have several names (see Introduction) but are here referred to as WM neurons. The data are obtained from rat and presented in paper II. Our focus turned to this subgroup due to the localisation of NT-fibers to the white matter and layer 6 as well as their previously described role in cortical orexin modulation.

Nearly all WM neurons tested responded to NT (n=114/116) comprising neurons from both somatosensory and visual cortex. In the majority of neurons, NT application was followed by depolarization from a quiescent baseline, resulting either in sustained tonic discharge of APs (n=36), or a transient depolarization followed by occasional APs (n=40; APs in 7/40 cells), or in the induction of rhythmic bistability with alternating periods of hyperpolarization and depolarized episodes of firing (n=21). In the final group of neurons application of NT resulted in a biphasic response: brief, subthreshold depolarization followed by sustained hyperpolarization (n=17).

In voltage clamp, a current (I_{NT}) in the inward direction could be observed subsequent to NT application in all WM neurons tested. This current was reduced by TTX suggesting either the involvement of sodium currents or excitatory synaptic input mediating part of the excitation. White matter neurons are primarily excitatory (Hoerder-Suabedissen & Molnár 2013; DeFelipe et al. 2010) and are interconnected at least in development (Hanganu et al. 2002). Consequently, a loss of recurrent excitation could explain the effect of TTX on the inward current. A positive shift in the distribution of PSCs in the presence of NT further strengthened the theory of excitatory interconnectivity. A broad NTR antagonist revealed the specificity of the NT effect as the inward current was completely abolished. A selective NTR1 antagonist suggests that both NTR1 and NTR2 are involved since the effect was only partially reduced.

These data, in combination with previous studies (Bayer et al. 2004; Chung et al. 2009), suggest a role for WM neurons in the cortex beyond development as WM neurons are the only subgroup in the cortical network reported to respond to orexin. WM neurons additionally project as superficially as layer 1 and finally NT exerts a dramatic effect on WM neurons. This begs the questions; *do WM neurons form specific connections in the overlaying cortex? And if so, how do their activation by NT affect their targets?*

4.2.2 Interneurons

Since inhibition plays a dominant role in the awake cortex compared to cortical deep sleep, we hypothesized that one or several subgroups of interneurons could be recruited by NT.

4.2.2.1 Fast spiking and low threshold spiking interneurons

Fast spiking and LTS interneurons were identified using the lhx6-EGFP mouse (Lavdas et al. 1999), the two groups can readily be divided according to electrophysiological characteristics (Ascoli et al. 2008). Data investigating the FS and LTS interneurons responses to NT are presented in papers II and III.

The two subgroups were pooled since they interestingly share the same response characteristics to NT application. All neurons tested responded with a small, mostly subthreshold, depolarization and only occasional APs (figure 2B). A striking increase in synaptic activity could also be observed in both cell types. (In rats, FS interneurons responded equally with a depolarization and increase in synaptic input; paper II.) Using voltage clamp configuration, the excitation could be seen as an inward current that appears to be mediated by changes in the activity of a pump observed through a parallel shift of the I-V ramp. The dramatic increase of synaptic input is excitatory and, like the inward current, mediated via NTR2. When comparing the effect of NT throughout the cortical column, a stronger effect in deep layers compared to superficial emerged, indicating an NT-response gradient.

4.2.2.2 5HT3aR interneurons

5HT3aR interneurons were identified using a 5HT3aR-EGFP mouse and the data are presented in paper IV. The 5HT3aR-EGFP neurons encompass a heterogeneous group of interneurons (Lee et al. 2010) but have been analysed as a single group due to consistent responses to NT. All neurons tested displayed a depolarisation occasionally accompanied by APs in current clamp (figure 2C). In voltage clamp an inward current could be observed. Neurotensin appeared to have a direct, postsynaptic effect on these neurons since its effect could not be blocked by TTX. As opposed to FS/LTS interneurons, 5HT3aR interneurons appear to respond to NT via the NTR1 receptor as co-administration of an NTR1 antagonist and NT completely abolished the I_{NT}. Elucidating NT's mechanism of action through I-V ramps in the presence of TTX and with ion-substitution revealed a dependence on potassium and calcium. In contrast to FS and LTS neurons, 5HT3a interneurons did not display a response-gradient to NT when recording from different layers.

Since the two mouse lines used in these projects together encompass most (if not all) cortical interneurons one can reason that all interneuronal subtypes have been explored. It is noteworthy that all interneurons respond to NT with excitation suggesting a powerful role for NT in augmenting inhibition. The different interneuronal subtypes play different parts in

the cortical network thus NT is in a position to increase several aspects of inhibition such as feed-forward inhibition, lateral inhibition and inhibitory tone.

4.2.3 Pyramidal cells

Pyramidal cells were sampled primarily in layers V-VI and treated as one group due to similar response properties within the respective species. In rats, pyramidal cells hyperpolarized as a result of NT-induced $GABA_B$ receptor activation (figure 2D; paper II) whereas in mice, pyramidal cells depolarized in the presence of NT (figure 2E; paper III). Inhibitory synaptic input frequency was, however, similarly increased in both species while excitatory input amplitude was increased in mice alone.

Why do pyramidal cells respond differently to NT in the two species? The respective hyperpolarization/depolarization is quite small but could greatly impact the network in both cases. Dissimilarities could be due to differing receptor expression in the species but further experiments will have to be performed to confirm this theory. In spite of the in some cases opposing responses to NT it does not seem to affect the network behaviour at large with respect to the reduction in UP state frequency in both species in the presence of NT (mice control: 0.73±0.01 *vs* NT: 0.02±0.01; n=4; $p<0.05$; data not present in manuscripts).

Published electrophysiological findings on NT in the cortex are scarce but in a study by Audinat *et al.* (1989) pyramidal cells in the pfc of the rat were reported to depolarize when exposed to NT. In order to exclude that these differences could be attributed to different experimental procedures *e.g.* composition of aCSF, recordings were performed in the pfc as well as sensory cortices. Pyramidal cells of the pfc depolarised in the presence of NT also in our experiments confirming the results from Audinat *et al*'s study (Case et al. 2016).

Examples of single cell recordings from neuronal subgroups are presented in figure 2.

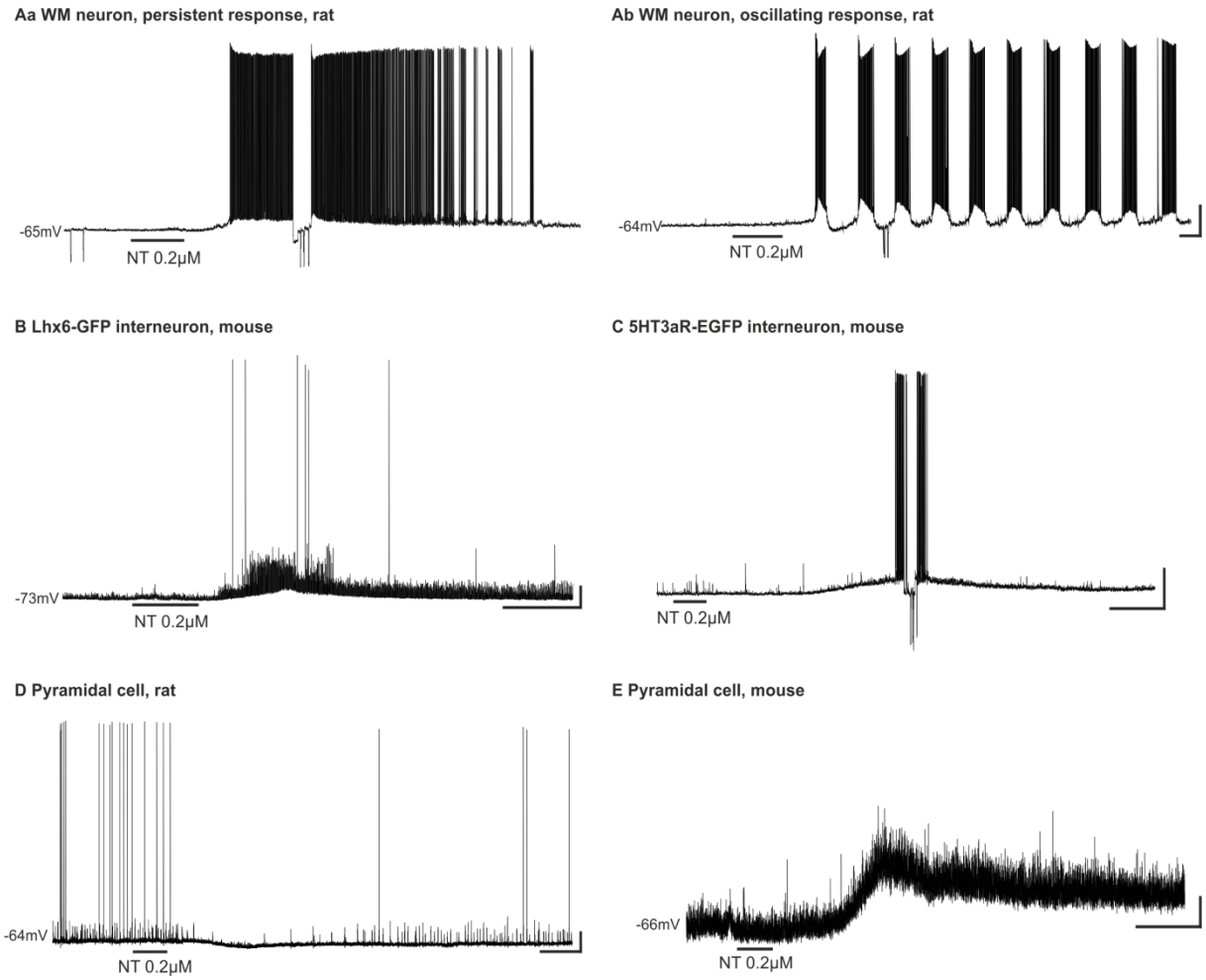

Figure 2. Example whole-cell recordings in current-clamp from neuronal subgroups: (Aa) rat WM neuron, (Ab) rat WM neuron, (B) Lhx6-GFP mouse interneuron, (C) 5HT3aR-EGFP mouse interneuron, (D) rat pyramidal cell, (E) mouse pyramidal cell.

4.3 Elucidating NT-action on microcircuitry

How can neuromodulators alter functional connectivity? Changing the probability of neurotransmitter release can strengthen or weaken of the synapse. In addition, this often leads to altered facilitation/ depression changing the behaviour of the communication between a neuron pair (Gu 2002). Gap junction connectivity can also be affected by neuromodulators either directly or indirectly through changes in synaptic input *e.g.* inhibitory shunting and NMDA mediated depression of electrical coupling (Sippy & Yuste 2013; Mathy et al. 2014). As we have observed significant effects on virtually all neurons tested we hypothesised that this would also be reflected in inter-neuronal communication.

4.3.1 Interneuron – pyramidal cell

Paired recordings of interneurons and pyramidal cells were performed in the lhx6-EGFP mouse to investigate the possibility of NT affecting their intercommunication (paper III). The recordings reveal increased amplitude of inhibitory synaptic currents from lhx6-GFP interneurons to pyramidal cells and the inverse in the opposite direction, *i.e.* decreased excitatory input to lhx6 interneurons by pyramidal cells (figure 3Aa-b). Further investigation of the modulation of lhx6 interneuron- pyramidal connectivity found the paired pulse ratio (PPR) to be significantly decreased. In this case, a decrease in PPR, together with an increase in amplitude of the first pulse, suggests an increased release probability of GABA in the presence of NT (Debanne et al. 1996). These data indicate a stronger role and influence over pyramidal cells for these interneurons in the microcircuitry in the presence of NT.

Paired recordings of 5HT3aR interneurons and pyramidal cells were attempted (paper IV) but due to low experimental yield and time restrictions these experiments were not pursued further. Pyramidal cells are mainly contacted by 5HT3aR neurons via their apical dendrites reflecting the stronger expression of this interneuronal subgroup in superficial layers (Marlin & Carter 2014). In this thesis I have focused on microcircuitry in deep cortical layers which could explain the comparatively low yield.

4.3.2 Gap junctions between Lhx6-GFP interneurons

The functional decoupling of FS and LTS interneurons can have large effects on the network; FS interneurons are well described drivers of synchronized gamma oscillations (Cardin et al. 2009), an activity that is dependent on gap junctions and reciprocal inhibition (Tamas et al. 2000). Disrupting the synchronizing mechanism for this oscillation would have extensive effects due to its involvement in cognitive functions. Desynchronizing LTS interneurons on the other hand could strongly affect the local network of both pyramidal cells and FS interneurons. LTS interneurons have been shown to impose slow synchronous firing (3-6Hz) on the local network seemingly dependent on gap junctions (Beierlein et al. 2000).

In the lhx6 mouse the FS and LTS interneurons display a large increase in synaptic "noise" in the presence of NT. Inspired by previous reports of the effects of inhibitory synaptic activity creating a shunting effect on gap junctional transmission of depolarizing impulses (Llinas et al. 1974), we hypothesized that functional electrical connectivity within these groups would be reduced as a result of the reduced input resistance caused by the barrage of excitatory synaptic input. To test this hypothesis, paired recordings of gap junction coupled interneurons were performed were pulses were injected into one cell and the resulting voltage deflections in the coupled cell were recorded (paper III). In control conditions the response in the coupled cell could clearly be distinguished as individual pulses whereas the application of NT led to an apparent loss of coupling in response to several pulses or occasionally a very large response (figure 3B). The average coupling strength between each pair was significantly reduced in the presence of NT but more striking; the coefficient of variation was greatly increased. Similarly; cross-correlating the cells showed a significantly greater correlation in control compared to NT. This apparent decrease in functional coupling could be due to direct effects rather than changes in the presynaptic input. To test this, the same experiment and analysis was performed under fast excitatory synaptic blockade. Under these conditions, neither the NT effects on gap junctional communication nor on input resistance were observed, supporting the role for synaptic input in reducing functional connectivity through a reduction in input resistance.

4.3.3 White matter neurons – Lhx6-interneurons

Performing paired recordings between WM neurons and interneurons located in close proximity above the WM neurons readily yielded connected pairs suggesting abundant connectivity. The WM neuron connected to the FS/LTS interneurons with an excitatory synapse that exhibited a relatively strong synapse (figure 3C) with fluctuating facilitation/depression at 20Hz. This is the first demonstration of a direct connection of WM neurons with an identified subgroup of cortical neurons revealing a role for WM neurons in the cortex beyond development. The connection provides an explanation for the increased excitatory input seen in FS/LTS interneurons in the presence of NT when additionally; the strength of the connection was amplified.

The WM neurons sampled in mice (paper III) were comparable to two of the subtypes of WM neurons that we found in the rat (paper II); the persistent responder and the transient responder. It should be noted, however, that only WM neurons that were found to connect to an interneuron were subsequently exposed to NT, thus the data do not exclude the existence of other subgroups of WM neurons in mice.

Neurotensin effects on cortical microcircuitry are presented and summarized in figure 3 and 4 respectively.

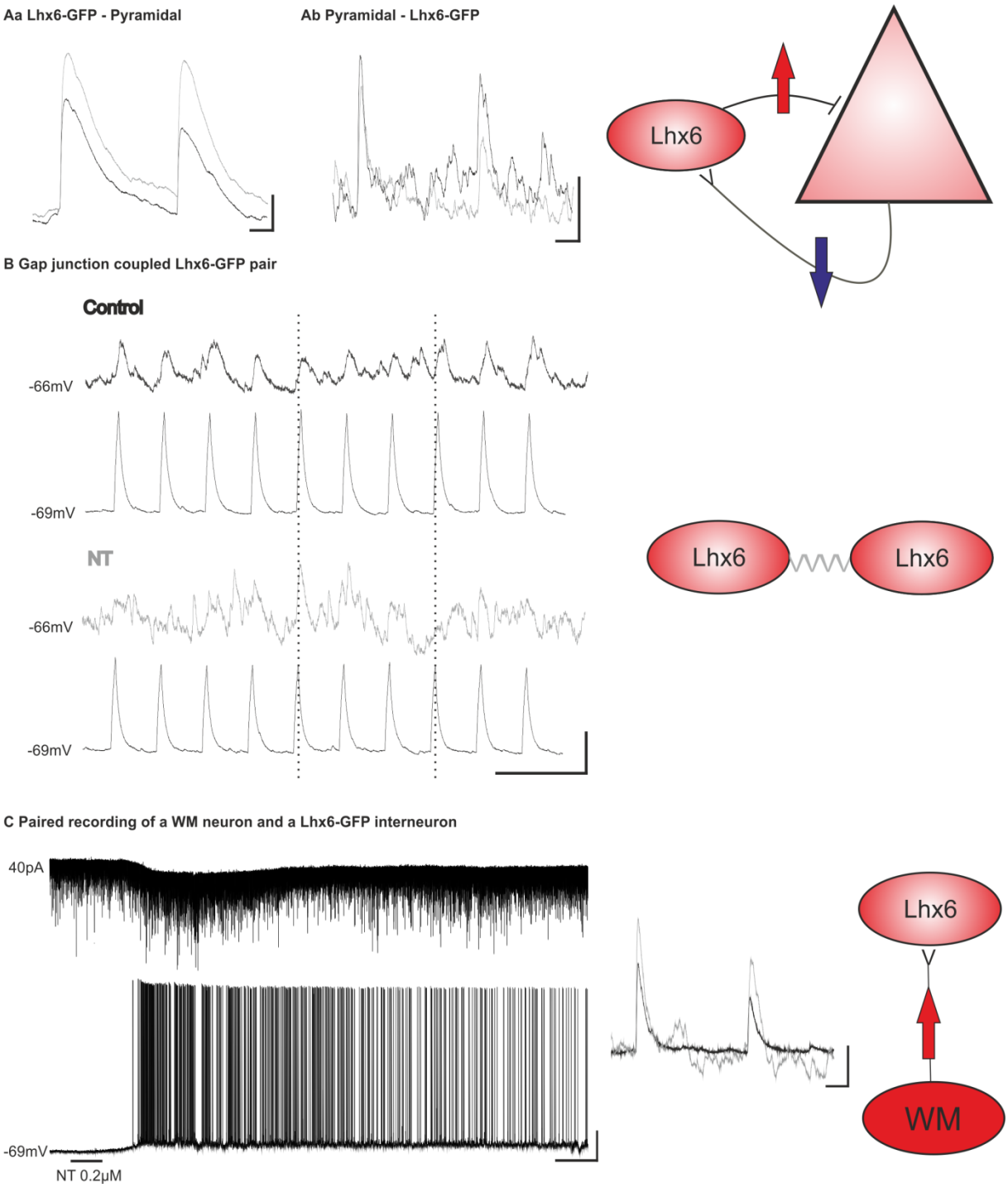

Figure 3. Examples of paired recordings between (A) pyramidal cell and Lhx6-GFP interneuron, (B) gap junction coupled Lhx6-GFP interneurons, (C) WM neuron to Lhx6-GFP interneuron. Red – excitation, blue – inhibition, Y – excitatory connection, T – inhibitory connection, WW – gap junction.

Summary of the effects of NT on the single neurons and microcircuitry

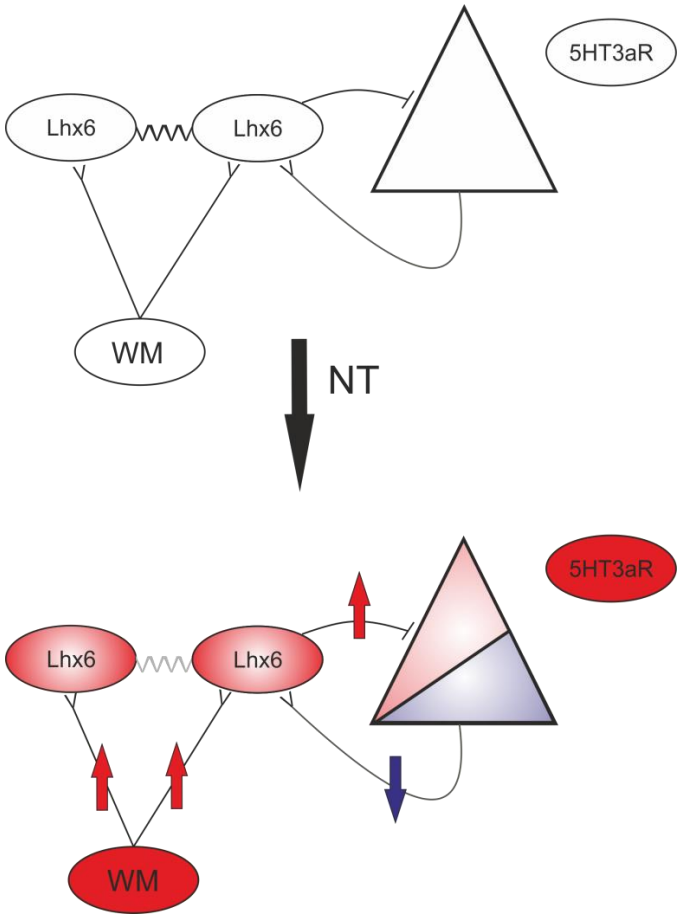

Figure 4. Schematic summary of NT effects on single neurons and microcircuitry from rats and mice. Red – excitation, blue – inhibition, T – inhibitory connection, Y – excitatory connection, WW – gap junction coupling.

4.4 Investigating a potential role of NT in modulating cortical state

The widespread actions of NT on the membrane properties and the interconnectivity of all the main cortical cell classes (see above) lead to the question: *can the peptide modulate overall state-dependent network activity?* The final data set that will be described addresses this outstanding question (Paper II).

4.4.1 The slow oscillation

A significant reduction in UP state frequency was observed in all slices where NT was applied at concentrations 0.2µM or higher. No significant difference was observed between visual and somatosensory cortex suggesting that NT network modulation is not specific to discrete cortical regions. The UP state frequency was reversible, often occurring before washout of NT, indicating desensitization possibly due to receptor internalization (Alonso et al. 1994). Bath application of NTR antagonists revealed a dependence on NTR2 to mediate the NT-induced reduction in UP state frequency. Dopamine modulation by NT did not appear to be involved since bath application of DA receptor (DAR) antagonists did not alter the effect of NT on the SO.

The SO involves all layers and the majority of cortical neurons (Sanchez-Vives & Mccormick 2000; Steriade, a Nuñez, et al. 1993). Thus, a neuromodulatory effect on the network can arise either from actions that are highly localized and discrete, or effects that are more broadly distributed. In our single neuron experiments we found that FS/LTS interneurons exhibited a response gradient with a stronger response in deep layers whereas 5HT3aR interneurons did not. To investigate the potential scope of NT cortical targets we therefore employed an approach involving systematic focal application. As the NT positive fibers are largely restricted to deep layers (Case et al. 2016; Jennes et al. 1982) and UP states are more likely to initiate in layer 5 (Sanchez-Vives & McCormick 2000) we hypothesised that focal application close to the white matter would be most effective at recapitulating the NT bath application. The experiments indeed revealed that only NT application in the deepest strata (the lowest segment of which included the white matter) was successful in recapitulating the effects of NT bath application on UP state frequency, a response that chimes with the strict confinement of NT-ir fibres to the deep layers.

4.4.2 The thalamo-cortical response

Since the cortical response to incoming sensory input differs between sleeping and waking (Haider et al. 2013) we hypothesised that this would be modelled in our experiments. To investigate the effects of NT on sensory processing we used the thalamocortical slice described in Agmon & Connors (1991). Whole-cell recordings were performed from pyramidal cells and FS cells of layer 6 that received direct thalamic input. To more closely

mimic *in vivo* conditions only slices where thalamic stimulation resulted in UP state generation in the form of a delayed network response were used.

In the presence of NT, the slope of repolarization was significantly steeper furthermore the area-under-the-curve calculated for the delayed network response ("UP state") was markedly decreased. While the average amplitude of response was not affected by NT, the standard deviation of the amplitude was significantly reduced, suggesting that the peptide decreases response variability across trials.

To investigate the network alterations responsible for the increased repolarizing slope the relative contributions of excitation and inhibition were examined. Measuring relative inhibitory and excitatory conductance offers a window onto the surrounding network; *is inhibition or excitation dominating or is there a balance?* Pyramidal and FS cells were recorded and their inhibitory and excitatory input conductances in response to thalamic stimulation were calculated. The proportions of excitatory and inhibitory conductances were not significantly different in control conditions. In the presence of NT, however, synaptic input unbalanced such that the relative excitatory conductance was significantly smaller than the inhibitory conductance.

The results described above following administration of NT are similar to what occurs *in vivo* when transitioning from sleep to waking; the temporally and spatially constrained response to thalamic input that can be gleaned from the whole-cell recordings, reduction in response-variability and finally an increase in relative inhibition compared to excitation (Haider et al. 2013).

5 Conclusions

This thesis focuses on the modulation of cortical functional networks and microcircuitry by NT. I have found that NT recruits inhibitory interneurons broadly and either depolarizes or hyperpolarizes pyramidal cells depending on species. Studying inter-neuronal connectivity revealed a shift in connectivity strength and reliability favouring inhibition and desynchronization, respectively. The global network activity associated with deep sleep was depressed in the presence of NT as was the evoked response to thalamic stimulation. The direct cortical response to thalamic input was modulated to be temporally more restricted and also less variable in amplitude. These findings all suggest a role for NT as a mediator of cortical arousal.

The increase in relative inhibition recorded in thalamocortical experiments can thus be explained through the single cell recordings from both mouse and rat where all inhibitory interneurons tested were depolarized in the presence of NT. This finding sets NT apart from several other neuromodulators that target selective interneuronal subgroups (Xiang et al. 1998; Kawaguchi & Shindou 1998; Gao et al. 2003). In addition, pyramidal cells either hyperpolarized and/or received an increase in inhibitory input. All FS and LTS interneurons recorded received an increase in excitatory input presumably mainly from WM neurons adding to the overall drive of inhibition.

The layer specificity of NT effects in SO experiments suggests a more important role for Lh6x-GFP interneurons since they also display a response gradient favouring deeper layers in contrast to 5HT3aR-EGFP interneurons. UP-states are more prone to initiate in layer 5 (Sanchez-Vives & Mccormick 2000) - where FS and LTS interneurons are abundant (Xu et al. 2010) – offering an explanation for the NT-induced decrease in recurrent excitation, which is thought to underlie UP state initiation and propagation (Sanchez-Vives & Mccormick 2000; Chauvette et al. 2010).

The decrease in global network oscillations induced by NT can also be understood through the changes in functional connectivity that we observed between Lhx6-GFP interneurons. It is known that gamma oscillatory activity can be detected within the UP states of the SO (Compte et al. 2008) and that FS interneurons are drivers of gamma (Cardin et al. 2009). Furthermore, FS interneurons extend axons horizontally beyond the column of the cell body (Xiang et al. 1998) indicating an importance in maintaining synchronized gamma activity in extensive and distant (Traub et al. 1996) patches of cortex from within layer 5. The powerful functional desynchronization of gap junction- coupled FS interneurons by NT can thus provide a rationale for the disassembly of the functional network underlying UP state synchrony throughout the cortical mantle. This relationship highlights the important, yet

until now rather unexplored, role of WM neurons since they are likely central to this effect on the FS interneuronal population.

Since gamma oscillations are associated with attention and sensory processing (Eckhorn et al. 1988; Fries 2001) it may seem counterintuitive for a purported wake-promoting peptide such as NT to attenuate the ability of interneurons to synchronize their activity. For efficient information processing, however, it is important that only the salient or relevant features of our surroundings are attended (Desimone & Duncan 1995). Thus a background of desynchronization becomes necessary.

In conclusion the data collected in this thesis all point in the direction of cortical arousal resulting from the activation of NT receptors; increased relative inhibition and desynchronization.

5.1 Future directions and questions

One of the main remaining questions regards the source of cortical neurotensin. A likely candidate in rat is the VTA since the fibers are TH positive (but DBH-negative) and the VTA has been shown to project to the cortex although as of yet only the pfc (BERGER et al. 1992). Recent studies have suggested a role for the VTA as an arousal mediator (Solt et al. 2014), primarily thought to act through DA but NT would presumably be co-released during the bursting behavior seen in aroused states (Gonon 1988). Assuming that the source would be the same in mice contradict the VTA as a potential candidate however, it has been shown to lack NT expression (Smits et al. 2004).

The data presented here suggest that current concepts of NT may need to be revised as the role of NT extends beyond that of modulating DA actions, and that its sphere of influence is not limited to the pfc. Our data indicates that care has to be taken to separate the effects of DA and NT when investigating the effects of the VTA on its projection targets in both rats and mice. Even though mice do not display the same co-localization of DA and NT as rats (Smits et al. 2004), NTR and DAR can be expressed by the same target neurons and influence DA transmission. In our experiments, the effect of NT on cortical network activity did not appear to involve DA transmission, however. It is important to note that in primates and humans NT and DA do not appear to be co-expressed in cortical fibers and NT fibers are not restricted to deep layers but are more widely expressed (Gaspar et al. 1990; Satoh & Matsumura 1990). Furthermore, in the VTA, humans do not exhibit co-localization between NT and DA (BERGER et al. 1992), suggesting a separation between the two modulators but perhaps an extended cortical role for NT.

Neurotensin has been implicated in the pathophysiology of schizophrenia, primarily through NT-DA interactions in the pfc (Richelson et al. 2005; Kinkead & Nemeroff 2004). Schizophrenic patients present low levels of NT in the CSF, and it is hypothesized that lower levels of NT will cause a weaker antagonistic interaction with the pfc D2 receptor effectively decreasing DA release (Garver et al. 1991). Additional evidence comes from the observation that the NT CSF levels can be reversed by treatment with haloperidol, an antipsychotic agent (Garver et al. 1991). In addition, gamma-band activity is reduced in schizophrenic patients suggesting an underlying role for FS interneuron malfunction in this disorder (Woo et al. 2010; Spencer et al. 2003). Since focus on the involvement of NT in schizophrenia has been on interactions with DA, NTR1 has been the favored receptor and even the target of a clinical trial that yielded no suitable drug-candidate. Perhaps attention has been on the wrong receptor and in too narrow a cortical region? The effects of NT on FS interneurons and the SO are mediated via NTR2 and appear to span the cortical mantle. It would be interesting to explore the possibility of NTs involvement in the pathophysiology of schizophrenia with this in mind.

Performing experiments *in vitro* has obvious benefits but also limitations; the environment is artificial and "sensory stimulation" is applied manually. The ability to investigate one modulator in isolation as in the case of NT, to study it and to carefully dissect the effects on single neurons and connectivity is doubtless valuable but to be certain that NT can indeed "awaken the cortex" an *in vivo* model would be necessary. To test the results from my studies it would therefore be very interesting to use one or more of the recently developed techniques such as optogenetics (Boyden et al. 2005) and/or Designer Receptors Exclusively Activated by Designer Drugs (DREADD; Mueller et al. 2005); perhaps stimulating the release of NT optogenetically to study arousal and/or sensory processing alternatively inhibiting the activity of WM neurons using DREADD to dissect the functional role of WM neurons. The difficulty using these *in vivo* techniques will be to specifically investigate NT without the involvement of co-released modulators such as DA and the potential subdivision of WM neurons. Conditional NTR-knockouts in specific neuronal subgroups such as NTR2 in FS/LTS interneurons could potentially yield more precise results. Alternatively one could measure NT release under different conditions such as sleeping, waking and attentive states to correlate NT levels to arousal.

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