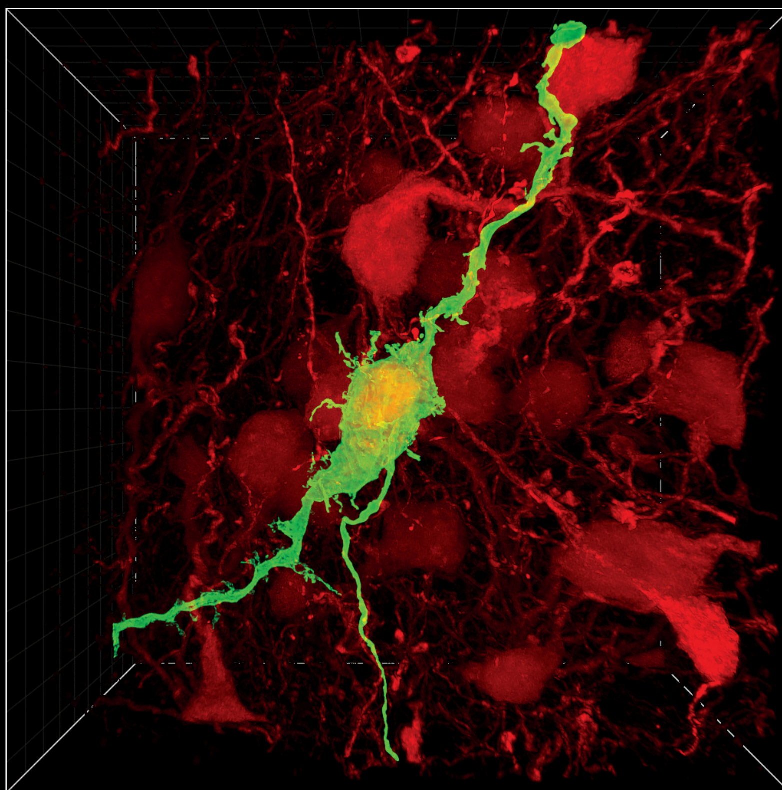
Thesis for doctoral degree (Ph.D.)
2018

Anatomical studies of the dopamine system in the hypothalamus and the pituitary gland



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ANATOMICAL STUDIES OF THE DOPAMINE SYSTEM IN THE HYPOTHALAMUS AND THE PITUITARY GLAND

Arash Hellysaz

Karolinska Institutet

Stockholm 2018

FRONT COVER ILLUSTRATION

High power maximum intensity projection Airyscan confocal micrographs of neurobiotin filled (green) tuberoinfundiblar (TIDA) neuron, from the arcuate nucleus of a juvenile mouse, immunostained for tyrosine hydroxylase (red), the rate-limiting enzyme in catecholamine biosynthesis. The green channel has been deconvolved and filtered for noise and staining artefacts. These kinds of high-resolution 3D micrographs of single neurons have been used in the studies included in this thesis, to analyze the morphological features of TIDA neurons and to determine their neurochemical profile. At this resolution, both somatic and dendritic spines are clearly visualized and can undergo detailed analysis.

BACK COVER ILLUSTRATION

Low power fluoresence micrograph of a coronal section from a juvenile rat, immunostained for tyrosine hydroxylase (red), to visualize A13 dopamine neurons of zona incerta.

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ISBN 978-91-7831-229-0

INSTITUTION FÖR NEUROVETENSKAP

Anatomical studies of the dopamine system in the hypothalamus and the pituitary gland

AKADEMISK AVHANDLING

som för avläggande av medicine doktorsexamen vid Karolinska Institutet
offentligen försvaras i *Samuelssonsalen*, Tomtebodavägen 6, Solna

Måndag den 17 december 2018, kl 10:00

av

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STOCKHOLM 2018

To my mother
my father
and my beloved sister

BRAIN: THE FINAL FRONTIER

THESE ARE THE VOYAGES OF THE PH.D. STUDENT ARASH HELLYSAZ

ITS FOUR-YEAR MISSION: TO EXPLORE STRANGE NEW NEURONS

TO SEEK OUT THEIR MORPHOLOGY AND THEIR CHARACTERISTICS

TO BOLDLY STAIN, WHAT NO MAN HAS STAINED BEFORE!

ABSTRACT

The hypothalamus is a small, evolutionarily conserved brain region, necessary for our survival as individuals and as a species. It collects various sensory inputs, process them to maintain homeostasis and to overcome stressors, and generates outputs that affect the autonomic nervous system, the endocrine system and somatomotor behaviors. Energy metabolism, fluid balance, thermoregulation, sleep, aggression and reproduction are examples of functions under direct and indirect hypothalamic control.

The neurochemical basis for these regulations involve different neurotransmitters and neuromodulators. The catecholamine dopamine is highly associated with various hypothalamic functions and behaviors, and has early on been shown to be present in intrinsic hypothalamic populations as well as incoming axon terminals. It acts on two types of receptors, excitatory D1-type and inhibitory D2-type, of which both have been reported to be expressed in the hypothalamus.

To increase our understanding of these circuitries, this thesis aims to investigate the dopamine system in the hypothalamus, and the structures closely related to its inputs and outputs, namely the circumventricular organs and the pituitary.

Immunohistochemical methods were used to generate a comprehensive distribution map of dopamine's two main receptors, D1 and D2, and the neurochemical identity of these dopamine-receptor expressing cells were characterized. While the D2 receptor was widely expressed, D1 expression was found to be sparse. The suprachiasmatic nucleus, however, showed the contrary expression pattern. The D2 receptor could be localized to parvocellular neurons as well as endocrine cells of the pituitary. Little evidence for dopamine receptor expression on the magnocellular neurons could, however, be observed. Evidence for D1 receptor expression was also found in the subcommissural organ and a sub-cluster of ependymal cells in the third ventricle.

Tuberoinfundibular dopamine (TIDA) neurons, which release dopamine in the portal vessels and thereby inhibit lactotrophs and prolactin release, were investigated in greater detail with regards to modulatory input, and morphological features. Anatomical substrate for innervation by serotonin and hypocretin/orexin on TIDA cell body and dendrites was identified together with electrophysiological evidence for excitation and suppression by hypocretin/orexin and serotonin or selective serotonin reuptake inhibitors, respectively.

Morphological studies of male mice and rat TIDA neurons were done on tissue section and marker filled neurons by means of immunohistochemistry. TIDA neurons were

found to preferentially extend dendrites towards the third ventricle, possibly even into the ventricle. Axon terminals were found in the median eminence, but collateral branches oriented laterally could also be detected. An intermingling subcellular distribution of inhibitory and excitatory synapses, on somatic and dendritic level, was also identified. No significant differences could be observed in most morphological properties of mouse and rat TIDA neurons. However, rats exhibited a higher total number of TIDA neurons and a lower spine density than mice.

Finally, the expression of three different calcium binding proteins, *i.e.* calbindin-D28k, calretinin and parvalbumin, were investigated within the arcuate nucleus. While both calbindin-D28k and calretinin could be detected in the arcuate nucleus, little evidence for parvalbumin expression was observed. None of the proteins were expressed in TIDA neurons or other investigated populations, except for proopiomelanocortin neurons that expressed both calbindin-D28k and calretinin. These neurons showed a rostrocaudal segregation of the two calcium binding proteins that resulted in two separate subpopulations.

Overall, the studies presented in this thesis reveal a previously unappreciated abundance of dopaminergic involvement in the hypothalamic circuitries which will increase our understanding of mammalian homeostatic and endocrine control.

LIST OF SCIENTIFIC PAPERS

The work in this thesis consists of the following communications, which are referred to in the text by their Roman numerals.

I. Kylie S. Foo*, **Arash Hellysaz*** and Christian Broberger (2014) Expression and colocalization patterns of calbindin-D28k, calretinin and parvalbumin in the rat hypothalamic arcuate nucleus. *Journal of Chemical Neuroanatomy* 61:20–32.

II. **Arash Hellysaz,** Marta Garo, Rachida Ammari, David J. Lyons and Christian Broberger (2018) Morphological characterization of tuberoinfundibular dopamine (TIDA) neurons in rodents. *Manuscript*.

III. David J. Lyons, Rachida Ammari, **Arash Hellysaz** and Christian Broberger (2016) Serotonin and antidepressant SSRIs inhibit rat neuroendocrine dopamine neurons: Parallel actions in the lactotrophic axis. *Journal of Neuroscience* 36:7392–7406.

IV. David J. Lyons, **Arash Hellysaz**, Rachida Ammari and Christian Broberger (2017) Hypocretin/orexin peptides excite rat neuroendocrine dopamine neurons through orexin 2 receptor-mediated activation of a mixed cation current. *Scientific Reports* 7:41535.

V. **Arash Hellysaz**, Alessandra Bonito-Oliva and Christian Broberger (2018) Distribution and neurochemical characterization of dopamine D2 receptor expressing neurons in the hypothalamus and pituitary gland of *Drd2*-EGFP transgenic mice. *Manuscript*.

VI. **Arash Hellysaz**, Qingyun Wen and Christian Broberger (2018) Distribution and neurochemical characterization of dopamine D1 receptor expressing cells in the hypothalamus, circumventricular organs and pituitary gland in mice. *Manuscript*.

* Equal contribution.

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Additional published communications not included in this thesis.

i. Lyons DJ, **Hellysaz A**, Broberger C (2012) Prolactin regulates tuberoinfundibular dopamine neuron discharge pattern: novel feedback control mechanisms in the lactotrophic axis. *The Journal of Neuroscience* 32:8074–8083.

ii. Horjales-Araujo E, **Hellysaz A**, Broberger C (2014) Lateral hypothalamic thyrotropin-releasing hormone neurons: Distribution and relationship to histochemically defined cell populations in the rat. *Neuroscience* 277:87–102.

iii. Tulke S, Williams P, **Hellysaz A**, Ilegems E, Wendel E, Broberger C (2016) Nucleobindin 1 (NUCB1) is a Golgi-resident marker of neurons. *Neuroscience* 314:179–188.

iv. Bonito-Oliva A, Södersten E, Spigolon G, Hu X, **Hellysaz A**, Falconi A, Gomes A-L, Broberger C, Hansen K, Fisone G (2016) Differential regulation of the phosphorylation of Trimethyl-lysine27 histone H3 at serine 28 in distinct populations of striatal projection neurons. *Neuropharmacology* 107:89–99.

v. Romanov RA, Zeisel A, Bakker J, Girach F, **Hellysaz A**, Tomer R, Alpár A, Mulder J, Clotman F, Keimpema E, Hsueh B, Crow AK, Martens H, Schwindling C, Calvigioni D, Bains J, Máté Z, Szabó G, Yanagawa Y, Zhang MD, Rendeiro A, Farlik M, Uhlén M, Wulff P, Bock C, Broberger C, Deisseroth K, Hökfelt T, Linnarsson S, Horvath TL, Harkany T (2017) Molecular interrogation of hypothalamic organization reveals distinct dopamine neuronal subtypes. *Nature Neuroscience* 20:176–188.

vi. Stagkourakis S, Pérez CT, **Hellysaz A**, Ammari R, Broberger C (2018) Network oscillation rules imposed by species-specific electrical coupling. *eLife* 7: e33144.

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LIST OF ABBREVIATIONS

3V	third ventricle
α-MSH	alpha melanocyte-stimulating hormone
β-END	beta-endorphin
ACTH	adrenocorticotrophic hormone
AgRP	agouti-related peptide
ANS	autonomic nervous system
Arc	arcuate nucleus of the hypothalamus
AVP	vasopressin
CaBP	calcium binding proteins
Calnuc	see NUCB1
CART	cocaine and amphetamine regulated transcript
CB	calbindin-D28k
CNS	central nervous system
CRH	corticotrophin-releasing hormone
DA	dopamine
DMH	dorsomedial hypothalamic nucleus
EGFP	enhanced green fluorescent protein
ENK	enkephalin
f	fornix
FITC	fluorescein isothiocyanate
FSH	follicle-stimulating hormone
GABA	gamma-aminobutyric acid
GH	growth hormone
GHRH	growth hormone-releasing hormone
GnRH	gonadotrophin-releasing hormone
H/O	hypocretin/orexin
IHC	immunohistochemistry
ISH	*in situ* hybridization
LH	luteinizing hormone
LHA	lateral hypothalamic area
MCH	melanin concentrating hormone
ME	median eminence
mRNA	messenger ribonucleic acid
mt	mammillothalamic tract
NPY	neuropeptide tyrosine
NUCB1	nucleobindin-1
NUCB2	nucleobindin-2
OXT	oxytocin
PFA	paraformaldehyde
POMC	proopiomelanocortin
PRL	prolactin
PV	parvalbumin
PVH	paraventricular hypothalamic nucleus
SON	supraoptic nucleus
SSRI	selective serotonin reuptake inhibitor
SST	somatostatin
TH	tyrosine hydroxylase
TIDA	tuberoinfundibular dopamine
TRH	thyrotropin-releasing hormone
TSA	tyramide signal amplification
TSH	thyroid-stimulating hormone
VIP	vasoactive intestinal peptide
VMH	ventromedial hypothalamic nucleus

CHAPTER 1

INTRODUCTION

The human brain is a marvelous machinery that has been estimated to harbor more than 80 billion neurons and more or less equal number of other cell types. The earliest known record that mentions the human brain dates back to the 17th century B.C. Egypt, where the word "brain" written on papyrus in hieroglyphs (see Figure 1), was used to describe the symptoms, diagnosis, and prognosis of traumatic injuries of the head (Gross, 1987). Aristotle suggested the brain to be a cooling mechanism for the blood. Galen, however, concluded that it controls muscles and sensory processing. Although, not far from the truth, these conclusions were based on completely false assumptions of density differences between cerebrum and cerebellum.

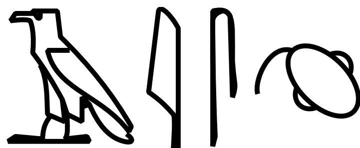

Figure 1. The hieroglyph for "brain" from 17th century B.C. ancient Egypt. Adapted from the work of Riccardo Metere as presented on Wikipedia. Licensed under the creative commons attribution-share alike 4.0 international license.

The modern view of the brain, where the neuron is seen as the functional unit, did not take shape until Camillo Golgi managed to stain neurons in the late 19th century (Golgi, 1873), and thereby provided the foundation that could be used by Santiago Ramón y Cajal to formulate the neuron doctrine (De Carlos and Borrell, 2007). Since then, our understanding of the brain and how it "really" functions has increased exponentially.

Yet remarkably numerous aspects of the brain, even at the most basic levels, remain unknown. In the same spirit as Golgi and Cajal, the studies included in this thesis, mainly focus on basic building blocks, *i.e.* neurons, neurotransmitters, neuromodulators and their receptors, in one of the evolutionarily oldest parts of the brain, the hypothalamus, and use anatomy and cytoarchitechtonics to explore functions and pathways and, thereby, to increase our understanding of this organ.

1.1 THE HYPOTHALAMUS

The hypothalamus is a relatively small but evolutionarily old part of the brain, which, as the name suggests, is located just below the thalamus. In an adult human brain, it is roughly the size of a walnut and constitutes less than one percent of the total brain weight (see Saper and Lowell, 2014). Anatomically, the hypothalamus is divided into a number of scattered smaller nuclei, and thus presents an – at first glance – unstructured organization relative to many other brain regions. The two hemispheres of the hypothalamus are separated in the midline by the third ventricle (3V) and rostro-caudally, it is usually subdivided into three parts.

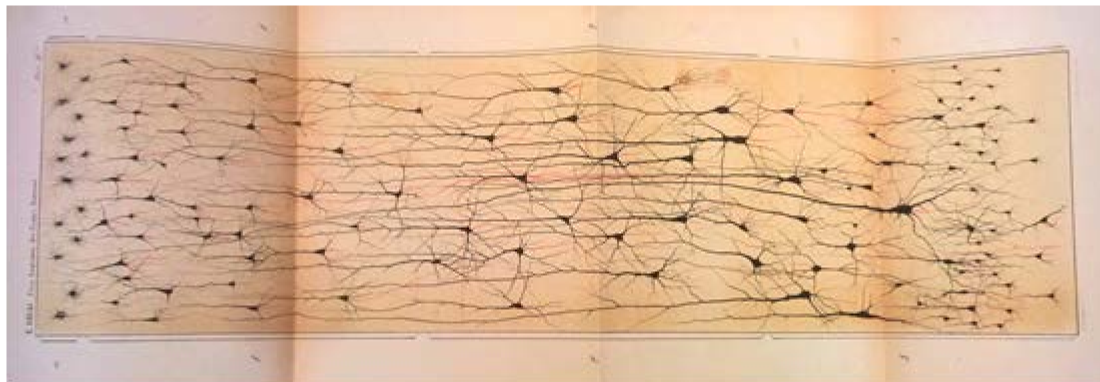

Figure 2. Drawing of neurons, by Camillo Golgi. From *"Sulla fina anatomia degli organi centrali del sistema nervoso"*, 1885. Original copy available at The Hagströmer Medico-Historical Library, Solna, Sweden.

The rostral part, known as the *preoptic area*, is located dorsal to the optic chiasm and includes the different preoptic areas and nuclei and the suprachiasmatic nucleus. In the mid part, known as the *tuberal hypothalamus*, the pituitary stalk (a.k.a. the infundibulum) that connects the hypothalamus and the posterior pituitary, emerges from the ventral surface. The anterior and lateral hypothalamic areas but also the dorsomedial, ventromedial, paraventricular, supraoptic, and arcuate nuclei are included in this part. Finally, in the caudal aspect, the *posterior hypothalamus* contains the mammillary bodies nuclei and areas dorsal to them, like the posterior hypothalamic nuclei (see Saper, 2012).

Despite its modest size, the hypothalamus is essential for the survival of the individual as well as the species as a whole, as it contains highly conserved neuronal circuitries that control basic functions. Energy metabolism, fluid balance, thermoregulation, sleep, aggression and reproduction are some examples of functions under direct and indirect hypothalamic control. The hypothalamus has an integrative role, where intero- and exteroceptive sensory inputs are collected and processed for activation of responses that maintain homeostasis and help the organism to overcome stressors and capitalize on opportunities for survival. These outputs are mediated through three major pathways: behavior through the somatomotor system, the autonomic nervous system and the endocrine system (see Swanson, 2000).

1.1.1 Behavioral outputs from the hypothalamus

As stimulation of different hypothalamic nuclei can rapidly result in fully formed somatomotoric behaviors, whilst lesion in the same area would inhibit the same behavior, it was initially thought that the hypothalamus contained different behavioral centers, *e.g.* for feeding (Anand and Brobeck, 1951a), drinking (Andersson et al., 1975) or attacking (Wasman and Flynn, 1962). This dual center hypothesis, as proposed by Eliot Stellar (1954), explained behavioral outputs from the hypothalamus as motivational states that direct the attention of an organism towards completing specific goals. Thus, early experiments identified *e.g.* the lateral hypothalamus as hunger center and ventromedial hypothalamus as a satiety center (Anand and Brobeck, 1951a, 1951b).

Current thought suggests, however, that behaviors arise through an interplay of different brain regions and cannot be pinpointed to a discrete nucleus. The hypothalamus directs a certain behavior by increasing the likelihood to engage it (see Plata-Salamán, 1998), rather than activating a set of motor functions. Hunger and wakefulness can for instance increase the organism's likelihood to eat, and more complex behaviors can be driven by *e.g.* sense of pleasure or disgust towards a certain odor or taste (see Saper and Lowell, 2014).

Furthermore, different hypothalamic nuclei have been identified to be involved in a multitude of functions. For instance, in addition to promoting satiety, it was found that electrical stimulation of the ventromedial hypothalamus, in addition to its role in feeding, also could elicit aggression (Siegel and Skog, 1970; Kruk et al., 1979; also see Hashikawa et al., 2017), and more recent investigations have identified the ventral premammillary nucleus to be involved in both leptin mediated onset of puberty (Donato et al., 2011; Ratra and Elias, 2014) and intermale aggression (Stagkourakis et al., 2018b).

1.1.2 Autonomic outputs from the hypothalamus

In the autonomic nervous system, preganglionic neurons of both sympathetic and parasympathetic pathways are directly and indirectly innervated by hypothalamic neurons located in the paraventricular nucleus, the lateral hypothalamic area and the arcuate nucleus (see Saper, 2002). Interestingly, endocrine glands in the periphery are also innervated, and hypothalamic neurons exert their regulation on *e.g.* insulin and glucagon by autonomic innervation of pancreas (Porte et al., 1975).

1.1.3 Endocrine outputs from the hypothalamus

The endocrine outputs of the hypothalamus are mainly subdivided into two routes. In the first route, magnocellular neurons (from Latin *magnus*, meaning "great" or "large") that exhibit large cell bodies, are located in the supraoptic and paraventricular nuclei and extend axons through the pituitary stalk to the blood vessels in the posterior pituitary, where they secrete the hormones vasopressin (Vigneaud et al., 1953a) or

oxytocin (Vigneaud et al., 1953b) directly into the general circulation (see Flament-Durand, 1980).

In the second route, parvocellular neurons (from Latin *parvus*, meaning “small”), which exhibit smaller cell bodies than magnocellular neurons, extend axons that terminate on the first portal capillary blood vessels in the median eminence and release messenger molecules in the portal capillary system. These so called factors are subsequently transported to the second portal capillary bed in the anterior pituitary where they either excite or inhibit endocrine cells (see Flament-Durand, 1980). Parvocellular neurons are located in close proximity to the third ventricle from the preoptic area towards the infundibulum and can mainly be found in the medial preoptic area and the paraventricular, periventricular and arcuate nuclei. As magno- and parvocellular neurons secrete directly into the bloodstream, they are also known as neuroendocrine neurons. This thesis focuses particularly on the parvocellular system, which is further described in the section below.

1.1.4 The parvocellular system

The view of hypothalamus controlling the release of pituitary hormones has, not always been a dogma. In the 1930s it was generally believed that blood would flow from the pituitary to the hypothalamus, and that it was the pituitary that would control the hypothalamus (see Watts, 2011). The contemporary view of the parvocellular system could be established thanks to pioneering studies conducted by Geoffrey Harris (1948), who could demonstrate the correct blood flow direction with compelling evidence, and suggest hypothalamus as the controlling body, and subsequent identification of

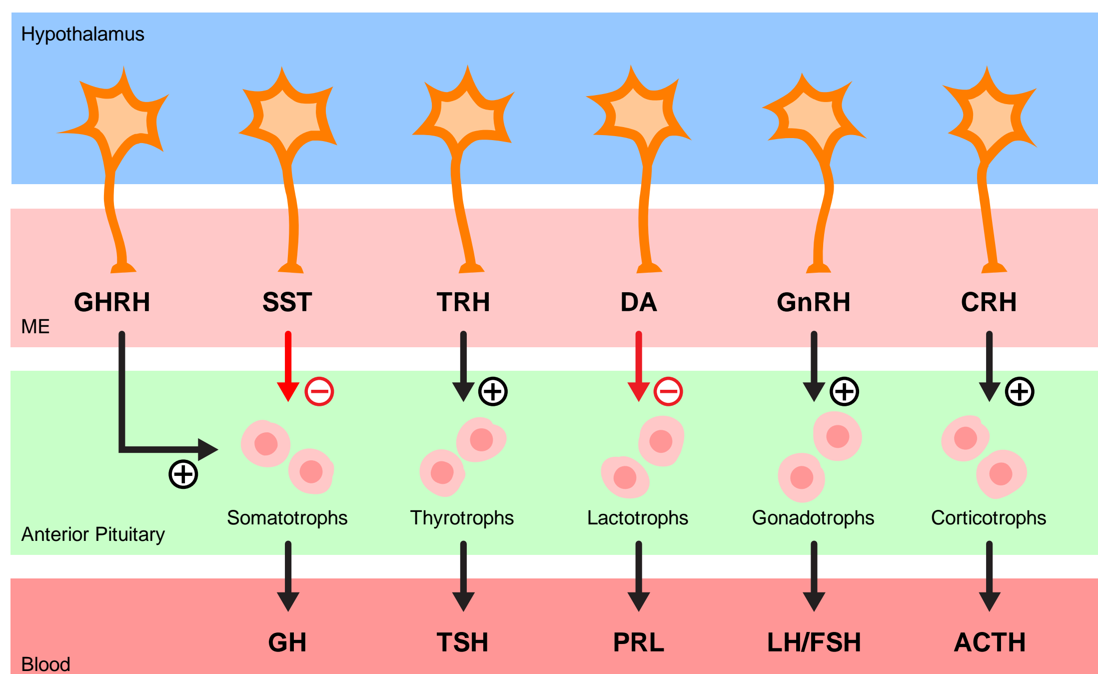

Figure 3. Schematic presentation of parvocellular neurons. Parvocellular neurons of the hypothalamus terminate in the median eminence, where they secrete factors into first capillary blood vessels. These factors are transported by the blood to the anterior pituitary and either excite (+) or inhibit (-) endocrine cells that release hormones into the systemic blood. See list for abbreviations.

releasing factors in 1968 by Roger Guillemin (Burgus et al., 1969) and Andrew V. Schally (Bøler et al., 1969).

The parvocellular system includes five axes defined by their target endocrine cell in the pituitary. These are the thyro-, cortico-, gonado-, somato-, and lactotrophic axes. Each axis is mainly associated with a distinct cell population in the hypothalamus with specific releasing and inhibiting factors, although cross-talk has been suggested to occur. For example, in addition to its powerful effects on the thyrotrophic axis, thyrotropin-releasing hormone also stimulate the release of prolactin in the lactotrophic axis (Bowers et al., 1971; Lamberts and Macleod, 1990). The lactotrophic axis, which is the main circuitry controlling the secretion of prolactin, has been investigated in several of the studies in this thesis and will be discussed further in the following section.

1.1.5 The lactotrophic axis

In the lactotrophic axis, the release of the polypeptide hormone prolactin, is controlled. Prolactin, which was first discovered in animals (Stricker and Grueter, 1928; Riddle et al., 1933), and much later confirmed to exist in humans (Friesen et al., 1970), is best known for initiating lactation in female mammals (see Freeman et al., 2000; also see Grattan, 2015). Investigations have however identified prolactin as a pleotropic hormone that is involved in several hundreds of different process, in both humans and other vertebrates, *e.g.* stimulation of paternal behavior in birds (Buntin et al., 1991) or promotion of gonadal activity (Rubin et al., 1976; Buntin and Tesch, 1985; Gunasekar et al., 1988). Its receptor is widely expressed throughout the body of both sexes, and it is difficult to find a tissue that does not express prolactin receptor (see Bole-Feysot et al., 1998).

Many early interrogations of the lactotrophic axis failed to identify a "prolactin releasing factor" similar to what is found in the other parvocellular axes. These initial failures turned out to be due to two reasons. First, lactotrophs are primarily controlled by inhibition, rather than stimulation. This is illustrated in pituitary stalk transection experiments causing a general drop in serum pituitary hormone levels, with the exception of prolactin, which rises (Gust et al., 1987), and in the anterior pituitary grafts transplantations under the kidney capsule, which results in an increase of prolactin, but not other pituitary hormones (Everett, 1954; Nikitovitch-Winer and Everett, 1958, 1959).

Second, the lactotrophic axis utilizes a biogenic amine rather than a neuropeptide as a hypothalamic inhibiting factor. Thus, it was found that parvocellular tuberoinfundibular dopamine (TIDA) neurons, located in the hypothalamic arcuate nucleus (Fuxe, 1964; Hökfelt, 1967; Björklund et al., 1973), provide inhibition by releasing dopamine into the portal vessels. Released dopamine is, as described previously, transported to the anterior pituitary where it acts on inhibitory D2-type receptors on the lactotrophs

(compare section 1.2.3), to inhibit Ca^{2+}-dependent exocytosis of prolactin (see Ben-Jonathan and Hnasko, 2001).

Dopamine-mediated inhibition on lactotrophs results in a powerful suppression of prolactin release, so that lactation only occurs in nursing females. The strength of this dopamine inhibition is illustrated by the potency of dopamine agonists in the treatment of hyperprolactinemia and prolactinomas (Molitch et al., 1985) which also lead to common sexual side effects like reduced libido and menstrual cycle abnormalities (Ghadirian et al., 1982) or less common side effects like galactorrhea, in antipsychotic drug treatments with dopamine antagonists (Polishuk and Kulcsar, 1956; Clemens et al., 1974).

1.1.6 Tuberoinfundibular dopamine neurons

More than half a century ago, TIDA neurons were first identified with Falck-Hillarp visualization (see section 1.2.2), and were subsequently investigated by their immunoreactivity for tyrosine hydroxylase (TH, see section 1.2.1), and their morphological features (Chan-Palay et al., 1984; van den Pol et al., 1984) and projection to the median eminence was described (Lichtensteiger and Langemann, 1965; Fuxe and Hökfelt, 1966; Björklund et al., 1970, 1973).

More recent *ex vivo* studies, first by Lyons et al. (2010), on the electrophysiological cellular and network properties of TIDA neurons have revealed a peculiar membrane potential oscillation (see also *e.g.* Brown et al., 2012; Yang et al., 2012; Romanò et al., 2013; Zhang and van den Pol, 2015; Stagkourakis et al., 2018a), where the cells rhythmically alternate between periods of quiescence (*DOWN state*) and phasic (*UP state*) firing (see Figure 4). In rats, these states are synchronized through a mechanism involving gap junctions, which provide a strong electrical coupling between TIDA neurons (Stagkourakis et al., 2018a); mouse TIDA neurons, on the other hand, are not synchronized and show complete lack of gap junctions.

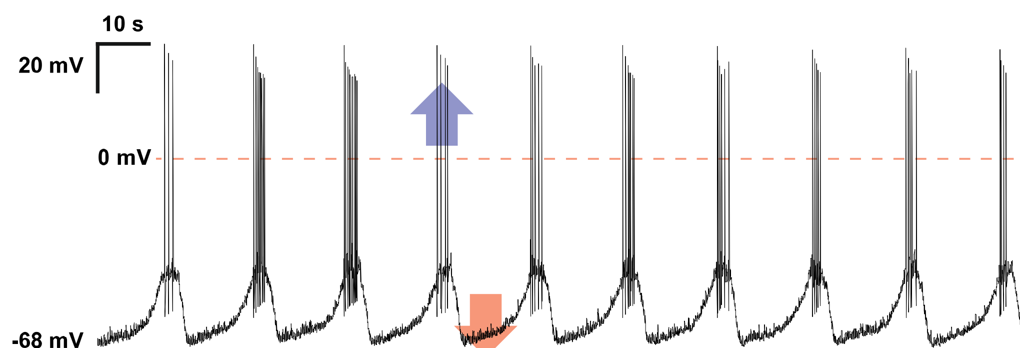

Figure 4. Electrophysiological properties of rat TIDA neurons. Current clamp whole-cell recording of a TIDA neuron from a juvenile male rat show a rhythmic oscillation that alternates between up (🡅) and down (🡇) states.

Since prolactin has been found to provide feedback and exert direct effects on TIDA neurons (Lyons et al., 2012), it is likely that the central regulation of serum prolactin

resides within the TIDA neurons themselves, which makes these cells particularly interesting to study in conditions where the systemic prolactin level is altered. Sexual side effects, including amenorrhea, infertility, anorgasmia, impotence, and impaired libido, are common in patients under antidepressant selective serotonin reuptake inhibitor (SSRI) treatments (Clayton et al., 2002) and are likely caused by systematic elevation of prolactin (Safarinejad, 2008; Madhusoodanan et al., 2010). As such, the effects of serotonin and SSRIs on TIDA neurons were investigated in study III.

Prolactin secretion is also variable over the day, with plasma levels in rats (Clark and Baker, 1964; Dunn et al., 1980), as well as humans (Nokin et al., 1972; Sassin et al., 1972) increasing during sleep. Based on these observations, the effects of two molecules that regulate the sleep-wake cycles, *i.e.* the wakefulness promoting hypothalamic peptide, hypocretin/orexin (see de Lecea et al., 1998; Sakurai et al., 1998; Martin-Fardon et al., 2018), and the sleep-regulating pineal hormone, melatonin (Johnston and Skene, 2015), were investigated in study IV.

In addition to the modulators mentioned above, several other messenger molecules have been identified in other studies (*e.g.* Lyons et al., 2010; Briffaud et al., 2015; Stagkourakis et al., 2016) to affect TIDA electrophysiology. These findings together raise a number of questions regarding the morphological and neurochemical properties of these cells, which have been addressed by histochemical techniques in the second study of this thesis.

1.2 DOPAMINE AND DOPAMINE RECEPTORS

Dihydroxyphenethylamine, more commonly known as dopamine, is an organic compound belonging to the catecholamine family. It was first synthesized in 1910 (according to Hornykiewicz, 2002) and identified in the human brain as a precursor to noradrenaline in the 1950s (Carlsson et al., 1957, 1958; Montagu, 1957). Subsequently, in 1958, Arvid Carlsson and Nils-Åke Hillarp identified it to act as a neurotransmitter in its own right (see Björklund and Dunnett, 2007); a discovery that resulted in the 2000 Nobel Prize in Physiology and Medicine being awarded to Dr. Carlsson.

Additionally, dopamine has also been found to be an active compound in many plants, where the fruit pulp of bananas contain the highest concentrations observed (Kulma and Szopa, 2007). The underlying neuronal circuitries involving dopamine actions in bananas have, however, not been established yet. Contrary to its precursor L-3,4-dihydroxyphenylalanine (L-DOPA, a.k.a. levodopa), dopamine cannot cross the blood-brain barrier, and therefore cannot act on the brain when consumed orally (Bertler et al., 1966).

1.2.1 Catecholamine biosynthesis

In the brain, dopamine is mainly synthesized from L-DOPA by the enzyme DOPA decarboxylase (DDC; a.k.a. aromatic amino acid decarboxylase, AADC). L-DOPA is

converted from the non-essential amino acid L-tyrosine by the enzyme tyrosine hydroxylase (see Figure 5). Dopamine by itself is a precursor to other catecholamines (see Figure 6), as it can be converted to the neurotransmitter norepinephrine (a.k.a. noradrenaline) by presence of the enzyme dopamine β-hydroxylase, which can be further processed by the enzyme phenylethanolamine N-methyltransferase (a.k.a. phenylalanine hydroxylase) to form epinephrine, a.k.a. adrenalin (see Daubner et al., 2011).

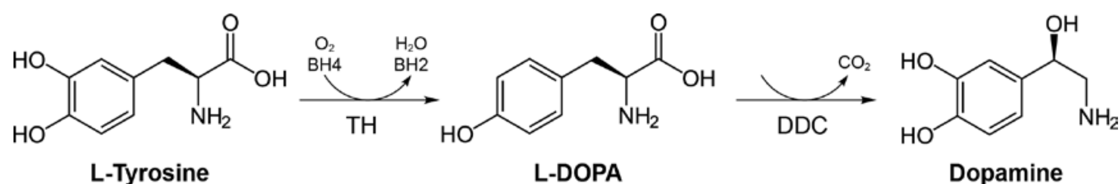

Figure 5. Dopamine biosynthesis. Dopamine is synthesized from L-tyrosine in two enzymatically catalyzed steps. The first reaction, where tyrosine hydroxylase catalyzes the production of L-DOPA is the rate limiting step. BH4, tetrahydrobiopterin; BH2, dihydrobiopterin; TH, tyrosine hydroxylase; L-DOPA, L-3,4-dihydroxyphenylalanine; DDC, DOPA decarboxylase.

Tyrosine hydroxylase, is the rate limiting enzyme of the biosynthesis of these catecholamines (Nagatsu et al., 1964). It has four different serine residues, and the enzyme activity can be regulated through phosphorylation and dephosphorylation of these serine sites by kinases and phosphatases (see Daubner et al., 2011). The enzyme is further regulated by a phosphorylation dependent feedback mechanism, where the catecholamines themselves (Daubner et al., 1992) can compete with the cofactor tetrahydrobiopterin and inhibit the enzyme activity.

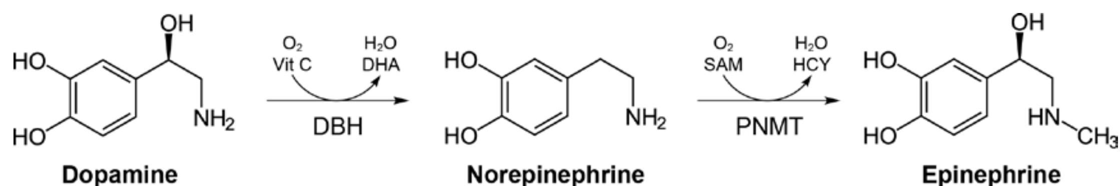

Figure 6. Norepinephrine and epinephrine biosynthesis. Cells that lack DBH cannot produce norepinephrine or epinephrine as these molecules are synthesized from dopamine. Vit C, vitamin C; DHA, dehydroascorbic acid; SAM, S-adenosyl methionine HCY, homocysteine; DBH, dopamine β-hydroxylase; PNMT, phenylethanolamine N-methyltransferase.

1.2.2 Dopamine expression in the brain

In the early 1960s, Bengt Falck and Nils-Åke Hillarp introduced a method that enabled fluorescence detection of catecholamines (Falck, 1962; Falck and Torp, 1962; Falck et al., 1982). This Falck-Hillarp visualization technique was used by Annica Dahlström and Kjell Fuxe (Dahlström and Fuxe, 1964) to present the first mapping of catecholamine expressing neurons in the brain. As these cell populations did not always adhere to classical neuroanatomical boundaries, an alphanumerical classification of the catecholamine expressing neurons was introduced. Thus, dopamine and norepinephrine expressing neurons were categorized in the A-groups, and serotonin

and adrenaline expressing neurons were categorized in B- and C-groups respectively and numbered sequentially according to their rostro-caudal distribution from medulla oblongata to the diencephalon.

The initial mapping was, however, mostly focused on the midbrain, and did not cover the entire hypothalamus. The remaining dopamine producing neurons in the brain were later described by their immunoreactivity for tyrosine hydroxylase (Pickel et al., 1975; Hökfelt et al., 1976; Chan-Palay et al., 1984; van den Pol et al., 1984) and their axonal projections were investigated subsequently with the retrograde tracer horseradish peroxidase (Ljungdahl et al., 1975).

There are three different midbrain dopamine cell populations: the cells in the retrorubral field (A8), the cells in the substantia nigra (A9) and the cells in the ventral tegmental area (A10). These cells have undergone substantial anatomical (*e.g.* German and Manaye, 1993) and physiological investigations and found to play vital roles in the regulation of voluntary movement (Iversen and Koob, 1977), emotion (Nestler and Carlezon, 2006) and reward (see Tzschentke and Schmidt, 2000). For instance, loss of dopamine neurons in the substantia nigra pars compacta is the main pathological characteristic of Parkinson's disease (see *e.g.* Björklund and Dunnett, 2007; Lees et al., 2009; Sveinbjornsdottir, 2016).

In the hypothalamus, there are five major dopaminergic cell populations (A11-A15), where the parvocellular neuroendocrine TIDA neurons (compare 1.1.6) belong to the A12 group. It should be emphasized that not all hypothalamic tyrosine hydroxylase expressing neurons are dopaminergic, as immunohistochemical investigations of the ventral A12 cells in the arcuate nucleus for instance, has revealed a lack of the enzyme AADC (Meister et al., 1988). Another dopaminergic cell group can also be found in the olfactory bulb (A16).

1.2.3 Dopamine receptors

Dopamine acts on two major types of G-protein coupled receptors: the excitatory D1-like, constituting of the D1 and D5 subtypes, and the inhibitory D2-like, constituting of D2, D3 and D4 subtypes (Spano et al., 1978; Kebabian and Calne, 1979). The D2 receptor has also been identified to exist in two different isoforms; a long (D2L) that is most abundant in the brain, and a short (D2S) that is predominant in the brainstem (Montmayeur et al., 1991). Dopamine receptors exert their effect on the cell by either increasing (D1-like) or decreasing (D2-like) the formation of the second messenger cyclic adenosine monophosphate (cAMP) through adenylyl cyclase mediated mechanisms.

Dopamine receptors are expressed throughout the brain, with the D1 and the D2 subtypes being the most abundant, and both these receptors have been identified to be expressed within the hypothalamus (Mansour et al., 1990). Although, most studied for their involvement in the mesolimbic (*e.g.* Sawaguchi and Goldman-Rakic, 1991; Tran et

al., 2005) and nigrostriatal pathways (see Korchounov et al., 2010), dopamine receptors have been identified in a variety of hypothalamically mediated functions and behaviors. These include for instance food intake (Parada et al., 1988; Caulliez et al., 1996; Chen et al., 2014; Mirmohammadsadeghi et al., 2018), circadian rhythm (Yamada and Martin-Iverson, 1991; Viswanathan et al., 1994; Duffield et al., 1998), pituitary hormone secretion (Bluet-Pajot et al., 1990; Crowley et al., 1991; Borowsky and Kuhn, 1992), as well as defensive (Filibeck et al., 1988) and reproductive (Fabre-Nys et al., 2003) behaviors. The broad role of dopamine in the hypothalamus is further illustrated by the observed side effects like tachycardia and hypotension in patients undergoing dopamine agonist based treatments (Rosell et al., 2015).

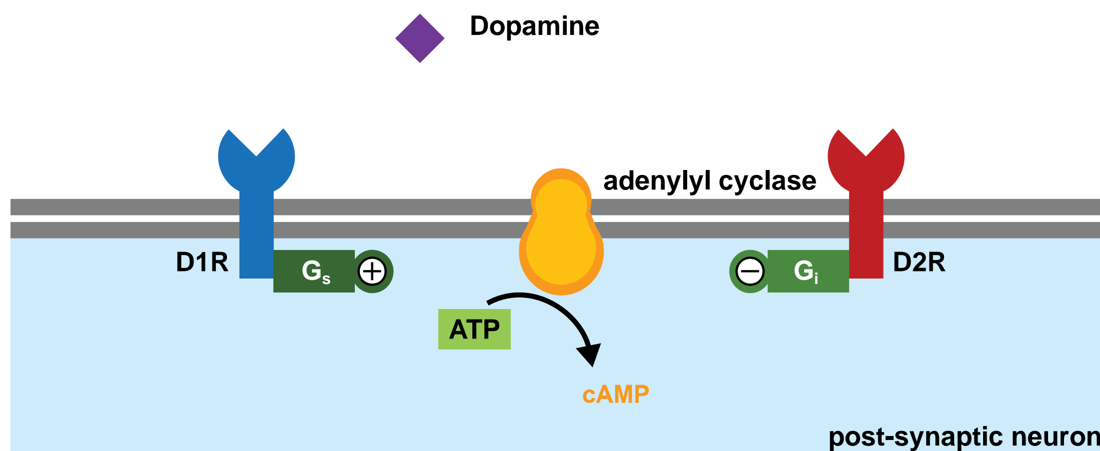

Figure 7. Dopamine actions are mediated through two types of G-protein coupled receptors. When dopamine binds to one of its receptors, it induces a conformational change that allows the receptor to act with G-proteins to either activate (D1) or inhibit (D2) adenylyl cyclase, which catalyzes the creation of the second messenger cAMP. ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate.

These studies suggest a broader role for dopamine in homeostatic and endocrine functions in the hypothalamus and implies the existence of several other dopamine dependent circuitries in addition to the lactotrophic axis. The dopaminergic influences in the hypothalamus can be explained by the distribution of its receptor among different hypothalamic cell populations. Yet, the expression of these two receptors in the hypothalamus has previously not been investigated in greater detail. Two of the studies included in this thesis (studies V and VI), address this lack of information and provide a thorough map of cells expressing D1 or D2 receptors within the hypothalamus and the structures that are closely related to it; namely the pituitary and the circumventricular organs.

1.3 CALCIUM BINDING PROTEINS

In addition to TIDA neurons that have been discussed in the previous sections and studied in this thesis, several other neuronal populations occupy the arcuate nucleus. This includes for instance agouti-related protein/neuropeptide tyrosine (AgRP/NPY) and proopiomelanocortin/cocaine- and amphetamine-regulated transcript (POMC/CART) -expressing neurons involved in metabolic regulation (Broberger et al.,

1998), somatostatin (SST) and growth hormone-releasing hormone (GHRH) expressing neurons involved in the somatotrophic axis and kisspeptin (KSP) expressing neurons involved in the gonadotrophic axis (see Jameson and Harrison, 2013 p.17). As these neurons are involved in distinct hypothalamic circuitries and exhibit different neurochemical, physiological (*e.g.* Spanswick et al., 1997; Cowley et al., 2001, 2003; Ohkura et al., 2009) and morphological properties, knowledge about these cells' molecular repertoires and the differences among them can provide valuable insight on how several properties of the cells, *e.g.* the membrane properties are regulated.

The neuronal signal transduction machinery relies on an action potential mediated calcium rise, and utilizes several proteins that allow vesicles to fuse to the membrane and release neurotransmitters (see Brini et al., 2014). Calcium, which acts as a second messenger, binds to one of the key components, synaptotagmin-1, and induces the neurotransmitter release (Geppert et al., 1994); a discovery for which Thomas C Südhof shared the Nobel Prize in Physiology or Medicine in 2013. Consequently, the intracellular calcium levels in neurons are tightly regulated by various kinds of calcium pumps/channels and binding proteins (see Kramer, 2016). Although these calcium binding proteins have been classified as either sensors, which facilitate signal transduction, or buffers, which maintain a stable calcium level in the cytosol (Dalgarno et al., 1984; Heizmann, 1993; Burgoyne, 2007), it is becoming apparent that several of these proteins are involved in both functions, and pure calcium buffers are less common (Schwaller, 2009).

In the mammalian brain, several different calcium binding proteins are expressed and they have been used as a histochemical tool to identify and classify different neuronal populations (Baimbridge et al., 1992). Three different calcium binding proteins, belonging to the "EF-hand" family (Moncrief et al., 1990; Lee et al., 1991; Nakayama et al., 1992), namely calbindin D-28k (Taylor and Wasserman, 1967; Jande et al., 1981; also see Schmidt, 2012), calretinin (Rogers, 1987; see Schwaller, 2014) and parvalbumin (Henrotte, 1952; Celio and Heizmann, 1981) have been used in the first study of this thesis to investigate different neuronal populations in the arcuate nucleus. Of these three proteins, parvalbumin is the only that has not been identified to have additional calcium sensor functions, and is possibly the only pure calcium buffer (Schwaller, 2009).

1.4 THE PITUITARY

The pituitary gland, classically known as the hypophysis, is usually referred to as the master endocrine organ and is present, albeit with some structural variabilities, in all vertebrates. In humans it is roughly about the size of a pea and weighs about 0.5 grams. It is positioned ventral to the hypothalamus and is directly connected to the brain through the pituitary stalk. The pituitary is divided into two main lobes, the anterior and the posterior. In some species like mice, rats and fish in general, a third intermediate

lobe located in-between the anterior and the posterior lobe, is also present. This lobe is absent in birds and rudimentary in humans (see *e.g.* Melmed, 2011). To examine the possible role of dopamine in the pituitary, the expression of the D1 and D2 receptors has been investigated in study IV and V included in this thesis.

1.4.1 The anterior pituitary

The anterior pituitary, also known as adenohypophysis, is the glandular part of the pituitary and harbors several different types of endocrine cells (see Strand, 1999) that are regulated centrally by neurotrophic factors originating from parvocellular neuroendocrine neurons of the hypothalamus (see section 1.1.4).

1.4.2 The intermediate lobe

In mice and rats, and to some degree in humans, although less developed and often considered part of the anterior pituitary, the intermediate lobe mostly constitutes of melanotrophs, which process the precursor molecule POMC, and releases α-melanocyte stimulating hormone (α-MSH) and β-endorphin (Mains and Eipper, 1979; Jackson and Lowry, 1983; Chang and Loh, 1984). This processing is different than what is found in the anterior lobe where the main released hormones are adrenocorticotrophic hormone (ACTH) and β-endorphin (Strand, 1999).

The melanotrophs of the intermediate lobe are directly innervated by a subpopulation of A14 dopamine neurons located in the periventricular nucleus of the hypothalamus (Goudreau et al., 1992, 1995; Demaria et al., 2000). It should, however, be mentioned that a review of older literature reveals some ambiguity about the nomenclature and anatomical location of these neurons (Björklund et al., 1973; also see Saland, 2001).

In amphibians, reptiles and fishes (Hogben and Slome, 1931; Kawauchi et al., 1983), α-MSH is involved in pigmentation of the skin. Although the exact function of the intermediate lobe melanotrophs in humans has not been fully elucidated, α-MSH has been suggested to be involved in pigmentation (Krude et al., 2003) as well as in pivotal control of the hypothalamic-pituitary axes (see Saland, 2001).

1.4.3 The posterior pituitary

The posterior pituitary (see Flament-Durand, 1980), classically known as neurohypophysis, does not synthesize any hormones by itself, but mainly contains axon terminals from magnocellular neuroendocrine neurons, which release the hormones oxytocin and vasopressin directly into the bloodstream (see section 1.1.3).

1.5 CIRCUMVENTRICULAR ORGANS

In 1958, the term "circumventricular organs" was proposed by the Austrian anatomist Helmut Hofer to describe the structures around the ventricular system of the brain (Hofer, 1958). Circumventricular organs are in contact with both the blood and the

cerebrospinal fluid and are not restricted by the blood brain barrier, thus constituting an interface between the brain and the rest of the body (see Gross, 1992).

Circumventricular organs can be either sensory, and provide a windows through which the endocrine or autonomic nervous system receives systemic feedback, or secretory, and act as a site of hormonal release. While median eminence, the neurohypophysis, the intermediate lobe of the pituitary gland, the pineal gland and the subcommisural organ are considered to belong to secretory, the subfornical organ, the organum vasculosum of the lamina terminalis and area postrema are considered to belong to sensory circumventricular organs (see Fry and Ferguson, 2007).

Circumventricular organs are partly composed by ependymal cells, a type of neuroglia, which line the ventricle walls. A specific type of ependymal cells, known as tanycytes, are found in the third ventricle. These cells exhibit long processes extending into the arcuate nucleus and have recently been identified to gate leptin into the brain (Balland et al., 2014). The close proximity to, and direct interaction with, the hypothalamus makes circumventricular organs an integral part of hypothalamic circuitries.

CHAPTER 2

MAIN OBJECTIVES

Based on the surge of new discoveries on different cellular, physiological and network properties of hypothalamic neurons, and the lack of fundamental understanding for the molecular basis of these discoveries, this thesis aims to investigate the dopamine system in the hypothalamus, the pituitary and the circumventricular organs, from a molecular and histochemical perspective, with specific focus on dopamine D1 and D2 receptors and the tuberoinfundibular dopamine (TIDA) neurons, in the arcuate nucleus.

The specific aims were to:

- define the expression pattern of three major calcium-binding proteins, namely calbindin-D28k, calretinin and parvalbumin, in the arcuate nucleus (study I)
- describe the morphological features of TIDA neurons in male rodents and identify potential species differences (study II)
- determine the serotonergic innervation pattern of TIDA neurons (study III)
- determine the hypocretin-/orexinergic innervation pattern of TIDA neurons (study IV)
- provide a comprehensive distribution map and neurochemical characterization of D1 (study VI) and D2 (study V) receptor expressing cells in the hypothalamus, the pituitary and certain circumventricular organs

CHAPTER 3

ASPECTS OF METHODOLOGY

Detailed descriptions of materials and methods used in the studies included in this thesis have been presented in the corresponding section of each paper (see studies I-VI). The following sections provide an overview of these methods and consider the specific choice, and their strengths and limitations.

3.1 ANIMAL MODELS

These studies aim to investigate the hypothalamic dopamine system in the mammalian brain. Mice and rats are two extensively studied models that have been frequently used in research and have a well characterized brain anatomy (Paxinos and Franklin, 2001; Paxinos and Watson, 2007). As the hypothalamus also is evolutionally well preserved compared to other brain regions and exhibit conserved anatomy across vertebrates (see Xie and Dorsky, 2017), these two species constitute good models for the aims of our investigations. Additionally, the relatively short gestation time is beneficial when studying animals through the life cycle and when breeding is required (*e.g.* in genetically modified animals; see below).

It should be noted that early on, Baker *et al.* (1983) could identify variations in number of dopamine neurons in the hypothalamus of two different mouse strains (BALB/c and CBA). The functional implications of this difference remains, however, unknown. As our aim is to investigate the dopamine systems in the hypothalamus, rather than quantify strain differences, we have adhered, throughout our studies, to a single strain from each species; *i.e.* C57BL/6 for mice and Sprague Dawley (SD) for rats.

For obvious reasons, explainable by the nature of each study, several different transgenic mouse lines (see following sections below) had to be used. These strains are all derived from C57BL/6 mice. For rats, on the other hand, only wild type animals were used. The somehow restricted repertoire of animal strains provide a reduction of unwanted variables across studies, which makes it easier to understand the dopaminergic system in the hypothalamus, and is highly desirable at this level of basic interrogation. This reduced biological diversity might, however, overlook important factors that could have

crucial impact in for instance drug development. Future studies should therefore further investigate to what extend these data can actually be extrapolated to either other strains or species (*e.g.* humans).

3.1.1 Transgenic reporter animals

Certain receptors and membrane proteins can be expressed at lower levels compared to other cell proteins, such as neuropeptides, that are constantly secreted. The mRNA expression level can also vary greatly among different cell populations (compare Paper V). These features make it challenging to identify, map and characterize these cells only by the means of *in situ* hybridization (ISH) techniques. As neurotransmitter and neuropeptide receptors are mainly localized at the synapse on the cell membrane, they are also difficult to target with conventional immunohistochemistry (IHC) techniques with a satisfactory resolution (also compare sections 3.3 and 3.4). It is therefore not possible to reach the aims of this thesis exclusively by means of ISH and/or IHC techniques on wild type animals, and it is so necessary to use bioengineered tools.

Transgenic animals (Jaenisch and Mintz, 1974) expressing a reporter gene under the control of a certain promoter, *i.e.* a receptor promoter, provide a good opportunity to identify those cells that specifically express the protein of interest (see Spergel et al., 2001). Using transgenic animals for this purpose is, however, not a straightforward procedure as there is a risk for both false positive (*i.e.* cells that express the reporter but not the receptor gene) caused by the influence of endogenous upstream promoters (see Conlon, 2011), and false negative expression (*i.e.* cells that express the receptor but not the reporter gene; compare A15 dopamine cells in study V). Furthermore, the random integration of the insert into the genome may affect the neighboring genes or interrupt the function of endogenous genes (see Babinet et al., 1989), which could lead to undesirable changes in phenotype. To minimize the negative side effects of such event, hemizygous animals, that carry the transgene only in one chromosome, are used. As the transcriptional profile of different neuronal populations are variable, correct expression in one brain region does not necessarily extrapolate to other brain regions, and to be able to draw valid conclusions, the expression of the reporter gene must be properly validated.

3.1.2 *Drd1*/*Drd2*-EGFP BAC transgenic mice

To investigate the distribution of D1 and D2 receptor expressing cells in the hypothalamus, circumventricular organs and the pituitary gland, two transgenic mouse lines expressing enhanced green fluorescence protein (EGFP) under either *Drd1* or *Drd2* promoter, within a bacterial artificial chromosome (BAC), were used. Both of these lines were developed by the Gene Expression Nervous System Atlas (GENSAT; www.gensat.org) program at Rockefeller University (Gong et al., 2003) and maintained in our own breeding facility. These transgenic animals will be referred to as *Drd1*- and *Drd2*-EGFP mice.

A BAC has the ability to carry a DNA fragment of several hundred kilobases and can thus be used to insert a large portion of a gene's promoter and upstream regulatory elements into a transgene (see Heintz, 2001). Thus, it is possible to rapidly create transgenic animals without any *a priori* knowledge of the exact fragment and function of the regulatory elements. This lack of information is not completely unproblematic as the insert could carry regulatory elements that affect gene expression independently of the transgene itself and lead to altered cellular phenotype. Such issues have been reported by Kramer *et al.* (2011), who observed altered physiology and behavior in the Swiss Webster (SW) *Drd2*-EGFP BAC transgenic mice. Later studies suggested, however, that these abnormal phenotypes were likely raised, because that specific transgene, was backcrossed on the outbred SW strain and were homozygous for the *Drd2*-EGFP BAC (Chan et al., 2012). The same study could not identify any abnormalities in the inbred C57BL/6 hemizygous transgene.

It should be noted that both *Drd1*- and *Drd2*-EGFP transgenic mice used in the studies included in this thesis have been backcrossed for at least three generations in the inbred C57BL/6 mouse before use. These strains have been maintained for almost a decade and no abnormal behaviors have been observed. The behavior and neurochemical phenotype of both strains have also been validated in the basal ganglia in other studies (Bonito-Oliva et al., 2016), and in current studies included in this thesis, the strains have been further validated by the means of multiplex ISH. These transgenic animals have thereby undergone the extensive characterization that is the prerequisite for obtaining reliable data with transgenic animals. It should however be noted that this obtained confidence in these two strains, cannot automatically be extrapolated to other transgenes.

3.1.3 DAT-tdTomato knock-in mice

To identify neurons expressing the dopamine transporter (DAT) in the arcuate nucleus, DAT-*cre* knock-in mice were crossed with a tdTomato floxed reporter mice to create the DAT-tdTomato strain. Contrary to the strains used to identify dopamine receptors, the DAT-*cre* strain was generated with a knock-in strategy with a fully identified insert (Zhuang et al., 2005), an therefore does not present concerns with regulatory elements as it is the case with BAC transgenic animals.

As this strain was not used for a mapping study, but only to identify a discrete cell population, a full validation and characterization of the transgene was not considered necessary. Nevertheless, it should be noted that the specific strain has been validated in other studies and extensively used to identify non-hypothalamic dopaminergic cells (Zhuang et al., 2005; Ekstrand et al., 2007; Turiault et al., 2007) as well as TIDA neurons (Stagkourakis et al., 2018a).

Following the reporter expression validation in the arcuate nucleus, these animals also made it possible to visualize and directly identify TIDA neurons in electrophysiological

experiments by fluorescence, which tremendously increased the success rate of the experiments, and enabled much faster data collection (see study II).

3.1.4 EGFP and tdTomato reporters

Together with transgenic animals, two different reporter proteins, namely EGFP and tdTomato, have been used to visualize the cells of interest. Both of these reporter proteins are fluorescent, but each of them have certain unique properties and structural differences that can affect the way they can be used in experiments.

Ever since the first isolation of GFP from the jelly fish *Aequorea Victoria* by Shimomura et al. (1962), the protein and its variants have been extensively used as reporters for visualization of various cell types, including mammalian neurons (*e.g.* Cowley et al., 2001; van den Pol et al., 2009). Numerous variants have also been engineered, there among EGFP, which contains chromophore mutations that makes the protein 35 times brighter than the wild type and is codon optimized for higher expression in mammalian cells (Zhang et al., 1996). Nevertheless, initial pilot experiments (data not included) showed that endogenous EGFP fluorescence was greatly reduced in tissues treated with conventional formaldehyde based fixatives. This is most likely caused by the creation of cross-links (see section 3.2.2) between the fixative and the EGFP, which nullifies the protein's fluorescence ability (Becker et al., 2012). To counter this limitation, immunofluorescence was used to visualize the EGFP. This enabled a robust and reliable recovery of the signal, and with the addition of the TSA protocol (see section 3.4.3), the signal could be substantially amplified.

The red fluorescent protein (DsRed) from *Discosoma* sp. (Dietrich and Maiss, 2002) is another extensively used reporter protein. Similar to EGFP, it has undergone several enhancements. By introduction of four point mutations (V22M, Q66M, V105L and F124M), the "dead-end" green component has been substantially reduced. A dimer has been constructed for faster and more complete maturation with greater fluorescent brightness, and the fusion of two of these dimers into a tandem dimer (td) has resulted in a non-aggregating tag. This enhanced version of DsRed has been denoted tdTomato (Shaner et al., 2004).

Contrary to EGFP, the endogenous fluorescence activity of tdTomato is not lost by tissue fixation. However, the fluorescence intensity is greatly reduced when conventional 2.5% DABCO in 100% glycerol is used as mounting medium. Introduction of ProLong® Gold as mounting medium resulted in virtually full recovery of fluorescence intensity and added the benefit of increased resistivity against photobleaching (see section 3.6.2).

3.2 TISSUE PREPARATION

Successful ISH and IHC assays rely intensely on proper tissue preparation. Fresh frozen tissues are appropriate for ISH (Dagerlind et al., 1992), and could potentially also be used in IHC. However, to be able to draw accurate spatial conclusions, it is necessary

that antigens in the tissue are immobilized. Many antibodies are also raised to recognize the covalently bound fixative-protein epitope. Therefore, the majority of immuno-histochemical experiments are conducted on fixed tissues.

In the studies included in this thesis, fresh frozen tissues have exclusively been used for ISH experiments, whereas all IHC experiments have been conducted on fixed tissues. It should be noted that a single fixation protocol does not suit all antibodies, and for optimal visualization of the targets, different protocols had to be used. The differences in these protocols and their implications are discussed in the following sections.

3.2.1 Fresh frozen tissues

For the process of any kind of *postmortem* tissue conservation, it should be noted that enzymatic activities and cellular processes will continue, even after blood circulation has ceased. This includes, ribonuclease (Kunitz, 1939) activity, which breaks down poly ribonucleotides (Findlay et al., 1961; see also Cuchillo et al., 2011), *i.e.* the target mRNA in ISH experiments. Therefore, it is important that tissue is frozen (a process that abolishes enzymatic activity) as soon as possible after euthanasia.

Tools that come in direct contact with the brain should also be free from ribonuclease. This is easily achieved by cleaning them in bleach or other commercially available nuclease decontamination reagents like RNase AWAY™ or RNaseZAP®. To minimize enzymatic activity prior to freezing, the brain can also be dissected into ice-cold saline containing ribonuclease inhibitors. By these precautions, up to four brains can been dissected sequentially and frozen together without any apparent loss of ISH signal, in our experience.

Theoretically, any rapid freezing techniques could be used. However, most techniques are less practical. For instance, dry ice in isopropanol provides rapid freezing but submerging a brain into a liquid makes subsequent mounting, embedding and storage challenging, carbon dioxide gas is difficult to manage in animal facilities and extremely fast freezing with liquid nitrogen often cause the brains to crack. The brains are best frozen mounted on aluminum foil, directly on dry ice. To make the freezing process more rapid, dry ice can also be crushed into fine powder and poured over the brain.

3.2.2 Fixed tissues

Cross-linking and coagulating fixatives are two types of fixatives extensively used in histology. Coagulating fixatives are not used in the studies included in this thesis and therefore the following section only focuses on cross-linking based fixation protocols. These fixatives are based on chemical reagents that react with the tissue and consequently polymerizes. They create, as the name suggests, cross-links and thereby preserve antigen positions and prevent their degradation. These cross-links also makes the tissue more rigid and easier to handle during subsequent staining protocols (see Ramos-Vara, 2005).

To obtain high quality immunostainings, it is crucial that the amount of cross-link is optimal. If the cross-link density is too high, antigens will be compromised and antibody penetration will be hindered. On the contrary, if the cross-link density is too low, antigens might not be preserved well and could even be washed away when processed for immunostaining. As mentioned earlier, many antibodies are raised to recognize a part of the protein that is cross-linked with the fixative. Therefore the choice of the chemical reagent that constitute the fixative is also important, as the wrong chemical would fail to give raise to the specific epitope recognizable by that specific antibody.

The cross-link density created by the fixative is determined by the fixative's composition and the fixation time. Aldehyde-based fixatives, in particular formaldehyde, is the most commonly used and the majority of the experiments in this thesis were performed on tissues from animals that were transcardially perfused with a 4% formalin and 0.2% picric acid-based fixative diluted in phosphate buffer according to Zamboni and Demartino (1967). This fixative worked very well with most of the antibodies used in these studies. It is, however, not appropriate for preserving smaller peptides like *i.e.* the neuropeptide thyrotropin-releasing hormone (TRH) which only consists of three amino acids.

To successfully stain for TRH in mouse brains, a final concentration of 0.05% glutaraldehyde had to be included in the fixative, similar to what was used by Horjales-Araujo *et al.* (2014) in rat brains. Contrary to formaldehyde, glutaraldehyde side chains will also polymerize and thereby create a denser cross-link, which prevents smaller peptides from being washed out. Unfortunately this fixation will also leave behind a much higher degree of free aldehyde (-CHO) groups, bound to the tissue. These free aldehyde groups can react with the tissue and give rise to green auto-fluorescence and radically reduce the signal-to-noise ratio. Free untreated aldehydes can also bind antibodies during subsequent antisera incubations and substantially reduce the quality of the immunostaining. To prevent this, tissues fixed with glutaraldehyde must be blocked with for instance 0.1% sodium borohydride (Clancy and Cauller, 1998) before freezing.

For even smaller molecules, like amino acid neurotransmitters, addition of 0.05% glutaraldehyde does not suffice. To obtain successful immunostainings against serotonin (compare Paper II) and γ-aminobutyric acid (GABA; compare Paper III) a cacodylate-based fixative containing 5% glutaraldehyde was used according to Yamada *et al.* (2007). Tissue was subsequently treated with 1% sodium borohydride. The high density cross-links created by this fixation protocol also affected antisera penetration substantially and much longer incubation times had to be applied.

Spine analysis of TIDA neurons conducted in the second study of this thesis, posed a different kind of challenge. In this case, ultrastructural preservation of the cell was the most crucial. By adding sucrose and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic

acid (HEPES) to the standard 4% formalin based fixative, as suggested by Fasoli *et al.* (2017), the spine morphology of the neurons could be properly preserved.

3.2.3 Colchicine pre-treatment

Neurochemical characterization of cell populations by IHC largely relies on visualization of target peptides in the cell body. Certain neuropeptides (and their precursors), however, are rapidly transported to the axon terminal in order to be released, and therefore cannot be visualized in the cell body in naïve animals. This axonal transportation can be inhibited by some neuronal toxins. Colchicine is one such toxin, which results in accumulation of neuropeptides in the soma enhancing its visualization by IHC (Hökfelt and Dahlström, 1971). It is derived from the plant *Colchicum autumnale,* and was first isolated by Pelletier and Caventou (1820) and purified and named by Geiger (1833). Colchicine inhibits the polymerization of tubulin into microtubules (Eigsti, 1938), which form the cytoskeleton of axons, and thereby suppresses microtubules-dependent axonal transportation.

As colchicine disrupts the cytoskeleton, the cell morphology is also altered (Eigsti, 1938) and detectability of axons and dendrites are reduced. Different neuronal populations have also shown different susceptibilities to colchicine (Goldschmidt and Steward, 1982) and both increased and decreased transcription levels have been observed in certain neuronal populations (Cortés et al., 1990). Thus, data collected from colchicine-treated animals should be interpreted with caution. The neuronal populations identified under colchicine treatment which are included in the studies in this thesis, have been validated in other studies. Thus, these undesirable side effects were taken into account in order to validate the conclusions in this thesis.

3.3 *IN SITU* HYBRIDIZATION

Expressed mRNA can be detected directly on tissue sections, using labeled nucleotide probes complementary to the mRNA of interest (Pardue and Gall, 1969). Classically, this *in situ* hybridization (ISH) technique has been performed with several different strategies in regards to probe design and detection methods, where each method has its own benefits and drawbacks (Jin and Lloyd, 1997). However, relative to quite recent development of the RNAScope platform (Wang et al., 2012), ISH has been a difficult method with long and tedious procedures that require optimization for individual probes.

Because of this, ISH targeting mRNA is rarely used in clinical settings, despite the technique's ability to detect pathological biomarkers (Warford, 2016). However, the strength of the technique is clearly apparent in initiatives like the Allen Brain Institute where ISH is used to create a full expression map of different transcripts in the mouse brain. In research settings ISH can serve as a substitute to immunohistochemistry (see section 3.4), whenever there is a lack of specific antibody, or if the target has a subcellular location that makes it difficult to identify.

Additionally, ISH is sometimes also used to confirm that a specific antisera used in immunohistochemistry (see section 3.4) is targeting the right cell population. It should however be emphasized that these two techniques are inherently different; while ISH is targeting a transcripted mRNA, IHC is (usually) targeting a translated protein. The correlation of the transcriptome and the proteome depend on a series of regulatory processes, including mRNA stability (Salton et al., 1988) and degradation (Casey et al., 1988), and a cell's transcriptome and proteome is not always coupled.

In the studies included in this thesis, two different ISH methods, *i.e.* radioactive oligo probes and RNAScope®, have been used as a complement to validate observations made by immunofluorescence. These two methods are discussed in the following sections.

3.3.1 Radioactive oligo probe in situ hybridization ISH

This method identifies the target mRNA with several complementary DNA oligo probes. Each oligo probe consist of around 50 bases of complementary DNA and a 10-15 base long ^{35}S-labeled poly-A tail at the 3'-end (Dagerlind et al., 1992; Wilcox, 1993). Following hybridization, the radioactive beta particles originating from the probe, is detected by exposure to a nuclear track plate emulsion, which after development generates silver grains (Buongiorno-Nardelli and Amaldi, 1970). Exposure can last from a couple of days to a couple of weeks (Woodruff, 1998). Although silver grains are visible in bright field microscopy at high magnifications, they are usually investigated at lower magnification powers with dark field microscopy (see section 3.6.1). As this provides a wider field of view, anatomical orientation is easier.

By increasing the number of oligo probes, signal intensity can be enhanced linearly (Young et al., 2016). It should, however, be noted that the poly-A tail creates a steric hindrance and oligo probes cannot target sequences that are overlapping or positioned too close to each other. Great consideration must also be taken into the design of the oligo probe in regards to its sequence and GC-content, to minimize the risk of hybridization of the probe to untargeted sequences. Signal intensity can also be enhanced by increased exposure time. This, however, has a negative impact on the background noise caused by cosmic radiation and poorly hybridized probes. The method is difficult to multiplex.

More recently, radioactive ISH has become less popular due to several reasons. It is a long process and involves many steps, where a single poor condition in one step jeopardizes the entire experiment. There are several mid-process control assays, like counting radioactivity of the labeled probe or confirming hybridization with phosphoimaging. Although quite valuable check-points, these controls only provide limited validation and true success of the experiment can only be established at the very end, under the microscope.

The use of radioactive materials is also regulated and the method is only allowed to be executed by trained personnel, in designated certified areas. Furthermore, the vast

increase of antisera availability has led to a decreased demand for ISH techniques in general, which in turn has led to substantially increase of material and equipment costs and availability. The bankruptcy of Kodak in 2012, the company that provided two of the key components, *i.e.* the NTB emulsion and the D-19 developer, was the final nail in the coffin for radioactive oligo probe ISH.

Nevertheless, radioactive oligo probe ISH was successfully used in study I to confirm expression of calcium binding proteins in the arcuate nucleus and address discrepancies observed among different parvalbumin antisera.

3.3.2 RNAScope®

RNAScope® is a proprietary platform, owned by ACD Bio Techne, and quite a recent addition to the ISH repertoire. The technique uses oligo probes to target mRNA and utilizes several intermediate DNA amplifier fragments to generate a highly amplified signal. The platform is provided as kits, that are available in both chromogenic and fluorescence. The entire process can be done in one or two days, and fluorescence kits are easily multiplexed. Probes are also proprietary. As of 2018, the company provides over 10,000 unique probes targeting several species including human, bovine, dog, monkey, mouse, pig, rabbit, rat and zebrafish. Custom probes are designed and made available for an additional charge.

Overall, RNAScope has addressed many of the issues previously associated with ISH (see above). The amplification steps render very favorable signal-to-noise ratios, fluorescence multiplexing is straight-forward and results are acquired within a day or two. The cost, however, remains a major drawback which greatly limits how much it can be used. From a cost-benefit point of view, mapping and neurochemical characterization of larger brain regions, like hypothalamus, cannot currently be justified with this method.

The fact that all the components, including the probes, are proprietary can also lead to concerns as the researcher has a very limited knowledge and control over the assay. This has truly been an issue in several assays, where a specific probe has failed to identify the target. Yet, RNAScope has a promising future and the potential to become a standard method in many laboratories.

As mentioned earlier, it is necessary to validate the EGFP expression in the BAC transgenic animal models, and due the lack of reliable antibodies (which is one of the reasons why transgenic animals are used in the first place), this had to be done through ISH. Unfortunately, endogenous EGFP fluorescence is lost when tissue is processed for ISH, and multiplex targeting is therefore necessary. RNAScope has provided the means to properly conduct these validations.

3.3.3 Other multiplex *in situ* hybridization techniques

It should be mentioned here that before the introduction of RNAScope, which is a method that only became available lately, many efforts were spent seeking a successful multiplex fluorescence ISH procedure to validate the BAC transgenic animals. First, multiplex oligo probe ISH, where one probe (targeting *Drd2*) labeled with biotin and the other probe (targeting *Egfp*) labeled with digoxigenin, or *vice versa*, and subsequently detected with fluorescent labeled streptavidin and antisera against digoxigenin, was evaluated. Enzymatic amplification steps by introduction of horseradish peroxidase and alkaline phosphatase conjugates were also assessed.

Each probe, by itself, yielded satisfactory results, but would fail in multiplex. Multiplex hybridization followed by sequential detection also failed to detect both targets simultaneously. Next, sequential IHC/ISH, where EGFP was detected by the means of IHC and *Drd2* was detected by the means of ISH, was evaluated with no success, regardless if the sequence started with IHC or ISH. Finally, usage of riboprobes spanning around 500 bases of the target gene was evaluated, also with no success.

The fact that *Drd2* has a relatively low expression level in the hypothalamus (compare Paper V) has a crucial role in these failures as detection is not possible with suboptimal conditions and there is little margin for methodological compromises. It was therefore concluded that it is not possible to combine the material and methods of the two detection methods evaluated in such a way that detectable signal intensity for both targets are preserved. Although the above mentioned approaches might pose a viable strategy for multiple detection of other target pairs that are expressed at higher levels and are less sensitive to suboptimal conditions, RNAScope nowadays provides an easier and more straightforward approach, and therefore renders these strategies obsolete.

3.4 IMMUNOHISTOCHEMISTRY

Labeled antibodies can be used to target specific antigens on tissue for visualization (Coons et al., 1941; Coons and Kaplan, 1950). This is known as direct immunohistochemistry. As this direct detection method involves labeling of antibodies, a process that result in loss of rare and precious immunoglobulins, it is rarely used. Most commonly, an indirect detection method that utilizes labeled secondary antibodies that recognize the primary antigen-complementary antibody, is used (see Ramos-Vara, 2005). Signal intensity can be amplified by conjugating an enzyme to the secondary antibody, which synthetizes a detectable precipitate or fluorophore. When detection is done with fluorescent dyes, the technique can also be referred to as immunofluorescence. In the studies included in this thesis, immunofluorescence techniques have been used to identify and profile cells, map neuronal populations and investigate neuronal connectivity.

3.4.1 Monoclonal and polyclonal primary antibodies

Primary antibodies are raised by immunizing a host animal with a specific antigen. The host is subsequently bled and the sera, which contain the antibody of interest, as well as other antibodies, is separated. To minimize background noise and reduce unspecific binding, the immunizing antigen can be used to purify and concentrate the antibody of interest from the sera. When immunizing an animal with *i.e.* a peptide, the host will most likely produce antibodies against several different regions of it, and purification will yield a mixture of several antibody clones, *i.e.* a polyclonal mixture, where different clones recognize the same target antigen, but not necessarily the same epitope (see Ramos-Vara, 2005).

Polyclonal antisera have two major disadvantages. First, since the mixture of antibodies in the sera is unknown, using different bleeding will result in different antibody mixtures, and unwanted variability is inevitably introduced in the experiment. Second, some assays, *i.e.* when targeting a specific phosphorylation site of a protein, require a single antibody that only recognizes a specific region of the protein. In such case, detection would not be possible with a polyclonal mix.

To circumvent these issues, the process of creating monoclonal antibodies has been developed, where antibody producing B-cells from the spleen of the immunized host is fused to myeloma cells to create hybridomas (Schwaber and Cohen, 1973; Köhler and Milstein, 1975). The hybridomas are then separated and a single clone that produces the antibody of interest is selected. A hybridoma clone will only produce a single type of immunoglobulin against a single epitope. When the abundance of the target is low, it is more favorable to use polyclonal antibodies that recognize multiple sites of the target and thereby yield a stronger signal (see Ramos-Vara, 2005). Monoclonal antibodies, on the other hand, can be produced in larger quantities and have less batch-to-batch variability.

3.4.2 Secondary antibodies

As primary antibodies originate from a specific species, secondary antibodies are raised to recognize the species specific constant region of primary antibodies. Purified secondary antisera against different species, conjugated to a variety of enzymes or fluorescent dyes are widely available. By using primary antibodies from different species, it is possible to multiplex an immunofluorescence assay and detect multiple targets on the same tissue at the same time. Caution must, however, be taken against species cross-reactivity (see Ramos-Vara, 2005).

3.4.3 Tyramide Signal Amplification

Following oxidation/radicalization, tyramine can chemically bind to solid substrates (Gross and Sizer, 1959) and since this reaction is short-lived, tyramine will stay in close proximity to the site of reaction (Hayat, 2002). This process was adapted to histology by Adams (1992) who used biotinylated tyramide together with horseradish peroxidase

conjugated antisera to create biotinylated epitopes on the tissue. Biotinylated epitopes were subsequently visualized with labeled avidin, yielding a highly amplified signal.

A less complex variation, where fluorescein (rather than biotin) is conjugated to tyramide, is used in the studies in this thesis. Test assays (data not included) have showed that this more direct tyramide signal amplification (TSA) approach generates about ten-fold stronger signal than conventional indirect fluorescence detection methods and the use of biotinylated tyramide does not improve this further. On the contrary, using biotinylated tyramide sometimes yielded a less intense signal, most likely since the size of the avidin-HRP caused steric hindrance and prevented further amplification.

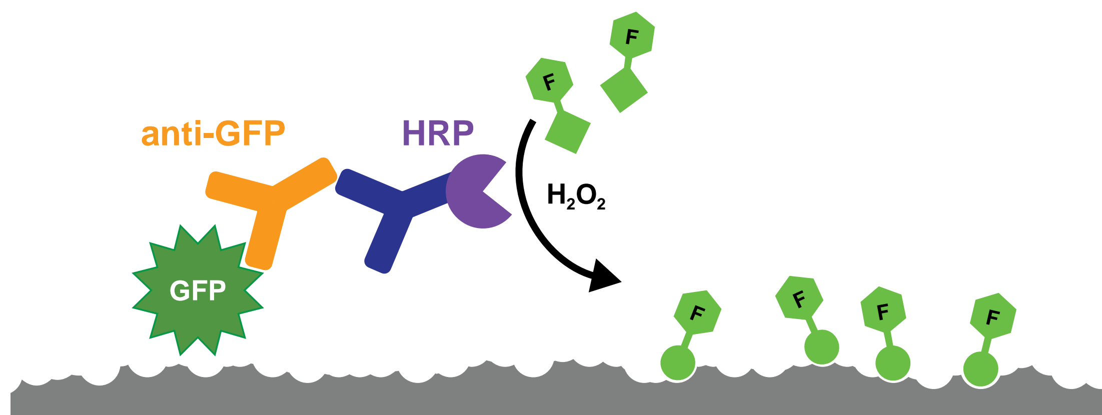

Figure 8. Basic principle for tyramide signal amplification. Horseradish peroxidase catalyzes the reaction to create reactive tyramide that can covalently bind to the tissue. This leads to deposition of a large number of fluorescent molecules in close vicinity of the target antigen, greatly amplifying the signal. HRP, horseradish peroxidase; GFP, green fluorescence protein; F, fluorescein isothiocyanate.

With TSA, it is possible to use antisera at *ca.* ten time lower concentration, without losing signal intensity. The use of lower concentration also leads to decreased background caused by unspecific binding. This makes it beneficial to combine TSA with unpurified polyclonal antisera, as lower antisera concentration result in a better signal-to-noise ratio. TSA is also particularly well suited for detecting low abundant targets, where stronger signal cannot be generated with higher immunoglobulin concentrations.

Due to the nature of the method, the resolution of the staining is, however, reduced. This is not visible at lower magnifications, but at higher magnifications, staining can appear "granular" and TSA is therefore less suitable for investigating finer structures of the cell. It has also been less successful when combined with primary antisera targeting the extracellular portion of membrane proteins as the staining appears diffuse. Compared to the cytosol, the extracellular matrix probably presents fewer reactive sites for the reactive tyramide to bind to, which causes a longer trace and gives rise to the diffuse staining pattern.

3.5 ELECTROPHYSIOLOGY

The different ion concentration between the inside and outside of a cell, produced mainly by actions of the Na^+/K^+-ATPase pump, generates a voltage difference across the membrane. The driving force thus created is particularly important in cells with excitable membranes, such as neurons, and can be used to produce action potentials, as described by Hodgkin and Huxley (1952), which in turn allow for the electrical triggering of chemical neurotransmission between neurons. In addition to a cell's morphology and transcriptional profile, knowledge of its active and passive membrane properties and electrophysiological behavior is thus necessary for understanding how ensemble of neurons work in networks to ultimately control the organism.

The electrophysiology of hypothalamic neurons is not the main focus of this thesis. Nevertheless, electrophysiological methods and properties are complementary to the molecular biological methods and properties. They provided data necessary for the overall understanding of the cell and how it respond to neurotransmitters and/or neuromodulators. Electrophysiology was also an integral part of some of the experimental procedures, as a mean to identify and fill TIDA neurons with markers for subsequent histochemical visualization of the cell (see below). These methods are briefly discussed in the following sections.

3.5.1 Whole-cell patch clamp recording

The electrophysiology of individual cells can be monitored by whole-cell patch clamp recordings where a glass micropipette with an electrode is used, to record the cell's active and passive membrane properties and how they change in response to messenger molecules (Neher and Sakmann, 1976; Hamill et al., 1981). In these experiments, the pipette, drawn to a fine open tip, is brought into direct contact with the cell membrane so that a so-called giga-seal is establish. The seal is then ruptured using suction, which gives the pipette direct access to the cell's interior. This is known as the whole-cell configuration.

In this configuration, electrical events across the membrane can be studied. By clamping the voltage, the current crossing the cell membrane can be recorded (Cole and Moore, 1960). Alternatively, the current can be clamped, and the voltage is consequently recorded. In the whole-cell configuration, it is important that the internal solution of the patch pipette has the right composition in regards to ions, pH and osmolality, and that it is as similar as possible to the cell's cytosol. Otherwise, the risk of dialysis is imminent, which can lead to electrical artefacts and cell death.

3.5.2 Filling patched neurons

Molecules added to a patch pipette's internal solution can be transferred into a cell during whole-cell patch clamp recordings. This process has been used to fill recorded cells with markers for subsequent IHC and visualization of the recorded cell, which

enables identification of both the intrinsic electrical properties and neurochemical identity of the same cell. After successful recording, the patch pipette from a filled cell must be removed in such a way that the cell body is not damaged.

To be able to completely fill and recover a patched neuron, in regard to its axon and dendritic tree, several parameters must be optimized. A patched cell can be filled with several different kinds of markers. It is important that the marker of choice does not pose any toxic effects to the cell and leaves the electrical recordings unaffected. Fluorescence dyes like Lucifer Yellow or different versions of Alexa dyes are useful for direct visualization, but are not suited if the slice is to be fixed for immunohistochemical investigation. Biotin-derivatives that can be detected with avidin are, on the other hand, well suited for this purpose. In the studies included in this thesis, neurobiotin has largely been used. Biocytin has also been investigated. No apparent differences could be observed between these two markers.

Other factors that affect the staining quality is the concentration of neurobiotin in the patch pipette (neurobiotin concentration), the time the pipette is allowed to fill the neuron by passive diffusion (fill time) and the time the slice is allowed to recover before fixation (relaxation time). For best visualization of TIDA neurons, the neurobiotin concentration was optimized to 0.2%, fill time to 60 min, and relaxation time to 15 minutes. It should be mentioned that these parameters are not necessarily optimal for investigating other neurons with different cell morphology.

3.5.3 Staining filled neurons

After a cell is filled, it has to be fixed, stained and processed for IHC. As electrophysiological slices (*ca.* 250 µm in these studies) are substantially thicker than conventional sections used for histology (*ca.* 14 µm), several procedural steps must be reconsidered for obtaining adequate results. A thick slice will result in considerably higher levels of fixative induced fluorescence and will make immunoglobulin penetration more difficult. Therefore, the slice should not be fixed for longer than 18 hours. All incubation and wash times must also be increased to ascertain adequate tissue penetration.

In the studies included in this paper, recorded cells have been successfully stained with both fluorescence and chromogen dyes. The latter utilized HRP conjugated streptavidin with 3,3-diaminobenzidine (DAB) and nickel as substrate. Chromogen detection has the benefit of being prone to photobleaching (see section 3.6.2), but cannot be used with confocal microscopy (compare section 3.6.3), nor with multiplex immunostaining. It was initially theorized that resistance to photo bleaching, would be more favorable and make it easier to maintain and identify finer structures like axons and spines. This, however, turned out to be false, mainly due to the complexity of the structures and the thickness of the slice. Nevertheless, data from DAB-Ni stained TIDA neurons showed that photobleaching did not have a negative impact on the reconstructions of the

dendritic trees of TIDA neurons (compare section 3.7.2). It should, however, be noted that it is likely that photobleaching will impact reconstructions of neurons possessing more complex dendritic trees negatively.

3.6 MICROSCOPY

The majority of experiments included in this thesis heavily rely on light microscopy, and several different microscopy techniques have been used to address different questions. These different microscopy techniques are discussed in the following sections.

3.6.1 Bright field and dark field microscopy

Although it has undergone major technological improvements in regards to both construction and optics, the basic principle of bright field microscopy has remained the same since its first description by Antonie van Leeuwenhoek in the 1600s (see Houtzager, 1983). In the studies in this thesis, it has been used at low power to investigate non-fluorescent tissues and to determine Bregma levels of tissue sections, counter-stained with toluidine blue, an acidophilic thiazine metachromatic dye (Augulis and Sepinwall, 1969; see also Sridharan and Shankar, 2012). At higher power, it has been used to investigate the morphology of filled TIDA neurons, which have been stained with DAB-Ni. The technique is rather straight-forward, although, it should be emphasized that correct Köhler illumination (Köhler, 1893) greatly improve image quality.

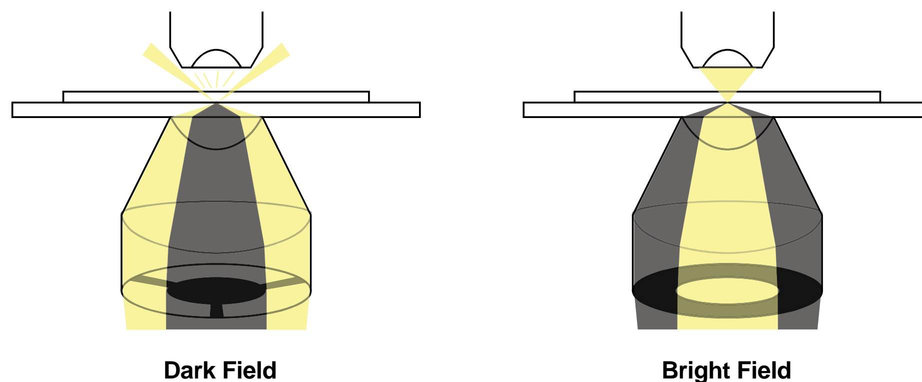

Figure 9. **Basic concept of bright field and dark field microscopy.** In dark field microscopy, the majority of the transmitted light is filtered out, and only scattered light is collected. Adapted from a drawing from the internet with unknown author.

In dark field microscopy (see *e.g.* Edmunds, 1877), a method used by Fritz Schaudinn and Erich Hoffmann in 1905 to identify the syphilis' causing bacterium, *Treponema pallidum* (Schaudinn and Hoffmann, 1905), the light that hits the specimen is aligned and angled in a way so that the amount of transmitted light is minimized. Only scattered light is collected (see Figure 9). This technique can improve image contrast dramatically and is therefore commonly used to visualize transparent specimen. Dark field microscopy is also used when investigating radioactive ISH experiments, as sparsely positioned, small sized, silver grains are difficult to detect in bright field, but will scatter

light very well. Although this technique makes the silver grains readily detectable, it is very sensitive to impurities, as dust on the cover glass, which also scatters light, will cause interference.

3.6.2 Fluorescence microscopy

Investigation of fluorescent dyes is usually done with epifluorescence microscopy, where the specimen is illuminated from above. This technique utilizes the fact that fluorescent molecules, when illuminated, will emit light of lower frequency than those they absorb. As the excitation and emission lights are different, they can be filtered, so that only the emitted light is collected, and thereby, only the structure of interest, which has been labeled with the fluorescence dye, is visualized. Using different fluorescent dyes with separated excitation/emission wavelengths, it is possible to stain different structures, visualize them individually and superpose them to create a multiplex image.

Although recent technical developments have made it possible to use single wavelength light sources produced from light-emitting diodes, the majority of microscopes, including the ones used in the studies in this thesis, uses a broad spectrum light source and rely on filter sets for light separation. Correct dye/filter set matching is crucial for successful image acquisition. If there is a mismatch in the excitation wavelength, the dye is not excited and little light is emitted, and if there is a mismatch in the emission wavelength, emitted light is not collected.

Unless intentional, which is the case when utilizing double filters for simultaneous observation of different dyes in the oculars, filter sets that fail to separate the light from different dyes, can have a more serious outcome. This phenomenon, known as “bleed-through”, will cause fluorescence signal to be attributed to the wrong structure, and thereby lead to false conclusions. Additional knowledge about the spatial distribution of the stained structures can provide necessary information to handle this. For instance, while neurobiotin will fill the entire cell, tyrosine hydroxylase (TH) is mostly void from the nucleus. TH signal in the nucleus can therefore alert the observer that the image is suffering from bleed through.

Lack of optical control in the z-axis leads to another type of concern in fluorescence microscopy. When the light hits the specimen, all fluorescent molecules absorbing the specific wavelength will get excited, regardless if they are in the focal plane or not. As the reflected light originates from both in-focus and out-of-focus structures, the image is rendered blurry. This interference can also causes fluorescence signal from two distinct structures, which are superposed in the plane of depth, to appear as a single structure. Together, these caveats make conventional florescence microscopy less suitable for co-localization studies.

3.6.3 Laser scanning confocal microscopy

Laser scanning confocal microscopy was patented by Marvin Minsky (1957) as a microscopy apparatus providing the "*means for producing a point source of light*" and an *"optical system capable of rejecting all scattered light except the emanating from the central focal point"*, *i.e.* an apparatus that made it possible to optically slice a specimen. This is done by introduction of a spatial pinhole that blocks out the light that is out focus. As a large portion of the light from the sample is blocked, a sensitive detector, usually a photomultiplier, is used to transform the light to electrical signal which can be handled by a computer.

To acquire a multidimensional image, the point beam has to scan across the sample. If the sample is scanned both laterally, and horizontally, the data can be put together into a 3D image. When multiple fluorophores are used, they can either be excited simultaneously or sequentially, depending on the setup of the microscope. Simultaneous excitation substantially reduces the scan time. This strategy can, however, generate bleed-through (see section 3.6.2) and should be avoided. Slower scan speeds also provide better signal-to-noise, but in multidimensional acquisitions can lead to bleaching. It can also become time-consuming to an extent that makes the acquisition unfeasible (compare Figure 10).

By the means of optical slicing and separation of the fluorescence signal through sequential scanning and filtering, confocal microscopy provides the necessary criteria to reliably separate multiple structures in space. Co-localization should therefore only be established with this technique.

3.6.4 Airyscan super resolution microscopy

In recent years, several microscopy techniques, like stimulated emission depletion microscopy (Hell and Wichmann, 1994), stochastic optical reconstruction microscopy (Rust et al., 2006) or photoactivated localization microscopy (Betzig et al., 2006), have been developed which makes it possible to visualize structures with a higher resolution than is imposed by the diffraction limit (about 250 nm for green light), as proposed by Abbe (1873). The Airyscan detector from ZEISS (Huff, 2015) is another recently developed technique, which has been used in the second study included in this thesis.

Airyscan utilizes a detector for confocal microscopy, consisting of an array of 32 photomultiplier tubes, where each detector element work as a single pinhole. By combining knowledge about the beam path and the spatial distribution of the detectors, improved signal-to-noise ratio and super-resolution is acquired (see Figure 10). Other advantages of the Airyscan system is that it does not require any changes to the staining protocols, the acquisition time is not increased and specimen is not bleached more than what is the case with conventional confocal microscopy.

A major disadvantage is that the image files generated by the acquisitions are 32-times larger, and that these images need to be post-processed to generate the final super

resolution image. Processing thicker stacks is CPU-consuming and pushes most desktop computers to their limits. Both acquisition and processing of larger regions of interest by the means of tiling require workstation-class computers. Computer power is today a limiting factor that will likely become obsolete in a near future.

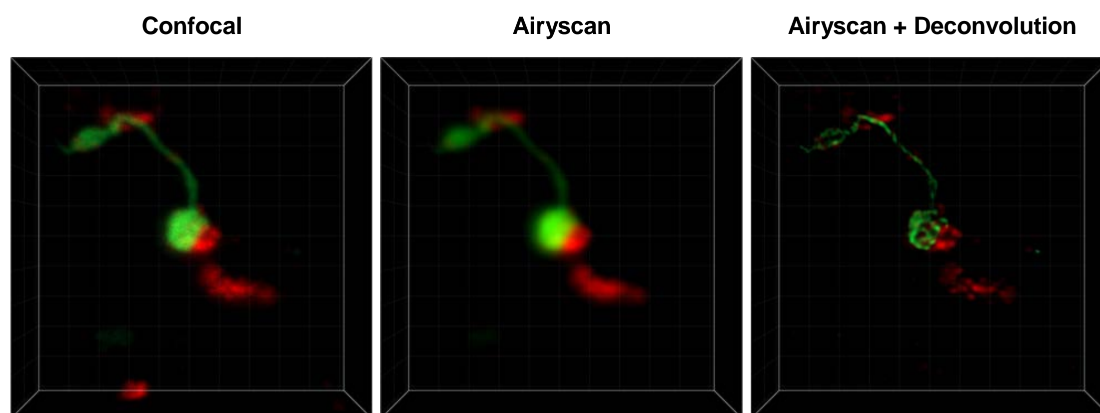

Figure 10. **Increased resolution with Airyscan confocal microscopy.** High power maximum intensity projection confocal micrographs of neurobiotin filled (green) TIDA neuron, immunostained for tyrosine hydroxylase (red), visualizing a putative synapse from the ventrolateral aspect of the arcuate nucleus and provide anatomical substrate for TIDA neurons innervating other dopamine neurons (n = 1). Image acquired with a ZEISS Airyscan LSM 700 microscope with a 63x oil objective at 9x zoom. To achieve optimal signal-to-noise ratio, a pixel time of 0.11 ms was utilized. Compared to confocal (left), the Airyscan processed image (middle) is smoother, with less noise and with higher resolution. After deconvolution (right), the subcellular distribution of neurobiotin and tyrosine hydroxylase can be identified. Although these settings are optimal for image quality, they are not practical as it would be too time consuming to scan a 30 μm portion of a dendrite in 3D (which was used for spine analysis in study II). Grid size = 1 μm.

3.6.5 Photobleaching of fluorescence samples

Photo-bleaching, or fading, caused by a reaction between the excited fluorophore and molecules in the environment, like oxygen, is a major concern when working with fluorescence samples. This is minimized by limiting the exposure to light and reducing its intensity, which unfortunately, also reduces the specimen visibility. Usage of anti-fading mounting medium can also significantly minimize photo-bleaching (Ono et al., 2001). Stained samples should be stored at -20°C, as lower temperatures will slow down the reactions and preserve the samples for longer period of time. Many samples included in this thesis have retained their fluorescence after several years of storage at this temperature.

3.7 ANALYSIS

In this section, certain aspects of analytical methods used have been discussed.

3.7.1 Close apposition and co-localization

Although confocal microscopy provides the criteria necessary to differentiate multidimensional structures (see section 3.6.3), this differentiation can be completely nullified by incorrect data handling and presentation. As we lack the true ability to present data in 3D, it is projected in 2D. Maximum intensity projections are commonly utilized to visualize stacks of confocal micrographs. These micrographs must be used

with extreme caution, as two structures separated in space, in the plane of depth, can appear as a single object, similar to what is observed in the oculars. Likewise, depending on the angle, objects that are far away in the plane of depth, can appear closer than they really are. This must be taken into consideration during data analysis, and close appositions and co-localizations must always be confirmed in a single optical slice and in all three planes.

3.7.2 Reconstruction of neurons in Neurolucida

Camera Lucida, which was patented by William Hyde Wollaston in 1806 (see Marien, 2014 p.7), provides the means to manually draw and create a proportionally accurate record from a live image. This has been a major advantage in neuroscience as image acquisition that covers the entire axonal and dendritic tree of neurons has not been technically possible. Thus, camera lucida drawings have for decades been a major technique for histologists to record and present their microscopy data. The computer software MBF® Neurolucida (Glaser and Glaser, 1990), is essentially a computer aided version of camera lucida, and provides the means to trace neurons from a live microscope image. This live image tracing does, however, have certain disadvantages, especially, on fluorescent specimens.

Photo-bleaching, as described previously (see section 3.6.5) is a major issue, particularly at high power magnifications. This basically means the examiner only has "a single shot" to trace a neuron, and optimization and future confirmation of a trace is thereby not possible. Photo-bleaching can also make tracing challenging when multiple neurites are in the same field of view, as all neurites are bleached when one neurite is being traced. This has been less of an issue for TIDA neurons due to their less complex dendritic tree.

The thickness and volume of the dendrites can also be integrated when tracing neurons with Neurolucida. These estimates depend highly on proper light and camera settings. If the signal from the process that is being traced is saturated, the thickness and the volume will be overestimated, and if the signal is too weak, they will be underestimated. This, together with the continuous bleaching of the tissue can make it difficult to maintain a uniform tracing, and a certain level of error is introduced in the data. In less complex structures, however, this error is not particularly significant.

It should be mentioned that recent developments in tissue clearing, like CLARITY (Chung et al., 2013) or iDISCO (Renier et al., 2014), and light sheet microscopy (see Elisa et al., 2018), have enabled acquisition of entire mouse and rat brains, albeit at lower resolutions. Given the computer power that is available at the time when this thesis is being written, handling these huge datasets is problematic. Development, in regard to computer power as well as light sheet microscopy resolution, should however make it possible to trace entire neurons from acquired micrographs, which should resolve many of the live image tracing issues.

3.7.3 Spine analysis in Imaris

The spine analysis that is part of the second study of this thesis has been conducted in BitPlane® Imaris. This computer software provides a fully automatic pipeline, which rarely generates realistic results, most likely because the algorithms lack the sophistication that is necessary to detect complex and highly variable structures like spines. Also, the company has mainly used medium spiny neurons from the striatum to develop their algorithms. As TIDA neurons exhibit extensions that does not always adhere to classical definitions (compare study II), it is plausible that the parameters used by the algorithms are not optimal for spine detection in the hypothalamus.

Using a semi-automatic pipeline, where several steps heavily rely on manually defined thresholds, satisfactory results can, however, be generated. As it is not possible to use equivalent thresholds across samples and maintain adequate reconstruction of the spines at the same time, the analysis can introduce unwanted variability in the results. Given the power of the data and observed differences between groups, these variabilities are, however, not of a size that would affect the study's conclusions.

It should be mentioned that BitPlane® is continuously developing their computer software and future versions might provide faster and more accurate solutions. However, as Imaris is a proprietary software, certain aspects of the functions, parameters and algorithms used in the program will remain unknown.

CHAPTER 4

RESULTS AND DISCUSSION

The following sections provide an overview of the results from the studies included in this thesis, and discuss the overall implications of them. For a complete insight in the results, and comprehensive discussion of the individual studies, the reader is referred to the respective communications.

4.1 TUBEROINFUNDIBULAR DOPAMINE NEURONS

The anatomical features of male mouse and rat TIDA neurons, at the level of population as well as individual cells, including axonal and dendritic arborization, somatic parameters and spines, were interrogated in study II, by the means of conventional immunofluorescence on brain sections, as well as by the visualization of patch clamp recorded neurons filled with marker-molecules. Multiplex immunofluorescence was also used to investigate subcellular distribution of glutamatergic and GABAergic synapses on TIDA neurons. Together, these strategies enabled us to describe TIDA neurons with higher resolution and certainty than what had been done previously.

Histochemical techniques, in combination with electrophysiology, were also used in studies III and IV to investigate if, and how, TIDA neurons are externally modulated by various different modulators. In study III, the anatomical substrate for serotonergic innervation of TIDA neurons was examined and the electrophysiological changes induced by serotonin and selective serotonin reuptake inhibitors (SSRIs) were investigated. Similarly, in study IV, a possible mechanism underlying the circadian rhythmicity of circulating prolactin was examined, by investigating the effects of hypocretin/orexin (H/O) and melatonin on TIDA neurons.

4.1.1 TIDA neurons are modulated by serotonin, SSRI's and H/O, but not melatonin

Studies III and IV present both anatomical and electrophysiological evidence for direct effects of serotonin and SSRIs as well as H/O, but not melatonin, on TIDA neurons. Other modulatory molecules like TRH (Lyons et al., 2010), prolactin (Lyons et al., 2012), oxytocin (Briffaud et al., 2015), and even dopamine (Stagkourakis et al., 2016) have also in other studies been identified to modify the electrophysiology of TIDA neurons, in

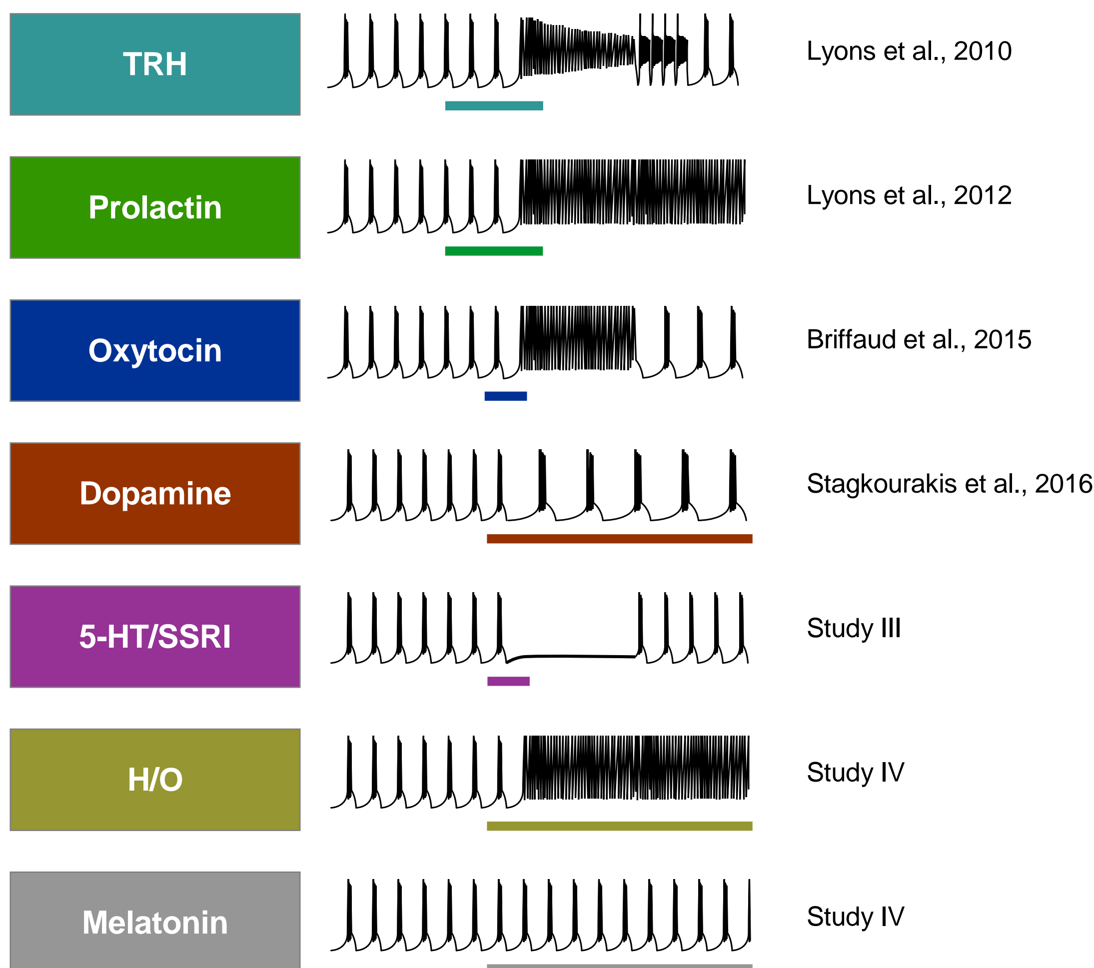

Figure 11. Different modulator's effect on the electrophysiology of TIDA neurons. Schematic drawings of *in vitro* current clamp recordings from TIDA neurons during bath application of different modulators. See list for abbreviations.

different ways (see Figure 11). For a more detailed description on how the different modulators affect the electrophysiology of the TIDA neurons, the reader is referred to the respective publication. Together these data show that the central dopaminergic control of prolactin secretion is highly susceptible to both physiological and iatrogenic modulations.

4.1.2 Oscillations might protect TIDA neurons from calcium overload

Ever since the oscillatory firing pattern of TIDA neurons were first identified and described (Lyons et al., 2010), the underlying functional explanation for this pattern has been under investigation. As adequate suppression of prolactin requires a constant tone of dopamine, TIDA neurons must remain active, and release dopamine, over long periods of time. During sustained high frequency discharge, excessive calcium influx can have damaging effects (Scharfman and Schwartzkroin, 1989; Sloviter, 1989; Iacopino et al., 1992), which the cell can counter with calcium binding proteins. This correlation has been observed in fast spiking neurons of both hippocampus and cortex (Kawaguchi et al., 1987; Kawaguchi and Kubota, 1993).

Although TIDA neurons does not discharge in the same high rate as fast spiking neurons, they sustain a high level of activity over longer periods of time, and one would therefore expect presence of buffers that protect the cell from excessive calcium influx. Yet, our investigations in study I could not identify neither calbindin-D28k, calretinin nor parvalbumin in rat TIDA neurons. Other studies have identified two members of the nucleobindin family, *i.e.* nucleobindin-1 and nucleobindin-2, to be expressed in TIDA neurons (Foo et al., 2008; Tulke et al., 2016). These two proteins are homologous multidomain calcium and DNA binding proteins, and were initially identified as transcription factors, since they bind DNA fragments *in vitro* (Miura et al., 1992). Due to their expression of the calcium binding EF-hand motif, they could potentially buffer calcium as well.

However, nucleobindin-1 is a pan neuronal marker associated with the Golgi apparatus (Tulke et al., 2016) and has been identified to bind G-proteins and function in signal transduction (Kapoor et al., 2010). Likewise nucleobindin-2, is many times associated with its DNA binding functions and identified by other names like calnuc (*e.g.* Lin et al., 2000). It has also been proposed that nucleobindin-2 is cleaved and secreted (Oh-I et al., 2006; Stengel et al., 2009a), although it remains controversial if cleavage and exocytosis occurs endogenously (Foo et al., 2008, 2010; Stengel et al., 2009b). Furthermore, it has been shown that nucleobindin-2 is virtually absent in axon terminals (Foo et al., 2008). Thus, these observations provide little evidence for nucleobindin proteins to buffer calcium in TIDA axon terminals.

Although the proteins mentioned above do not account for all calcium binding proteins, the data do suggest that TIDA neurons might be able to sustain firing by utilizing an alternative mechanism, independent of calcium binding proteins. The quiescence period following phasic firing could be the necessary component that gives the cell time to handle excessive intracellular calcium, and make it possible for TIDA neurons to sustain a constant dopamine inhibition on lactotrophs.

4.1.3 Morphological properties of mouse and rat TIDA neurons are largely similar

The morphological features investigated in the second study revealed that mouse and rat TIDA neurons exhibit similar properties in regard to their distribution, cell body size and axonal and dendritic arborization. Thus, if this finding is generalizable across other CNS regions, the larger brain volume in rats is a consequence of higher number of neurons, rather than larger cells.

TIDA neurons of both species showed a preference to extend dendrites towards, and in some cases probably into, the third ventricle (see Figure 12). This finding raised the question if TIDA neurons are able to circumvent the blood-brain-barrier to access different kinds of molecules, like ions or hormones, directly from the cerebrospinal fluid. Non-canonical neuronal structural features like somatic spines (*e.g.* Campbell et al., 2005) and extension of axons from dendrites rather than from soma (*e.g.* Herde and

Herbison, 2015) were also identified in both mouse and rat TIDA neurons. The functional implications of these observations remain, however, to be investigated.

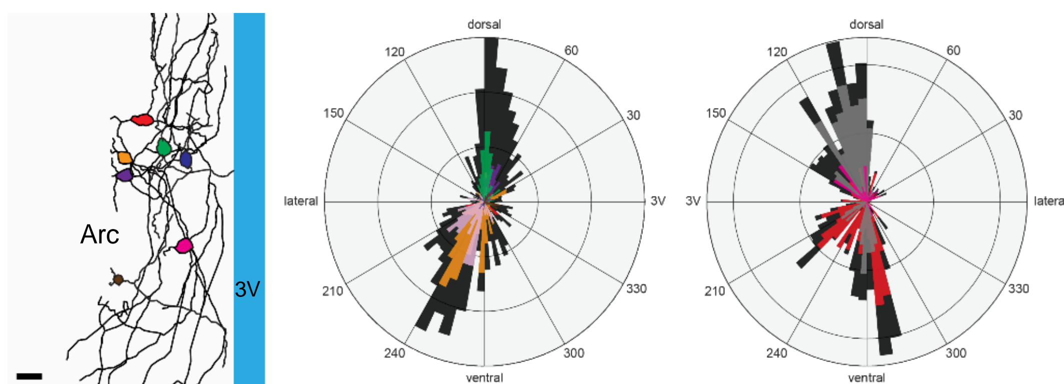

Figure 12. Reconstruction of neurobiotin filled rat TIDA neurons. Virtual reconstruction (A) as well as polar histograms (B) of the dendritic trees of TIDA neurons illustrates the preference towards the ventricle side. See list for abbreviations. Scale bar in (A) = 25 µm.

4.1.4 TIDA neurons are more spiny in mice than in rats

Spine analysis on mouse and rat TIDA neurons revealed species differences. Mouse TIDA neurons commonly exhibited a higher density of dendritic spines, compared to rat dendrites (compare Table 1). Interestingly, previous investigations have identified mouse and rat TIDA neurons to be governed by different network mechanisms. While rat TIDA neurons exhibit gap junctions and are electrically coupled, mouse TIDA neurons lack gap junctions and fire asynchronously (Stagkourakis et al., 2018a). The higher prevalence of spines in mouse TIDA neurons could be related to these other observations. See study II for more details about spines on TIDA neurons and their differences between mice and rats.

Table 1. Comparison of mouse and rat TIDA neurons

MICE	RATS
Asynchronous firing[1]	Synchronous firing[1]
No gap junctions[1]	Gap junctions[1]
High spine density	Low spine density

[1] Original data presented by Stagkourakis *et al.* (2018a).

4.2 DOPAMINE RECEPTORS IN THE HYPOTHALAMUS

In studies V and VI included in this thesis, two transgenic mouse lines were used to generate a comprehensive map of cells expressing D1 and D2 receptors in the hypothalamus, the pituitary and select circumventricular organs. The neurochemical identity of these cells was also investigated. Our data confirm previous reports (*e.g.* Meador-Woodruff et al., 1989, 1991; Mansour et al., 1990; Brouwer et al., 1992; Mengod et al., 1992; Yokoyama et al., 1994), and add substantial details.

The expression patterns of D1 and D2 receptors showed a striking contrast. While the D2 receptor was widely expressed in the hypothalamus and could be found in many hypothalamic nuclei, the D1 receptor expression was sparse and restricted to a few areas, suggesting that the dopaminergic control that is exerted on the hypothalamus is mainly inhibitory. The suprachiasmatic nucleus, however, constitutes a clear exception, as this area contained many D1 receptor expressing neurons, but was completely void of D2.

These dopaminergic inputs (compare section 1.2.2) could proceed from local hypothalamic neurons (*i.e.* A11-A15), or originate from either the mesencephalon (*i.e.* A8-A10) or the olfactory bulb (*i.e.* A16). The local hypothalamic circuitries could also involve auto-receptors. However, the dopamine source providing innervation to the hypothalamus was not addressed in these studies, and needs to be evaluated in future studies.

4.2.1 Dopamine receptors on dopaminergic neurons

The alphanumerical classification of catecholamine neuron, which includes A11-15 in the hypothalamus was used to identify dopamine neurons (see section 1.2.2). D2 receptor was found to be expressed in subpopulations of all these groups. Although *Drd2*-EGFP immunofluorescence could not be found in the A13 dopamine neurons of zona incerta, closer examination by the mean of multiplex *in situ* hybridization could identify *Drd2* mRNA in this population as well, confirming previous D2 receptor ligand binding autoradiography studies (Yokoyama et al., 1994). The lack of immunofluorescence in A13 dopamine cells is thus most likely a transgenic artefact (see section 3.1.1). As detailed information about the included regulatory elements in the BAC construct is lacking, the exact cause of this artefact cannot be identified.

D1 receptor expressing neurons were also found in subpopulations of A12-A14 dopamine neurons, but in fewer numbers. Although controversial, previous immunohistochemical investigations have identified co-expression of D1 and D2 receptors in the same cells of striatal neurons (Aizman et al., 2000) as well as avian hypothalamic neurons (Chaiseha et al., 2003). Our current results from these two studies raise the question if such co-expression also occurs in the mouse hypothalamus. Nevertheless, our current data clearly indicate that hypothalamic dopamine control in both A12 and A14 dopamine neurons is mainly mediated by the D2 receptor.

4.2.2 Magnocellular neurons lack dopamine D1 and D2 receptors

Our data provide little evidence for direct dopaminergic modulation of the magnocellular system, as no significant co-expression could be observed with either D1 or D2, on the magnocellular neurons in the supraoptic or the paraventricular nuclei. Surprisingly, EGFP signal resembling axon terminals could, however, be observed in the posterior pituitary of *Drd1*-EGFP mice, but the neurochemical identity of these terminals is currently not determined. It should be mentioned that a single vasopressin

expressing neuron (*i.e.* one neuron in one section), was occasionally observed in the supraoptic nucleus, but the density of the fibers in the posterior pituitary was too high to originate from a single neuron.

4.2.3 Dopamine receptors are involved in the majority of the hypothalamic-pituitary axes

Dopamine can control endocrine release at hypothalamic and/or pituitary levels. D1 receptor was only observed in dopaminergic parvocellular neurons (see section 4.2.1), and the anterior pituitary was void of D1 receptor. In the hypothalamus, D2 receptor was expressed in a subpopulation of both somatostatin and TRH expressing neurons whereas in the pituitary lacto-, thyro- and gonadotroph cells expressed D2 receptor. No conclusions could be drawn regarding growth hormone-releasing hormone (GHRH) expressing neurons in the hypothalamus due to the lack of specific antisera.

These data indicate a broad role for the D2 receptor in the hypothalamic endocrine output, and could provide a neuroanatomical explanation to various conditions, for instance side effects observed in patients under pharmacological treatments, such as antipsychotics, that target the D2 receptor. It also illustrates the caveats of delivering drugs at systemic level and thereby highlights the importance of direct targeting. These new insights could thus be the foundation of new D2 mediated therapeutic strategies for treating hormonal disorders and/or pituitary adenomas that give rise to *e.g.* hyperprolactinemia, hyperthyroidism (see Singh and Hershman, 2017) or acromegaly, but likely not for instance Cushing's disease (see Störmann and Schopohl, 2018); a condition caused by dysregulations in corticotropin-releasing hormone expressing neurons or corticotrophs, where no D2 receptor could be identified (see study V).

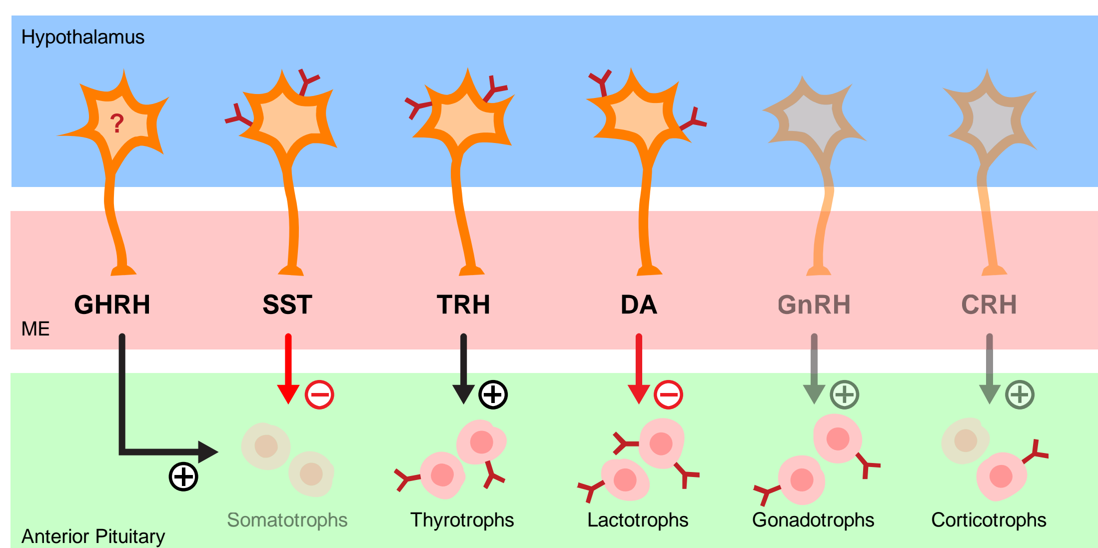

Figure 13. Schematic presentation of dopamine D2 receptor expression in parvocellular neurons of hypothalamus and endocrine cells of anterior pituitary. Cells that express D2 receptor have been marked with (Y), and cells that doesn't express D2 receptor have been faded. See list for abbreviations.

4.2.4 Involvement of D2 receptor in acromegaly

Acromegaly is a hormonal disorder, caused by excessive growth hormone release during adulthood, which causes bones and body extremities to grow abnormally (see Melmed, 2009). It is usually caused by pituitary adenomas and is treated with surgery, somatostatin analogs or D2 agonists (see Zahr and Fleseriu, 2018). By studying the dopamine receptors in the hypothalamus and the pituitary, we found D2 receptor expression in a subpopulation of somatostatin expressing neurons in the hypothalamus, but not in the somatotropic cells of the pituitary.

Activation of D2 receptors in the hypothalamus would be predicted to impair the inhibitory effects of somatostatin on somatotrophs, and thus lead to increased growth hormone release, which makes our data, at an initial glance, difficult to reconcile with clinical observations. However, investigations of pituitary adenomas, have revealed expression of D2 receptors on somatotropinomas from acromegaly patients (Neto et al., 2009), which would provide an explanation why patients respond to D2 agonist treatments. Notably, the literature also reports D2 receptor expression on normal somatotrophs (*e.g.* see Ben-Shlomo et al., 2017). This however, does not seem to be supported by the original data provided by Neto et al. (2009), which did not distinguish somatotrophs from the rest of the endocrine cells in the pituitary.

It should be mentioned that bromocriptine treatments, in line with our results, does not always suppress growth hormone secretion in acromegaly patients, and the opposite where the treatment leads to increase secretion has also been reported (Arihara et al., 2014). This illustrates why understanding of the entire system is necessary to be able to provide effective treatments for different patients. Finally, it should be noted that our data currently lack information about dopamine receptor involvement on GHRH expressing neurons which could have implications on the somatotrophic axis.

4.2.5 D1 receptors are expressed in ependymal cells

Possible D1 receptor expression was observed in a subpopulation of the ependymal cells of the third ventricle, lining the wall of the arcuate nucleus. This is a curious finding since previous records of these cells in the literature are absent. These results demand further investigation and confirmation. Considering the methodology in use, the signal could have three different explanations. The first explanation is that these cell do in fact express D1 receptors, which could be confirmed by *in situ* hybridization experiments. The second explanation is that observed signal is a transgenic artefact (see section 3.1.2). In such case, the strong expression of EGFP can, nevertheless, be used to target these highly underappreciated cells for further anatomical and electrophysiological interrogations.

A third possibility is gap junction mediated EGFP translocation, from D1 receptor expressing neurons to the ependymal cells. As EGFP is too large to passively diffuse through a gap junction pore (see Alexander and Goldberg, 2003), this translocation

would utilize a different mechanism. Three different gap junction dependent mechanisms, have been described in the literature, which are through internalization (Jordan et al., 2001), cytoplasmic bridges (Bukauskas et al., 1992) or tunneling nanotubes (Wang et al., 2010). This would constitute a novel circuitry involving ependymal cells, and provide hypothalamic neurons access to larger proteins in the blood and the cerebrospinal fluid.

4.3 CALCIUM BINDING PROTEINS IN THE ARCUATE NUCLEUS

In the first study included in this thesis, the expression pattern and neurochemical identity of three calcium binding proteins, *i.e.* calbindin-D28k, calretinin and parvalbumin, in the arcuate nucleus have been investigated. Both mRNA and immunoreactivity were detected for calbindin-D28k and calretinin. Results from one parvalbumin antiserum was, however, ambiguous as it, contrary to *in situ* hybridization experiments and other parvalbumin antiserum, showed immunoreactivity in the arcuate nucleus. The exact reason for this discrepancy could not be elucidated from the study. It should be mentioned that the majority of the data from study I, together with data from other studies (Celio, 1990; Fortin and Parent, 1997), provide evidence against expression of parvalbumin in the arcuate nucleus of juvenile male rats.

4.3.1 Possible antisera cross-reactivity

As all three calcium binding proteins investigated in study I belong to the EF-hand family, and thereby exhibit similar motifs in their 3D structure, it is plausible that some (but not necessarily all) immunoglobulins in the polyclonal parvalbumin antisera fail to distinguish these structures in fixed tissues. Closer examination of multiplex immunostainings did show overlap between the two antisera targeting calbindin-D28k and parvalbumin to some extent.

The co-expression is, however, not absolute, which is usually expected when antisera cross-react. By staining the two targets separately, cross-reactivity at the level of secondary antisera was ruled out. The fact that parvalbumin was targeted with a polyclonal antiserum could, however, provide an explanation. If only a subpopulation of the immunoglobulins cross-react, the antisera, that has been titrated to be used when all immunoglobulins are active, will be less potent and mainly detect areas with a high calbindin-D28k concentrations. This diminished signal was exactly what was observed in the experiments where antisera PV-25 was used.

4.3.2 The functional role of calcium binding proteins in the arcuate

Many calcium binding proteins, including calbindin-D28k and calretinin, have been identified to function in signal transduction, in addition to calcium buffering (compare section 1.3). To our best knowledge, parvalbumin, has so far not been identified to be involved in signal transduction, and is considered to be a “pure” calcium buffer. Although the functional role of these calcium binding proteins were not elucidated in

this study, it is interesting that the calcium binding protein that is known to only act as buffer, was absent. Future studies should investigate if the role of calcium binding proteins in the arcuate nucleus is limited or not to signal transduction.

4.3.3 Calcium binding proteins are expressed in POMC neurons

Of all the neuronal populations in the arcuate nucleus that were investigated, POMC neurons were the only ones that expressed calcium binding proteins. Interestingly, the expression was not uniform in the population, but both a rostro-caudal and a medial-lateral differentiation could be observed.

Diversity in arcuate POMC neurons has previously been identified in regards to projection patterns (Swanson and Kuypers, 1980; Baker and Herkenham, 1995; Elias et al., 1998, 1999), sensitivity to metabolic hormones (Williams et al., 2010) and amino acid transmitter phenotype (Collin et al., 2003; Meister, 2007; Hentges et al., 2009; Jarvie and Hentges, 2012; Wittmann et al., 2013). The data from all these studies together with current findings presented in study I can be summarized as follows: The rostral POMC neurons express calretinin, respond to insulin and project caudally to the autonomic areas. The caudal POMC neurons express calbindin-D28k, project within the hypothalamus and respond to leptin. It should be emphasized that the correlation of these data is not confirmed yet and remains to be determined in the future.

CHAPTER 5

CONCLUDING REMARKS

In the studies presented in this thesis, we have investigated the dopamine system in the hypothalamus with a particular focus on the TIDA neurons. We have identified an anatomical substrate for innervation by serotonin and hypocretin/orexin and showed that these modulators do alter the electrophysiological behavior of the cells. We have also provided a comprehensive map of D1 and D2 expressing cells in the hypothalamus, the pituitary and some of the circumventricular organs. We identified cell populations that express either D1 or D2 receptors. Finally we provided evidence that both calbindin and calretinin are expressed in the arcuate nucleus, particularly in POMC neurons, which are subdivided into two groups based on the expression of these two calcium binding proteins.

These data provide evidence to understand the underlying circuitries that enable the hypothalamus to work, in order to maintain homeostasis and drive motivated behaviors, which for instance can be used to explain physiological features in hormonal control, predict pharmaceutical agents' actions and adverse effects. This understanding also provide a solid basis for future development of treatments in diverse areas of medicine.

CHAPTER 6

ACKNOWLEDGEMENTS

Now when I have made it this far, I have been active at Karolinska Institutet (KI) for over a decade. It has been quite an eventful time and I have met a lot of people in the line of research, as a representative in different boards and committees or at Medicinska Föreningen (MF) – our beloved student union. These people have all been important to me and have had an impact on my personal and professional development in one way or another. I would like to express my sincere gratitude to all of them.

Christian Broberger, my supervisor, for providing this opportunity and introducing me to the world of neuroscience, and particularly the hypothalamus. His genuine love for these subjects are truly contagious. The many meetings we had were mostly fun, but what I truly enjoyed was our microscopy sessions, where I always learned something new from him. Thank you Christian for caring, for listening, for teaching, and for always being available.

Karl Hult, my external mentor, for making time for me and guiding me. His knowledge and experience is almost beyond comprehension, and it is always a pleasure discussing things with him regardless of the subject. Thank you for your insightful comments and for broadening my perspectives.

Charlotte Björnberg, my other mentor, for fun and interesting discussion and for giving me the non-academic perspective of the world.

My co-supervisors, **Per Uhlén** for introducing me to Imaris before most people had heard of it, and **Marie Björnholm** for inviting me to her lab's journal clubs and for keeping her door open and discuss scientific questions with me.

I would like to express my gratitude to **Joel Elmquist** at UT Southwestern for inviting me to his lab, and **Charlotte Lee** for teaching me the art of riboprobe *in situ* hybridization and for showing me where to get the finest food in Dallas. Swedish beef just doesn't taste anything to me ever since.

Special thanks to **Jeffrey Friedman** for his limitless hospitality and for having me in his laboratory at the Rockefeller University in numerous occasions. It is always a pleasure listening

to him, as his way of thinking and interpreting the data, is what truly help us move science forward. From the noisy underground, to the top of the Rock, or even higher in the sky, there were many things I experienced in Manhattan. The Friedman lab with **all its members**, especially **Mats Ekstrand**, **Zhiying Li**, who is one of the kindest person and best teachers I have met, and **Kaamashri Latcha**, was certainly one of my best experiences in the Big Apple.

I am also grateful to have had the opportunity to collaborate with **Roman Romanov** and **Tibor Harkany** within my field of interest and am thankful for Tibor's mindfulness and attentiveness.

Additional collaborations with **Lennart Svensson** and **Marie Hagbom** from Linköping University, directed my attention towards the enteric nervous system and got me interested in the rotavirus and its effects on the central nervous system. Their genuine enthusiasm also makes it extra fun to work on these projects and I look forward to future studies in these subjects.

The Department of Neuroscience at KI has the honor to host several emeriti, including **Tomas Hökfelt**, **Gunnar Grant** and **Sven Ove Ögren**, who has during several decades built up the faculty and the scientific foundation we stand on today. I am thankful these people are still around to help, to guide, to inspire and to share their knowledge and experiences.

Staffan Cullheim, **Mårten Risling**, **Mia Lindskog**, **Roman Chrast**, **Eva Hedlund**, **Nils-Göran Larsson**, **Gilberto Fisone** and their respective **lab members** for sharing lab space, equipment, reagents but most importantly a positive and friendly attitude that provide a pleasant, fun and relaxed environment to work in. Lunch and coffee brakes are also always fun when these people are around and constitute a necessary cheerful moment in life, especially in the line of research where one many times face failed experiments.

Members of my halftime committee, **Sophie Bensing**, **Jens Hjerling-Leffler** and **Jan Mulder**, for providing many insightful comments and feedback that were very useful to me, and helped improving my research.

Current and past members of the **Blueberry** (read Broberger) **family**, for all the fun we had inside and outside the lab. **David Lyons** for mentoring me when I started in the lab, for showing me how electrophysiology is done, and for driving me with scientific questions. **Emilia Horjales-Araújo** for introducing me to immunohistochemistry, and providing the foundation which I have based this thesis on. **Kylie Foo**, for sharing valuable tips and tricks about how to run different experiment and assays. **Lovisa Case**, for her invaluable scientific insights, rapid responses and willingness to share her thoughts and experiences. **Siobhan Connor**, for being friendly and for discussing scientific questions. **Stefanos Stagkourakis** for his scientific mindset, and for zealously perusing his scientific goals. **Paul Williams** for his insightful questions and ability to identify proper controls. **Carolina Thorn Perez** for her energy and for not being satisfied with later and driving people to work harder. **Virginie Briffaud** for making everyone in the lab to keep things tidy and clean; every lab should have a Briffaud! **Laura Heikkinen** and **Roksana Khalid** for help setting things up in Biomedicum. **Olof Lagerlöf** for providing clinical expertise particularly regarding pharmaceuticals.

A very special thanks also goes to **Rachida Ammari** and **Jimena Ferraris** for the elaborate scientific discussions we have had, for their friendliness, but also for proofreading this thesis, actually more than once, and providing invaluable suggestions on how to improve it. Without

their passionate input this thesis would have been of a lesser quality, and maybe even frustrating to read.

During my time at KI, I also had the pleasure to help supervising several undergraduate students including **Susanne Tulke**, **Paul Williams**, who later actually joined the lab, **Justine Courty**, **Renee Neal**, **Henrik Åslund**, **Marta Garo**, **Qingyun Wen**, who singlehandedly ran our own "Q" division, and **Zoe Grivet**. These opportunities were not completely effortless, but have been very fruitful, partly because the students helped generate a lot of meaningful data, but more importantly as their way of thinking and questioning helped me towards a deeper understanding of the subject, developing my personally and producing better scientific outcomes.

Elin Dahlberg. I only wish she had joined us sooner. She is kind, helpful, diligent and an absolute ace in cryosectioning. She is also the fairy that makes the genotyping in the Excel sheets magically appear.

Abdel El Manira and **Gilad Silberberg** for being really cool professors, easy to talk to, and for their scientific reasoning and for asking inspiring questions.

Csaba Adori and **Nicolas Renier** for scientific discussions and help with iDISCO, although, the data could not make it to this thesis.

Alessandra Bonito Oliva for teaching me how to use the stereotaxic equipment and how to do intracerebroventricular injections.

In MF I would like thank the speaker of the council **Jens Andersson** for keeping things in order with his unending stamina, the members of the presidia throughout the years **Johan Hilm**, **Felicia Fixell**, **Erik Hellsing**, **Veronica Hansson**, **Karin Böttiger**, **Elin Ekeroth**, **Jennie Sporre**, **Andrea Montes** and **Frida Hellström**, who have been a support to me when I was acting chairman of the Doctoral Students' Association (DSA), all the other students whom I have worked together with in different workgroups and committees and the Doctoral students' ombudsmen, particularly **John Steen**, who was truly trustworthy and an invaluable support in difficult times.

Also thanks to all the board members in DSA, especially my deputy **Hugo Zeberg**, but also **Åsa Samuelsson**, **Arnika Wagner**, **Jayesh Pandya**, **Fei Yang**, **Daniel Holl**, **Håkan Nero**, **Javad Jafari**, **Amir Mahani**, **Ranjita Dutta Roy**, **Susanna Bächle**, **Agata Agnieszka Korecka**, **Sonal Pendharkar**, **Cheng Xu**, **Emma Ahlén Bergman**, **Ulrika Norin**, **Yuan Xu**, **Eva Hesselmark** and **Matilda Liljedahl** who made those late meetings so interesting I nearly always lost the time.

Anders Gustafsson, **Marianne Schultzberg** and all the **other members** of the Board of Doctoral Education, for listening to my suggestions, caring and working to improve the doctoral education.

In *Konsistoriet*, fellow student representatives, representatives from the faculty, including **Elias Arnér**, **Lena von Koch**, **Anna Karlsson** and representatives from the union. And a special thanks from the deep of my heart to the external members **Annika Andersson**, **Dan Andersson**, **Anders Blanck**, **Susana Borrás**, **Eskil Franck**, **Torbjörn Rosdahl** and **Charlotte Strömberg**, who always worked for the best interest of KI, and who helped me correct myself when I was in error. I am also grateful for the internal revision with **Bo Myrup** and **Peter Ambroson** and to **Per Bengtsson**, who supported and helped me to participate in the meetings, and together with the others made the university board room feel less intimidating for us rookies. Last but not least, **Lars Leijonborg**, who let the discussions flow and give rise to well-founded decisions.

All my friends and co-workers in the department, including **Rebecka Björnfors**, **Elham Jalalvand**, **Song Jianren**, **Maya Ketzef**, **Shreyas Suryanarayana**, **Giada Spigolon**, **Andreas Kardamakis**, **Adolfo Talpalar**, **Matthijs Dorst**, **Josina Van Lunteren**, **Joanne Bakker**, **Manideep Vemula**, **Salvatore Magara**, **Teresa Femenia**, **Jean-Jacques Medard**, **Luca Bartesaghi**, **Filipa Boucanova**, **Hasna Baloui**, **Enric Domenech-Estevez**, **Ivar Dehnisch**, **Marta Gómez**, **Caitlin DuPont**, **Mike Feyder**, **Peter Löw** and **Konstantinos Ampatzis**, thanks for making a pleasant work environment.

All the technical and administrative staff, like **Boje Persson**, **Karin Lagerman**, **Karin Vågstrand**, **Maya Petrén**, **Axel Bergwik**, **Niklas Lilja**, **Claes-Olov Fälth**, **Sandra Azami** that make sure everything is actually up and running.

My mom **Zahra Lapour**, her husband **Peter Hanspers**, my dad **Bahman Hellysaz**, my sister **Isabell Yalda Hellysaz**, her husband **Fredrik Marmbrandt** and my lovely little nephew **Leon**, and my friends outside KI, **Alireza Tavakoli Targhi** and **Amirhossein Banuazizi**, who helped me not getting lost in the rabbit hole.

Believe it or not, the list is not exhaustive, but I am afraid I have to truncate here.

CHAPTER 7

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CHAPTER 8

NEUROART

Acquiring all the micrographs that has been used as a basic foundation for the studies included in this thesis, required countless of hours in the dark room and in the basement, where I had to either look in the oculars to investigate the results, or stare at the computer screen to optimize acquisition settings. Had it not been for the beautiful world visualized in the microscope, the workload had not been endurable. As a way to share the marvelous world of neuroscience and fluorescence microscopy, and to raise interest in the general public, I used some of the data in a more artistic way in my Christmas greetings every year.

Some of these greetings have been included in this sections. These Christmas cards are a manifestation to how fun science can be, but also demonstrate technical as well as my personal developments in the last decade, as the resolution of the micrographs I have acquired has throughout the years increased tremendously.

2010 This was the very first NeuroCard I made. It is an immunofluorescence visualization of parvalbumin neurons (see study I), not in the hypothalamus, but in cortex. The underlying micrograph originates from the very first immunostaining I have done on brain sections.

2011 A cortical neuron identified by fluorescence microscopy, is shining up the sky and guiding the three wise men to baby Jesus. A neuron is the functional unit of the brain and the very basic building block that give rise to our rational and intelligence. This greeting was my way to proclaim that rational is the only path to transcendence, and that it is lighten up by science.

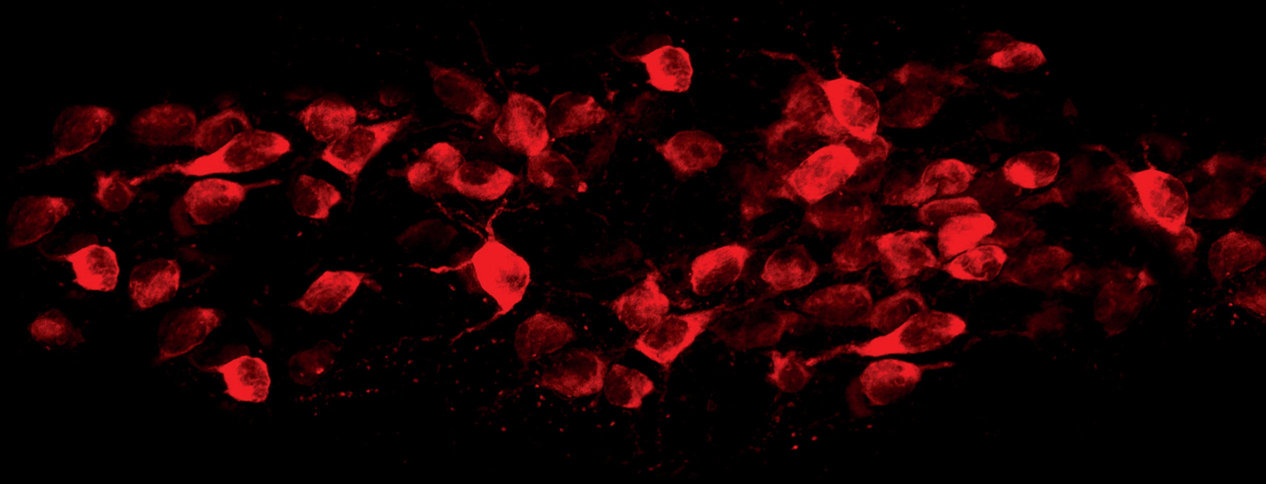
2012 The tree, the star and the decorations are all different kind of neurons from cortex as well as from hypothalamus, which has been visualized and used in real experiments. A fully decorated tree and a merry life is only possible, if they are all present, in the right place, and interconnected correctly.

2013 The arborization of axon and dendrites of cortical neurons can be quite complex. To generate a full reconstruction of a complex neurons through manual tracing in Neurolucida (see section 3.7.2) can take a couple of days of work. Fortunately, the studies in this thesis focused on TIDA neurons, which are far less complex. Nonetheless, I have traced quite a few cortical neurons as well. The Christmas decoration on this greeting is a reflection of the amount of hard work that lays behind a merry celebration.

2014 Inspired by the Nobel prizes in Chemistry as well as Physiology or Medicine in 2014, the motives on the card originate from cortical neurons, dendrites and dendritic spines and are acquired with super resolution STED microscopy. The dendritic spines are pseudo colored.

2016 Inspired by the vast number of in situ hybridization experiments I have done for the past years. The stars are actually experimental data, *i.e.* visualization of fluorescent mRNA of dopamine receptor D2 in the hypothalamus (see study V), magnified about 1000x. To better understand the role of dopamine in hypothalamus may aid us identify new therapeutic targets as well as understand the mechanism (and side effects) of existing therapeutic agents.

2017 Inspired by recent development in microscopy resolution and real research data, dendritic spines are lighting up the path to baby Jesus, sheltered under a neuron tree. The stars are also background staining from the brain, magnified more than a thousand times. Seemingly countless and insignificant in the sky, each dot in fact harbors an entire world. Our ability to investigate the brain in such detail enables us to better understand how individual physiological changes through life are in fact linked with changes in our brains.



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