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Anatomical Studies of Hypothalamic Thyrotropin-Releasing Hormone: Implications for Motivated States

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Stockholm 2010

Cover Image: Confocal micrograph of coronal section from the rat lateral hypothalamic area processed for single immunofluorescence to visualize cell bodies containing thyrotropin-releasing hormone (TRH).

Published by Karolinska Institutet. Printed by Larserics Digital Print AB

ISBN 978-91-7409-916-4

ABSTRACT

The survival of every animal as an organism and species depends on three motivated states: ingestive, defensive and reproductive. Regulation of these states is coordinated by the hypothalamus through the coordination of three principal output pathways: behavioural, endocrine and autonomic. An additional key component required for motivated behaviour is arousal. Imbalances within this intricate system have widespread clinical consequences as observed in *e.g.* endocrine disorders. A messenger molecule implicated in all three output pathways is the peptide, Thyrotropin-Releasing Hormone (TRH). While TRH has primarily been studied as a releasing factor in the hypothalamo-pituitary-thyroid axis, it is now well recognized that TRH also has a variety of neuromodulatory effects beyond pituitary regulation. The processes affected by TRH include the sleep-wake cycle, lactation, food intake and thermogenesis. The aim of this thesis was investigate the circuitry and mechanisms underlying the effects of hypothalamic TRH in arousal, lactation and metabolism.

In paper I, we explored the effect of TRH on cells in the lateral hypothalamic area (LHA) that express the peptide hypocretin (a.k.a. orexin), which exerts potent arousal-promoting actions. We found that TRH depolarized and increased the firing rate of hypocretin neurons through activation of cation currents. By double-label immunofluorescence, we observed close appositions between TRH-immunoreactive (-ir) nerve terminals and hypocretin-ir cell bodies. These results identify a new potential modulator of hypocretin cell firing, and suggest that hypocretin cell excitation may contribute to the arousal-enhancing actions of TRH.

In paper II, the population of cells located in the LHA that expresses TRH was anatomically defined and histochemically characterized. Occupying most of the ventrolateral hypothalamus in its full rostro-caudal axis, the TRH-ir population in the LHA displayed little co-existence with other hypothalamic neuronal markers, suggesting a unique neuronal population. The TRH-ir cells were innervated by terminals containing Agouti Gene-Related Peptide and α-Melanocyte- Stimulating Hormone, suggesting that they form a downstream target for the hypothalamic metabolic sensor in the arcuate nucleus.

In paper III, a population of cells located in the dorsomedial arcuate nucleus was demonstrated, which discharge in rhythmic oscillations. Oscillator neurons were histochemically identified as tuberoinfundibular dopamine (TIDA) neurons, which provide tonic inhibition of pituitary prolactin release. The oscillation was shown to be robustly synchronized between neurons via electrotonic gap junction coupling. Terminals containing TRH were observed in close apposition to TIDA cells, and when TRH was applied oscillating TIDA neurons switched from phasic to tonic discharge. These results suggest a novel mode of regulation of lactation where a change in TIDA network from oscillations to tonic discharge switches dopamine function from an antagonist to an agonist. Furthermore, our findings suggest that the lactation-releasing actions of TRH may take place at the hypothalamic, rather than the pituitary level.

LIST OF PUBLICATIONS

I. González JA, **Horjales-Araújo E**, Fugger L, Broberger C, Burdakov D (2009) Stimulation of orexin/hypocretin neurones by Thyrotropin-Releasing Hormone.
J Physiol 587(Pt 6):1179-86

II. **Horjales-Araújo E**, Broberger C (2010) Lateral Hypothalamic Thyrotropin-Releasing Hormone (TRH) neurons: distribution and relationship to chemically defined cell populations in rodent.
Manuscript.

III. Lyons DJ, **Horjales-Araújo E**, Broberger C (2010) Synchronized Network Oscillations in Rat Tuberoinfundibular Dopamine Neurons: Switch to Tonic Discharge by Thyrotropin- Releasing Hormone.
Neuron 65(2):217-29

CONTENTS

LIST OF ABBREVIATIONS

α-MSH	alpha- Melanocyte Stimulating Hormone
ACTH	Adrenocorticotropic Hormone
AGRP	Agouti Gene-Related Peptide
Arc	Arcuate nucleus
CART	Cocaine- and Amphetamine-Regulated Transcript
CNS	Central Nervous System
CRH	Corticotropin- Releasing Hormone
DMH	Dorsomedial Hypothalamic nucleus
Enk	Enkephalin
fx	fornix
FITC	Fluorescein Isothiocyanate
FSH	Follicle Stimulating Hormone
GABA	γ- Aminobutyric Acid
GFP	Green Fluorescent Protein
GnRH	Gonadotropin- Releasing Hormone
Hcrt	Hypocretin
HRP	Horseradish Peroxidase
-ir	immunoreactive
LH	Luteinizing Hormone
LHA	Lateral Hypothalamic Area
MCH	Melanin Concentrating Hormone
ME	Median Eminence
nNOS	neuronal Nitric Oxide Synthase
NPY	Neuropeptide Y
PACAP	Pituitary Adenylate- Cyclase Activating Polyeptide
PFA	Paraformaldehyde
ppTRH	Prepro-TRH
PVH	Paraventricular ucleus
Som	Somatostatin
T3	Triiodothyronin
T4	Thyroxin
TH	Tyrosine Hydroxylase
TIDA	Tuberoinfundibular Dopamine
TRH	Thyrotropin- Releasing Hormone
TSA	Tyramide Signal Amplification
TSH	Thyroid Stimulating Hormone
TTX	Tetrodoxin
TuLHA	Tuberal region of the Lateral Hypothalamic Area
VMH	Ventromedial Hypothalamic nucleus
3v	Third ventricle

1. INTRODUCTION

Our survival depends critically on the ability of the brain to respond when tissue health is challenged or when an opportunity to improve the viability of the animal or the species presents itself. This is accomplished by the promotion of motivational states that focus the attention and resources of the central nervous system onto the completion of specific goals (Stellar, 1954). Classically, these survival-oriented motivational states include defense, ingestion (broadly defined as the maintenance of energy balance) and reproduction (see Swanson 2000). Motivation is a key feature of how the brain initiates, sustains and directs behaviour. In biological terms, motivation can be seen as a generalized state whereby the brain orients and coordinates all its actions to the completion of a particular goal.

The ultimate purpose of goal-oriented behaviour is to maintain balance - **homeostasis**- within the variables (*e.g.* temperature, ion concentration, availability of metabolic fuel) that allow optimal functioning of proteins and the tissues in which they act. The basis of the homeostasis concept can be traced back to Claude Bernard (1865; 1876) who proposed the importance of an invariable *milieu intérieur* enclosed by a constantly changing *milieu extérieur*. The continuous compensation that enables the constancy of the *milieu intérieur* was further elaborated by Walter Cannon (1929) who proposed the term "homeostasis" to describe the mechanisms underlying this compensation. While homeostatic balance is partly accomplished by local effects at the cellular and tissue level, they also need to be coordinated between organs and within the animal as a whole. The role of master coordinator of homeostasis and of initiating, organizing and terminating motivational states is played by the hypothalamus.

1.1. The hypothalamus and the regulation of homeostasis

To accomplish homeostasis, the hypothalamus works as an integrator (Fig 1), receiving input from external sensory systems and internal feedback about the state of the body and then comparing them with a brain-encoded set point. If a discrepancy is detected in some variable (controlled system), the hypothalamus generates an error signal that activates the controlling elements, resulting in three types of responses: endocrine, autonomic and behavioural (Risold *et al.*, 1997; Swanson 2000; Swanson 2005). Through the combined action of these output pathways, the variable is brought back towards the set point; the comparison and corrective measures are continuous, to adjust to changing environmental conditions (Cannon, 1929).

Ingestive behaviour provides an illustration of how the homeostatic flow chart works. Thus, in starvation, the depletion of energy stores is reflected in the blood as falling levels of *e.g.* glucose, insulin and leptin. Specialized hypothalamic neurons in the Arcuate nucleus (Arc) (and, to some extent, in the brainstem) compare the relative energy availability encoded in these signals with a predetermined set point

(which is often described as corresponding to 70 kg body weight in the average grown man). If the perceived level of energy stores is below that of the set point, Arc neurons generate an error signal through output to the downstream neurons that constitute the controlling elements. Through projections to *e.g.* thyrotropic neuroendocrine neurons in the paraventricular nucleus, thyroid hormone output can be shut down to decrease energy expenditure. Through projections to *e.g.* preganglionic sympathetic neurons, glycogenolysis can be activated to make more glucose available for the tissues. Through projections to *e.g.* other subcortical nuclei, the pattern generator for seeking out and consuming food can be switched on. These three elements generate a change in the whole-body energy level (controlled system), which in time will be reflected in adjusted blood levels of *e.g.* glucose, leptin and insulin, whose correspondence to the set point will set the parameters for changes in the output of the controlling elements.

It should be mentioned that non-homeostatic factors can interfere with this process, causing deviations from the set point. One example is the hedonic value of food (encoded in *e.g.* taste) that facilitates consumption beyond what homeostatic satiety mechanisms would allow. Although not elaborated further in this thesis, such factors are likely central to the development of obesity (reviewed in Saper et al., 2002).

Of particular relevance to this thesis is the hypothalamic neuroendocrine response, which will now be described in detail. For in-depth discussions on the autonomic and behavioural responses, see Saper (2002) and Swanson (2000), respectively.

1.2. The neuroendocrine response

The neuroendocrine system of the hypothalamus is composed of cells that incorporate properties of both neurons (as they are composed by a cell soma, dendrites and axon and generate action potentials in response to electrical excitation) and of endocrine cells (as they secrete their signal substances - hormones - into the blood stream). Neuroendocrine cells can be classified into magno- and parvocellular cells.

The larger magnocellular cells are located in the paraventricular and supraoptic nuclei (Fig 2). Their axons project directly to the posterior pituitary gland where they secrete the peptide hormones, oxytocin (which acts primarily on uterine and mammary smooth muscle to cause parturition or milk ejection, respectively) or vasopressin (which regulates water homeostasis via the kidney) directly into the general circulation.

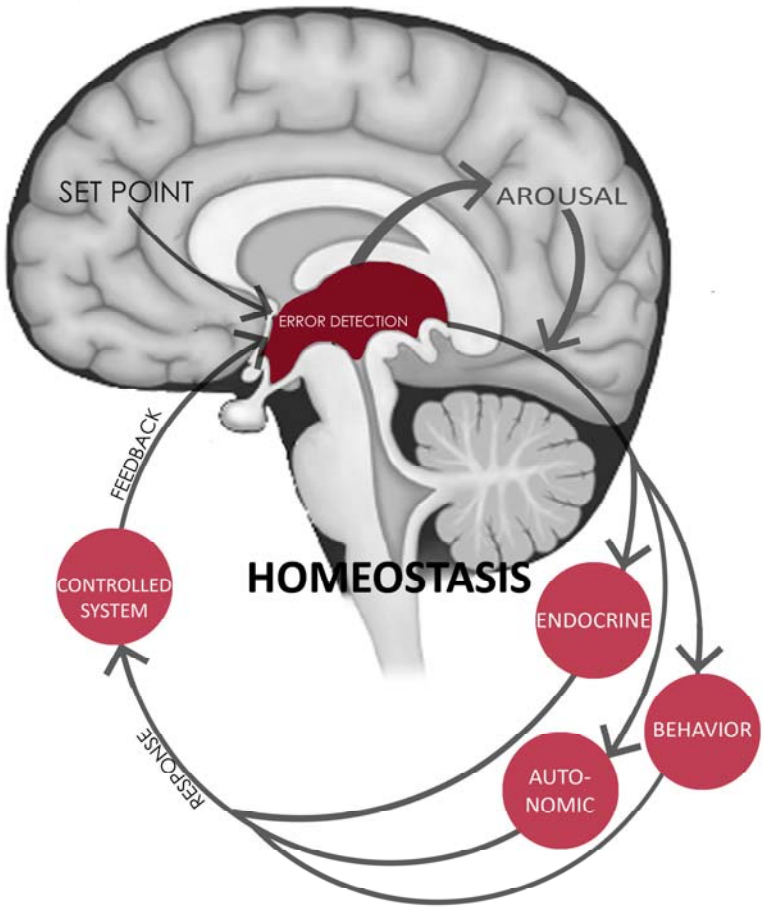

Figure 1. Homeostatic flow chart. To ensure that variables that determine the viability of the tissues, e.g. energy availability, salt concentration, blood pressure, are maintained at optimal levels, he hypothalamus compares feedback signals coming from the body with a previously encoded set point. If a discrepancy is detected, endocrine, autonomic and behavioural responses are generated to restore the variable towards the set point. This monitoring is continuous to cope with a wide spectrum of environmental conditions. Additional non-homeostatic system can affect the controlling systems; motivated states require arousal. In addition, the reward value of stimuli influences the magnitude of the behavioural response (Figure by Ximena Horjales).

The smaller parvocellular cells are located around the third ventricle in the paraventricular, arcuate and periventricular nuclei of the hypothalamus (Fig 2). These neurons secrete hypophysiotropic hormones to the first capillary bed of the hypophyseal portal vessels located in the median eminence on the ventral surface of the brain (Harris, 1948). In the anterior pituitary, the hypothalamic releasing and inhibiting factors are released from the blood in the second capillary bed of the portal vessels to act on their endocrine target cells. The hypothalamic-pituitary

system includes five principal hormonal axes (Fig 3), which can be characterized by the actions of their pituitary hormone: somatotrophs (synthesizing somatotropin or Growth Hormone), lactotrophs (Prolactin), corticotrophs (Adrenocorticotropic Hormone), gonadotrophs (Luteinizing Hormone and Follicle-Stimulating Hormone) and thyrotrophs (Thyroid-Stimulating Hormone) (reviewed in Nussey and Whitehead, 2001). In the present thesis, the regulation of the lactotrophic axis has been studied in detail.

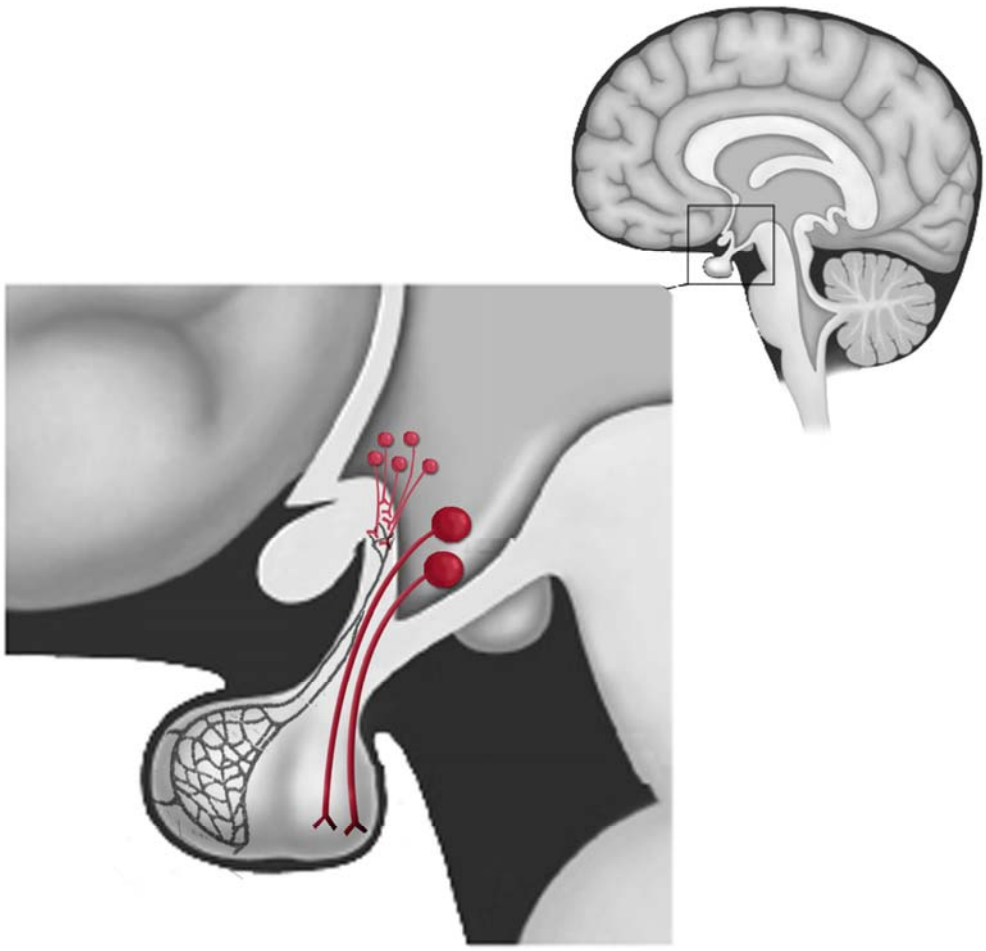

Fig 2: The hypothalamic neuroendocrine system. Magnocellular neurons (in dark red) projecting from the hypothalamus to the posterior pituitary releasing either vasopressin or oxytocin. The hypophysiotropic hormones from the parvocellular neurons (in pink) are brought to the anterior pituitary through a portal vessel system (Figure by Ximena Horjales).

1.2.1. Regulation of prolactin secretion

Prolactin is a pituitary polypeptide hormone which plays a central role in reproductive states by shifting the focus from procreation to caring for offspring after conception. This is accomplished by triggering lactation, but also by inhibiting sexual arousal and fertility (Freeman *et al*, 2000; Fitzgerald and Dinan, 2008).

Prolactin secretion, in contrast to the other pituitary hormones, is dominated by hypothalamic inhibition, rather than stimulation. Thus, under non lactating conditions, hormone release is suppressed via the dopamine D2-receptor of lactotrophs, from the parvocellular hypothalamic tuberoinfundibular dopamine (TIDA) neurons in the Arc (Fuxe, 1964). Tuberoinfundibular DA cells release dopamine through the median eminence to the blood stream, which transports it to the anterior pituitary gland (Hökfelt, 1967).

The dominant role of (dopaminergic) inhibition in the control of prolactin release is illustrated by the fact that while pituitary stalk transection causes a general drop in serum levels of pituitary hormones, the levels of prolactin spike (Gust et al., 1987). Attempts to identify prolactin-releasing modulators have met with varying success; *e.g.* the peptide originally named Prolactin-Releasing Peptide has later been shown to be only a weak stimulant of lactation (Swinnen, 2005). To date, the most potent stimulant of prolactin secretion appears to be the hypothalamic releasing factor Thyrotropin-Releasing Hormone (TRH; Jacobs et al., 1971; Schams, 1972; Lu et al., 1972; see Samson et al., 2003), introduced in detail below.

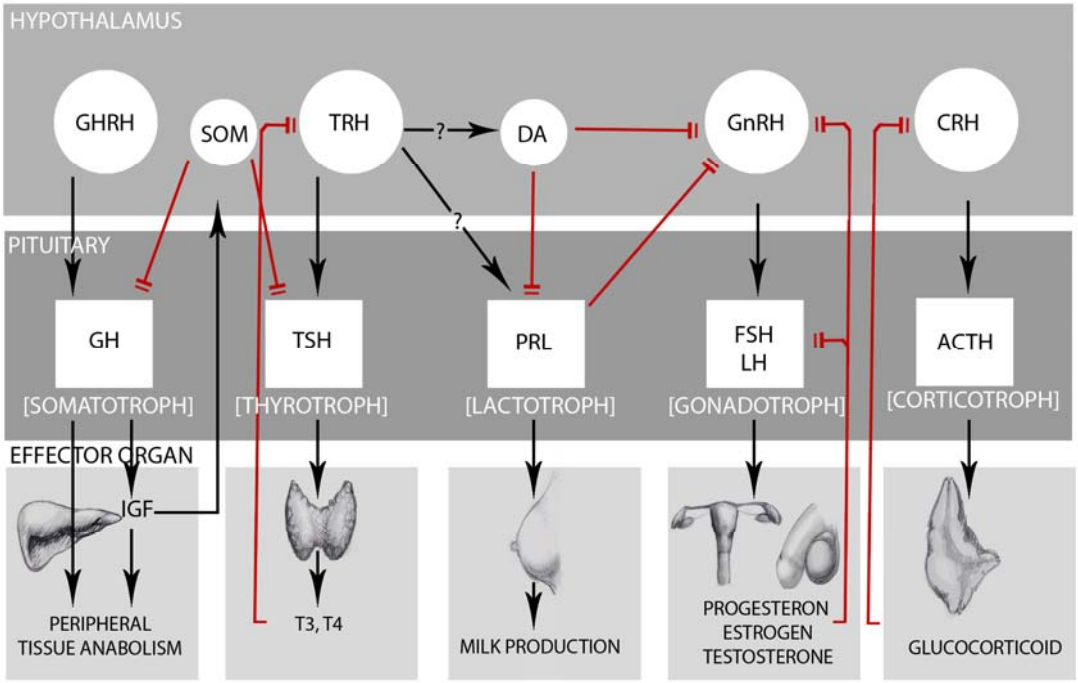

Fig 3: Hormonal axes regulated by parvocellular cells in the hypothalamus. GHRH, Growth Hormone- Releasing Hormone; GH, growth hormone, Som, somatostatin; TRH, Thyrotropin-Releasing Hormone; TSH, Thyroid- Stimulating Hormone; T3; Triiodothyronin; T4, Thyroxin; DA, dopamine; PRL, prolactin; GnRH, Gonadotropin- Releasing Hormone; FSH, Follicle- Stimulating Hormone; LH, Luteinizing Hormone; CRH, Corticotropin-Releasing Hormone; ACTH, Adrenocorticotropic Hormone (Figure by Ximena Horjales).

1.3. Arousal and motivational states

In 1951, Anand and Brobeck - in a classical lesion study - building on earlier observations by Hetherington and Ranson (1940) and Brooks and colleagues (1946), generated severe obesity and hyperphagia in rats by ventromedial hypothalamic lesions (likely by removal of the Arc). In contrast, electrolytic lesions in the lateral hypothalamic area (LHA; see Fig 4) generated anorexia, weight loss and, in some cases, death by starvation. In conjunction with other studies (Ranson, 1937; Nauta, 1946), these studies have been interpreted to mean that components of the LHA are crucial for providing the arousal component required for motivated behavior, including food intake.

Arousal can be briefly defined as a state of responsiveness to sensory stimulation or excitability. The arousal concept includes sleep-awake states, but also consciousness and alertness. Thus, being aroused implies that the animal is awake, but to be awake does not necessary means be aroused.

In the brain, wakefulness and arousal are represented as specific patterns of cortical activity (Steriade et al., 1993). In slow-wave sleep, the cortical network discharges in slow, synchronized oscillations, whereas in wakefulness the same neurons take on a more individual profile, firing action potentials in stochastic patterns to generate an internal representation of the external world. However, while the state of consciousness is a cortical phenomenon, it is the de- or hyperpolarizing influence of ascending subcortical projections that determine the functional configuration of the cortical network.

Classical cortically ascending pathways use norepinephrine, dopamine, serotonin, histamine and acetylcholine as transmitters (Jones and Beaudet, 1987; Jones and Webster, 1988; Jacobs and Azmitia, 1992; Lewis et al, 1998; Jones, 2002; Pfaff and Banavar, 2007). It is becoming increasingly evident, however, that in addition to amines and other classical transmitters, neuropeptide modulators can also exert a powerful influence over vigilance (Adamantidis and de Lecea, 2008). This work has identified the LHA - the heterogeneous area first implicated in the above-mentioned lesion studies - as a key site for peptidergic control of arousal.

1.3.1. Hypocretin/orexin regulation of arousal

In 1998, a new family of neuropeptides was independently and simultaneously discovered by two research teams (de Lecea et al., 1998; Sakurai et al., 1998). The hypocretin (Hcrt; a.k.a. orexin) peptides are, in the brain, expressed exclusively in the LHA and have been ascribed roles in energy metabolism and arousal phenomena.

The neurons producing these peptides - named orexin A and B (by Sakurai and colleagues; 1998) and hypocretin 1 and 2 (by de Lecea and colleagues; 1998) project

both to the hypothalamus itself and to a wide range of extra-hypothalamic brain nuclei that include key sites involved in arousal: septum, locus coeruleus, raphe nuclei, the pontine region, medullary reticular formation, cortex, amygdala and hippocampus (Peyron et al., 1998; Chen et al., 1999; Date et al., 1999). In the late 1990s, it was shown that narcolepsy (a condition characterized by sudden and uncontrollable episodes of sleep) could occur in dogs and mice as a result of genetic lack of Hcrt peptides or functional Hcrt receptor 2 (Lin et al., 1999; Chemelli et al., 1999). In addition, narcoleptic patients have severe deficits of Hcrt mRNA expression and peptide content (Peyron et al., 2000; Thannickal et al., 2000). These findings, along with the stimulant effects observed upon central Hcrt administration (Hagan et al., 1999) suggest that the Hcrt system is necessary for sleep regulation. Relatively little is, however, known about the upstream inputs that modulate Hcrt neuronal output.

1.4. Thyrotropin-Releasing Hormone

Similar to Hcrt, the peptide Thyrotropin- Releasing Hormone (TRH) was purified and characterized simultaneously and independently as L-pyroglutamyl-L-histidine-L-proline amide by two teams led by Guillemin (Burgus et al., 1969) and Schally (Boler et al., 1969) in 1969. Using purified extracts from hypothalami from millions of pigs or sheep, isolated TRH was found to release thyroid-stimulating hormone (TSH) from the pituitary, which, in turn, leads to activation of the thyroid gland.

The powerful endocrine effects of TRH captured much of the attention early after its discovery. Evidence for widespread brain expression (Hökfelt et al., 1975; Hökfelt et al., 1989) and other actions (Renaud et al., 1975) soon emerged, however. The wide range of TRH actions include the stimulation of prolactin release (Bowers et al, 1971; Lamberts *et al*., 1990), inhibition of food intake (Suzuki et al., 1982; Choi et al., 2002; Lechan and Fekete, 2006), stimulation of cholinergic and monoaminergic turnover (Taché et al., 1980; Jackson, 1982), depolarization of spinal motoneurons (Winokur *et al*., 1989), modulation of locomotor activity (Segal and Mandell, 1974; Miyamoto and Nagawa, 1977), regulation of respiration and modulation of pain perception (Yehuda, 1987; Nie and Liu, 1990), but also, and important for this thesis, TRH is a potent inducer of arousal.

1.4.1. Thyrotropin-Releasing Hormone and arousal

The tri-peptide has well-documented analeptic effects (Breese et al; 1975) illustrated by its antagonistic action of the sedative actions of depressant drugs like ethanol, barbiturates and benzodiazepines (Prange et al., 1974; Jackson, 1982; 1983; Burt and Sharif, 1984). Thyrotropin-Releasing Hormone also induces locomotor activity (Segal and Mandell, 1974; Andrews and Sahgal, 1983; Ushijima et al., 1984), mediated by dopamine release into the nucleus accumbens (Heal and Green, 1979; Masserano and King, 1981). In narcoleptic dogs, which carry a mutation in the Hcrt

2 receptor, TRH and TRH analogs can increase wakefulness, decrease sleep and reduce cataplexy (Nishino et al., 1997). In clinical and experimental studies, it has been suggested that epileptic seizures, which, like sleep involves large-scale synchronization of the cortical network, can be inhibited by TRH (Nemeroff et al., 1975; Matsumoto *et al.*, 1987; Momiyama *et al.*, 1996). The mechanisms whereby TRH influences arousal are, however, not well understood, nor the CNS sites where this regulation occurs

1.4.2.Thyrotropin-Releasing Hormone and prolactin release

Thyrotropin- Releasing Hormone has also been described as a prolactin-releasing agent. This idea was proposed when administration of exogenous TRH was found to result in elevated serum levels of prolactin in humans and animals (Bowers et al, 1971; Jacobs et al., 1971; Lamberts *et al.*, 1990). Although it is not well understood how TRH regulates prolactin release, it has generally been assumed that the tri-peptide acts directly on pituitary lactotrophs, in concert with TRH action on thyrotropic TSH-secreting cells. Indeed, such actions have been shown on isolated pituitary preparations (Smith and Convey, 1975). The biological importance of such mechanisms is, however, not established, and complicated by the fact that only a minority of lactotrophs express TRH receptors (Burt and Synder, 1975; Konaka et al., 1997), and that serum levels of TRH and prolactin do not show a consistent relationship (Gautvik et al., 1973).

Thus, TRH has a wide range of actions in the regulation of homeostatic processes. The purpose of this thesis is to analyze some elements of how TRH is implicated in the regulation of motivated states by its interaction in different stages on the homeostatic flow chart, with particular emphasis on anatomical aspects.

2. SPECIFIC AIMS

The overall objective of this thesis is to increase our understanding of the role played by TRH in some different aspects of homeostasis and motivation. The work includes three studies with the following specific aims:

- To investigate the anatomical and physiological substrate for potential interactions between TRH and the Hcrt system (paper I).

- To histochemically characterize a population of cells located in the LHA that expresses TRH (paper II).

- To identify the network behaviour of the prolactin regulating TIDA population and evaluate the possibility of direct interactions between TRH and these neurons (paper III).

3. METHODOLOGICAL CONSIDERATIONS

The studies in this thesis are based on several methods:

- Single and double immunofluorescence staining
- Confocal laser scanning microscopy
- *In situ* hybridization
- Reconstruction of dye-filled cells
- Whole cell patch-clamp recording

Specific protocols are detailed in the corresponding papers. Here I want to highlight some of the advantages and limitations of these methods.

3.1. Immunofluorescence staining

Immunofluorescence is used to anatomically localize molecules - most commonly peptides and proteins - in tissues and cells through the binding with specific antibodies conjugated with a fluorescent dye (papers I, II and III; Coons and Kaplan, 1950). This is a powerful method that has contributed significantly to the paradigm shift in our understanding of the connectivity and pharmacology of the brain in the past four decades. The usefulness of immunofluorescence, however, is directly dependent on the sensitivity and specificity of the antisera and antibodies used (Saper, 2009).

Antisera can be produced by immunizing animals (*e.g.* rabbit, goat and chicken) with a purified antigen, often along with stimulants of the immune response. After some weeks, the animal is bled and the resulting serum, if successful, will contain antibodies against the injected antigen. While it is hoped that the serum will primarily contain this specific antibody, due to the generalized immune response and the unselected plasma cell population in the immunized animal, the serum will most likely include a mix of antibodies. Such antiserum is thus referred to as 'polyclonal' (Ramos-Vara, 2005). Monoclonal antibodies (Köhler and Milstein, 1976), on the other hand, are produced by injecting mice with immunogen, and then selecting specific antibody-producing B lymphocytes to produce hybridomas (reviewed in Ramos-Vara, 2005). Because all antibodies originate from a single clone, monoclonal antibodies offer greater specificity.

Neural tissue offers particular sensitivity challenges for immunofluorescence. Often, neuropeptides are synthesized in the cell soma, rapidly transported through the axon and released in the synapse. The high turnover rate of many peptides can thus complicate the visualization of the molecule in the soma, and make it difficult to identify the origin of terminal plexa.

To overcome this problem, cell body levels of peptide can be experimentally increased by intracerebroventricular injection of colchicine prior to perfusion. Colchicine - discovered in 1820 by Pelletier and Caventon (1820) – is a natural toxic secondary metabolite extracted from the plant, *Colchicum autumnale*, which inhibits microtubule polymerization by binding to tubulin, which, in turn, blocks microtubule-dependent processes such as mitosis and axo-plasmatic transport (Eigsti and Dustin, 1938; Hanson and Edström, 1978). Due to the inhibition of the retrograde and anterograde axonal transport (Hökfelt and Dahlström, 1971; Hanson and Edström, 1978; Grafstein and Forman, 1980; Ochs, 1982) the neurotransmitter is not transported to the terminal and accumulates in the cell soma.

Colchicine injection is a commonly used method that has revealed numerous previously undetected cell body populations. Treatment with this compound has, however, additional effects that need to be considered. For instance, different neuronal populations have different susceptibilities to the neurotoxic effect of colchicine (Goldschmidt and Steward, 1982). Also, the cytoplasm of the cell is composed of microtubules (Eigsti and Dustin, 1938; Goldschmidt and Steward, 1980; Goldschmidt and Steward, 1982). Caution is therefore warranted when cell morphology is studied in animals treated with colchicine. A final consideration is the potential effect of colchicine on transcription, since different mRNA species in different brain regions can sometimes be either increased or decreased (or not affected) by colchicine treatment (Cortés et al., 1990).

While colchicine can affect the subcellular distribution of peptides, another important sensitivity issue is the overall preservation of antigen within the cell. In general, fixation of tissues is necessary to preserve cellular components, prevent autolysis and displacement of cell constituents and stabilize cellular materials (reviewed in Ramos-Vera, 2005). Formaldehyde is the fixative most commonly used for histology and immunofluorescence studies through its ability to crosslink proteins and thus stabilize tissue.

Low-molecular-weight molecules, such as TRH, require a more aggressive fixation to remain in the tissue during processing. The addition of glutaraldehyde (Webster and Collins, 1964) or acrolein (Ceccarelli and Pensa, 1968), due to their particular potency in the formation of cross-linkage (Hopwood, 1967; Jameela and Jayakrishnan, 1995), has been introduced to increase visualization of TRH in immunohistochemical studies (Lechan and Jackson, 1982; Nishiyama et al., 1985; Tsuruo et al., 1987; Hökfelt et al., 1989). Fixation with glutaraldehyde (as with aldehydes in general) results in relatively high levels of background fluorescence, but this artifact can be mitigated by treating the tissue $NaBH_4$ (Hökfelt et al., 1989: Clancy and Cauller, 1998).

Sensitivity can be further improved by the Tyramide Signal Amplification (TSA) protocol (Bobrow et al., 1989). TSA is an enzyme-mediated detection method that uses the catalytic activity of horseradish peroxidase (HRP) to generate high density labeling of a molecule in three steps: binding of a primary antibody to the molecule of interest; binding of a secondary antibody conjugated to HRP to the first

antibody; and activation of a tyramide conjugated to fluorescent dye by HRP, which, through tyramide interacting with protein residue in the tissue, results in the incorporation of fluorescence around the molecule of interest.

The TSA protocol enables substantially increased sensitivity (Bobrow et al., 1989; Adams, 1992; Berghorn et al., 1994; Merz et al., 1995), while using ten-fold lower concentrations of primary antisera. The size of the molecular complex involved in detection does, however, need to be taken into account when analyzing staining patterns, as the fluorescent marker may be deposited in a radius around the epitope, sometimes giving the staining a more 'fuzzy' appearance than with conventional indirect immunofluorescence. Thus, absolute *sizes* of epitope-containing structures may thus be artificially large. The increased sensitivity of the method, on the other hand, and thus ability to detect epitope-containing structures (e.g. cell bodies, terminals), is improved, resulting in more accurate absolute *numbers*.

3.2.*In situ* hybridization.

An additional method to visualize cells in tissue based on their neurochemical repertoire is *in situ* hybridization (Pardue and Gall, 1969). In this technique, specific mRNAs are detected using complementary nucleic acids conjugated to a marker molecule (Young III and Mezey, 2003). Two major probe types exist. Oligoprobes (usually 40-50 base pairs in length) are produced synthetically by automated chemical synthesis. These probes are highly stable and easy to produce. The combination of several probes directed against different stretches of the target mRNA can often increase sensitivity (Lewis et al., 1985; Emson, 1989). Riboprobes (RNA probes) are produced by a more labour-intensive process; full-length transcription of a complementary copy of the targeted mRNA. This yields, however, very thermo-stable hybrids, which allow for more stringent washing conditions, providing better signal-to-noise ratio (Lewis et al., 1985; Emson, 1989).

To detect the nucleic acid hybrids, radioactive or non-radioactive (enzymatic) methods can be used. Radioactive *in situ* hybridization uses probes commonly labeled with either ^{33}P or ^{35}S and detected with photographic emulsion. Non-radioactive markers include enzyme-based techniques (*e.g.* HRP or alkaline phophsatase) resulting in coloured precipitates or fluorescence (Emson, 1989).

In situ hybridization offers, in a sense, a greater versatility than immunochemistry, since our knowledge of the genetic code now allows for the construction of probes against virtually any gene, whereas the production of antibodies involves biological processes beyond strict experimental control and in the absence of an established 'protein code'. Especially radioactive *in situ* hybridization also shows a better linear relationship between tissue levels of the molecules of interest (mRNA) and detected signal than immunofluorescence and is thus more reliable for quantitative purposes (Feldmann and Bernuau, 1989). On the other hand, as *in situ* hybridization only visualizes mRNA it is, at best, an indirect method to study proteins.

3.3. Dye-filled cell reconstruction

Cellular morphology is highly related to the functional properties of neurons and can be studied by reconstruction of dye-filled cells. This method is particularly powerful when applied on neurons that have been subjected to intracellular or whole-cell electrophysiological recording (see below).

During patch clamp or intracellular recording with traditional 'sharp' electrodes the cell can be filled with a marker such as neurobiotin which diffuses easily into the cell. After recording, the tissue is fixed and biotin detected through binding with avidin conjugated with a fluorescent dye, or through enzyme-based techniques. (Directly fluorescent compounds such as Alexa dyes or Lucifer Yellow can also be introduced to the cell during recording). Under optimal conditions, this allows visualization of the full dendritic tree, including spines (if present) and sometimes also the axonal arborization. Through specialized software, the three-dimensional shape of the cell can be reconstructed. In addition, this method, combined with immunofluorescence, can determine the transmitter identity of the cell and also visualize the presence of transmitter-identified terminals upon the recorded cell. Thus, the anatomical and functional properties can be studied in the one and same cell. The main limitations of the technique lie in its low yield – only one cell can be studied at a time and cell staining and reconstruction are labor-intensive – and the fact that tissue preservation must be optimized primarily for electrophysiology rather than histochemistry.

3.4. Whole-cell patch clamp recording

The operations of neuronal networks are the product of both connectivity and the electrical properties of the connected neurons. Thus, a powerful complement to neuroanatomy (which is the focus of this thesis) in brain circuit analysis is electrophysiology. To understand the physiology of individual cells, whole-cell patch clamp (Neher and Sakmann, 1976; Hamill et al., 1981) allows the study of both passive and active membrane properties and the reaction of a cell to messenger molecules. This methodology was vital in determining the behavior of hypothalamic neurons and their response to TRH in papers I (Gonzalez et al., 2009) and III (Lyons et al., 2010).

In patch clamp, a high-resistance seal is formed between a recording glass micropipette electrode and the cell membrane. For the whole-cell configuration, the membrane is then ruptured, allowing high access to the cell and electrical control of its interior. Through the high sensitivity of the technique, very small currents and potentials over the membrane can be detected and quantified. The recent development of transgenically engineered mice that express fluorescent markers (*e.g.* Green Fluorescent Protein, GFP; Yeh et al., 1995; Okabe et al., 1997) under the control of a chosen promoter allows the targeted recording of specific cell populations, also in the cellularly heterogenic hypothalamus (Paper I).

The limitations of the patch clamp technique include the risk of dialyzing the cell's interior through the large pore formed from the membrane rupture. Thus, the intracellular levels of ions and messenger molecules may be changed, resulting in artificial electrical properties and sometimes cell death. It is therefore important that the intracellular recording solution is as similar to the actual composition of the cellular interior as possible. It should also be kept in mind that, although clamp conditions are improved compared to traditional intracellular techniques, a full control of the voltage and currents of the most distant parts of the dendrites - which compose a large part of total cell volume - is unlikely to be achieved.

4. RESULTS

4.1. Stimulation of orexin/hypocretin neurons by Thyrotropin-Releasing Hormone (paper I)

Paper I describes experiments exploring a potential anatomical and physiological relationship between the arousal-promoter peptides Hcrt and TRH.

Using double-label immunofluorescence and confocal microscopy on mouse hypothalamus, we were able to visualize TRH-immunoreactive (-ir) terminals in close apposition on cell bodies and proximal dendrites of about 30% of the Hcrt-ir neurons in the LHA. It was noted, however, that the Hcrt-ir cell body population and the TRH-ir terminal-field only displayed partial overlap in the LHA.

The potential functional implications of this innervation were then explored by electrophysiology. Hypocretin-expressing neurons were identified by transgenic expression of eGFP. Whole-cell patch clamp recordings were performed to identify the effect of TRH on passive and active membrane properties of Hcrt cells.

Bath application of TRH (250 nM) resulted in a reversible membrane depolarization and increased action potential discharge in Hcrt cells. This effect was dose dependent, and could not be observed using a biologically inactive TRH analogue. The depolarizing effect of TRH on Hcrt cells persisted during blockade of action potential discharge through the Na^+ channel antagonist, tetrodoxin (TTX), suggesting a direct, post-synaptic action of the peptide. This conclusion was further supported by the demonstration that the TRH-induced depolarization and stimulation of firing also persisted in a '*low* Ca^{2+} / *high* Mg^{2+}' extracellular solution.

The conductance(s) mediating the depolarization was next investigated through manipulation of intra- and extra-cellular ion concentrations. A chloride component was excluded since no difference in depolarizing response to TRH application was seen when using either potassium chloride or potassium gluconate-based intracellular pipette solutions.

In contrast, reducing the Na^+ concentration in the extracellular solution (by substitution of Na^+ with N-methyl-D-glucamine) altered the TRH-evoked depolarization by 80%, suggesting the involvement of a substantial Na^+ component. The exact mechanism of Na^+ influx could not be determined, but did not appear to be through either the hyperpolarization-activated mixed cation current I_h or the Na^+/Ca^{2+} exchanger, since the antagonists ZD7288 and KB-R7943 failed to suppress the TRH-induced depolarization. The calculated reversal potential, however, for this depolarization was ca. -25 mV, typical of a mixed cation current.

Using a low K^+ intracellular pipette solution, the net TRH-induced membrane conductance was reduced, suggesting that the TRH effect on Hcrt cells involves K^+ components, in addition to the previously identified Na^+ contribution.

4.2. Lateral Hypothalamic Thyrotropin-Releasing Hormone (TRH) Neurons: Distribution and Relationship to Histochemically Defined Cell Populations in the Rodent (paper II)

In Paper II we describe and histochemically characterize a population of cells located in the LHA that expresses TRH.

By radioactive *in situ* hybridization and immunofluorescence staining a population of cells expressing TRH was visualized in the LHA of mice and rats, respectively. The TRH-ir cells in the LHA occupy a large part of the ventral hypothalamus in almost its entire rostrocaudal extension. Thus, rostrally, a small cluster of TRH-ir cell bodies are first seen in the ventral part of the ventrolateral preoptic nucleus, co-extensive with the suprachiasmatic nucleus (Bregma -0,5mm).

Further caudally, the number of TRH-ir cells increases, spreading dorsolaterally to occupy the lateral part of the preoptic area and the peduncular part of the lateral hypothalamus. At this level (~Bregma –1,40mm) a less intensely immunofluorescent subpopulation of TRH is also observed in the LHA. Co-extensive with the neuroendocrine TRH-ir cells in the paraventricular nucleus (PVH), the number of TRH-ir cell bodies in the LHA increases further. These two TRH-ir populations connect via the fornix, forming a ring-like structure around the central part of the anterior hypothalamic area. Proceeding caudally, the population of TRH-ir cell bodies concentrates in the tuberal region of the lateral hypothalamus (TuLHA). While the ventromedial hypothalamic nucleus (VMH) contains no TRH-ir neurons, several cell bodies and a dense terminal plexus occupy the dorsomedial hypothalamic nucleus (DMH). These cells form a diagonal (interrupted by the fornix) with the TRH-ir cells in the TuLHA. Caudal of this level, the number of ir cells diminishes and disappear at Bregma~ –3,60mm.

The TRH-ir cells in the LHA (15-20 μm diameter) present two distinct shapes: a fusiform type cell, extending two primary dendrites, and a multipolar type, presenting between three and four primary dendrites. These two types of TRH-ir cells intermingle through the LHA.

Double immunofluorescence staining with different neuronal markers was performed in order to study the potential co-existence with TRH-ir LHA cells. No co-existence was found between these cells and neurons immunoreactive to Cocaine- and Amphetamine-Regulated Transcript (CART), Melanin Concentrating Hormone (MCH), Hcrt, Neurotensin, Somatostatin, neuronal Nitric Oxide Synthase, Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) and Tyrosine Hydroxylase.

By double immunofluorescence staining a unique group of TRH-ir cells in LHA- specifically located in the juxtaparaventricular region- was found co-expressed with urocortin3 and enkephalin, suggesting the existence of subpopulation of cells. To evaluate the possibility of innervation from the Arc to the TRH-ir cells, double immunofluorescence was performed for TRH and Agouti Gene-Related Peptide (AGRP) and α-Melanocyte- Stimulating Hormone. Terminals immunoreactive for these neuronal markers were observed in close apposition to soma and proximal dendrites in TRH-ir cells.

4.3. Synchronized Network Oscillations in Rat Tuberoinfundibular Dopamine Neurons: Switch to Tonic Discharge by Thyrotropin-Releasing Hormone (paper III)

In paper III, the electrical behavior of tuberoinfundibular dopamine (TIDA) neurons and its possible modulation by TRH was studied.

Using whole-cell patch clamp recordings, a population of cells in the Arc was observed to discharge in highly regular oscillations, characterized by periods of hyperpolarization (DOWN) alternating with periods of depolarization (UP) and action potential firing.

Oscillator cells were encountered exclusively in the dorsal part of the Arc. By reconstruction of recorded dye-filled oscillator cells, their morphological characteristics were observed to include a cell body of 20-30 μm diameter and extend between three and five primary dendrites, extending both ventrally and dorsally along the ventricular wall.

To identify the neurochemical phenotype of the oscillator cells, immunofluorescence was performed on recorded and reconstructed dye-filled cells. All recovered oscillator cells were found to contain tyrosine hydroxylase (TH)-like immunoreactivity (-LI), while none of the recovered non-oscillator cells in the Arc were TH-ir. As TH is a histochemical marker for monoaminergic neurons, this finding strongly implicates TIDA cells as the oscillator population. This conclusion was further strengthened by the positive uptake of Evans Blue in oscillator cells; Evans Blue is a fluorescent dye that, when injected intravenously, is taken up only by neurons located outside the blood-brain barrier action (Weiss and Cobbett, 1992).

Next, the network properties of TIDA neurons were investigated. Simultaneous dual recording from oscillator cells demonstrated an exceptional synchrony between neurons. Surprisingly, the oscillation does not appear to be mediated via conventional chemical synapses since it persisted under voltage clamp conditions, as well as when synaptic transmission was blocked by bathing the preparation in a '*low* Ca^{2+}/*high* Mg^{2+}' solution or by application of antagonists of fast (ionotropic) glutamate and GABA transmission. In contrast, application of gap junction

blockers (carbenoxolone and 18β-glycyrrhetinic acid) abolished the oscillation, indicating that it is transmitted between cells through electrotonic coupling.

As described above TRH is a potent prolactin-releasing factor (see Samson et al., 2003), an effect that has been assumed to take place in the pituitary through TRH released from parvocellular neurons in the PVH. Here, we explored the possibility that such effects may be mediated via hypothalamic actions. Immunofluorescence staining revealed that TH-ir (TIDA) cells in Arc are densely innervated by TRH-ir terminals on both soma and proximal dendrites. In electrophysiological recordings of TIDA cells, the application of TRH (1 μM) resulted in reversible depolarization and a switch from phasic to tonic discharge in all neurons recorded was observed. The TRH-induced depolarization included a direct post-synaptic (TTX-insensitive) component. In addition, modulation of synaptic input to TIDA cells was also observed. TRH induced an increase in the frequency and amplitude of excitatory postsynaptic currents (EPSCs). A small increase in the frequency of inhibitory PSCs was also observed, but the amplitude of these events was reduced.

5. DISCUSSION

Thyrotropin- Releasing Hormone was the first hypothalamic releasing factor to be purified and characterized, and not surprisingly, initial work focused on its role as master regulator of the thyroid axis (Burgus et al., 1969; Boler et al., 1969). Subsequent studies identified several additional TRHergic neuronal populations (in addition to the neuroendocrine paraventricular cells; Brownstein et al., 1974; Hökfelt et al., 1975) as well as functional effects within the CNS (Plotnikoff et al., 1972; Dyer and Dyball., 1974; Renaud et al., 1975). The peptide has thus been implicated in the regulation of circadian rhythms (Kruse, 1977; Beale at al., 1977), arousal (Breese et al., 1975; Andry and Horita, 1977; Stanton et al., 1980; Yamamura et al., 1991), seizure activity (Matsuishi, 1983), autonomic function (Taché et al., 1980; Tonoue et al., 1982; Horita and Carino, 1982), energy metabolism (Suzuki et al., 1982), body temperature (Metcalf, 1974; Lin et al., 1980), mood (Prange and Wilson, 1972; Prange et al., 1972; Coppen et al., 1974; Betts et al., 1976; Kastin et al., 1972) and pain perception (Yehuda, 1987; Nie and Liu, 1990), In this thesis, the role of TRH in relation to some aspects of CNS control of homeostasis has been studied.

5.1. Thyrotropin-Releasing Hormone in the regulation of arousal

Earlier studies have shown that TRH can promote arousal in experimental animals and humans, both during natural sleep and drug-induced narcosis (Prange et al., 1974; Breese et al., 1975; Stanton et al., 1980; 1981; Chihara et al., 1984). The mechanism(s) and anatomical substrate(s) underlying these analeptic effects have, however, not been conclusively determined. Recent studies have identified several potential candidates.

Given the prominent role of Hcrt in sustaining normal wakefulness, we were interested to find out if Hcrt cells are targets for TRH. Notably, dogs suffering from narcolepsy due to a mutation in the Hcrt 2 receptor gene (Lin et al., 1999) can be successfully treated with TRH or TRH analogs, resulting in a reduction of cataplexy and daytime sleep (Nishino et al., 1997; Riehl et al., 2000).

The results in paper I indicate direct anatomical and physiological interactions between TRH and Hcrt cells. Histochemically, TRH-ir terminals were seen on a large part of the Hcrt-ir cells. Physiologically, Hcrt cells responded to TRH application by depolarization mediated through activation of cation currents.

Almost all (27/28) Hcrt cells recorded were directly responsive to TRH. In contrast, immunofluorescence revealed TRH-ir terminals in close apposition to only about a third of the Hcrt-ir cells in the LHA. The lower proportion of TRH-contacted cells may be due to the inherent limitations of histological methods (see *Methodological Considerations*) and the fact that TH immunofluorescence only allows visualization of cell somata and proximal dendrites; TRH terminals innervating the distal dendrites of Hcrt neurons would not be detected with the current protocol. Since

neuropeptides, like TRH, signal partly via extrasynaptic mechanisms and 'volume transmission' (see Agnati et al., 2010), direct synaptic contact may, however, not be a requirement for TRH to act on Hcrt neurons.

A concurrent study by Hara and colleagues (2009) yielded similar functional results, demonstrating a depolarizing effect of TRH on Hcrt cells in the lateral hypothalamus. Hara *et al.* (2009) also analyzed the behavioural effect of TRH injection into the LHA. Thus, local injection of TRH into the LHA increased locomotor activity in wild type mice but not in orexin/ataxin-3 mice (a transgenic strain in which the Hcrt cells degenerate postnatally), suggesting that the ambulatory TRH effect is mediated by Hcrt cells.

Together, these two studies identify TRH as a potential modulator of Hcrt output in the control of arousal. Future studies will be necessary to determine to what extent this is a pharmacological effect (which may still be of therapeutic relevance), or if endogenous release of TRH contributes to Hcrt-dependent maintenance of arousal in the intact animal and man. It also remains to identify, through tract tracing methods, the source of TRH terminals on Hcrt cell bodies. Possible candidates include the cells located in the LHA (see paper II) but also those in the DMH (Chou et al., 2003).

Our findings and those by Hara *et al.* (2009) expand the list of potential sites of action for TRH-induced arousal. Recent research has offered several such anatomical loci. Histaminergic tuberomammillary neurons play an important role in sleep-waking regulation. In freely moving animals, histaminergic neurons discharge tonically during waking states (Steininger et al., 1999), and increased histaminergic transmission promotes wakefulness (Lin et al., 1988). TRH was recently shown to be able to depolarize histaminergic neurons (Parmentier et al. 2009). Noradrenergic neurons in the locus coeruleus discharge during wakefulness (Aston-Jones et al., 1991) and direct stimulation of the locus coeruleus desynchronizes cortical EEG (Berridge and Foote, 1991). Ishibashi et al. (2009) have shown that these cells can be excited by TRH. In addition to these ascending arousal-promoting systems, in the thalamus, whose intimate connections with the cortical mantle forms the very corticothalamic loop where state changes result in sensory awareness (wakefulness) or disconnect from the outside world (sleep), application of TRH causes depolarization of neurons (Broberger and McCormick, 2005). This action, mediated through attenuation of K^+ leak currents, switches the network from rhythmic oscillations typical of slow-wave sleep to the tonic firing conducive to sensory processing in the awake state.

These recently uncovered mechanisms whereby TRH could regulate arousal are not mutually exclusively; indeed, they could all act in parallel, a scenario of obvious attractiveness for sleep disorder therapy. At the end of the day, any arousal-promoting agent must accomplish, directly or after relay via ascending systems, state changes in functional cortical network configuration (reviewed in Aston-Jones, 2005; Pfaff and Banavar, 2007). Thyrotropin-Releasing Hormone appears to be anatomically positioned (and functionally capable) of achieving both.

5.2. Thyrotropin-Releasing Hormone: a possible connection between arousal and energy metabolism?

In paper II we explore the anatomical boundaries and transmitter properties of one of the potential sources of TRH terminals in the hypothalamus, namely a group of neurons in the LHA itself. This population of TRH cells extends widely through the hypothalamus (from Bregma -0,5 to -3,60 mm) and appears, from our observations, to be more numerous than the well described LHA Hcrt population (Peyron et al., 1998), and possibly on par with the large MCH population (Knigge et al., 1996). The lack of co-existence of these cells with several other prominently expressed hypothalamic transmitter markers suggests a unique characteristic of this substantial cellular population.

The LHA projects *i.a.* to the brain stem (Leibowitz and Brown, 1980), septum (Guillery, 1957), cortex (Saper et al., 1979), amygdala (Ono et al., 1985) and hippocampus (Köhler et al, 1984). The LHA is also known to be involved in feeding behaviour (Anand and Brobeck, 1951) and other aspects of the regulation of energy metabolism and the autonomic system (see Bernardis and Bellinger, 1993; Swanson, 2000; Thompson and Swanson, 2003). Lesion studies (Ranson, 1937; Nauta, 1946) established that the integrity of the LHA is required for wakefulness, suggesting a role of the LHA in the regulation of arousal. Also, cells located in the LHA expressing Hcrt (Peyron et al., 1988; Sutcliffe and de Lecea, 2000) and MCH (Bittencourt et al., 1992) exert prominent effects on vigilance (Chemelli et al., 1999; Lin et al., 1999; Verret et al., 2003). Thus, several converging lines of evidence suggest that the LHA, and peptides therein, are key players in the global control of consciousness.

Accordingly, the arousal-promoting actions of TRH (summarized in *Introduction*) and the its presence in the LHA raise the possibility of a possible role of the TRH LHA in arousal regulation, specifically – perhaps - in the regulation of arousal required for goal-oriented behaviour. This speculation is based on the innervations patterns we observed in our study.

The TRH- expressing cells in LHA received dense innervation of from both AGRP- and α-MSH-ir terminals. These peptides are produced by the Arc (Watson et al., 1977; Shutter et al., 1997; Broberger et al., 1998) and derive from neurons that act as sensors for the metabolic state of the body but play opposite roles in energy metabolism with AGRP/NPY neurons promoting food intake and decreasing energy expenditure and the α-MSH (melanocortin) neurons promoting catabolic actions (reviewed in Broberger, 2005). Thus, the Arc-LHA pathway may be a means of recruiting arousal to motivated behaviour in periods of starvation. Indeed, Anand and Brobeck (1951; see Fig 4) observed that the hyperphagia and obesity generated by lesions of the ventromedial hypothalamus (likely the Arc), could be abolished by subsequent destruction of the LHA, resulting in weight loss and, in extreme cases, death by starvation. This finding provided early evidence that the

LHA lies downstream of the Arc and that the connection between the two is required for metabolic signals to be translated into an appropriate behavioural response.

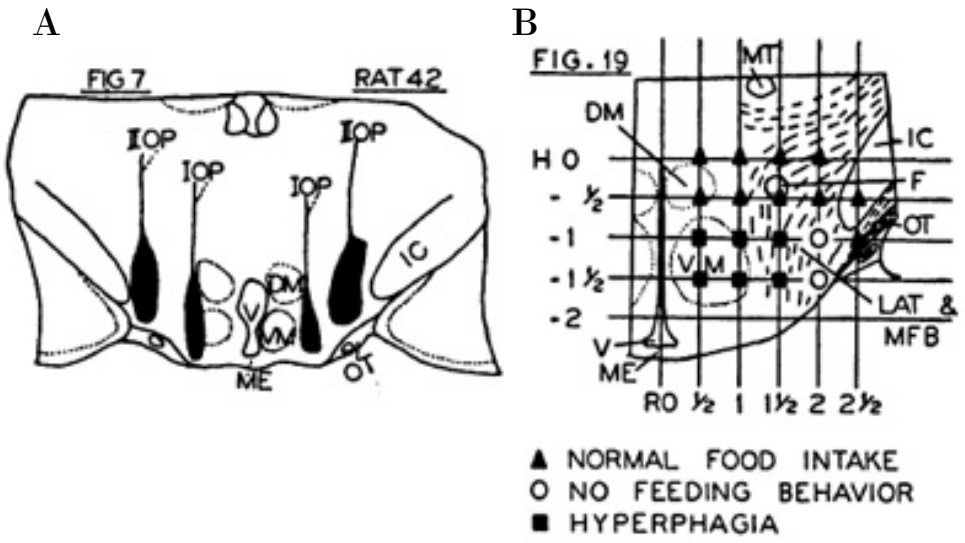

Figure 4. Lateral hypothalamic lesions disrupt food intake. Over half a century ago, Anand and Brobeck (1951) demonstrated that anatomically distinct lesions of the rat hypothalamus affect feeding behaviour in specific ways. A) In this animal (brain shown in coronal cross-section), bilateral lesions of the ventromedial hypothalamus (IOp) caused hyperphagia and obesity. Subsequent lateral lesions (IIOp) completely abolished food intake. B) Summary diagram from several experiments show rat hypothalamus in coronal section with Horsley-Clarke coordinates superimposed. Behvioural effect as a function of location is indicated by filled triangles (unaltered feeding behavior), open circles (aphagia) or filled squares (increased feeding). Note the abolishment of food intake caused by destruction of the lateral-most aspects of the lateral hypothalamus. Reproduced with permission from Anand and Brobeck, 1951. DM, dorsomedial nucleus; IC, inferior colliculus; F, fornix; LAT, lateral hypothalamus; ME, median eminence; MFB, median forebrain bundle ; MT, mammillothalamic tract; OT, optic chiasm; V, third ventricle; VM, ventromedial nucleus.

5.3. Thyrotropin-Releasing Hormone in the regulation of endocrine response

Prolactin plays a central role in reproduction as the signal that shifts focus from procreation to the caring for offspring. This is accomplished by stimulation of lactation, attenuation of sexual libido and inhibition of fertility (reviewed in Freeman et al., 2000; Fitzgerald and Dinan, 2008). In contrast to other pituitary hormones, prolactin is primarily regulated by hypothalamic inhibition, rather than stimulation. Thus, under non lactating periods, TIDA neurons provide suppression of prolactin release. Despite first having been described almost half a century ago (Fuxe, 1964), remarkably little is known about the TIDA neuronal properties that underlie this inhibition.

In paper III, we present the first recordings of the electrical behavior of identified TIDA cells which reveal a highly synchronized rhythmic oscillatory firing pattern.

This behavior persists in the absence of conventional (chemical) synaptic communication. Rather, inter-neuronal phase-locking appears to be accomplished via electrotonic coupling, since the oscillation was abolished by gap junction blockade. In our reconstructions of oscillating Arc cells, not only did we observe a consistent immunoreactivity for the TIDA marker Tyrosine Hydroxylase (TH), but we could also see that TIDA cells as a rule extend their dendrites within the region occupied by the cluster of TH-positive cells. This finding provides anatomical support for the concept of dense interneuronal communication within this cell network. We hypothesize that gap junction-dependent coordination of bursting inside the TIDA population serves to maximize population output of hormone into the portal circulation, ensuring the blockade of lactotroph exocytosis. Indeed, in *magnocellular* neuroendocrine neurons (Dutton and Dyball, 1979), as well as mesencephalic dopamine systems (Gonon, 1988) phasic firing has proven a substantially more efficient means of transmitter release than tonic discharge.

Notably, the endocrine role of TRH is not reduced to the regulation of the thyroid hormones; the tri-peptide can also influence other hypothalamic endocrine responses. One prominent example of this is the actions of TRH in prolactin release: i.v. administration of TRH results in a prominent surge in plasma prolactin (Bowers et al., 1971; Jacobs et al., 1971). Based on the observation that the peptide can stimulate prolactin secretion from cultured lactotrophs (Smith and Convey, 1975) and the presence of TRH receptors in the anterior pituitary gland (Konaka et al., 1997), it has been assumed that the TRH regulation of prolactin release happens in the pituitary gland in parallel with TSH release. However, this conclusion is not uncontroversial. Firstly, only a minority of lactotrophs – indeed only half of the percentage of somatotrophs – express TRH receptor mRNA (Konaka et al., 1997). Secondly, TSH and prolactin secretion do not consistently correlate (Gautvik et al., 1973). Other mechanisms- and sites-of-actions must therefore be considered.

In paper III, we demonstrate that TIDA neurons are densely targeted by TRH terminals on cell bodies and proximal dendrites. By electrophysiological recordings we show that application of TRH dramatically changes the firing pattern of TIDA cells from oscillation to tonic discharge. This result provides evidence that TRH can modulate TIDA cell discharge at the hypothalamic level. Pituitary actions cannot be excluded. As discussed above, in relation to arousal, parallel (hypothalamic and pituitary) actions with similar functional end products (*i.e.* promotion of milk production) may well rather be operating together.

Our findings suggest a novel model of the regulation of lactation where the electrical properties of TIDA neurons form an important site of state control in the lactotrophic axis. Thus, under baseline conditions, high (oscillation-dependent) release of dopamine ensures that prolactin release is suppressed. With the switch to tonic discharge, dopamine output from the network is reduced and the brake on prolactin secretion (and lactation) is removed. Intriguingly, there is both *in vivo* (Denef et al., 1980; Tagawa et al., 1992; Arey et al., 1993; Burris and Freeman, 1993) and computational (Tabak et al., 2007) data showing that low dopamine concentrations in the pituitary not only results in a loss of D2-mediated inhibition, but rather that allows remaining dopamine to act as a stimulant of lactotroph exocytosis. Future studies that measure the dopamine release from the TIDA systems during different discharge patterns (through *e.g.* electrochemistry; Makos et

al., 2009), and *in vivo* recordings of TIDA activity in different states of the reproductive cycle will be required to validate this hypothesis. It also remains to determine the source of TRH terminals upon TIDA neurons. One candidate is a previously identified projection from the PVH to the Arc (Armstrong and Hatton, 1980; Tóth and Palkovits, 1998), but the transmitter phenotype of these neurons is not yet known.

5.4. Therapeutic potential of TRH

To what extent does our current understanding of the TRH system(s), and the additional information contributed by the present studies, allow us to speculate about treatment in disorders where functions modulated by TRH are affected? The first application that comes to mind is sleep disorders. Disorders of insufficient, excessive as well as inappropriate sleep are as a group exceptionally common and limit both quality-of-life and productivity (see Schenck et al., 2003). Overall, treatment options for insomnia, but also narcolepsy, are inadequate as currently available compounds fail to restore normal sleep patterns and are plagued with addictive properties (Ramakrishnan and Scheid, 2007). There is a clear need for new therapies that lack these adverse effects. The potent analeptic actions of TRH in experimental animals are promising in this regard. The possibility of functionally converging actions in several CNS arousal systems (discussed above) provides additional justification for such optimism. In comparison to findings in animals, however, the few studies in healthy humans suggest less impressive effects (*e.g.* Chihara et al., 1977; Glue et al., 1992; Hemmeter et al., 1998). (A systematic evaluation of TRH as an analeptic drug in humans has not been found in the literature.) It remains to be evaluated if TRH may exert more potent actions when the sleep-wake cycle is compromised.

In this context, encouraging findings on antiepileptic actions of TRH in pediatric epilepsy (in particular West syndrome) can be noted. Thus, systemic administration of TRH to otherwise medically refractory patients decreased, and in some cases abolished, seizures, to an extent comparable to adrenocorticotropic hormone (Matsumoto et al., 1987). Though these studies include relatively few patients, they are interesting since epileptic seizures, like slow-wave sleep, is a state of wide-spread – sometimes global – cortical synchrony. It has also been pointed out that as an antiepileptic compound, TRH is an attractive alternative because of its general activating properties (Takeuchi et al., 2001). In contrast, most current medications increase inhibition, which, in turn, may underlie the fatigue and learning disabilities seen in many children on such medications.

A second potential application of relevance to the present results is the use of TRH-based therapies in prolactin-related disorders. Here, TRH could be used to increase prolactin levels. Pathophysiologically, however, such situations appear rare. In contrast, hyperprolactinaemia is a significant medical problem and is ubiquitous in patients medicated with dopamine antagonist anti-psychotic drugs (Bushe and Shaw, 2007.), as well as in patients suffering from prolactinoma, the most common form of functional pituitary tumor (Burrow et al., 1981). Hyperprolactinaemia,

though less commonly causing over galactorrhea, can result in decreased libido, amenorrhea and infertility, and obesity (reviewed in Colao, 2009). Since the increased prolactin secretion in such patients is, however, related to decrease (relative or absolute) potency of dopaminergic inhibition, it is unclear if addition of a TRH antagonist (which, it should be noted, is currently not available) would be meaningful. Decreasing TRHergic suppression of TIDA action in a situation where the dopaminergic brake on the lactotroph has already been bypassed may be functionally irrelevant.

Today, the clinical application of TRH is primarily restricted to its use in provoking hormonal release (including prolactin) in endocrine investigations (Faglia, 1998). The powerful biological effects of TRH, however, prompted several studies on its therapeutic potential in the first decades following its discovery (*e.g.* Metcalf and Dettmar, 1981). Thus, TRH was tested on a range of conditions, including depression (Callahan et al., 1997), respiratory distress syndrome (Ikegami et al., 1987) and, though encouraging, most of these reports showed a relatively limited duration of positive effects (Molchan et al, 1990). Several factors may contribute to this, including the brief biological half-time (minutes) of TRH (see Metcalf, 1982). Therefore more biologically stable analogs of TRH have been developed (Yamamura et al, 1991). Experimentally, a rapid desensitization has been noted in many systems (*e.g.* Broberger and McCormick, 2005), which is likely due to receptor internalization (Nussenzveig et al., 1993; Ashworth et al., 1995). Finally, several studies have noted a limited access to the CNS (see Metcalf, 1982), although the physiological relevance of this restriction is debatable since systemic injection of TRH has been shown to modulate central functions such as arousal without affecting the peripheral thyroid axis (Nemeroff et al., 1975; Nishino et al., 1997).

In summary, though a range of therapeutic areas of interest related to TRH can be identified, several issues regarding the indications, administration and properties of TRH-based therapies remain to be addressed. Importantly, the widening repertoire of biological functions where TRH is implicated also means additional potential side effects to take into consideration. What is less in doubt is the hope that also studies on basic neuroanatomy, such as those presented in this thesis, can contribute to the ultimate goal of improved diagnoses, treatments and cures for disorders of the brain.

6. CONCLUSION

In this thesis the role played by TRH in some different aspects of homeostasis and motivation was studied. It can be concluded that:

- Thyrotropin- Releasing Hormone is in anatomical position and excerts functional effects that allow stimulation Hcrt neurons in the LHA, providing a potential mechanism for TRH-induced arousal.

- Thyrotropin- Releasing Hormone-expressing cells form a large and potentially unique LHA population that may serve as a link between central metabolic sensor and arousal.

- Tuberoinfundibular dopamine cells constitute an electrotonically synchronized network that discharges in robust oscillations. The TIDA network switches to tonic discharge in response to the lactogenic peptide TRH, suggesting a novel model for the control of prolactin release based on neuroendocrine firing pattern where lactation-modulatory agents act at the hypothalamic, in addition to or *in lieu* , of the pituitary level.

7. ACKNOWLEDGEMENTS

This thesis was performed at the Department of Neuroscience of Karolinska Institutet and was supported by Wenner-Gren Foundations, Knut och Alice Wallenbergs Foundation (KAW2004.0150), the Rut and Arvid Wolff Foundation, the Novo-Nordisk Foundation, the Magnus Bergvall Foundation, Swedish Research Council, the Åke Wiberg Foundation, the Royal Swedish Academy of Science, Längmanska Kulturfonden, Petrus and Augusta Hedlund's Foundation, the Axel Linder Foundation, the Golje Foundation,the Lars Hierta Memorial Foundation, the Hagberg Foundation, the Jeansson Foundations, the O.E. and Edla Johansson Foundation, the Fredrik and Ingrid Thuring Foundation and the European Commission Coordination Action ENINET(contract number LSHM-CT-2005-19063).

When I started this project two years ago, I had no idea how it would end. This thesis owes its existence to the help and support of many people, thanks to all of you.

I would like to thank the Department of Neuroscience and the staff that works there, for helping me day by day solving all kinds of practical issues.

Special thanks to Christian Broberger, for giving me the opportunity. For your constant challenges, pushing me to try new ideas, to save that one cell….to learn more. I would also like to extend my gratitude to the members of the Broberger lab (past and present): Kylie, Dave, Guncha, Michiel, Lovisa, Siobhan, Arash and Hanna: I have learned something from each and every one of you that I will not forget…

Many thanks to my co-supervisors: Hjalmar Brismar, for your time and patience while introducing me to the still quite mysterious world of microscopy; and Marie Björnholm, for being there whenever a question arose, thanks for your mails and coffees checking if I was alive.

I want to express my gratitude to Hans Eklund, my mentor; for your calm and warm presence; for having the right advice at the right moment.

Thanks to all the people in every corridor we have lived in during these years, for sharing your equipment and expertise with us and for making a good working atmosphere. Anita thanks for all your tips.

Many thanks to my KI friends, for so many lunches, coffees and talks in between experiments. Amanda, Susanne, Richard, Blanca, Hui, Noor, Karin and Vanessa: thank you very much for your tips, for giving me your different point of views and for have the patience to hear my complaints. You have done my time at KI more pleasant.

I also want to thank my non KI friends for distracting me from the lab, for reminding me that there is more in life than just science and experiments. Natt,

Shiva and Sussi, thanks for helping me always to see the big picture; Matte, for your patience helping me with this crazy language; Jimz, for giving me your friendship since my very first day in Stockholm.

To “the Patetsos”. Special thanks to Ziba and Andreas, for being there checking up on me; to Simon, thank you for all your help.

Very special thanks to the family Mira/Diaz: Sole, Meme, Violeta, Yerko, Andrea y Stefan, por su siempre calurosa bievenida, por hacerme sentir en casa y en familia.

I have no words enough to thank my parents. Papá y mamá, gracias por su apoyo constante e incondicional, pero sobre todo, gracias por ensñarme a luchar y perseguir mis sueños.

Big thanks to my sister Inés, por tu apoyo y motivación, por ser siempre mi fan número uno sin importar las tonterías que haga.

I would not have survived in Stockholm without the help of two persons:
Alle, thanks for your unconditional support and comprehension; for being my “secretario de asuntos aleatorios”; but specially thank you for adopting me as a sister.
Ximena, my big sister, what can I say? Thank you very much for everything……for being there since the very beginning, taking care of me since I was a zygote. This goes out to you!

Elias, mi corazón, thanks for all your support and motivation; you always know what to say to make me feel better. Thanks for being my oxygen every time I felt like I was drowning.
Σ 'αγαπώ πολύ!

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