IDENTIFICATION AND CHARACTERIZATION OF NEUROENDOCRINE PATHWAYS INVOLVED IN THE REGULATION OF SEASONAL BODY WEIGHT CYCLES

Animal Physiology
Department of Biology
Philipps University
Marburg

DISSERTATION
zur
Erlangung des Doktorgrades
der Naturwissenschaften
(Dr. rer. nat.)
vorgelegt von

Mohammad H. Khorooshi
Aus Mashad, Iran

Marburg/Lahn (2004)

Vom Fachbereich _____
der Philipps-Universität Marburg als Dissertation am _____
_____ angenommen.

Erstgutachter _____
Zweitgutachter _____
Tag der mündlichen Prüfung am _____

CONTENTS

GLOSSARY OF TERMS

ACTH	adrenocorticotropic hormone
AgRP	agouti-related peptide
a-MSH	a -melanocyte stimulating hormone
ARC	arcuate nucleus
CART	cocaine- and amphetamine-regulated transcript
CRH	corticotropin-releasing hormone
DLG	dorsal lateral geniculate nucleus
DMH	dorsomedial hypothalamic nucleus
DR	dorsal raphe nucleus
EW	Edinger-Westphal nucleus
F	fornix
GHT	geniculohypothalamic tract
IGL	intergeniculate leaflet
ir	immunoreactivity
JAK	janus kinase
LA	lateroanterior hypothalamic nucleus
LD	long day photoperiod (16:8 h light:dark)
LHA	lateral hypothalamic nucleus
MCH	melanin-concentrating hormone
MCR	melanocortin receptor
ME	median eminence
MnPO	median preoptic nucleus
MPO	medial preoptic nucleus
MR	median raphe nucleus
NPY	neuropeptide Y
OB-RB	leptin receptor long form
OXB	orexin-B
OXR	orexin receptor
PC	prohormone convertase
Pe	periventricular nucleus
peri-ARC	peri-arcuate nucleus
PFA	perifornical area

PG	pineal gland
PHA	posterior hypothalamic area
PMV	ventral prememmilary nucleus
POMC	proopiomelanocortin
PVN	paraventricular hypothalamic nucleus
PVT	paraventricular thalamic nucleus
RCH	retrochiasmatic area
RHT	retinohypothalamic tract
SCN	suprachiasmatic nucleus
SD	short day photoperiod (8:16 h light:dark)
SOCS-3	suppressor of cytokine signaling-3
SON	supraoptic nucleus
STAT3	signal transducer and activator of transcription-3
TMV	ventral tuberomammillary nucleus
TRH	thyrotropin-releasing hormone
VLG	ventral lateral geniculate nucleus
VLPO	ventrolateral preoptic nucleus
VMH	ventromedial hypothalamic nucleus
ZI	zona incerta

CHAPTER I

GENERAL INTRODUCTION

Current understanding of the neuroendocrine pathways involved in the regulation of energy balance has evolved from lesion studies, molecular genetics of obesity, standard laboratory rodents as well as the discovery of leptin (Barsh and Schwartz 2002;Kalra et al. 1999;Zhang et al. 1994). Beyond this, only limited information is available on the central regulatory mechanism of energy balance in mammals exhibiting seasonal cycles in body mass, driven either by circannual rhythmicity or triggered by natural changes in photoperiod (Morgan et al. 2003). The Djungarian hamster (*Phodopus sungorus*) is a well-known photoperiodic seasonal mammal and represents and ideal animal model to study the neuroendocrine basis of seasonal body weight regulation.

Seasonal regulation of the body weight

In response to transition from long day (LD) photoperiod (16:8 h light:dark) to short day (SD) photoperiod (8:16 h light:dark), hamsters spontaneously reduce food intake and body mass declines (Fig. 1), over a 12-week period, to a lower winter level (Steinlechner et al. 1983). The decrease in body mass is mainly due to fat depletion (Klingenspor et al. 2000). Hamsters remain in this winter acclimated state for up to 3 months. Thereafter, hamsters increase food intake and body mass to the summer level as they become refractory to short photoperiod. At any phase of this body mass cycle a proposed sliding set-point mechanism (Fig. 1) appears to encode the seasonally appropriate food intake and body mass (Steinlechner et al. 1983). The effectiveness of short photoperiod to trigger the sliding set-point decrease in body mass requires communication between neuronal components of the circadian timing system and neuroendocrine pathways involved in the regulation of energy balance.

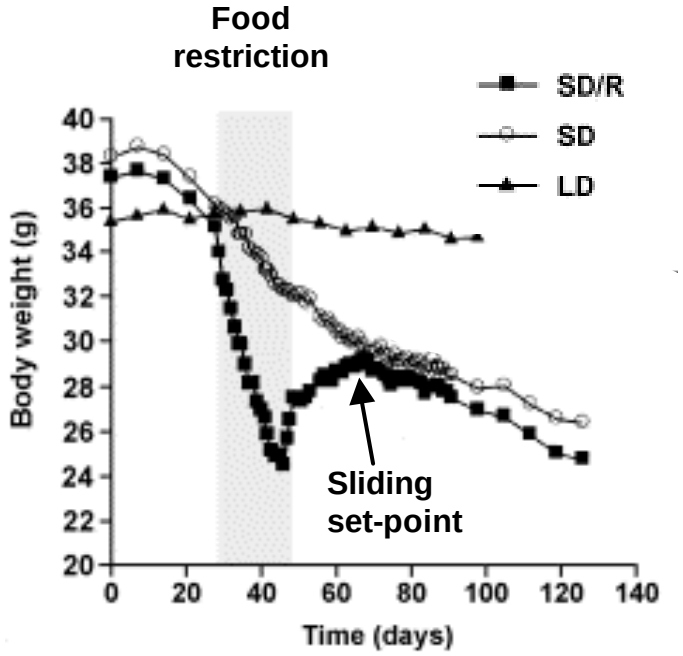

Figure 1. Body weight of hamsters fed ad libitum in SD, or held in short day length with restricted food SD/R (Shaded area). For comparison, a typical body weight trajectory of hamsters fed ad libitum in LD is shown. From (Mercer and Tups 2003).

Circadian timing system

Changes in photoperiod result in large variations of duration and amplitude of pineal melatonin secretion that influence the central regulatory mechanism by which energy balance is controlled (Morgan *et al.* 2003). Melatonin secreted from the pineal gland is the neuroendocrine transducer of photoperiod information acting on its receptors to regulate both mammalian circadian and seasonal biological rhythms (Goldman and Darrow 1983). The suprachiasmatic nucleus (SCN) is a major site of melatonin binding in the rodent brain. It contains the master circadian biological clock and plays an essential role in the generation and maintenance of a wide variety of circadian rhythms (Moore 1983). The central role of the SCN in feeding regulation is well documented and lesions of the SCN abolish short day mediated decrease of food intake and body mass (Bittman et al. 1991). The SCN is part of the neural components of the circadian timing system that forms a network coordinating the temporal organization of physiological processes and behaviour. The intergeniculate leaflet (IGL), the median raphe nucleus (MR) and the dorsal raphe nucleus (DR) are also considered primary nodes of the circadian timing network. The SCN receives photic input from the retina through the retinohypothalamic tract (RHT) and the IGL through the geniculohypothalamic tract (GHT), and non-photic input from the midbrain raphe nuclei (Meyer-Bernstein and Morin 1996;Meyer-Bernstein and Morin 1998;Morin and Blanchard 1991;Morin 1999). Serotonergic cells of the median raphe nucleus (MR) and dorsal raphe nucleus (DR) project to the SCN and IGL,

respectively, influencing circadian rhythm regulation (Meyer-Bernstein and Morin 1996;Morin and Blanchard 1995). The neuronal link between raphe nuclei and the SCN is further demonstrated by the presence of serotonin receptors in the rat SCN (Moyer and Kennaway 1999). In contrast, the IGL neurons contain neuropeptide Y (NPY) and enkephalin that through GHT project to the SCN (Morin et al. 1992;Morin and Blanchard 1995;Smale et al. 1991). This directly influences the circadian timekeeping processes by supplying the SCN with both photic and non-photic information (Fig. 2).

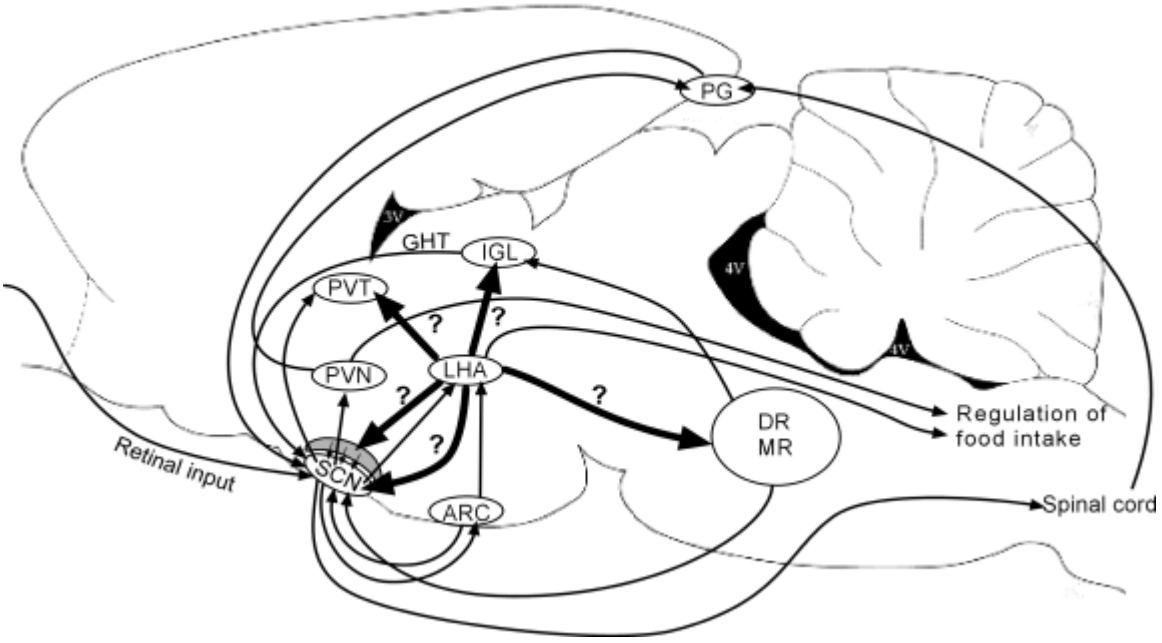

Figure 2. Diagram of neuronal connections between brain structures implicated in the regulation of energy balance and neuronal components of the circadian timing system (sagittal section of the rat brain). The shaded area above the SCN indicates the peripheral zone of the SCN. Whether LHA neurons project to the neuronal components of the circadian timing system is not known. ARC, arcuate nucleus; DR, dorsal raphe nucleus; GHT, geniculohypothalamic tract; IGL, intergeniculate leaflet, LHA, lateral hypothalamic area; MR, median raphe nucleus; PG, pineal gland; PVN, paraventricular hypothalamic nucleus; PVT, paraventricular thalamic nucleus; SCN, suprachiasmatic nucleus.

The SCN, in turn, projects to several brain regions including the pineal gland, paraventricular hypothalamic nucleus (PVN), arcuate nucleus (ARC), paraventricular thalamic nucleus (PVT), Edinger-Westphal nucleus (EW) and posterior hypothalamic area. The SCN is connected to the pineal gland, controlling the rhythm of melatonin

synthesis, by a multisynaptic pathway including neurons of the PVN, noradrenergic sympathetic neurons of the superior cervical ganglion and sympathetic preganglionic neurons of the intermediolateral cell column of the spinal cord (Bittman et al. 1989;Klein et al. 1983;Larsen et al. 1998).

The SCN sends direct and indirect neuronal information to the ARC, which in turn provides the SCN with excitatory and inhibitory inputs (Saeb-Parsy et al. 2000). Arginine vasopressin and vasoactive intestinal polypeptide containing neurons of the SCN project to the PVT, which transmits information to several cortical regions (Abrahamson and Moore 2001;Sylvester et al. 2002). The PVT plays an important role in regulation of arousal and maintaining wakefulness (Novak et al. 2000). Furthermore, substance-P positive neurons of the SCN project to the EW, that contains parasympathetic preganglionic neurons projecting to the ciliary ganglion. The EW innervates the iris sphincter muscle and mediates pupillary constriction and lens accommodation (Gamlin et al. 1982;Gamlin and Reiner 1991;Pickard et al. 2002;Sekiya et al. 1984). In addition, the SCN is associated with brain structures involved in energy balance regulation (Abrahamson et al. 2001). This may prove a functional link between the circadian timing system and the central nervous system that translates the photoperiodic information in order to integrate it for energy balance regulating processes. However, a neuroanatomical basis for components of the neuroendocrine pathway to influence or feedback to circadian timing processes has not been yet identified (Fig. 2).

Neuroendocrine pathways involved in the regulation of energy balance

The neuroendocrine pathway involved in regulation of energy balance receives hormonal and neuronal information from the periphery and other regions of the central nervous system about the status of energy stores. It then adjusts the activity of the autonomic nervous system to optimize energy conservation. Leptin, a peripheral derived hormone, has been considered as a potential input for controlling neuroendocrine pathways involved in the regulation of body weight. Leptin is mainly synthesized by adipocytes and it's circulating concentration is proportional to body fat mass. It plays an important role in regulation of feeding and energy expenditure via neural circuits located in the hypothalamus (Ahima et al. 1996;Halaas et al. 1995;Levin et al. 1996). The injection of leptin into mice or rats reduces food intake and increases energy expenditure.

The action of leptin within the central nervous system is mediated through a long form receptor (OB-Rb), which belongs to the cytokine-receptor super-family possessing intracellular JAk2-tyrosine kinase- signal transducer and activator of transcription-3 (STAT3) signaling pathway (Tartaglia et al. 1995;Tartaglia 1997;Baumann et al. 1996;Bjorbaek et al. 1997). The intracellular signaling pathway is activated by binding of leptin to OB-Rb leading to phosphorylation, dimerization, nuclear translocation and binding of STAT3 to DNA. This finally leads to activation of leptin-dependent gene transcription including the suppressor of cytokine signaling-3 (SOCS-3), which inhibits JAK/STAT activity and subsequent signal transduction (Bjorbak et al. 2000;da Silva et al. 1998;Baskin et al. 2000;Banks et al. 2000;Bjorbak et al. 2000;Imada and Leonard 2000).

OB-Rb mRNA expression is mainly detected within hypothalamic structures including arcuate nucleus (ARC), ventromedial hypothalamic nucleus (VMH), dorsomedial hypothalamic nucleus (DMH), paraventricular hypothalamic nucleus (PVN) and lateral hypothalamic area (LHA) in rats and hamsters (Mercer et al. 2000;Elmquist et al. 1998;Mercer et al. 1998c;Buyse et al. 2001). These structures form a complex of neuronal networks (Schwartz et al. 2000), that regulates energy balance (Fig. 3).

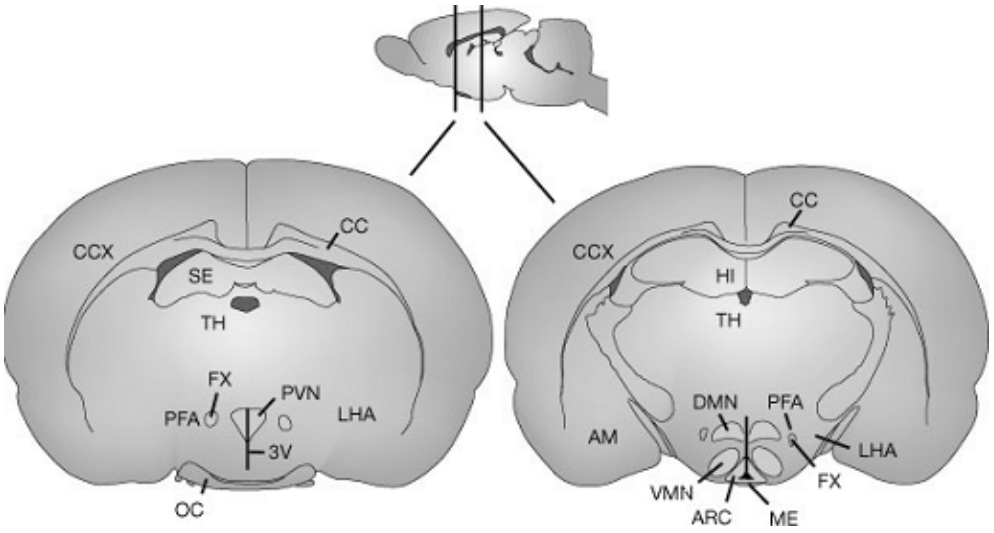

Figure 3. Schematic representation of the hypothalamic nuclei implicated in the regulation of food intake. AM, amygdala; ARC, arcuate nucleus; CC, corpus callosum; CCX, cerebral cortex; DMN, dorsomedial nucleus; HI, hippocampus; ME, median eminence; OC, optic chiasm; PVN, paraventricular hypothalamic nucleus; SE, septum; TH, thalamus; VMN, ventromedial nucleus; 3V, third ventricle. From (Schwartz et al. 2000).

Several neuropeptides are associated with this complex neuronal network, that are categorized as orexigenic or anorexigenic, respectively, based on their stimulatory and inhibitory effect on food intake (Schwartz et al. 2000). Orexigenic neuropeptides include neuropeptide Y (NPY), agouti-related peptide (AgRP), melanin-concentrating hormone (MCH) and orexin (Fig. 4). Anorexigenic neuropeptides include proopiomelanocortin (POMC), cocaine- and amphetamine-regulated transcript (CART), thyrotropin-releasing hormone (TRH) and corticotropin-releasing hormone (CRH) (Fig. 4). Leptin receptors have been identified in NPY/AgRP- and POMC/CART-containing neurons of the ARC, and in MCH- and orexin- containing neurons of the LHA. Functional studies show that leptin alters the tone of orexigenic and/or anorexigenic drive towards activation of catabolic pathways resulting in decreased food intake. Mice lacking leptin and leptin receptors exhibit elevated levels of NPY/AGRP mRNA and/or decreased levels of POMC/CART mRNA within the ARC (Mizuno et al. 1998). The expression level of CART- and POMC-mRNA in fasted rats is reduced, which after leptin administration returns to normal levels (Schwartz et al. 1997;Vrang et al. 1999). Furthermore, leptin treatment in rats induces cellular activity and STAT3 nuclear translocation within POMC/CART neurons in the ventrolateral part of the ARC (Elias et al. 1998;Elias et al. 1999;Hubschle et al. 2001). POMC/CART neurons in the ARC project to several areas including the spinal sympathetic preganglionic neurons, the PVN, and orexin/MCH neurons in the LHA (Elias et al. 1998;Elias et al. 1999;Elias et al. 2000;Elmquist 2001). This creates a functional link between adipose tissue and the central nervous system that translates information about body fat stores provided by leptin to input into energy balance regulating processes. For instance, the synaptic contacts between the CART-containing neuronal system and PVN (Fig. 4) provide a link between leptin and TRH and CRH in the PVN (Schwartz et al. 2000). In addition, CART projections to LHA neurons containing MCH and orexin provide a route for leptin to adjust the activity of the autonomic nervous system (Schwartz et al. 2000) (Fig. 4).

The CART-MCH-orexin system is of special interest, since a link between the SCN and MCH/orexin containing neurons in the LHA has been identified in rat and human (Abrahamson et al. 2001) (Fig. 2). This link may create a basis for the circadian timing system to influence the regulation of energy balance. However, it is not clear whether CART-MCH-orexin pathways in turn feedback or influence the generation of

circadian rhythmicity. In fact, a neuroanatomical basis for the CART-MCH-orexin system to influence circadian timing processes has not been yet identified (Fig. 2).

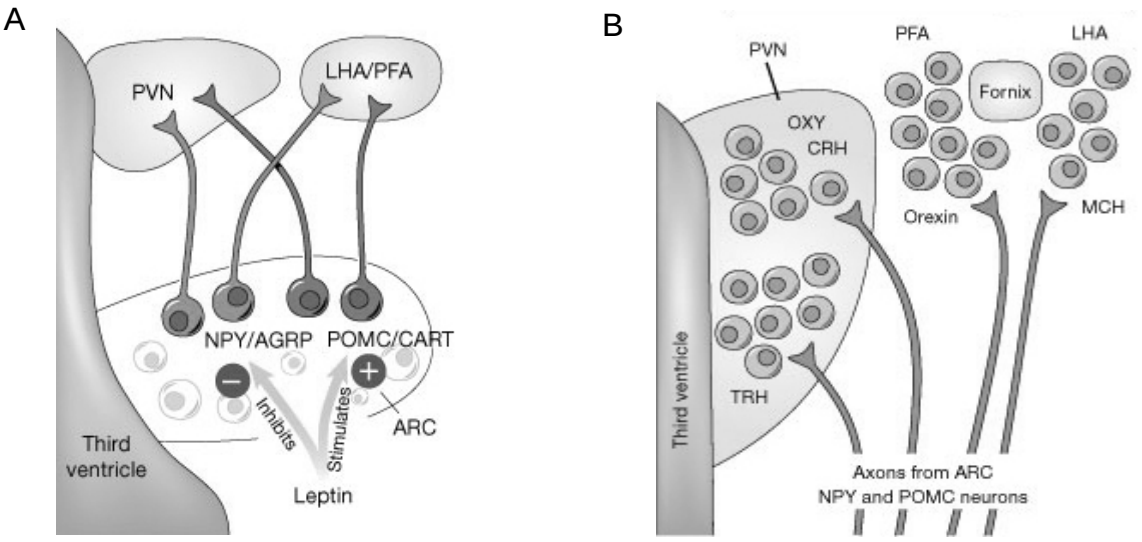

Figure 4. A) NPY/AGRP and POMC/CART neurons in the arcuate nucleus project to the PVN and to the LHA and PFA. B) The PVN and the LHA and PFA neurons contain CRH, TRH, MCH and orexin involved in the regulation of food intake and energy homeostasis. ARC, arcuate nucleus; AgRP, agouti-related peptide; CART, cocaine- and amphetamine-regulated transcript; CRH, corticotropin-releasing hormone; LHA, lateral hypothalamic area; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; TRH, thyrotropin-releasing hormone; OXY, oxytocin; POMC, proopiomelanocortin; PFA, perifornical area; PVN, paraventricular hypothalamic nucleus. From Schwartz et al. 2000.

CART-, MCH- and Orexin neuronal system

Known from non-photic laboratory animals, CART peptide is an anorexigenic neuropeptide localized within hypothalamic- and extrahypothalamic structures (Vrang et al. 1999a;Koylu et al. 1997;Koylu et al. 1998;Koylu et al. 1999;Couceyro et al. 1997;Hurd and Fagergren 2000). Central administration of CART in rats conveys an inhibitory effect on food intake (Stanley et al. 2001) and induces fos-immunoreactivity (ir) in brain structures involved in feeding behavior (Vrang et al. 1999b). Food deprivation has been demonstrated to reduce CART mRNA expression, whereas leptin treatment stimulated CART gene expression (Kristensen et al. 1998). Together, these findings emphasize the important role of CART in controlling food intake. Furthermore, CART-containing neurons mediate leptin action through extensive innervation of other brain areas including the MCH/orexin system in the LHA (Fig. 4B).

MCH synthesizing neurons are a population of hypothalamic neurons with stimulatory effects on food intake. Intracerebroventricular administration of MCH in rats has been shown to stimulate food intake in a dose dependent manner (Rossi et al. 1997). The stimulatory effect of MCH on food intake was further confirmed in transgenic mice in which over-expression of MCH induce obesity (Ludwig et al. 2001), and mice lacking MCH were lean (Shimada et al. 1998). Also, it has been demonstrated in mice that leptin administration blunts the fasting-induced rise in MCH mRNA, indicating that leptin may regulate MCH expression (Tritos et al. 2001).

Orexins (OXA and OXB), also referred to as hypocretin (hypocretin 1 and 2), are orexigenic neuropeptides which stimulate food intake when injected into the brain ventricle (Edwards et al. 1999;Sakurai et al. 1998;Sahu 2002). Although the anatomical localization of orexin cell bodies has been shown to be restricted within the LHA, the fibers emerging from the orexin perikarya have been found throughout the brain (Peyron et al. 1998;Van Dijk et al. 1997;Cutler et al. 1999). This is consistent with the localization of two recently identified receptors for orexins (orexin receptor 1 and 2), that are widely expressed in the central nervous system (Lu et al. 2000;Trivedi et al. 1998), specifically in brain areas involved with sleep/wake cycle, arousal, circadian rhytmicity, pain, sympathetic and parasympathetic functions. For instance, the role of orexins and receptors in the sleep/wake cycle is supported by recent findings in animal models, where defective orexin system signaling is linked to the sleep/wake disorder, narcolepsy (Lin et al. 1999).

These studies have furthered the understanding of leptin function and neuropeptides in non-photoperiodic animals, where food intake and energy metabolism can be manipulated by fasting and food deprivation. However, similar information is sparse concerning Djungarian hamsters, who can change body weight in response to photoperiod. Thus, in seasonal animals, the function of leptin and neuropeptides in the neuronal network for controlling energy balance is not well understood (Ebling et al. 1998;Morgan et al. 2003).

In response to short photoperiod, body fat content and serum leptin levels of hamsters decrease, and their sensitivity to exogenous leptin increases (Atcha et al. 2000;Klingenspor et al. 2000;Rousseau et al. 2002). One major contributor to leptin sensitivity in hamsters is SOCS3 (Mercer and Tups 2003;Tups et al. 2003). SD induces down-regulation of SOCS3 mRNA in the ARC, and exogenous leptin stimulates SOCS3 mRNA only in SD hamsters (Mercer and Tups 2003;Tups et al.

2003). These results suggest that photoperiod modulates the sensitivity of neuroendocrine pathways in the hypothalamus to peripheral leptin. The ARC is the major hypothalamic structure in which gene expression is regulated by photoperiod (Adam and Mercer 2001). In contrast, leptin treatment in rats has been shown to induce cellular activity not only within the ARC but also in several hypothalamic areas including LHA, VMH, DMH and PVN that receive projections from the ARC (Elias et al. 1998;Elias et al. 1999;Elias et al. 2000). The ARC may therefore represent the central area in which photoperiod and leptin interact. However, a functional map of brain structures responding to leptin in hamsters has not been established. Currently, the mediation of leptin action is mainly based on localization of leptin receptors in the hamster's brain (Mercer et al. 1998a;Mercer et al. 1998b;Mercer and Speakman 2001) or on results obtained in non-photoperiodic animals (Halaas and Friedman 1997).

Current understanding of the neuronal circuit involved in photoperiodic regulation of energy balance has emerged, in part, from studies demonstrating the altered gene expression of neuropeptides. For instance, due to its ability to activate the catabolic pathway, CART gene expression has been studied in response to changes in photoperiod. Results of these studies are either controversial or represent overall CART gene expression changes in the ARC. In contrast to studies demonstrating no effect on CART-mRNA expression from changes in photoperiod (Robson *et al.* 2002), there is evidence demonstrating increased CART-mRNA expression in the ARC in response to SD (Mercer et al. 2003). Furthermore, changes in CART gene expression detected in the ARC cannot define a specific region within the ARC that may be responsible for mediation by changes in photoperiod. Therfore, analysis of CART peptide expression would significantly improve our knowledge about this anorexigenic signal in seasonal body weight regulation. In addition, the exact localisation of CART changes within the ARC in response to changes in photoperiod, if any, support the idea of a specific central regulatory mechanism involved in photoperiodic control of energy balance.

Specific aims

Knowledge about neuropeptides, their distribution, and their association with the circadian timing system as well as their response to changes in photoperiod and peripherally derived signals would further the understanding of neuroendocrine

pathways involved in seasonal body weight regulation in the Djungarian hamster. The specific aims of this thesis were:

1) To establish a suitable procedure to study the localisation and co-localisation of selective neuropeptides in the brain of the Djungarian hamster.

2) To study the distribution of MCH, CART and OXB as well as their morphological relationships in selected brain structures harbouring neuroendocrine pathways controlling energy balance and circadian timing system in the Djungarian hamster (Chapter II).

3) To further identify a pathway through which hypothalamic neuropeptides may interact with circadian timing processes (Chapter III).

4) To investigate whether seasonal acclimation alters CART peptide expression (Chapter IV).

5) To identify hypothalamic structures mediating the effect of leptin (Chapter V).

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CHAPTER II

Neuroanatomical basis for cross-talk of brain regions involved in the control of energy balance and circadian timing system in a seasonal mammal

Abstract

The distribution of melanin-concentrating hormone (MCH)-, cocaine- and amphetamine-regulated transcript (CART)- and orexin B (OXB)- immunoreactive (ir) elements as well as their morphological relationships in selected brain structures harbouring the neuroendocrine pathways controlling energy balance and circadian timing system in the Djungarian hamster (*Phodopus sungorus*) were studied. CART-(55-102) ir perikarya co-expressed MCH- ir in the lateral hypothalamic area (LHA), dorsomedial hypothalamic nucleus (DMH), zona incerta and posterior hypothalamic area. In addition, arcuate nucleus, hypothalamic periventricular nucleus, Edinger-Westphal nucleus, and rostral aspect of the dorsal raphe nucleus contained CART- ir cell bodies. OXB- ir perikarya were distributed in the LHA, DMH and retrochiasmatic area. Cells immunoreactive for OXB did not co-express MCH- ir, but OXB- ir fibers had close apposition to many MCH- ir cells. Whereas, MCH-, CART- and OXB-ir were absent in the SCN, OXB- and to a lesser extent MCH-. CART- ir fibers were present in the thalamic paraventricular nucleus, intergeniculate leaflet, and raphe nucleus. These observations in Djungarian hamsters indicate that MCH-, CART- and OXB- neuronal system are strongly conserved between species. In addition, the presence of fibers within part of the neuronal components of the circadian timing system suggests that they may indirectly influence the circadian timing processes.

Introduction

Knowledge on the complex neuroanatomy and physiology of central networks regulating energy balance is mainly based on studies in standard laboratory rodents. Many transgenic mouse models have been generated to test the function of selected neuropeptides and receptors (Barsh and Schwartz 2002). Beyond this, only a limited number of comparative studies have been performed on mammals that undergo pronounced seasonal cycles in food intake and body mass either driven by circannual rhythmicity or triggered by natural changes in photoperiod (Morgan et al. 2003).

The Djungarian hamster is a well known photoperiodic seasonal mammal. In response to a long to short photoperiod transition, Djungarian hamsters, in an apparent pre-programmed manner, decrease food intake and body mass to a lower winter level within 10-12 weeks (Steinlechner et al. 1983). Approximately 40% loss in body mass is mainly due to fat depletion (Klingenspor et al. 2000). Hamsters remain in this winter acclimated state for up to three months, and thereafter increase food intake and body mass to the summer level as they become refractory to short photoperiod. At any phase of this body mass cycle, a proposed sliding set-point mechanism appears to encode the seasonally appropriate food intake and body mass (Steinlechner et al. 1983). This rheostatic control of energy balance is associated with seasonal changes in sensitivity toward leptin (Klingenspor et al. 2000). However, the role of neuropeptides and receptors in the central nervous system identified so far as general components of the neuronal network controlling energy balance is not understood (Ebling et al. 1998;Morgan et al. 2003).

The effectiveness of the short photoperiod to trigger the sliding set-point event of decreasing body mass requires communication between neuronal components of the circadian system, photoperiodic time measurement and energy balance. However, limited information is available on the possible neuroanatomical basis for such cross-talk in seasonal mammals. The neuronal networks controlling energy balance are mostly located in hypothalamic structures including the arcuate nucleus (ARC), dorsomedial hypothalamic nucleus (DMH), the ventromedial hypothalamic nucleus (VMH), the lateral hypothalamic area (LHA) and the paraventricular hypothalamic nucleus (PVN).

Neuronal components of the circadian timing system are distributed throughout the brain, forming a network coordinating the temporal organization of physiological processes and behaviour. The primary nodes of this network include the suprachiasmatic nucleus (SCN), the intergeniculate leaflet (IGL), the median raphe nucleus (MR) and the dorsal raphe nucleus (DR). Circadian rhythms in physiology and behavior are generated by the SCN located in the hypothalamus. SCN, the pacemaker of the circadian timing system, receives photic input from the retina through the retinohypothalamic tract (RHT) and from the IGL through the geniculohypothalamic tract (GHT). In addition, non-photic input comes from the midbrain raphe nuclei (Meyer-Bernstein and Morin 1996;Meyer-Bernstein and Morin 1998;Morin and Blanchard 1991;Morin 1999). MR and DR influence circadian rhythmicity through innervations of the SCN and IGL, respectively (Meyer-Bernstein and Morin 1996;Morin and Blanchard 1995). The IGL neurons project through the GHT to the SCN and directly influence circadian timekeeping processes by supplying the SCN with both photic and non-photic information. The SCN efferences in turn transmit circadian rhythmicity to several brain regions including the paraventricular thalamic nucleus (PVT). This leads to transmission of time information to several cortical regions (Abrahamson and Moore 2001;Sylvester et al. 2002) and thus plays an important role in the regulation of arousal and maintenance of wakefulness (Novak et al. 2000).

The importance of orexigenic and anorexigenic neuropeptides in the photoperiodic control of energy balance in seasonal animals has been the focus of increasing interest. For example, several studies have investigated short day induced changes in the expression levels of leptin, neuropeptides and corresponding receptors (Klingenspor et al. 1996;Adam et al. 2000;Mercer et al. 2000;Klingenspor et al. 2000). In Djungarian hamsters acclimated to short photoperiod, small changes in gene expression of neuropeptide Y (NPY), agouti gene-related peptide (AGRP) and proopiomelanocortin (POMC) oppose reduced food intake, whereas the expression of the anorexigenic peptide cocaine- and amphetamine-regulated transcript (CART) in the ARC is increased (Adam et al. 2000;Mercer et al. 2000;Mercer and Speakman 2001;Mercer et al. 2003). Thus, CART may be a key factor in seasonal acclimation of energy balance.

In addition, the central role of the SCN in regulating food intake has been documented with lesion studies. For example, chemical lesions of the ARC does not have a major impact on short day mediated decrease of food intake and body mass (Ebling et al. 1998), whereas lesions of the SCN abolish this response (Bittman et al. 1991). Hence, the circadian timing system plays an important role in the regulation of seasonal body mass cycles. In addition, a direct projection from the SCN to perikarya of melanin-concentrating hormone (MCH) and orexins (hypocretins) producing neurons, two positive effectors of food intake, are found in the rat and human (Abrahamson et al. 2001). These findings suggest that the generation of circadian rhythmicity in feeding behaviour may in part involve the function of MCH and orexin containing neurons. Whether the neuronal network of MCH and orexin may in turn influence the circadian timing processes requires further investigation, specifically, in seasonal animals. Analysis of neuroanatomical distribution, if any, of these neuropeptides known to regulate feeding within neuronal networks of the circadian timing system may therefore provide a basis for such cross-talk. Thus, the present study was to investigate the immunohistochemical distribution of MCH, CART and OXB within selected brain areas implicated in the control of food intake and circadian timekeeping processes. In addition, dual-labeling immunostaining was performed to examine the relationship between these neuropeptides in Djungarian hamsters.

Materials and methods

Tissue preparation

Adult male Djungarian hamsters (*Phodopus sungorus*, n = 10) weighing 47-55 g were used for the study. Hamsters were housed individually in Macrolon cages under naturally occurring light (14:10 light:dark cycle, May), with free access to standard rodent chow (Altromin 7014) and water. Hamsters were deeply anaesthetized in a CO_2 atmosphere and killed by decapitation between 13:00 and 14:00 h in the afternoon. Brains were then removed, fixed in 4% paraformaldehyde (48 h, 4°C) and cryoprotected in 20% sucrose in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 24 hr at 4°C. The brains were then cut on a cryostat into 30 µm coronal sections. Free-floating sections were stored in PBS at 4°C prior to immunohostochemical procedures. All procedures were in accordance with German animal welfare regulation.

Immunostaining

To investigate the anatomical localization of MCH, CART and OXB in the brain of Djungarian hamsters, two different detection methods were used, namely immunofluorescence and HRP-peroxidase reaction.

Free-floating sections were rinsed in PBS and then in PBS containing 0.5% Triton-X 100 (PBS-TX). Following pre-incubation in blocking solution containing PBS-TX and 3% BSA, sections were incubated with primary rabbit anti- MCH (Phoenix Europe GmbH; H-070-47), CART (55-102; Phoenix Europe GmbH; H-003-62) and OXB (Phoenix Europe GmbH; H-003-32) antibodies each diluted 1:200 in blocking solution overnight at 4°C. Following washing in PBS-TX, sections were then incubated with Cy3 conjugated goat anti-rabbit antibody (Dianova, 112-165-144) or Alexa-fluor-488 conjugated goat anti-rabbit antibody (Molecular Probes, A-11034) each diluted 1:250 in blocking solution in dark at room temperature (RT). Sections were then rinsed in PBS, mounted on gelatin-coated slides, air-dried, dehydrated in graded alcohol, cleared in xylene and coverslipped with Enthelan (Merck).

To detect MCH-, CART- and OXB-containing cells by peroxidase reaction, the same procedures as described above were performed with a few modifications. Briefly, endogenous peroxidase activity was inhibited in sections using 80% PBS, 10% methanol and 10% H_2O_2. Primary antibodies were diluted 1:400 in blocking solution, and the secondary antibody, peroxidase-conjugated goat anti-rabbit antibody (Jackson Immunoresearch, 111-035-144), was diluted 1:500 in blocking solution. Using DAB-nickel/Substrate SG (Vector kit), the colour reaction resulted in dark-gray/blue immunostaining.

Dual-labeling immunofluorescence immunostaining

Dual labeling immunofluorescence was performed on free-floating coronal sections to investigate colocalization of MCH with CART and OXB. After rinsing in PBS and PBS-TX, sections were pre-incubated in blocking solution containing PBS-TX and 3% BSA at RT. Sections were then incubated with primary rabbit polyclonal MCH, CART and OXB antibodies diluted (1:200) in blocking solution. After washing in PBS-TX, sections were then incubated with goat Fab-fragment anti-rabbit antibody (Dianova, 111-007-003) diluted 1:75 in blocking solution. Sections were then washed in PBS-TX and incubated with Cy3 conjugated donkey anti-goat antibody (Dianova, 705-165-147) diluted 1:250 in blocking solution. Following washing, sections were

incubated with MCH, CART, and OXB antibodies each diluted 1:200 in blocking solution. Sections were then washed in PBS-TX and incubated with Alexa-fluor-488 conjugated goat anti-rabbit antibody (Molecular Probes, A-11034), each diluted 1:250 in blocking solution. Sections were then rinsed in PBS, mounted onto gelatin-coated slides, air-dried, dehydrated in graded alcohol, cleared in xylene and coverslipped with Enthelan (Merck).

Antibody specificity

To test the specificity of primary antibodies, adsorption controls were carried out by adding an excess of CART- (Phoenix Europe GmbH; 003-62), MCH- (Phoenix Europe GmbH; 070-047) and OXB- (Phoenix Europe GmbH; 003-32) peptides to primary antibodies for three hours at RT before application to sections. A second control reaction was performed by omission of the primary antibody from the described procedure.

Analysis of single/dual-labeling immunostaining

Sections were examined under a conventional Zeiss Axioskop epiflourescent microscope equipped with two separate filter cubes and a laser scanning confocal/multiphoton microscope (Leica TCS SP2). The excitation wavelength for Alexa- and Cy3-induced fluorescence was 488 nm and 543 nm, respectively. Images were taken by a Polaroid DMCe digital camera mounted on the Zeiss Axioskop epiflourescent microscope. The contrast was then adjusted using Adobe Photoshop version 7.0 software and annotated in Microsoft PowerPoint. The anatomical localization of neuropeptides within the brain of Djungarian hamsters was annotated according to the atlas of rat brain (Paxinos and Watson 1998).

Analysis of OXB-ir apposition on MCH-ir cell bodies and fibers were carried out by scanning a series (10-15) of 0.5 µm sections at both wave lengths. Apposition of OXB-ir fibers to MCH-ir perikarya were recorded, if the proximity of labelled profile were about 0.5 µm in several serial sections.

The relative intensity of OXB-ir fiber in various brain regions was scored by visual inspection. This was further verified by colleagues blind to the results of the first scoring. The density of OXB-ir fibers was categorized as high (+++), moderate (++), low (+) and very low/absent (-).

Results

Antibody Specificity

In control sections incubated with pre-adsorbed antibodies or without primary antibodies, the MCH-, CART- and OXB-ir were absent (Fig. 1a, 2a and 3a). In addition, comparing immunofluorescence and HRP-peroxidase detection a similar staining pattern was observed for all antisera. However the immunoreactivity for fibers and axon terminals appeared stronger by immunofluorescence.

MCH immunoreactivity

MCH-ir cell bodies were distributed exclusively within the hypothalamus including LHA, dorso medial hypothalamic nucleus (DMH), zona incerta (ZI), and the posterior hypothalamic area (PHA, Fig. 1A, D). MCH-ir was found in cell bodies with rarely more than three processes (Fig. 4A). The MCH-ir fibers and terminal boutons were distributed within hypothalamic and thalamic areas including DMH, ZI, LHA, internal zone of median eminence (ME, Fig 1C) and paraventricular thalamic nucleus (PVT). MCH-ir fibers with terminal boutons were also present to a lesser extent in the DR (Fig. 1F), the IGL (Fig. 1G) and few in the MR. Whereas MCH-ir fibers were merely absent in the SCN (Fig. 1E). Furthermore, the peripheral zone of the SCN (Fig. 1E) including lateroanterior hypothalamic nucleus (LA) contained few MCH-ir fibers with terminal boutons.

CART immunoreactivity

Large and dense CART-ir perikarya were localized within LHA, DMH, ZI, and PHA (Fig. 2A, C, H). CART-ir perikarya were most abundant within the medial aspect of LHA. In contrast, few small sized CART-ir perikarya were observed in periventricular hypothalamic nucleus (Pe; Fig. 2B), ventral premammilary nucleus (PMV), ARC and peri-ARC (Fig. 2C, E), The latter structure refers to the hypothalamic retrochiasmatic area (RCH) close to the rostral ARC, lateral to the ARC and ventral to the ventromedial hypothalamic nucleus. Based on the intensity of immunofluorescence in perikarya, the expression levels of CART-ir were lower in the ARC, PMV and Pe as compared to LHA, DMH, ZI, and PHA. Dense CART-ir cell bodies also appeared within the nucleus of Edinger-Westphal nucleus (EW; Fig. 2D). In the rostral part of the dorsal raphe nuclei only a few CART-ir perikarya were present (Fig. 2G). CART-ir processes with terminal boutons were distributed throughout the hypothalamus. The

external zone of the ME and ARC was enriched in CART-ir fibers with terminal boutons, whereas the internal zone of ME was sparsely innervated by CART-ir fibers (Fig. 2C, E, F). CART-ir fibers with terminal boutons appeared to a lesser extent in IGL (Fig. 2J) and DR (Fig. 2I), and only a few in the MR. In the SCN, CART-ir fibers were merely absent, whereas the brain structures surrounding the SCN contained few CART-ir fibers with terminal boutons. CART-ir fibers and axon terminals were also observed within the PVT. CART-ir cells exhibited the same shape as MCH-ir cells (Fig. 4C and 4F).

OXB immunoreactivity

OXB-ir perikarya were found exclusively in the hypothalamus. OXB-ir perikarya were most abundant in the LHA and PFA (Fig. 3A). Few OXB-ir cell bodies were also found in the brain structures including DMH (Fig. 3A) and RCH. OXB-ir cell bodies had no more than three processes. OXB-ir axons as well as its collaterals and terminal varicose were distributed with different densities within the examined brain regions (Fig. 3C-K). High density (+++) OXB-ir fibers and varicose terminals were found in the areas including anterior and posterior paraventricular thalamic nucleus (PVA and PVP; Fig. 3G, H), LHA (Fig. 3B), ventral tubermammilary nucleus (VTM; Fig. 3F), DR (Fig. 3J) and MR (Fig. 3K). Moderate density (++) OXB-ir fibers with varicose terminals were present in the RCH, hypothalamic paraventricular nucleus (PVN), lateroventral preoptic area (VLPO), DMH, supramammillary nucleus, lateroanterior hypothalamic nucleus, Pe, median preoptic nucleus (MnPO; Fig. 3C), and IGL (Fig. 3I). The low-density (+) OXB-ir fibers and terminals were observed in ARC, VMH and supraoptic nucleus (SON, Fig. 3D), and very low/absent (-) in the SCN (Fig. 3E). A summary of distribution density of OXB-ir fibers in hypothalamus, thalamus and raphe nuclei is given in Table 1.

MCH and CART dual-labeling immunofluorescence

Dual-labeling immunofluorescence analysis showed that nearly all MCH-ir were co-localized with CART-ir in DMH, ZI and within the medial part of LHA (Fig. 4). In the caudal and lateral aspect of LHA, only a few MCH-ir cell bodies co-expressed CART-ir.

MCH and OXB dual-labeling immunofluorescence

Dual-labeling immunofluorescence analysis of cells immunostained with MCH antibody showed no co-localization with OXB-ir. However, there was close apposition of OXB-ir fibers and varicose terminal boutons on many MCH-ir perikarya located in DMH and the medial and lateral aspects of LHA (Fig. 5D-L). Close apposition of OXB-ir/MCH-ir cell bodies on MCH-ir/OXB-ir cell bodies was also observed (Fig. 5A-C).

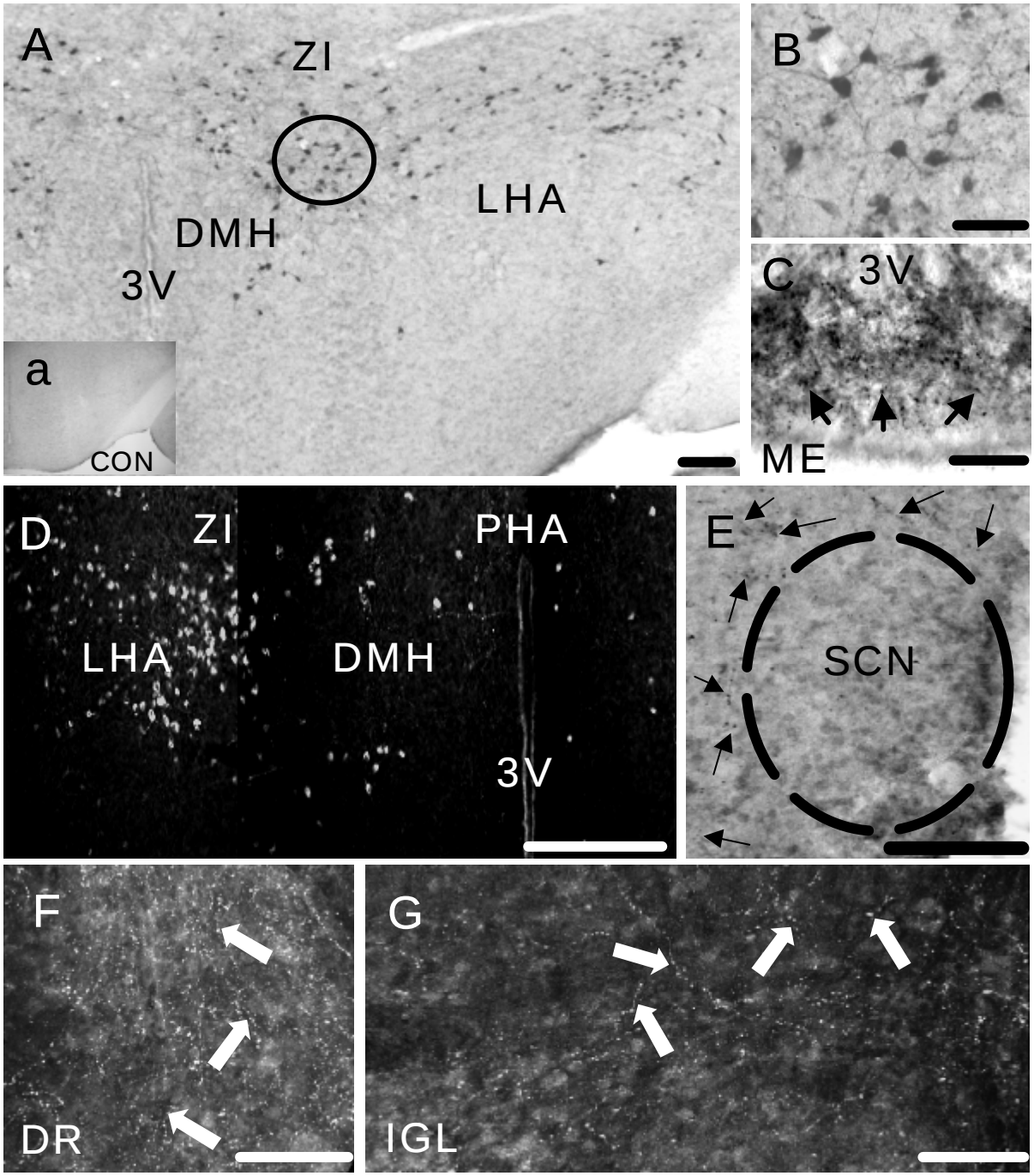

Figure 1. Photomicrographs of MCH-ir cell bodies and fibers in several brain structures of Djungarian hamster. A) Photomicrographs showing MCH-ir perikarya in the LHA, DMH and PHA. a) Image of section after incubation with MCH antibody preadsorbed with peptide showing a total absence of MCH-ir. B) Larger magnification of MCH-ir cell bodies in the outlined area in A. C) Photomicrograph showing the intense staining of MCH-ir only in the internal zone of ME (arrows). D) Immunofluorescence image showing the distribution of MCH-ir cell bodies ín the several hypothalamic nuclei. E) Whereas MCH-ir is absent from the SCN, the surrounding region of the SCN contain MCH-ir fibers and terminals (arrows). F and G) Immunofluorescence images showing MCH-ir fibers with terminal boutons in DR (arrows, F) and IGL (arrows, G). con, control; DMH, dorsomedial hypothalamic nucleus; DR, dorsal raphe nucleus; IGL, intergeniculate leaflet; LHA, lateral hypothalamic area; ME, median eminence; PHA, posterior hypothalamic area; SCN, suprachiasmatic nucleus; ZI, zona incerta; 3V, third ventricle. Scale bar, 100 µm (A-F), 150 µm (G).

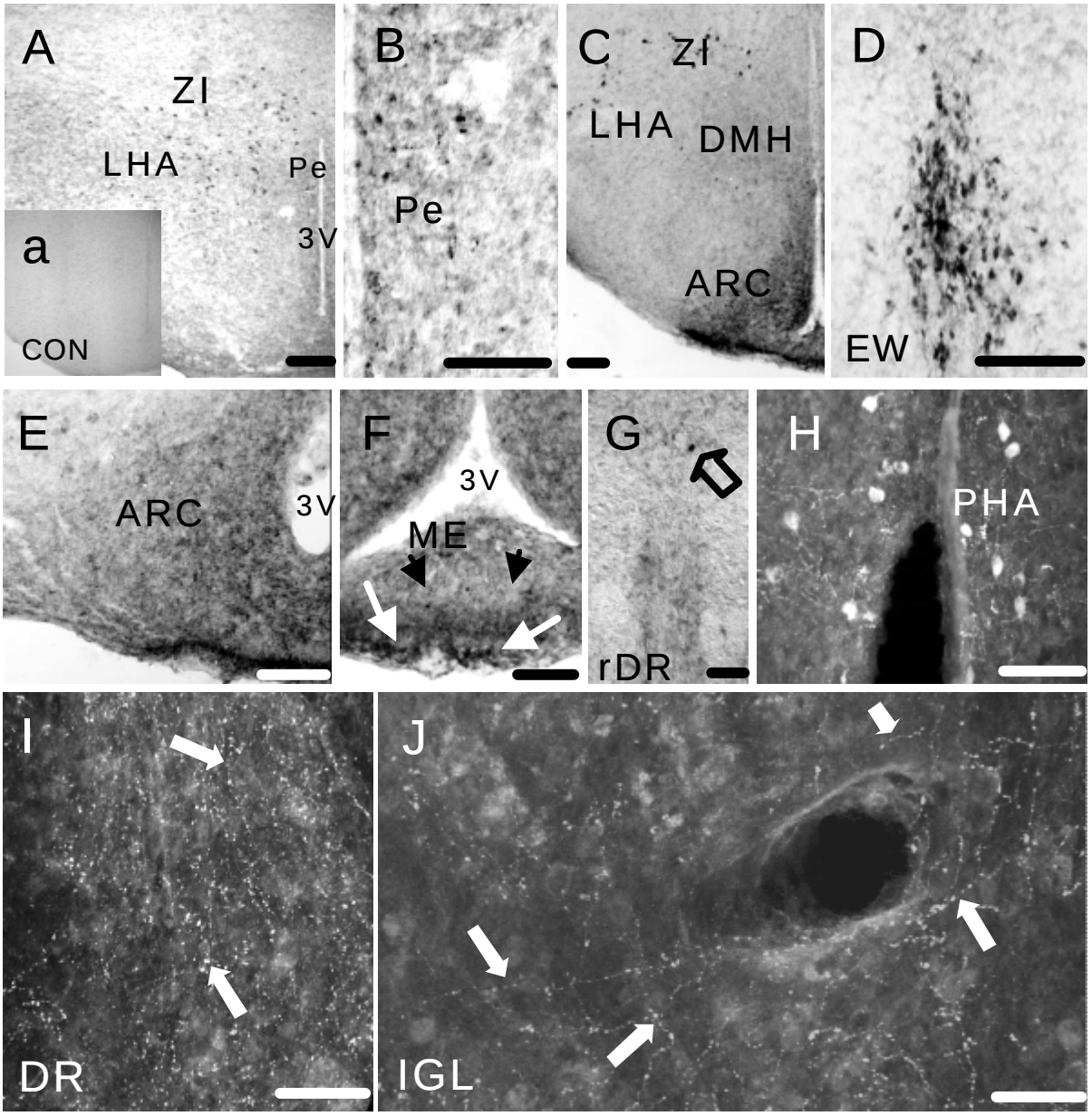

Figure 2. Photomicrographs of CART-ir in cell and fibers of the hypothalamic and extrahypothalamic brain structures of Djungarian hamster. Images showing the distribution of CART-ir in cell bodies of the ARC, Pe, LHA, PHA, DMH, ZI and EW (A-H). a) Image of section after incubation with CART antibody preadsorbed with peptide showing a total absence of CART-ir. E) Photomicrograph of CART-ir in rostral aspect of the ARC. Dense CART-ir fibers with terminal boutons in rostral (E) and caudal aspect (C) of the ARC are shown. F) The external zone of ME showing intens CART staining (White arrows), whereas the internal zone is sparsely innervated by CART-ir (black arrows). G) Open arrow showing a CART-ir cell body in rostal part of the DR. H-J) Immunofluorescence images showing CART-ir cell bodies in PHA (H) and CART-ir fibers with terminal boutons in the DR (I) and IGL (J). ARC, arcuate nucleus; con, control; DMH, dorsomedial hypothalamic nucleus; DR, dorsal raphe nucleus; EW, Edinger-Westphal nucleus; IGL, intergeniculate leaflet; LHA, lateral hypothalamic area; ME, median eminence; Pe, periventricular hypothalamic nucleus; PHA, posterior hypothalamic area; rDR, rostral dorsal raphe nucleus; ZI, zona incerta; 3V, third ventricle. Scale bar, 200 (A), 100 µm (B-F, H), 200 µm (G), 160 µm (I), 40 µm (J).

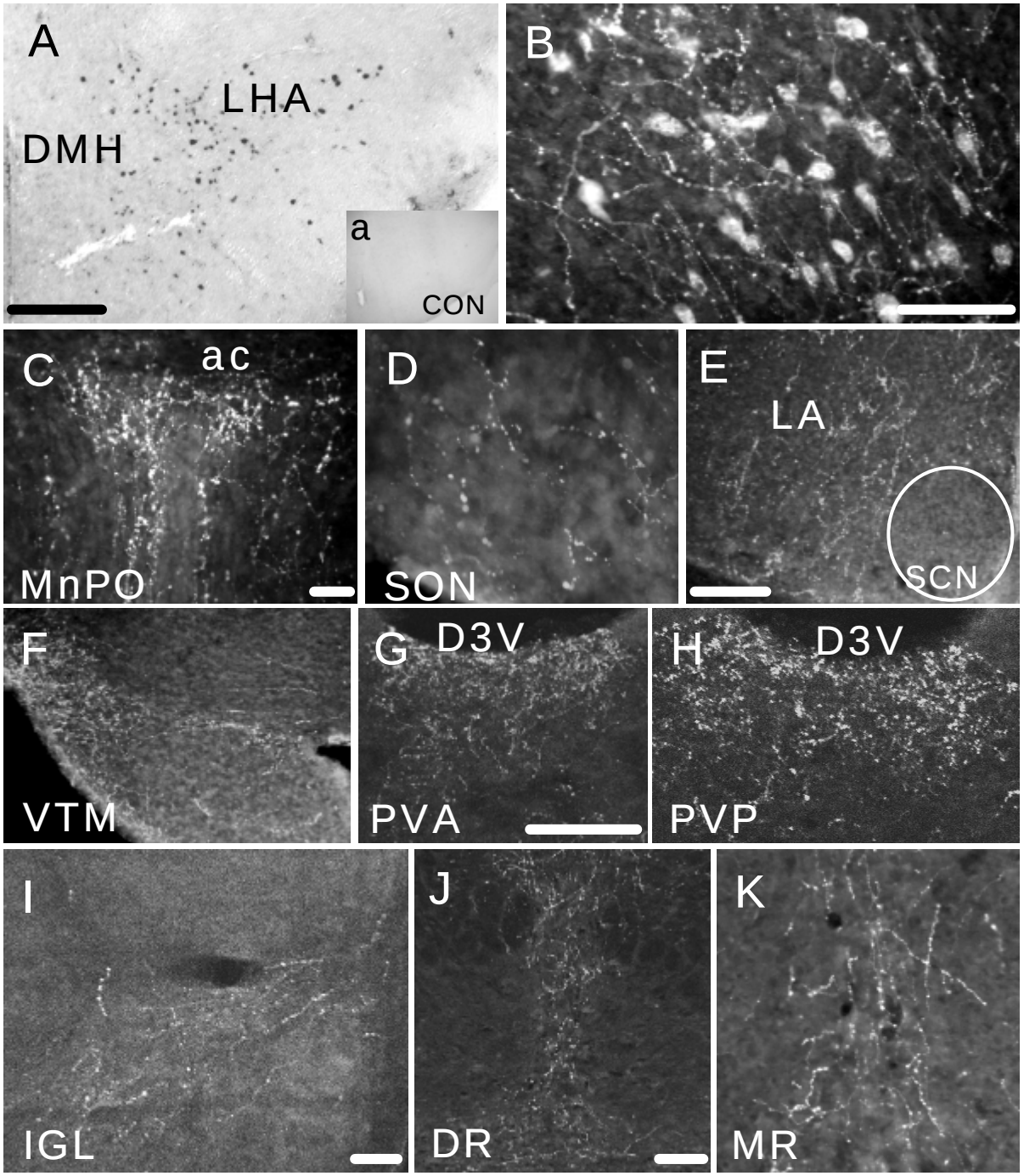

Figure 3. Photomicrographs of OXB-ir perikarya and fibers in the hypothalamic and extrahypothalamic brain structures of Djungarian hamster. A) Distribution of OXB- cell bodies within LHA and DMH are shown. a) Control section showing the absence of OXB-ir after incubation with OXB antibody preadsorbed with peptide. B) Immunofluorescence micrograph of OXB-ir cell bodies within the LHA. Immunofluorescence micrographs of OXB-ir fibers with terminal boutons and varicoses in MnPO (C), SON (D), SCN (E, marked by circle), VTM (F), PVA (G), PVP (H), IGL (I), DR (J) and MR (K). ac; anterior commissure; D3V, dorsal 3V; con, control; DMH, dorsomedial hypothalamic nucleus; DR, dorsal raphe nucleus; IGL, intergeniculate leaflet; LA, lateroanterior hypothalamic nucleus; LHA, lateral hypothalamic area; MnPO, median preoptic nucleus; MR, median raphe nucleus; PVA, paraventricular thalamic nucleus anterior; PVP, paraventricular thalamic nucleus posterior; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus, VTM, ventral tuberomammillary nucleus; 3V, third ventricle; Scale bar in A and B = 100 µm; in C, 50 µm (C, D); in E, 150 µm (E, F); in G, 50 µm (G, H); in I 80 µm, in J, 120 µm (J, K).

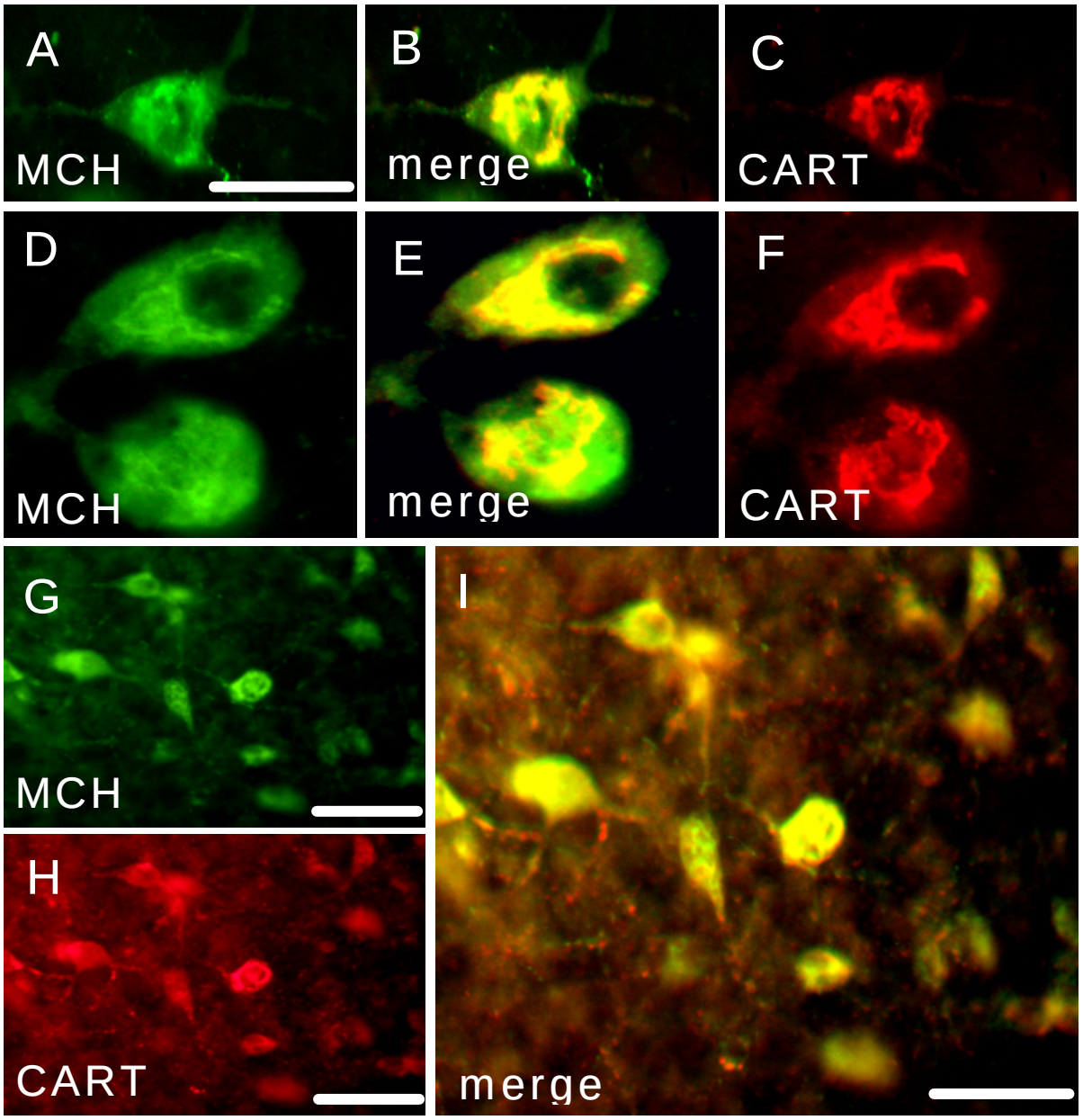

Figure 4. Immunofluorescence micrographs of MCH- (green) and CART-ir (red) in cells with more than three processes (A-C) and in oval form cells (D-F). G, H) Fluoresence micrograph of medial part of the LHA showing the co-localization of MCH-ir with CART-ir. B, E and I are the overlay images of A and C, D and F, and G and H, respectively. Scale bar, in A, 40 µm (A-F); 50 µm (G-I).

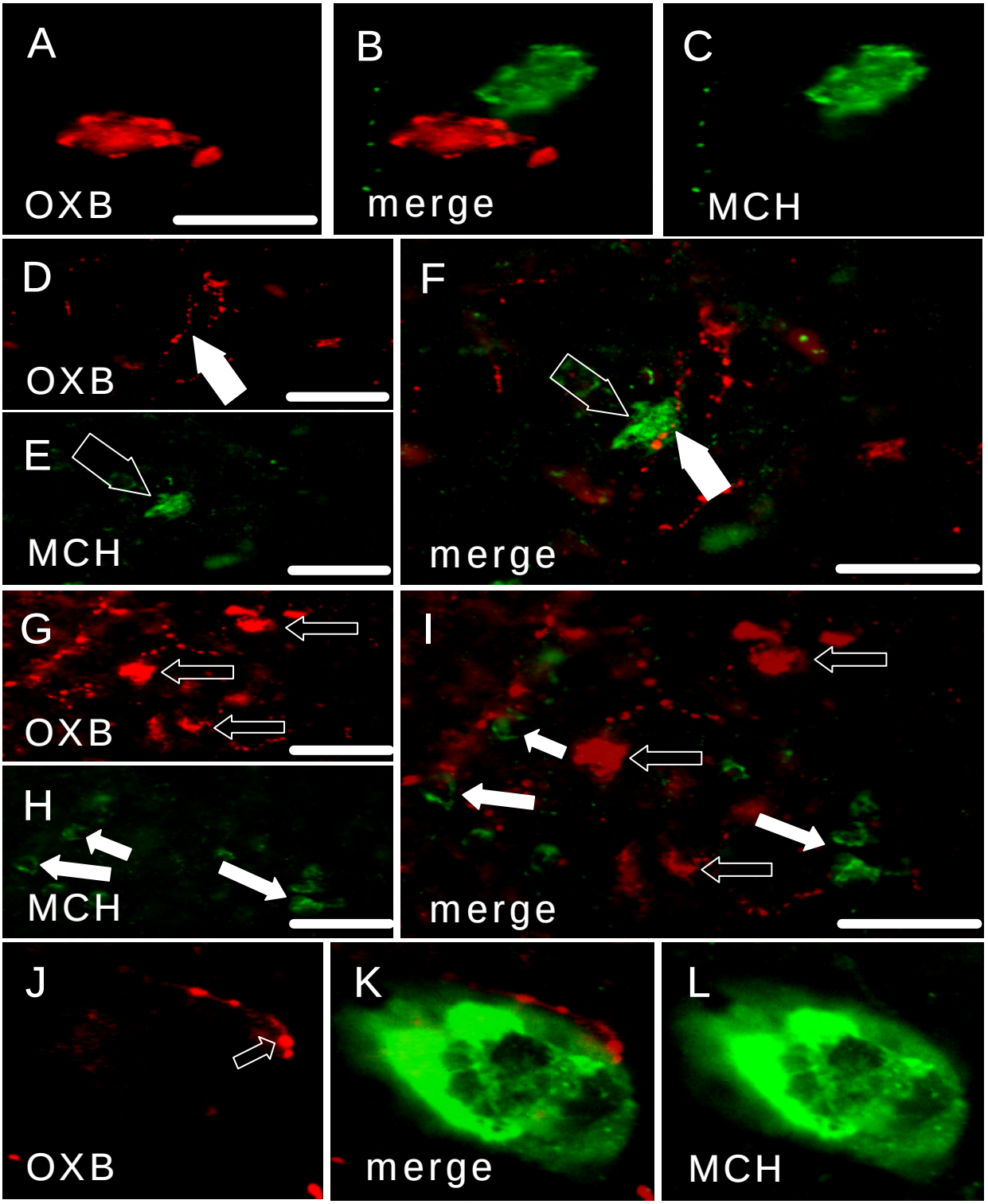

Figure 5. Immunofluorescence micrographs of OXB- (red) and MCH-ir (green) cell bodies and fibers with terminal boutons and varicoses. B) images showing the close contact between OXB-ir (A, red) and MCH-ir (C, green) perikarya. D) OXB-ir fibers with terminal boutons (close arrow) on MCH-ir cell body (E, open arrow). Fluorescence micrograph from the LHA showing no colocalisation between OXB-ir (G, open arrows) and MCH-ir cell bodies (H, close arrows). Fluorescence micrograph showing close apposition of OXB-ir varicose with terminal bouton (J, open arrow) on a MCH-ir cell body (L). B, F, I, and K are the overlay images from A and C, D and E, G and H, and J and L, respectively. Scale bar, in A, 40 µm (A-C, J-L); 100 µm (D-I).

Table 1. Qualitative estimation of the densities of OXB-ir fiber within the brain of Djungarian hamster.

Structure	Relative density
A. Hypothalamus	
Anterior hypothalamic area	++
Arcuate nucleus	+
Dorsomedial nucleus	++
Lateral hypothalamic area	+++
Lateral mammilary nucleus	-
Medial mammilary nucleus	-
Medial preoptic area	++
Medial preoptic nucleus	++
Median preoptic nucleus	++
Paraventricular nucleus,	++
Periventricular nucleus	++
Preoptic nucleus	+
Retrochiasmatic area	++
Suprachiasmatic nucleus	-
Supraoptic nucleus	+
ventral Premammillary nucleus	++
Ventral tuberomammillary nucleus	+++
Ventrolateral preoptic area	++
Ventromedial nucleus	+
B. Thalamus	
Anterodorsal nucleus	-
Anteriventral nucleus	-
Central medial thalamic nucleus	+
intergeniculate leaflet	++
Dorsal lateral geniculate nucleus	-
Lateral habenular nucleus	+
Paraventricular nucleus, Anterior	+++
Paraventricular nucleus, Posterior	+++
Mediodorsal nucleus, central	-
Mediodorsal nucleus, medial	-
Mediodorsal nucleus, lateral	-
Periventricular fiber system	++
Reuniens nucleus	+
Rhomboid nucleus	++
Zona Incerta	++
Ventral lateral geniculate nucleus	-
C. Raphe nuclei	
Dorsal Raphe nucleus	+++
Median Raphe nucleus	+++

Four-point scale; +++, high density; ++, moderate density; +, low density; -, no or very low density

Discussion

The distribution of MCH-, CART- and OXB-ir as well as their neuroanatomical relationships was examined in the Djungarian hamster, a seasonal photoperiodic mammal. We focused on selected brain regions, harbouring neuroendocrine pathways involved in the control of food intake or circadian rhythmicity.

In the current study, MCH-ir perikarya were present in several hypothalamic structures including LHA, ZI, DMH and PHA. A similar distribution pattern of CART-ir perikarya was also observed in these brain regions implicated in the regulation of energy balance. Several lines of evidence suggest that the release of MCH from neurons in the LHA stimulates food intake. Intracerebroventricular (ICV) administration of MCH in rats has been shown to stimulate food intake in a dose dependent manner (Rossi et al. 1997). The stimulatory effect of MCH on food intake was further confirmed in transgenic mice in which over-expression of MCH causes obesity (Ludwig et al. 2001), whereas mice lacking MCH are lean (Shimada et al. 1998). In contrast to MCH, the administration of CART peptides induces an anorectic effect (Kristensen et al. 1998;Stanley et al. 2001), and food deprivation causes decreased CART mRNA expression (Kristensen et al. 1998). In the Djungarian hamster, the exact function of MCH and CART in the neuronal network of the LHA is not clear. Furthermore, the change in photoperiod or food status has been demonstrated to cause no effect on their gene expression in the LHA (Mercer et al. 2000;Mercer and Speakman 2001;Robson et al. 2002). However, the existence of MCH- and CART-ir within the hypothalamic area involved with regulation of energy homeostasis is suggestive of a role for CART and MCH in the regulation of energy balance in Djungarian hamsters.

Using dual-label immunofluorescence, we found that nearly all MCH-ir co-localized with CART-ir within these regions. This is consistent with former rat and human studies (Broberger 1999;Elias et al. 2001). However, a physiological function for such co-expression is unclear. Rats with LHA lesions shift the set point for body weight to a lower level compared to non-lesioned controls (Mitchel and Keesey 1977). Possibly, the balance in expression levels of neuropeptides with antagonistic effects on food intake in selected neurons of the LHA contributes to the determination of a set point for body mass.

In the present study, CART- and MCH-ir projections and axon terminals were found in the PVT that is involved with control of sleep/wake cycle. Since CART-ir fibers are found in many brain regions, then the origins of CART-ir fibers in the PVT remain unknown. However, the presence of CART- and MCH-ir fibers in the PVT suggests a role for CART and MCH in the modulation of sleep/wake cycle. Furthermore, Abrahamson et al., 2001 demonstrated a direct projection from the SCN to MCH and orexin neuronal network in the rat and human hypothalamus, suggesting that the SCN control of sleep/wake cycles occurs, in part, through such a projection (Abrahamson et al. 2001). The co-expression of MCH-ir and CART-ir may also allow the SCN to influence the CART neural network in this area, which possibly innervates the PVT. CART- and MCH-ir fibers were to a lesser extent present in IGL, DR, but merely absent in the SCN. h the rostral part of DR, very few CART-ir cell bodies were present. Whether CART expression is responsible for activation of serotonergic neurons in the DR requires further investigation. Furthermore, *in vitro* studies failed to demonstrate release of serotonin (5-HT) from hypothalamic synaptosomes in response to CART peptide-(55-102) (Orlando et al. 2001). In addition, hypothalamic structures adjacent to the SCN contain MCH- and CART-ir fibers which suggest that CART and MCH may interact with afferent or efferent projections of SCN. However, the distribution of MCH- and CART-ir within neuronal components of the circadian timing system suggests that CART and MCH may indirectly influence circadian rhythms in physiology and behavior generated by the SCN.

Interestingly, we found very dense CART-ir perikarya in the rostro-caudal extension of the EW to concur with findings in the rat (Koylu et al. 1998). The EW contains parasympathetic preganglionic neurons projecting to the ciliary ganglion, which in turn innervates the iris sphincter muscle, ciliary muscle, and smooth muscle of choroidal blood vessels (Gamlin and Reiner 1991). In addition, EW is thought to have a neuronal connection with two neural components of the circadian timing system, SCN and IGL, in the hamster and pigeon (Gamlin et al. 1982;Pickard et al. 2002). The presence of CART-ir within the EW suggests that CART may not only influence the functioning of the eye's internal musculature, but also may interact with the projection conveying the circadian timing information.

In the current study, MCH-, CART-ir fibers and axon terminals were distributed within several hypothalamic brain structures including ME suggesting a role for MCH and

CART in neuropeptidergic regulation of the hypothalamic-pituitary axis. This is supported by the finding that administration of MCH into the ME and MPO stimulates release of luteinising hormone in rats (Gonzalez et al. 1997;Jezova et al. 1992). The highest density of CART-ir fibers were found in the external zone of ME. This indicates the important role for CART as a neuroendocrine-releasing factor that may influence hormonal secretion throughout the pituitary gland (Larsen et al. 2003).

A small number of CART-ir cell bodies were also found in the ARC, but staining intensity was lower than in the other hypothalamic regions. Furthermore, CART-ir cell bodies were not present in either the supraoptic nucleus (SON) or paraventricular hypothalamic nucleus (PVN). The pattern of CART-ir cells distribution overlap the CART-mRNA expression patterns described in the hamster (Robson et al. 2002). However, it is different from findings in the rat, where abundant expression of CART-mRNA and immunoreactivity have been demonstrated (Vrang et al. 1999). This discrepancy may be either due to species differences or methodological aspects, for example, using different CART antibodies and application of colchicine to block axonal transport. In addition, CART gene expression in ARC has been demonstrated to depend on photoperiod with lower levels in hamsters acclimated to long compared to short photoperiods (Adam et al. 2000). Whether the observed small number of CART-ir cell bodies in the ARC is due to the photoperiodic history of the experimental animals is not clear and requires further investigation.

In contrast to MCH- and CART-ir, fibers with varicose terminals immunostained with the OXB antibody were more abundant throughout the examined brain regions. Moreover, the distribution pattern of OXB-ir cell bodies is distinct from the expression pattern of MCH. In support of this observation, dual-label immunofluorescence examination revealed no co-expression of OXB-ir with MCH-ir, but showed a close apposition of OXB-ir fibers with varicose terminals on many MCH-ir cell bodies located in the examined brain regions. The precise function for such connections has not been investigated. However, the presence of neuronal connections between OXB and MCH suggest that OXB may act as a neurotransmitter to regulate the function of MCH containing neurons. Indeed, it has been demonstrated that MCH cell bodies co-express OXR1 in rats (Backberg et al. 2002).

Similar to MCH- and CART-ir, cell bodies and axon terminals immunoreactive for OXB-ir were present within hypothalamic structures implicated in the control of food

intake. This suggests a similar role for OXB in the regulation of feeding behavior for the Djungarian hamster as described in the rat and mouse (Nambu et al. 1999;Sakurai 1999).

In the current study, the dense OXB-ir fibers with axon terminals were found within DR, MR and IGL, whereas in the SCN, OXB-ir was almost absent. Moreover, OXB-ir fibers with axon terminals were also found in brain structures adjacent to the SCN. The presence of OXB-ir fibers with axon terminals within several neuronal components of the circadian timing system is suggestive that OXB may indirectly influence circadian timekeeping processes in the Djungarian hamster. Dense innervations by OXB-ir fibers and axon terminals were also found in the rostro-caudal extension of PVT, TMV and VLPO. The VLPO is involved in sleep regulation and bilateral VLPO lesions have been shown to cause insomnia (Lu et al. 2000). The presence of OXB-ir within these brain regions corresponds with the known involvement of OXB in regulation of the sleep/wake cycle. OXB-ir fibers with terminal varicose were also found in hypothalamic structures including ARC, MPO, MnPO, and PVN suggesting a role for OXB to influence the function of neurosecretory neurons located in these hypothalamic structures.

OXB and OXA are derived from a common pro-orexin precursor (Edwards et al. 1999;Sahu 2002;Sakurai et al. 1998) and their distribution pattern is similar in rats (Peyron et al. 1998). To establish whether Djungarian hamsters, often referred to as Siberian hamsters, also display a similar overlap between OXB and OXA, the distribution patterns of OXB-ir were compared with recent findings on OXA-ir in this species (McGranaghan and Piggins 2001;Peyron et al. 1998). The density of OXB-ir fiber distribution in some examined brain areas was different in relation to OXA-ir (McGranaghan and Piggins 2001). This may be due to the use of different antibodies. In addition, this difference may be also related to different physiological functions of OXB and OXA. For instance, in contrast to the mere absence of OXA-ir fibers in Mnpo, moderate OXB-ir fibers and axon terminals were observed in this structure. This is consistent with a similar finding on OXB-ir in Syrian hamsters (Mintz et al. 2001). Along with several other functions, the Mnpo is involved with the regulation of cardiovascular and autonomic function (O'Neill and Brody 1987), osmotic control of vasopressin secretion and water intake (Yasuda et al. 2000). Therefore, there is a possible role for OXB in the regulation of cardiovascular response, hormonal release

and drinking behavior (Kunii et al. 1999). In the present study, ARC appears to receive fewer OXB-ir fibers as compared to OXA-ir, demonstrated in a previous study of Djungarian hamsters. This finding supports the view that OXA may be a more potent orexigenic neuropeptide than OXB (Lubkin and Stricker-Krongrad 1998). In the present study, few OXB-ir fibers appeared within the SON, in which the absence of OXA-ir fibers has been reported in the Djungarian hamster. Our observation is consistent with a recent report demonstrating the presence of orexin receptors mRNA in the rat SON (Marcus et al. 2001). This could suggest that SON is a target for OXB. The differences in innervation patterns suggest that OXB and OXA may exert different effects within several brain structures.

In conclusion, the presence of MCH-, CART- and OXB-ir cells, fibers and terminal boutons within several brain structures suggests that they may act as neurotransmitter/neuromodulator in a broad range of physiological processes including neuroendocrine pathways related to feeding behavior in Djungarian hamsters. Specifically, the distribution of MCH-, CART- and OXB-ir fibers and terminal boutons within parts of neuronal components of the circadian timing system provides a neuroanatomical basis for their interaction with the circadian timing processes. We suggest that one pathway by which these neuropeptides may influence circadian timing processes could be through interaction with NPY-containing neurons and projections within the IGL and peripheral region of the SCN. However, the role of possible cross-talk between neuronal centers of energy and time management in seasonal photoperiodic acclimation processes is a challenging topic for further research.

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CHAPTER III

Orexin-B interacts with Neuropeptide Y neurons in the Intergeniculate Leaflet and in peripheral part of the Suprachiasmatic Nucleus of Djungarian hamsters (*Phodopus sungorus*)

Abstract

The intergeniculate leaflet (IGL) is part of the neuronal network involved in the regulation of circadian timing processes. The IGL receives photic- and non-photic-inputs from the retina and midbrain and projects this information to the suprachiasmatic nucleus (SCN) through the geniculohypothalamic tract (GHT) arising mostly from neuropeptide Y (NPY) containing neurons in the IGL. Based on the distribution of orexin B (OXB)-ir fibers and terminal boutons in the IGL, we suggest that at least one pathway by which OXB could influence circadian timing is through the interaction with NPY, the most abundant neuropeptide/neurotransmitter in the IGL of the Djungarian hamster. In order to test this hypothesis, we investigated the anatomical basis for such an interaction using dual-label immunofluorescence. OXB-ir fibers with terminals had close apposition on NPY-ir perikarya and fibers with terminal boutons in the rostro-caudal extension of the IGL. Furthermore, NPY-ir fibers in the peripheral structures of the SCN had apposition from OXB-ir fibers and terminals. These observations provide an anatomical basis for OXB to interact with NPY and influence the circadian timing system.

Introduction

The intergeniculate leaflet (IGL) is part of the neuronal network in the circadian timing system receiving photic and non-photic inputs from retina and midbrain. The IGL projects to the suprachiasmatic nucleus (SCN) through the geniculo-hypothalamic tract arising mostly from NPY containing neurons in the IGL. Based on the distribution of orexin B (OXB)-ir fibers and terminals in the IGL, we suggest that at least one pathway by which OXB could influence circadian timing processes is through the interaction with NPY neurons in the IGL. In order to test this hypothesis, the neuroanatomical basis for such an interaction was investigated in the Djungarian hamster using dual-label immunofluorescence.

Material and methods

Tissue preparation

Adult male Djungarian hamsters (*Phodopus sungorus*, n = 3) were housed individually in Macrolon cages under three different light conditions;

1- naturally occurring light (14:10 light:dark cycle, January).
2- long-day light (16:8 light:dark cycle at 23 °C).
3- short-day light (8:16 light:dark cycle, for four weeks at 16°C).

The hamsters had free access to standard rodent chow (Altromin 7014) and fresh water. Hamsters were deeply anaesthetized in a CO_2 atmosphere and killed by decapitation between 10:00 and 11:00 h in the morning. Brains were then carefully removed, fixed in 4% paraformaldehyde (48 h, 4°C) and cryoprotected in 20% sucrose in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 24 hr at 4°C. The brains were then cut on a cryostat into 30 µm coronal sections. Free-floating sections were stored in PBS at 4°C prior to immunohistochemical procedures. All procedures were performed in accordance with German animal welfare regulation.

Single immunostaining

To detect OXB- and NPY-ir by peroxidase reaction, the same procedures were performed as described in chapter II. In brief, the endogenous peroxidase activity was inhibited in sections using 80% PBS, 10% methanol and 10% H_2O_2. Primary antibodies were diluted 1:400 in blocking solution, and the secondary antibody, Peroxidase-conjugated goat anti-rabbit antibody (Jackson Immunoresearch, 111-

035-144), was diluted 1:500 in blocking solution. Using DAB-nickel/Substrate SG (Vector kit) the colour reaction produced dark-gray/blue immunostaining.

Dual labeling immunofluorescence

Dual labeling immunofluorescence was performed on free-floating coronal sections to investigate colocalization of OXB- with NPY-ir. After rinsing in PBS and PBS-TX, sections were pre-incubated in blocking solution containing TBS-TX and 3% BSA at RT. Sections were then incubated with primary rabbit polyclonal NPY antibody (Phoenix Europe GmbH; H-049-03) diluted (1:200) in blocking solution. After washing in PBS-TX, sections were then incubated with Fab conjugated Cy3 goat anti-rabbit antibody (Dianova, 111-165-006) diluted 1:200 in blocking solution for one hour at RT. Sections were rinsed in PBS-TX and incubated with goat Fab-fragment anti-rabbit antibody (Dianova, 111-007-003) diluted 1:50 in blocking solution for one hour at RT. Sections were then washed in PBS-TX and incubated with OXB antibody (Phoenix Europe GmbH; H-003-32) diluted 1:200 in blocking solution. Sections were then washed in PBS-TX and incubated with Alexa-fluor-488 conjugated goat anti-rabbit antibody (Molecular Probes, A-11034) diluted 1:250 in blocking solution. Sections were then rinsed in PBS, mounted onto gelatin-coated slides, air-dried, dehydrated in graded alcohol, cleared in xylene and coverslipped with Enthelan (Merck).

Analysis of single/dual-labeling immunostaining

Sections were analyzed as described in chapter II.

Results

NPY- and OXB-ir

The SCN and its peripheral structures had dense projections immunoreactive for NPY (Fig. 1). NPY-ir cell bodies and fibers were present within the rostro-caudal extension of the IGL (Fig. 1). Several hypothalamic brain structures including arcuate nucleus, dorsomedial hypothalamic nucleus, and lateral hypothalamic area had obviously dense NPY-ir projections (Fig. 1).

The distribution of OXB-ir were similar to that observed in the first experiment (chapter II).

In addition, light conditions had no effect on distribution or density of NPY- and OXB-ir.

Dual labeling immunofluorescence

Dual-labeling immunofluorescence revealed a close association between OXB-ir fibers with terminal and NPY-ir cell bodies and fibers in the IGL (Fig. 2). In the peripheral regions of the SCN, OXB-ir fibers had apposition on NPY-ir fibers (Fig 3).

Conclusion

The present study provides neuroanatomical basis for a possible interaction between OXB and NPY within the IGL. This may create a link allowing OXB to indirectly influence the generation of circadian rhythmicity.

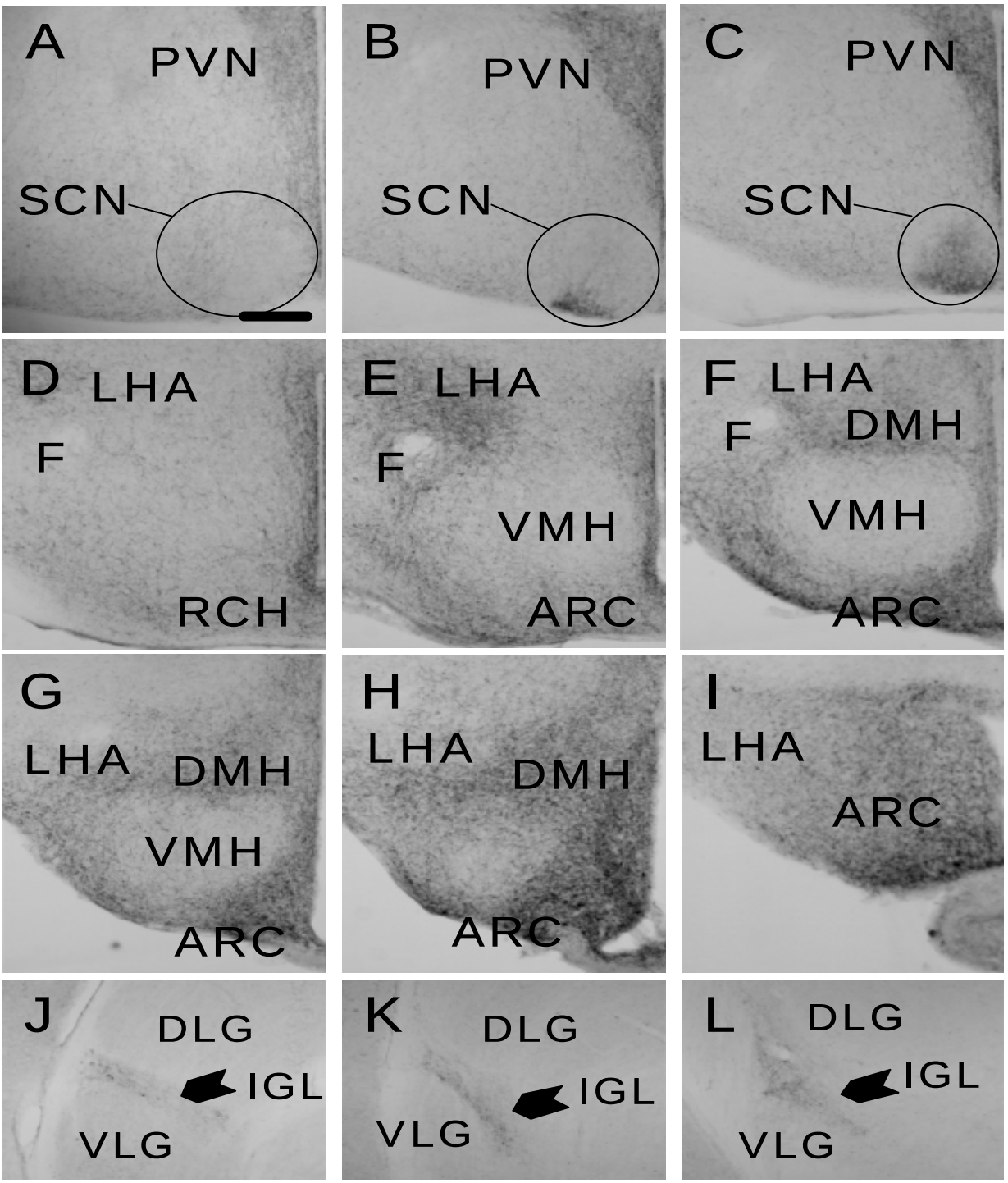

Figure 1. Images of sections immunostained with NPY antibody. (A-I) Distribution of NPY-ir fibers in the rostro-caudal extension of hypothalamus. (J-L) NPY-ir cell bodies and fibers in the IGL (arrow). ARC, arcuate nucleus; DLG, dorsal lateral geniculate nucleus; DMH, dorsomedial hypothalamic nucleus; F, fornix; IGL, intergeniculate leaflet; LHA, lateral hypothalamic area; RCH, retrochiasmatic nucleus; SCN, suprachiasmatic nucleus; VLG, ventral lateral geniculate nucleus; VMH, ventromedial hypothalamic nucleus;.3V, third ventricle. Scale bar in A is 400 µm for A-I, in A is 200 µm for J-L.

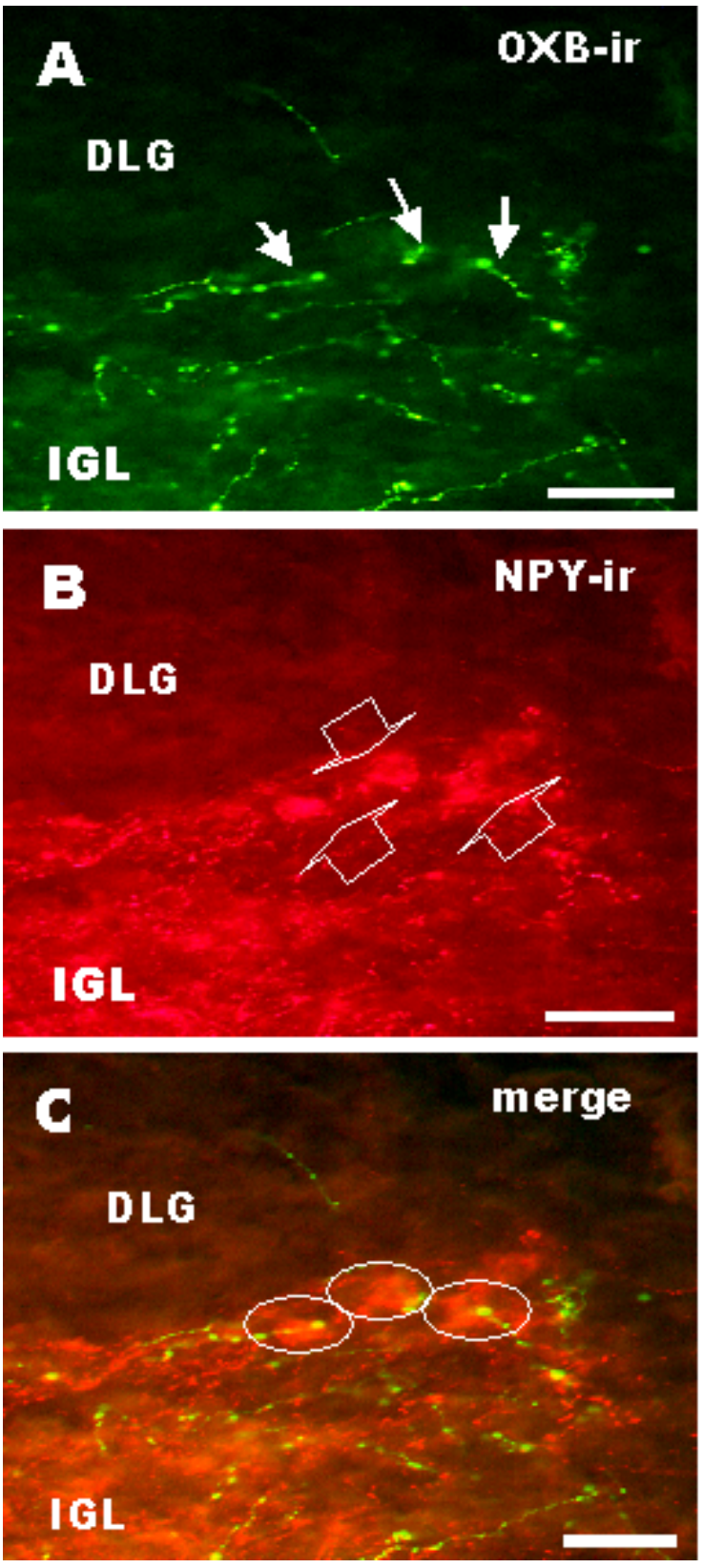

Figure 2. Fluorescence images of brain section immunostained with OXB- (A) and NPY- antibodies (B). Close apposition of OXB-ir fibers and terminals (close arrows, A) on NPY-ir neurons (open arrows, B) in the IGL. C) Overlay picture from A and B, showing close association (circle, yellow). DLG, dorsal lateral geniculate nucleus; IGL, intergeniculate leaflet. Scale bar = 40 µm

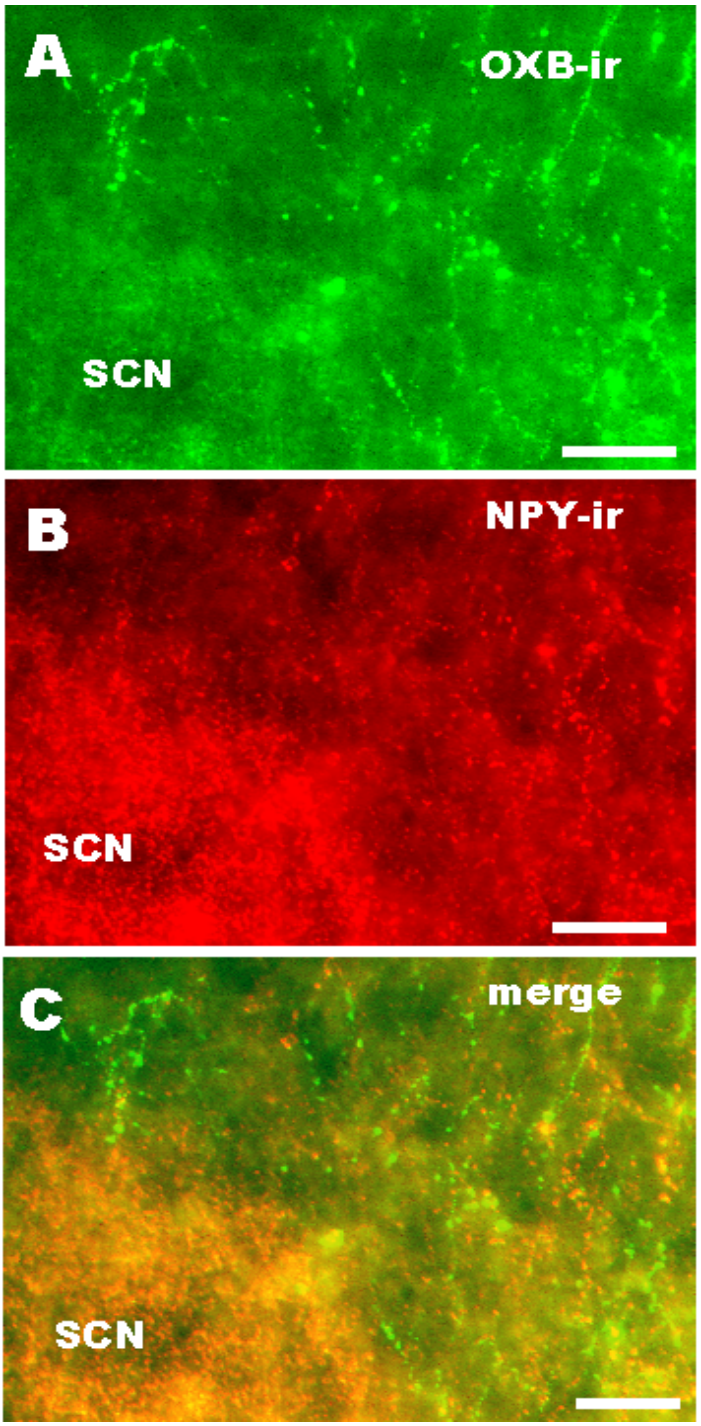

Figure 3. Fluorescence images of brain section immuno-stained with OXB- (A) and NPY- antibodies (B). A) whereas OB-ir fibers are absent from the SCN, the peripheral zone of the SCN contain OXB-ir. B) The SCN contains dense NPY-ir fibers. NPY-ir fibers are also present in the peripheral zone the SCN. Close apposition of OXB-ir fibers and terminals (A) on NPY-ir fibers (B) in the peripheral area of the SCN. C) Overlay picture from A and B, showing close association (yellow). SCN, suprachiasmatic nucleus. Scale bar = 40 µm

CHAPTER IV

CART neuronal system in the rostral arcuate nucleus mediates seasonal regulation of energy balance in the Djungarian hamster (Phodopus sungorus).

Abstract

In seasonal mammals, the hypothalamic neuropeptidergic system involved in photoperiodic control of seasonal cycles of body mass and food intake is poorly understood. In the present study, we investigated whether distribution and number of hypothalamic neuronal cell populations containing cocaine-amphetamine-related transcript (CART)- and adrenocorticotropic hormone (ACTH)-ir were influenced by seasonal acclimation and feeding status. Djungarian hamsters bred and reared in long-days (LD; at 23°C) were transferred to short-day photoperiod (SD; at 16°C). After four weeks in SD, uterus and body weight were decreased compared to controls maintained in LD. The number of CART-ir cells within the arcuate nucleus (ARC) was higher in SD hamsters compared with LD. The increase in CART-ir cell numbers observed in SD hamsters was restricted to the rostro to mid portion of the ARC; specifically, in the hypothalamic retrochiasmatic area close to the rostral part of the ARC and in the hypothalamic region lateral to the ARC and ventral to the ventromedial hypothalamic nucleus. In similar hypothalamic regions, food deprivation for 48 h decreased the number of CART-ir in SD hamsters. Seasonal acclimation and food deprivation had no effect on the number of CART-ir cells in the lateral hypothalamic area. The number of ACTH-ir cells in four representative and comparable sections in the rostro-caudal extension of the ARC were lower in the SD vs. LD hamsters. However, no differences in the number of ACTH-ir cells were observed in the entire rostro-caudal extension of the ARC. Similarly, food deprivation did not influence the number of ACTH-ir cells within the ARC. These findings indicate that the CART neuronal system within the rostral portion of the ARC may mediate seasonal and compensatory regulation of energy balance.

Introduction

Djungarian hamsters (*Phodopus sungorus*) exhibit seasonal adaptation in physiology and behaviour. The most prominent seasonal adaptations are changes in body weight, metabolism, pelage colour and reproductive status (Hoffmann 1973;Morgan et al. 2003). Hamsters transferred from long-photoperiod (LD) to short-photoperiod (SD) display lower food intake, body fat content and serum leptin (Adam et al. 2000Klingenspor et al. 2000;Mercer et al. 2000;Mercer and Speakman 2001). Moreover, shortening of photoperiod causes impairment of reproductive activities and triggers moulting to a winter pelage (Lerchl and Schlatt 1993). Imposed food restriction during SD induces additional body mass decline, which after re-feeding returns the hamsters to a seasonally programmed "sliding set-point" similar to SD control hamsters fed *ad lib* (Steinlechner *et al.* 1983). These remarkable observations in hamsters emphasize the importance of photoperiod in the regulation of energy homeostasis. Changes in photoperiod result in variations of pineal melatonin secretion, that influences central regulatory mechanisms controlling energy balance (Morgan et al. 2003). In non-photoperiodic animals, the adipose tissue derived hormone, leptin, has been considered as a potential input controlling neuroendocrine pathway involved in body weight regulation. Leptin acts in the central nervous system to activate catabolic pathways.

In both photoperiodic and non-photoperiodic animals, the hypothalamus mediates the action of centrally and peripherally derived signals. It consists of several neuropeptides and hormones acting within the central nervous system to affect energy metabolism. Acumulating evidence demonstrates that changes in photoperiod regulates hypothalamic gene expression for several hypothalamic neuropeptides (Mercer et al. 2000;Mercer and Speakman 2001). In response to SD, the expression of amphetamine-regulated transcript (CART)- mRNA in the hamster's hypothalamic arcuate nucleus (ARC) is elevated, whereas gene expression is reduced for pro-opiomelanocortin (POMC) and agouti-related peptide (AGRP) (Mercer et al. 2000;Mercer and Speakman 2001). Interestingly, CART and POMC coexist in a large proportion of neurons in the ARC that also express the leptin-receptor (Meister 2000). Leptin treatment in rats induces cellular activity within POMC/CART neurons of the ARC (Elias *et al.* 1998). Expression levels of CART- and POMC-mRNA in fasted rats and leptin deficient ob/ob mice is reduced, which after leptin administration returns to normal levels (Schwartz et al. 1997;Vrang et al. 1999a) .

In contrast to POMC, the expression of CART peptide and mRNA is also found in neurons of the lateral hypothalamic area (LHA) and the paraventricular hypothalamic nucleus (PVN) (Kristensen et al. 1998;Robson et al. 2002;Vrang et al. 1999a). The function of CART seems to be inhibition of food intake. Intracerebroventricular administration of CART induces cellular activity within several brain structures involved in the regulation of energy balance and causes inhibition of food intake in fasted mice (Thim et al. 1998;Vrang et al. 1999b;Vrang et al. 2000). Nevertheless, it was recently demonstrated that injection of CART (55-102) directly into discrete hypothalamic nuclei acutely stimulates food intake proposing an orexigenic function for CART (Abbott *et al.* 2001). In addition, CART is co-localized with melanin concentrating hormone (MCH), an orexigenic neuropeptide, in neurons of the LHA (Elias *et al.* 2001). These findings suggest that CART may as well act as an orexigenic neruopeptide. At present, the function of CART therefore remains controversial, although most findings support an anorexigenic function.

In addition to the ARC, POMC gene expression is also found in the pituitary gland. POMC is cleaved by prohormone convertase 1 (PC1) to generate several peptides including adrenocorticotropic hormone (ACTH) and ß-lipotrophin (Benjannet et al. 1991;Friedman et al. 1994;Thomas et al. 1991). Synthesis and release of ACTH in the anterior pituitary gland is regulated predominantly by corticotrophin-releasing hormone (CRH), known as an anorexigenic neuropeptide, synthesized in neurons of the paraventricular hypothalamic nucleus (PVN). ACTH stimulates the secretion of glucocorticoids from the adrenal cortex, which in turn inhibits CRH synthesis.

In the hypothalamus, ACTH-ir cell bodies are localised within the ARC and adjacent structures (Knigge *et al.* 1981). Moreover, a role for ACTH in regulating both neuroendocrine and autonomic activities has been suggested based on the presence of ACTH-ir fibers in the rat's PVN as well as neuroanatomical relationships with vasopressin-, oxytocin- and CRF-containing neurons within hypothalamic structures (Liposits et al. 1988;Piekut 1985). In addition, ACTH is further processed by prohormone convertase 2 (PC2) into alfa-melanocyte-stimulating hormone (alfa-MSH), known as an anorexigenic neuropeptide that acts on melanocortin receptors (MCR3-MCR4) to antagonize the effect of AGRP (Ollmann et al. 1997;Tatro 1996;Yang et al. 1999). Therefore, ACTH may play an important role within the neuroendocrine system either by direct interaction with the PVN neurons or indirectly as a precursor for a-MSH.

Recent advances in understanding the involvement of neuropeptides in the photoperiodic regulation of energy balance has emerged from studies demonstrating altered gene expression of neuropeptides. However in the hamster, gene expression profiles for several neuropeptides have either been controversial or unexpected. For instance, there have been controversial findings concerning gene expression profiles of CART in response to changes in photoperiod. In contrast to studies demonstrating no effect of changes in photoperiod on CART-mRNA expression (Robson *et al.* 2002), there is evidence demonstrating increased CART-mRNA expression in the ARC in response to SD (Mercer et al. 2003;Mercer and Tups 2003). In addition, POMC gene expression is reduced during SD (Mercer *et al.* 2000), an unexpected finding on the basis of its anorexigenic function. However, investigating the expression of CART and POMC- derived peptide (ACTH) in response to seasonal acclimation elucidates the central regulatory function of these neuropeptides on neural mechanisms controlling feeding behaviour. The present study aimed to investigate distribution and number of hypothalamic neuronal cell populations containing CART- and ACTH-ir in hamsters exposed to different photoperiod and feeding status.

Material and Methods

Animals and tissue preparation

Female Djungarian hamsters were housed individually in Macrolon cages, and reared in LD (16-h light, 8-h dark cycle, at 23°C). All hamsters had free access to standard rodent chow (Altromin 7014) and water. At seven months of age, hamsters were divided in two groups consisting of ten each. Group one hamsters were kept in LD, whereas group two were transferred to SD (8-h light, 16-h dark cycle) at 16°C. The lower ambient temperature was chosen in order to accelerate acclimation to declining photoperiod (Elliott et al. 1987;Ruf et al. 1993). Body weight of hamsters was measured at weekly intervals. After four weeks, half of the hamsters in each group were fasted for a period of 48-h. At the end of the experiment, pelage colour and body weight were assessed and all hamsters were deeply anaesthetized in a CO_2 atmosphere and killed by decapitation between 10:00 and 14:00 h. The uterus was rapidly removed and weighed. Brains were excised, fixed in 4% paraformaldehyde (48 h, 4°C), cryoprotected in 20% sucrose in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 24 hr at 4°C and cut on a cryostat into 30 µm coronal sections.

Free-floating sections were stored in PBS at 4°C prior to immunohistochemical procedures. All procedures were performed in accordance with German animal welfare regulation.

Immunostaining

The endogenous peroxidase activity was inhibited in sections using 80% PBS, 10% methanol and 10% H_2O_2 for 15 min at room temperature (RT). Free-floating sections were rinsed in PBS and in PBS containing 0.5% Triton-X 100 (PBS-TX). Following pre-incubation in a blocking solution containing PBS-TX and 3% BSA, sections were incubated with primary rabbit anti- CART (55-102; Phoenix Europe GmbH; H-003-62) and primary rabbit anti- ACTH (Phoenix Europe GmbH; H-001-21) antibodies each diluted 1:350 in blocking solution overnight at 4°C. Following washing in PBS-TX, sections were then incubated with peroxidase-conjugated goat anti-rabbit antibody (Jackson Immunoresearch, 111-035-144) diluted 1:500 in blocking solution for 1 hr at RT. Using substrate SG (Vector kit) the color reaction resulted in dark-gray/blue immunostaining. Sections were then rinsed in PBS, mounted on gelatin-coated slides, air dried, dehydrated in graded alcohol, cleared in xylene and coverslipped with Enthelan (Merck).

The specificity of primary antibodies was tested by adding an excess of CART- (Phoenix Europe GmbH; 003-62) and ACTH- (Phoenix Europe GmbH; 001-21) peptides to primary antibodies for 3 hrs at RT before application to sections, or by omission of the primary antibodies. Brain sections incubated either with preadsorbed primary antisera or in the absence of primary antibodies did not exhibit any CART- or ACTH-ir (data not shown).

Counting of cells and statistical analysis

Sections from each brain were arranged in the rostro-caudal extension according to Paxinos Atlas of Rat brain (Paxinos and Watson 1998). CART- and ACTH-ir cell bodies in the ARC and peri-ARC, referring to the hypothalamic retrochiasmatic area (RCH) close to the rostral ARC, lateral to the ARC and ventral to the ventromedial hypothalamic nuclei (VMH) were counted by two students blinded to the experimental protocol, using a Zeiss microscope (objective, 20X). The number of CART-ir cells in the LHA were counted in a similar manner. The mean values of both countings were calculated and either presented as the mean of the total number of cells found in 7-8

sections or the number of cells in four representative hypothalamic sections at levels corresponding to the bregma –1.8, -2.5, -3.3, and -4.1 mm (Paxinos and Watson 1998). Data were analyzed by ANOVA using SPSS 11.5 statistical software. Data are presented as means ± SEM, and differences between groups were considered significant at $p = 0.05$.

Result

Body and uterus weight

After four weeks, body weight in SD hamsters at 16°C had decreased by 5% compared to hamsters maintained in LD (Fig.1A). Food deprivation for 48-h resulted in approximately 15% body weight reduction in either group (Fig. 1B). The weights of uteri decreased significantly in SD hamsters and food restriction induced additional weight reduction (Fig. 1C). At the end of the experiment, no change was detected in the pelage colour of hamsters exposed to SD.

CART-ir and ACTH-ir

CART-ir cells and fibers were distributed throughout the hypothalamus. In addition to the ARC, the peri-ARC was found to contain small CART-ir cell bodies (Fig. 2). Small and low density CART-ir cells in the hypothalamus were also detected in the periventricular nucleus (Pe) and ventral premammillary nucleus. However, the densest and largest CART-ir cells in the hypothalamus were observed in the zona incerta (ZI), dorsomedial hypothalamic nucleus (DMH), and LHA (Fig. 2). The densest staining of fibers and axon terminals immunoreactive for CART was found in the external zone of median eminence (ME).

ACTH-ir cells were found in the hypothalamic ARC and peri-ARC (Fig. 2). Very few ACTH-ir cells appeared within VMH (Fig. 2). The fibers and axon terminals immunoreactive for ACTH were found with variable densities throughout the hypothalamus (Fig. 2). The pattern of ACTH-ir was similar to that described for CART-ir with a few differences. In the ARC and peri-ARC regions, the number of ACTH-ir cells were more abundant than cells immunoreactive for CART. The densest staining of fibers and axon terminals immunoreactive for ACTH was found in the PVN, ARC, DMH, Pe, and LHA (Fig. 2), whereas ACTH-ir fibers and terminals were almost absent from the VMH, the external zone of ME and the SCN (Fig. 2).

The effect of seasonal acclimation and food status on CART-ir

Seasonal acclimation resulted in a two-fold increase in the number of CART-ir cells in the ARC and in peri-ARC (Fig. 3). Further analysis resolving the regional distribution of CART-ir cells in the rostro-caudal extension revealed that increased cell numbers observed in SD hamsters was restricted to the rostro to mid portion of the ARC, specifically the peri-ARC (Fig. 4). In seasonal acclimated hamsters, the number of CART-ir cells in this region was 2-3 fold higher compared to LD, whereas similar numbers were counted in the caudal extension of the ARC (Fig. 4).

In SD hamsters fasted for 48 h, the mean number of CART-ir cells in the ARC and peri-ARC decreased significantly compared to SD hamsters fed *ad lib*. (Fig. 4). In fasted SD hamsters the number of CART-ir cells decreased to the level observed in LD hamsters either fed *ad lib*. or fasted (Fig. 4). Furthermore, the decrease in cell numbers observed in fasted SD hamsters was restricted to the rostro to mid portion of the ARC, specifically the peri-ARC (Fig. 4). Food deprivation had no effect on the number of CART-ir cells in LD hamsters.

In the LHA, no significant difference in the number of CART-ir cells was detected between hamsters fed *ad lib*. or fasted in LD and SD (Fig. 5).

The effect of seasonal acclimation and food status on ACTH-ir

As a result of acclimation no difference in the number of ACTH-ir cells was observed in the entire rostro-caudal extension of the ARC (Fig. 6A). However, a decrease in the ACTH-ir cells in SD hamsters was detectable when measuring the number of cells immunoreactive for ACTH counted in four sections arranged from rostral to caudal extension of ARC (Fig. 6B). These sections were approximately in the same level as those immunostained with CART antibody which are shown in Fig. 4. Food deprivation did not influence the number of ACTH-ir cells (Fig. 6A and B).

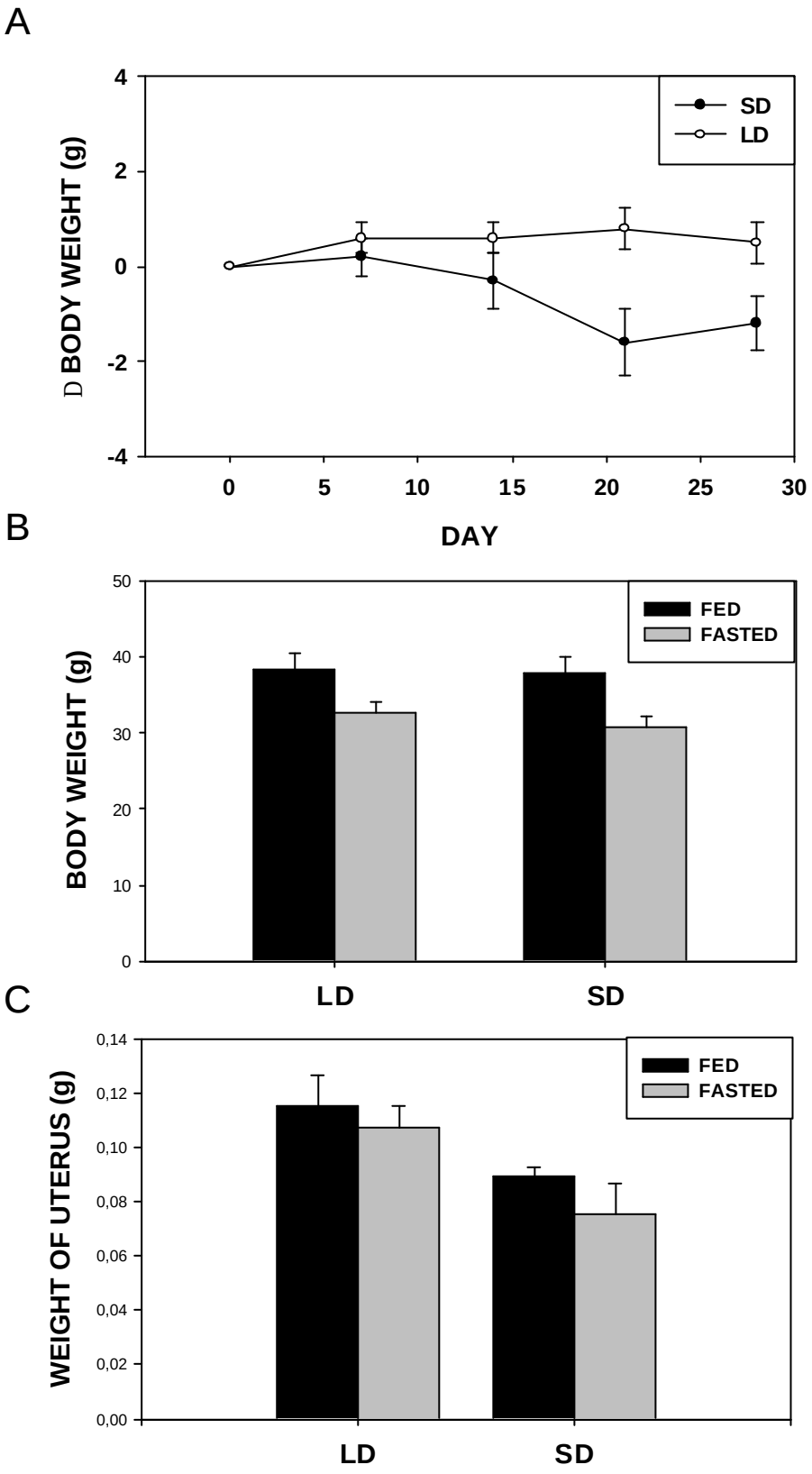

Figure 1. Body weight (A and B) and uterus weight (C) in adult female Djungarian hamsters transferred to SD at 16°C at day 0 or maintained in LD at 23°C. Data are presented as means ± SEM.

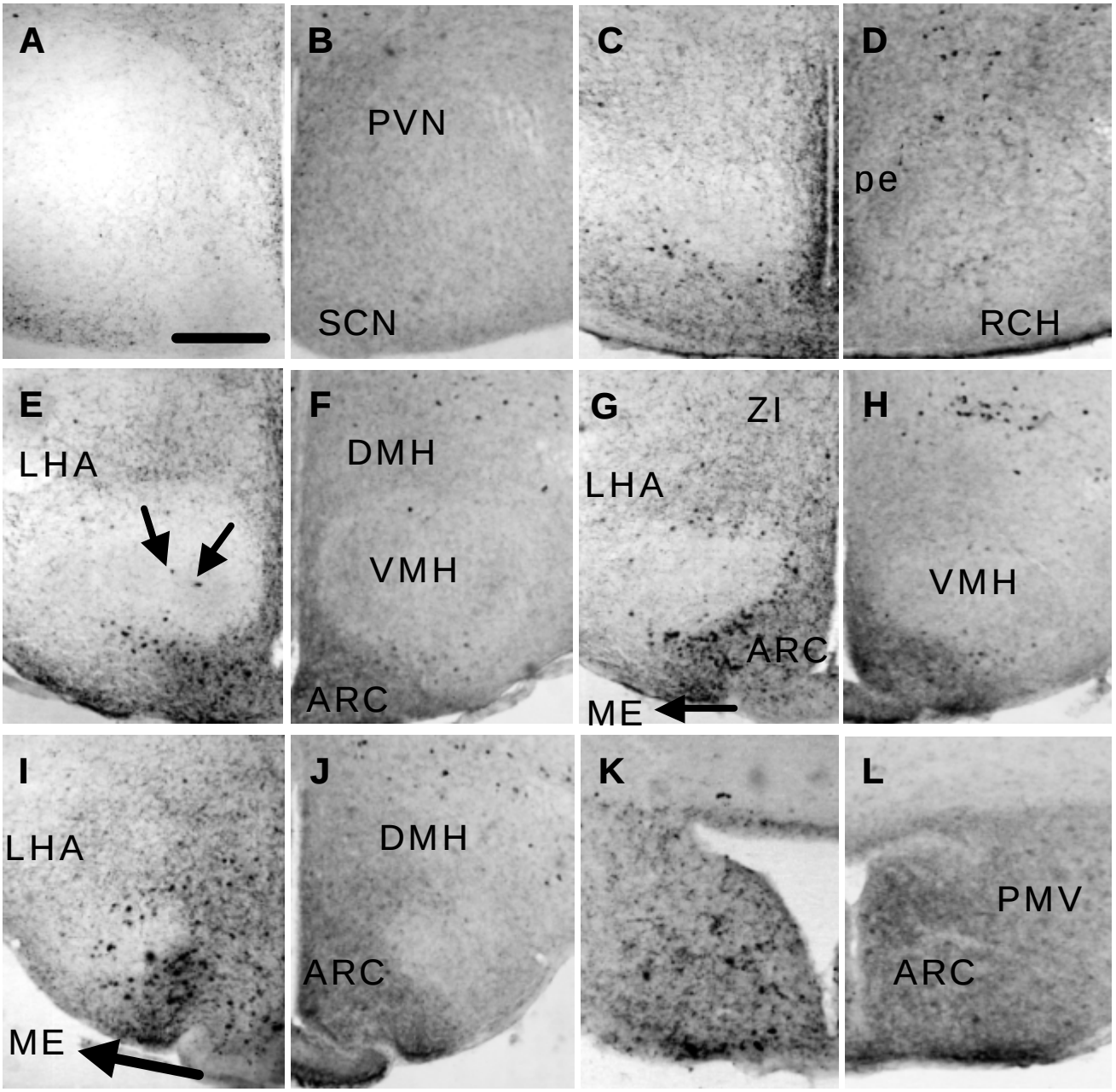

Figure 2. Distribution of CART- and ACTH-ir in the Djungarian hamster hypothalamus. Expression of ACTH-ir (A, C, E, G, I, K) is compared with CART-ir (B, D, F, H, J, L). Adjacent sections represent similar brain regions of each individual hamster. Sections immunostained with ACTH- and CART antibodies are organized from rostral (A) to caudal (L). Arrows in E showing ACTH-ir cells in the VMH. Note that the distribution pattern of ACTH-ir in the RCH and ARC is similar to CART-ir. G and I) In contrast to CART-ir, fibers and axon terminals stained with ACTH antibody are merely absent from external zone of ME. Arrows in G and I showing the ME. ARC, arcuate nucleus; LHA, lateral hypothalamic area; Pe, periventricular nucleus; PMV, ventral premammillary nucleus; PVN, paraventricular nucleus of the hypothalamus; RCH, retrochiasmatic area; SCN, suprachiasmatic nucleus; ZI, zona incerta. Scale bar = 300 µm.

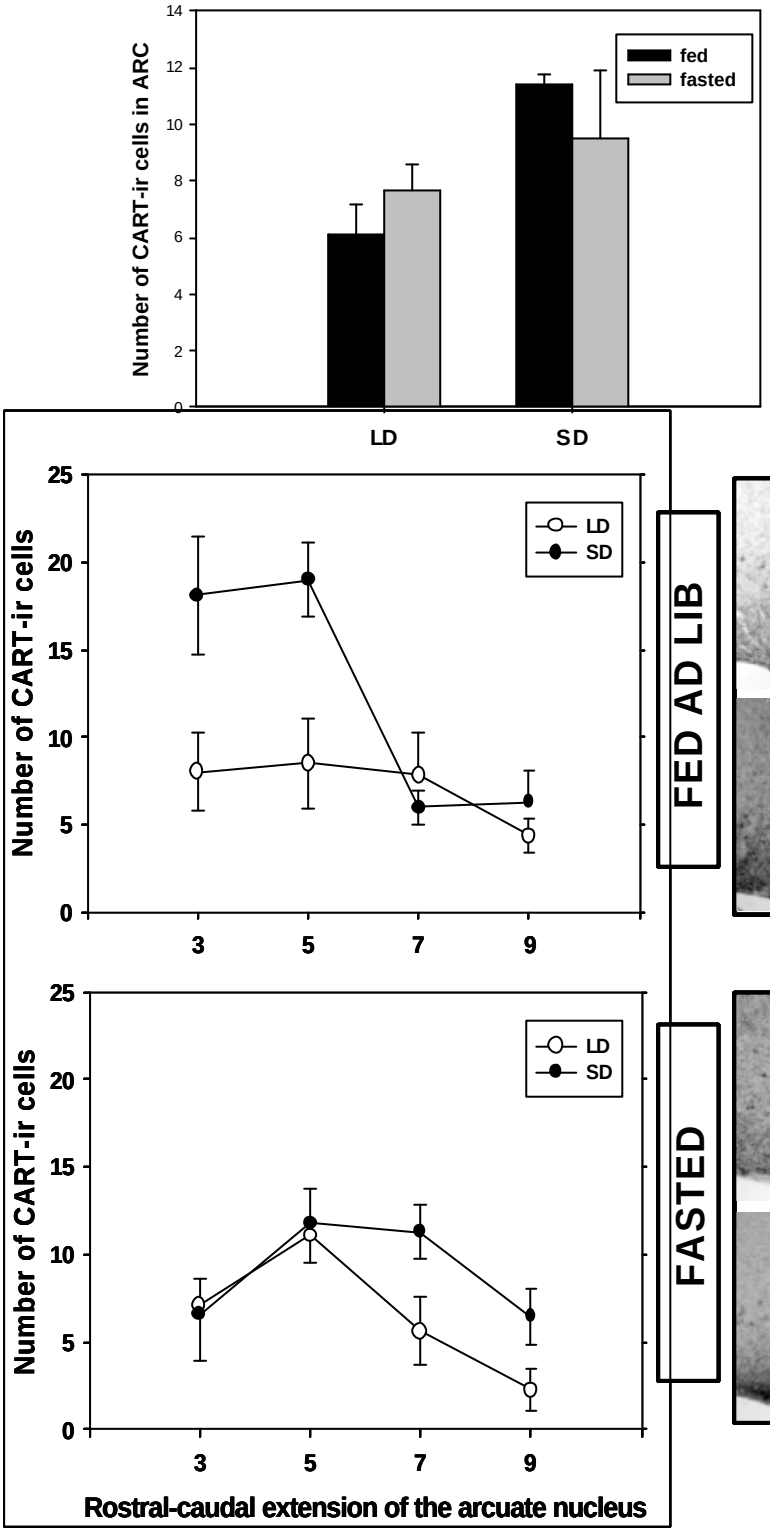

Figure 3. Quantitative analysis of the number of CART-ir cells in the entire rostro-caudal extension of the ARC. Data are presented as means ± SEM.

Figure 4. Quantitative analysis of the number of CART-ir cells (left) and photomicrographs of CART-ir (right) in the arcuate nucleus of LD- and SD-hamsters either fed ad lib or fasted for a period of 48 hrs. Right images) Arrows showing CART-ir cell bodies that are mainly present in rostral part of the ARC in SD fed ad lib hamsters. Numbers (3, 5, 7, and 9) each highlighting a representative and comparable sections in the rostro (3) to caudal (9) extension of the arcuate nucleus. Data are presented as means ± SEM.

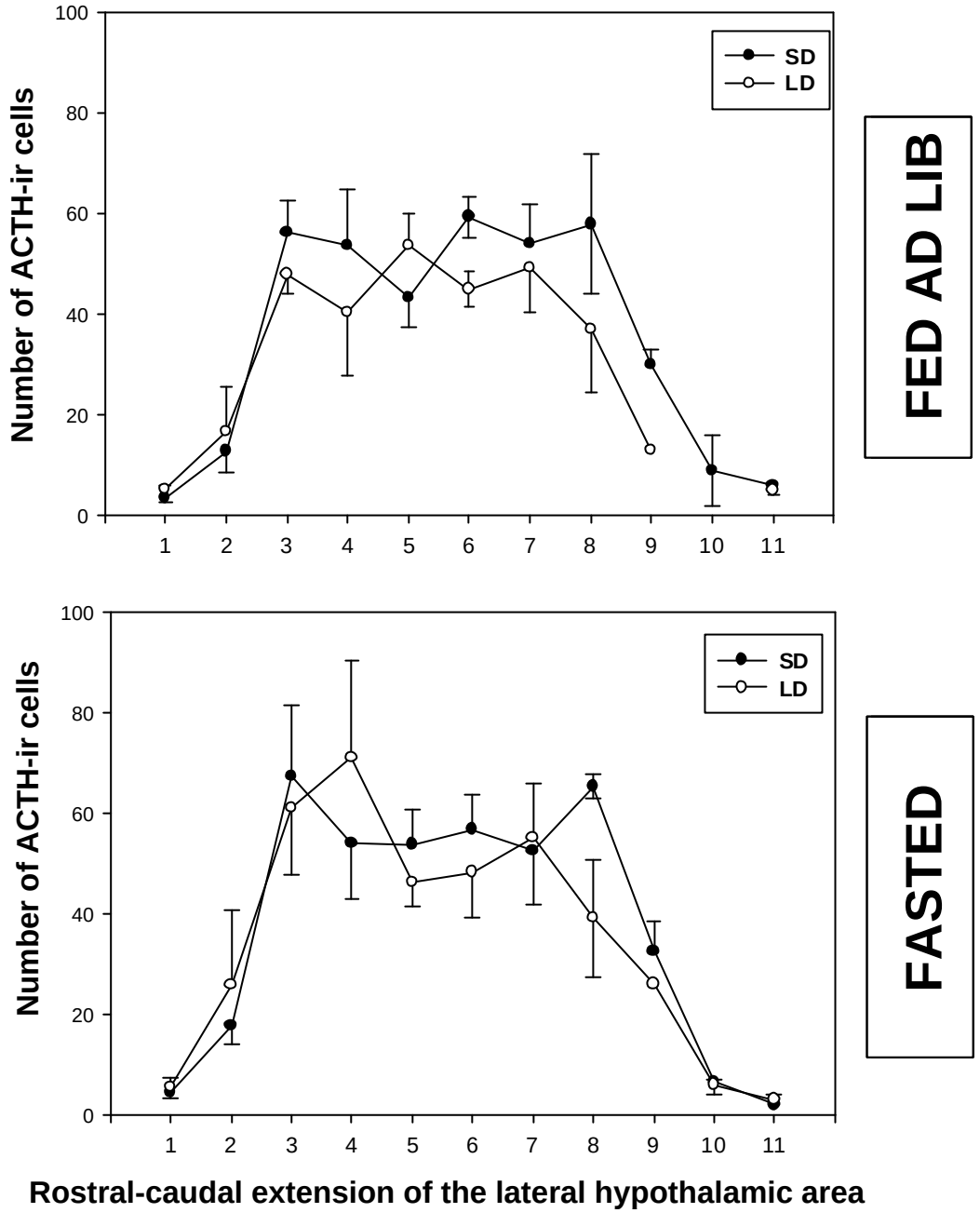

Figure 5. Quantitative analysis of the number of CART-ir cells in the lateral hypothalamic area of LD- and SD-hamsters either fed ad lib (top) or fasted (bottom) for a period of 48 hrs. Numbers (1-11) representing the rostro (1) to caudal (11) extension of the lateral hypothalamic area. Data are presented as means ± SEM.

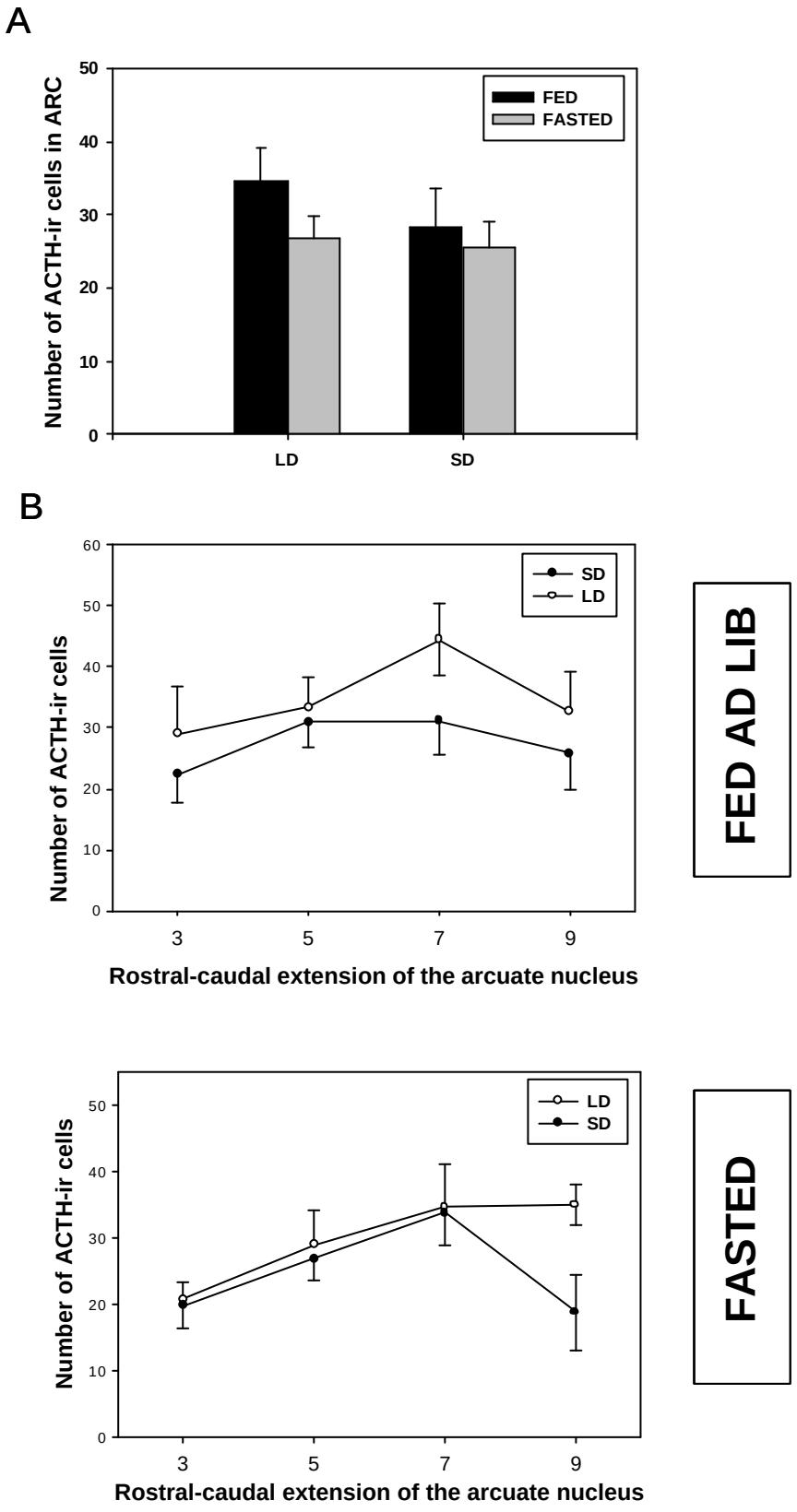

Figure 6. Quantitative analysis of the number of ACTH-ir cells. A) number of ACTH-ir cells in the entire arcuate nucleus and (B) in four representative sections arranged from rostral (3) to caudal (9) of LD- and SD-hamsters either fed ad lib or fasted for a period of 48 hrs. Numbers (3, 5, 7, 9) in B representing regions where ACTH-ir cell was counted. Data are presented as means ± SEM.

Discussion

Despite free access to food, the body weight of SD hamsters was lower than control hamsters maintained in LD. Moreover, uteri weights were significantly reduced in SD hamster, a change that may coincide with the initiation of reproductive quiescence in these animals. Thus, SD combined with low ambient temperature (16°C) appeared to alter the physiological activity in Djungarian hamsters, although a major change in pelage color was not detectable.

After 30 days in SD, Djungarian hamsters had elevated numbers of CART-ir cells in the ARC. The present finding is consistent with the result demonstrating an increased CART gene expression in the ARC of SD hamsters (Adam et al. 2000;Mercer et al. 2003). This is in contrast to a former study finding no significant difference in CART-mRNA expression between SD and LD hamsters (Robson et al. 2002). The present finding suggest a role for the CART neuronal system in seasonal regulation of energy homeostasis in the Djungarian hamster. Interestingly, in the present study, distribution patterns of CART-ir cell bodies in SD hamster matches the distribution pattern of CART neurons co-expressing fos -ir in rat ARC and peri-ARC after leptin injection (Elias et al. 1998;Elias et al. 1999). In these regions, CART containing neurons project to several areas including the spinal sympathetic preganglionic neurons, PVN, and the LHA (Elias et al. 1998;Elias et al. 1999;Elias et al. 2000;Elmquist 2001). These areas are known to be involved in the control of energy balance. Thus, one pathway used by leptin to directly activate neuroendocrine pathways involved in energy balance is via CART-ir neurons in these regions. However, plasma leptin levels in SD hamsters are decreased, and SD hamsters display lower food intake and body weight. Therefore in these brain regions of SD hamsters, it is unlikely that increased CART-ir expression is due to the direct action of leptin on these cells. However, SD hamsters are more sensitive to leptin compared to those kept in LD (Atcha et al. 2000;Klingenspor et al. 2000;Rousseau et al. 2002). Hence, it is more likely that changes in photoperiod alter feeding behavior by changing neuronal sensitivity to peripheral leptin. This is supported by the recent finding that suppressors of cytokine signaling molecules which inhibit leptin signaling appear to be a major mediator of changes in leptin sensitivity (Mercer and Tups 2003;Tups et al. 2003). In addition, the current findings suggest that CART containing neurons in the rostral-mid extension of ARC and peri-ARC, mediating leptin's effect in non-photoperiodic animals, may also be involved in mediating the

effect of leptin on the central mechanism involved in feed regulation in Djungarian hamsters.

Food deprivation caused reduction of CART-ir cell numbers measured in the rostro to mid portion of the ARC in SD hamsters. In fasted SD hamsters, the number of CART-ir cells decreased to the level observed in LD fed *ad lib* hamsters. In addition, food deprivation in LD had no effect on the number of CART-ir cells in the ARC. Food status is therefore part of the regulatory mechanism that induces a pronounced effect on CART-ir in hamsters. In part, this compensatory mechanism involves leptin, which is reduced due to the food deprivation. Leptin acts on CART containing neurons to activate catabolic pathways. In the present study, it is therefore likely that reduced numbers of CART-ir was due to lower plasma levels of leptin.

Considering the anatomical localization of CART containing neurons, together with the number of CART-ir cells in *ad lib* and fasted hamsters, we suggest that CART neurons in the rostral-mid ARC, specifically peri-ARC, play an important role in both seasonally programmed- and compensatory- regulation of energy balance. The results of the present study may provide further evidence supporting the anorexigenic function of CART in maintaining lower food intake, due to the increased number of CART-ir cell bodies during SD, lower food intake during SD and decreased CART-ir expression in fasted hamsters.

In the current study, we also quantified CART-ir in the LHA, a hypothalamic structure implicated in feed regulation. The number of CART-ir cells in the LHA appeared to be unaffected in response to either the change in photoperiod or fasting. This is consistent with a former experiment demonstrating that expression of CART-mRNA was unaffected by changes in photoperiod in hamsters (Robson et al. 2002). A role for CART in the LHA has not been defined. However, a neuroanatomical relation between the suprachiasmatic nucleus (SCN) and melanin-concentrating hormone (MCH)-containing neurons of the LHA has been found (Abrahamson *et al.* 2001). It is also known that MCH containing cells in the LHA co-express CART (Elias et al. 2001). Considering the former neuroanatomical findings together with present results, it is most likely that CART-containing neurons in the LHA either contribute to the balance between anorexigenic and orexigenic system or relay photoperiodic information emerging from the SCN to other brain structures.

In the present study, fibers and axon terminals immunoreactive for ACTH were also present in the LHA. The origin of these fibers may be ACTH-ir cell bodies found in the

ARC and peri-ARC. ACTH-ir fibers and axon terminals were also present in several hypothalamic areas implicated in food intake regulation.

The distribution pattern of cells immunoreactive for ACTH in the ARC and peri-ARC appear to overlap CART-ir. Since ACTH is derived from POMC co-localized in the majority of CART-neurons, it is therefore likely that CART and ACTH are co-expressed in the ARC. Surprisingly, in the present study the number of ACTH-ir cells was reduced in SD hamsters compared to LD hamsters. This observation is opposite to the result of CART-ir, despite co-localization of ACTH with CART in the ARC. Therefore, photoperiodic regulation of energy balance may involve different regulatory pathways within these cells. The reduced numbers of ACTH-ir cells may be due to decreased ACTH synthesis, increased ACTH transport from cell bodies, or reduced PC1/PC2 activities. However, our findings are consistent with results demonstrating decreased POMC gene expression in hamsters (Mercer et al. 2000) and reduced numbers of ß-endorphin-ir in ewes (Skinner and Herbison 1997) exposed to SD.

Axon terminals immunostained with ACTH antibody were abundant in the PVN, indicative of an important role for ACTH as a neurotransmitter in the regulation of neuroendocrine and autonomic activities in Djungarian hamsters. Interestingly, gene expression of CRH in the PVN were higher in voles kept in LD compared to SD (Peacock *et al.* 2004). This finding combined with the results of this study raises the possibility that ACTH in the hypothalamus controls CRH expression.

We cannot exclude the possibility that seasonal changes in CART and ACTH expression within the ARC are enhanced by exposing hamsters to SD combined with a lower ambient temperature. However, there are several lines of evidence to support that low ambient temperature itself does not affect the changes observed in the present study. Differential CART gene expression between LD and SD hamsters can be induced by changes in photoperiod with constant ambient temperature (Mercer et al. 2003). Second, in the present study, results of pelage colour and body weight were not distinct from those reported in hamsters exposed to SD and LD at a constant temperature (Robson et al. 2002). Third, Kawamoto et al 2000 (Kawamoto *et al.* 2000) investigated the effect of photoperiod and ambient temperature (7°C) on the number of gonadotropin-releasing hormone (GnRH) in the brain of gray hamsters. They demonstrated that low ambient temperature itself had no effect on

the number of GnRH-ir neurons, but in combination with SD enhanced the effect of SD-induced changes in GnRH neurons.

In conclusion, this study demonstrates that seasonal changes in photoperiod, ambient temperature and food status influence the distribution and number of CART-ir cells in the rostral part of the ARC. This finding indicates that the rostral CART neuronal system mediates seasonal regulation of energy balance in Djungarian hamsters.

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CHAPTER V

Leptin induces cellular activity within only subpopulations of hypothalamic cells containing STAT3 in Djungarian hamster (*Phodopus sungorus*)

Abstract

The present experiment was aimed to describe the distribution of STAT3 and identify hypothalamic structures involved in mediating leptin effects in Djungarian hamsters. In addition, dual labelling immunofluorescence was used to clarify whether changes in cellular activity were related to the action of leptin on target cells. STAT3 was detected within several hypothalamic nucleus including the arcuate nucleus (ARC), the ventromedial hypothalamic nucleus (VMH), the dorsomedial hypothalamic nucleus (DMH), lateral hypothalamic area (LHA), paraventricular hypothalamic nucleus (PVN), retrochiasmatic area (RCH) and supraoptic nucleus (SON). Leptin treatment in fasted hamsters induced fos-ir within several hypothalamic structures including the ARC, peri-ARC, RCH, dorsomedial part of the VMH, ventral part of the DMH, medial part of the LHA and parvocellular subdivision of the PVN. Co-localisation studies demonstrated fos-ir within only subpopulations of STAT3 positive cells. The present results suggest that the hypothalamus may act as the principle site in mediating the action of leptin in Djungarian hamsters. Also, leptin action within the central nervous system may lead to both activation and inhibition of subpopulations of hypothalamic nuclei involved in body weight regulation.

Introduction

Leptin secreted mainly by adipocytes plays an important role in the regulation of feeding, neuroendocrine status, thermogenesis, and energy expenditure (Ahima et al. 1996;Halaas et al. 1995;Levin et al. 1996). The profound effect of leptin is mediated through a long form receptor (OB-Rb), that belongs to the cytokine-receptor superfamily possessing intracellular JAk2/tyrosine kinase-STAT3 signaling pathway (Tartaglia et al. 1995;Tartaglia 1997;Baumann et al. 1996;Bjorbaek et al. 1997). Activation of the intracellular signaling pathway following binding of leptin to OB-Rb leads not only to induction of fos, but also phosphorylation, dimerization, nuclear translocation, binding of STAT3 to DNA, and finally activation of leptin-dependent gene transcription including suppressor of cytokine signaling-3 (SOCS-3) (Bjorbak et al. 2000;da Silva et al. 1998;Baskin et al. 2000;Banks et al. 2000;Bjorbak et al. 2000;Imada and Leonard 2000). Functional mapping of the neuronal circuit responsible for leptin action has been performed using *in situ* hybridization of OB-Rb mRNA, fos antibody as a cellular activity marker, STAT3/phospho-STAT3 antibody as a candidate to detect direct leptin action, and markers for expression of orexigenic and anorexigenic neuropeptides. OB-Rb mRNA has been localized in both hypothalamic and extrahypothalamic structures(Mercer et al. 2000;Elmquist et al. 1998b;Mercer et al. 1998;Buyse et al. 2001), suggesting that leptin action is mediated through a complex neuronal network. This was further confirmed by showing fos- and STAT3-ir in subpopulations of hypothalamic and extrahypothalamic brain structures responsible for body weight regulation (Woods and Stock 1996;Elmquist et al. 1997;Elmquist et al. 1998a;Niimi et al. 1999;Elias et al. 2000;Buyse et al. 2001;Hubschle et al. 2001). In addition, the complex neuronal network has been shown to be associated with more and more neurotransmitter/neuropeptides containing both orexigenic signals including neuropeptide Y (NPY), melanin concentrating hormone (MCH), Orexin A/B, agouti-related protein (AGRP), as well as anorexogenic signals including the cleavage product of proopiomelanocortin precursor (POMC), cocaine- and amphetamine-regulated transcript (CART), thyrotropin-releasing hormone (TRH), corticotropin-releasing hormone (CRH), and cholecystokinin (CCK). Experimental evidence has demonstrated that OB-Rb mRNA is co-expressed in ARC neurons containing

NPY/AGRP and POMC/CART mRNA(Cheung et al. 1997;Mercer et al. 2000;Mercer et al. 1996;Hahn et al. 1998;Baskin et al. 1999a). Furthermore, it has been shown that fasting increases the orexigenic signals, whereas leptin treatment causes a reduction of orexigenic signals versus anorexigenic signals within the hypothalamus (Mercer et al. 2000;Kristensen et al. 1998;Wang et al. 1997;Schwartz et al. 1997;Schwartz et al. 1997;Ahima and Hileman 2000). This favours neuronal activity in catabolic pathways, suggesting that the net effect of leptin in reducing food intake is a summation of inhibitory effects of leptin on orexigenic signals and activation of anorexigenic signal pathways. This is consistent with findings in mice lacking leptin and leptin receptors, which exhibit elevated levels of NPY/AGRP mRNA and/or decreased levels of POMC mRNA within the hypothalamus (Mizuno et al. 1998;Mizuno et al. 1998;Yamamoto et al. 1999;Mizuno and Mobbs 1999;Meister 2000). This information has been helpful to understand the effect of leptin in animals, where food intake and energy metabolism can be manipulated by fasting and food deprivation. Similar information is sparse in seasonal animals, such as Djungarian hamsters, that modify behaviour and body weight in response to photoperiod. Exposure of Djungarian hamsters to short day lengths (SD) causes a decreasing food intake, body fat content, serum leptin, and changes in neuroendocrine status, despite free access to food (Adam et al. 2000;Klingenspor et al. 2000;Klingenspor et al. 1996). Exposing hamsters to additional food restriction during SD induces further reductions of body weight. Providing the hamster with *ad libitum* re-feeding, returns body weight to a specific sliding set-point which is seen in SD under *ad libitum* food conditions; this indicates that photoperiod is the major signal in determining body weight (Steinlechner and Heldmaier 1982;Steinlechner et al. 1983). Although, the central pathway responsible for such changes is known to involve the neuroendocrine signal transducer hormone, melatonin, secreted by the pineal gland (Steinlechner and Heldmaier 1982;Steinlechner et al. 1983), the neuroendocrine mechanisms linking photoperiodic time measurement and energy metabolism are not known.

Several studies have investigated the effect of photoperiod on neuropeptide levels involved in energy balance regulation (Mercer et al. 1997;Mercer et al. 2000), but

there is paucity of information about a neuronal circuit responsible for mediating leptin action in seasonal animals.

The aim of present study was to i) anatomically localize STAT3-immunoreactivity in the hypothalamus, ii) to identify hypothalamic structures involved in mediating leptin effects, and iii) to characterize whether the changes in cellular activity could be related to the effect of leptin on target cells, by dual-labelling immunofluorescence of fos and STAT3 antibodies in the Djungarian hamster.

Material and Methods

Animals

Adult female Djungarian hamsters (n=12), weighing 19-31 g, were housed individually in Macrolon cages under natural lighting conditions (SD, 10:14 light-dark, February). Hamsters were divided into two groups. Group one included hamsters with free access to food and water, and group two included hamsters fasted for 48 hrs with access to water. All animal procedures were performed in accordance with the German animal welfare regulations. The weight of hamsters was monitored 48 hrs prior and during treatment with leptin and/or vehicle. At the time of treatment, fasted hamsters had lost almost 10% of their initial weight.

Treatment of hamsters with leptin and vehicle

Three hamsters from each group received an intraperitoneal injection of recombinant murine leptin (5 µg/g body weight, R&D SYSTEMS, cat#498-OB, dissolved in 15 mM HCL and 7,5 mM NaOH), 75 minutes prior to tissue preparation procedures, whereas control hamsters received an i.p injection of vehicle (15 mM HCL and 7,5 mM NaOH).

Tissue preparation

Hamsters were deeply anaesthetized by i.p. injection of 100mg/kg ketamine-hydrochloride (100 mg/ml, Ketavel, Pharmacia & Upjohn) and 3 mg/kg Rompun (2%, Bayer Vital, Germany). Anaesthetized hamsters were transcardially perfused with cold 4% paraformaldehyde (PFA) in 0.15 phosphate buffer (PB, pH 7.4) for five minutes. Brains were removed and post-fixed for two hours in the same type of

fixative, followed by fixation overnight at 4°C in phosphate buffer with 0.1% PFA. The brains were washed in PBS, gelatinised, and hypothalamic blocks were cut into 30 µm coronal sections. Free floating sections were collected in phosphate buffer on ice and kept at 4°C prior to immunohistochemical procedures.

Immunohistochemistry

From each animal, four sections from the rostral to caudal limit of the hypothalamic area were selected and processed for fos immunostaining.

The immunohistochemistry was carried out using the streptavidin-horseradish-peroxidase method as described below. Following rinsing of the free floated tissue sections in PBS, endogenous peroxidase activity was inhibited by incubation of the sections in a solution containing 80% PBS, 10% methanol, and 10% H_2O_2, for 15 min at room temperature (RT). Sections were then washed in PBS containing 1% Triton X-100 (PBT), and pre-incubated in 3% BSA (Sigma)-PBT at RT prior to 1 hr at RT and overnight at 4°C incubation of section with primary polyclonal rabbit anti fos antibody (Calbiochem, cat#PC38) diluted 1:20000 in 3% BSA-PBT. To examine the STAT3-immunoreactivity sections were incubated with rabbit polyclonal anti-STAT3 antibody (New England, cat#9132) diluted 1:400 in 3% BSA-PBT. After rinsing in PBT, the tissue sections were incubated with biotinylated goat anti-rabbit antibodies (Santa Cruz, cat#SC-2040) diluted 1:200 in 3% BSA-PBT. The tissue sections were then washed in PBT and incubated with HRP-conjugated streptavidin (Dako, cat#P0397) diluted 1:200 in 3% BSA-PBT. The tissue sections were then incubated with 0.05% Diaminobenzidine (DAB; Sigma, cat#D5637) and 0.033% H_2O_2 in PBS. The tissue sections were finally washed in PBS, mounted on gelatinised glass slides, air-dried, dehydrated in graded alcohol, cleared in Xylene and coverslipped with Enthelan (Merck). To identify the brain structures some of the brain sections were counter-stained with Hematoxylin (Vector, cat#H-3401) prior to dehydration procedure.

Dual-labeling immunofluorescence immunostaining

Four representative brain sections, from fasted hamsters after 75 minutes treatment with either a vehicle or leptin were selected to study the co-localization of fos antigen

with STAT3 using dual-labeling immunofluorescence immunostaining as described below. Following rinses in PBT, sections were preincubated in blocking buffer containing 3% BSA, 2% normal goat serum, and PBT for 30 min at RT, and incubated with primary polyclonal rabbit anti-fos antibody (Calbiochem, cat#PC38) diluted 1:5000 in blocking buffer overnight at 4°C. After rinsing in PBT, sections were incubated with Alexa-fluor-488 conjugated goat anti-rabbit antibody (Molecular Probes, cat#A-11034) diluted 1:300 in blocking buffer for 1 hr at RT. For double labelling, sections were washed in PBST and incubated with rabbit-gammaglobulin (dianova, cat#011-000-002) diluted 1:200 in blocking buffer for 1 hr at RT. After washing in PBT, sections were incubated with goat Fab-fragment anti-rabbit antibody (dianova, cat#111-007-003) diluted 1:200 in blocking buffer for 1 hr at RT. Following washing in PBT, sections were incubated with rabbit polyclonal anti-STAT3 antibody diluted 1:250 in blocking buffer for 2 hrs at RT. After 3 X 10 min rinsing, sections were then incubated with Cy3 conjugated goat anti-rabbit antibody (dianova, cat#112-165-144) diluted 1:200 in blocking buffer for 1 hr at RT. Sections were then rinsed 3 X 10 min in PBS, mounted on gelatin-coated slides, air dried, dehydrated in graded alcohol, cleared in Xylene and coverslipped with Permount (Fischerchemical, Lot, No. 976066-24).

Antibody specificity

To determine the specificity of fos immunostaining, several types of control reactions were performed on adjacent sections. The control reactions consisted of i) omission of the primary antibody in the above described protocol, ii) substitution of the primary antibody with fos-peptide (Calbiochem, cat#PP10), and iii) preincubation of 1 µl primary antibody with 10 µg/100 µl fos-peptide for 3 hrs at RT before application to sections. The absence of fos immunoreactivity in sections subjected to control reactions confirmed the specificity of immunostaining.

Control reactions for STAT3-immunostaining consisted of i) omission of primary antibodies, and ii) omission of secondary antibodies from the protocol. Control reactions resulted in no staining. The absence of STAT3-immunoreactivity in sections subjected to control reactions does not exclude the possibility that the primary

antibody only reacts specifically. Therefore, the term STAT3-immunoreactivity in the present study refers to STAT3-like immunoreactivity.

Analysis

A conventional Zeiss light microscope (Germany) equipped with a digital camera (Polaroid) and a Leica TCS SP2 laser scanning confocal microscope (CLSM, Germany) was used to analyse the distribution of STAT3- and fos immunostaining in sections developed by DAB, and to detect dual-labelling immunofluorescence. Fos and STAT3 immunofluorescence were visualised by switching between the two Alexa 488 and Cy3 filter system, respectively. To analyse the co-localization, an overlay image was produced from both colour channels. Contrast and sharpness of CLSM images and captured images from fos- and STAT3-immunostained sections were adjusted using Adobe Photoshop 7.0 software.

Results

STAT3-ir

STAT3-ir perikarya and processes were distributed throughout hypothalamic structures including ARC, periARC, VMH, DMH, PVN, LHA, RCH, SON, Pe and median eminence (EM) and ependymal lining of the third ventricle. The distribution patterns and density of STAT3-ir were not changed by either fasting and/or leptin treatment.

In the ARC, STAT3-ir cell bodies and their processes were found in both ventromedial and ventrolateral regions. STAT3-ir was also found in the rostro-caudal extension of an area lateral to ARC, peri-ARC (Fig. 1B, D and F). Whereas STAT3-ir cell bodies appeared within the internal zone of the ME, the external zone of the ME had mainly STAT3-ir process (Fig. 1G). STAT3-ir cell bodies were distributed along the rostro-caudal extension of VMH and DMH (Fig. 1B). STAT3-ir cells were also observed in the PVN, the RCH and the SON (Fig. 1A, C and E). In general, the density of STAT3-ir appeared stronger within ARC and Peri-ARC compared to other hypothalamic structures that showed STAT3-ir.

fos-ir

The qualitative comparison of sections showed that peripheral leptin treatment in fasted hamsters induced fos-ir within hypothalamic structures including ARC, peri-ARC, VMH, DMH, LHA and PVN (Fig. 2B, D-F), whereas a scattered fos-ir could be detected in *ad libitum* fed hamsters after leptin treatment.

Fos-ir was merely detected within rostral and ventrolateral part of the ARC and in the peri-ARC (Fig. 2D, E and F), In the VMH, and DMH, fos-ir appeared within the dorsal- and ventral parts, respectively (Fig. 2D). Leptin treatment induced fos-ir within the parvocellular subdivision of the PVN (Fig. 2B).

Counter-staining of the sections demonstrated that only subpopulations of nuclei in the above mentioned brain structures were stained with fos antibody (Fig. 2A and B).

Co-localization analysis

The dual-labelling experiment showed that fos-ir was co-localized within only a subpopulation of STAT3-positive cells (Fig. 3). In general, by changing from green- (fos-immunostaining) to red-channel (STAT3-ir), more STAT3- versus fos-ir could be detected within examined areas (Fig. 3)

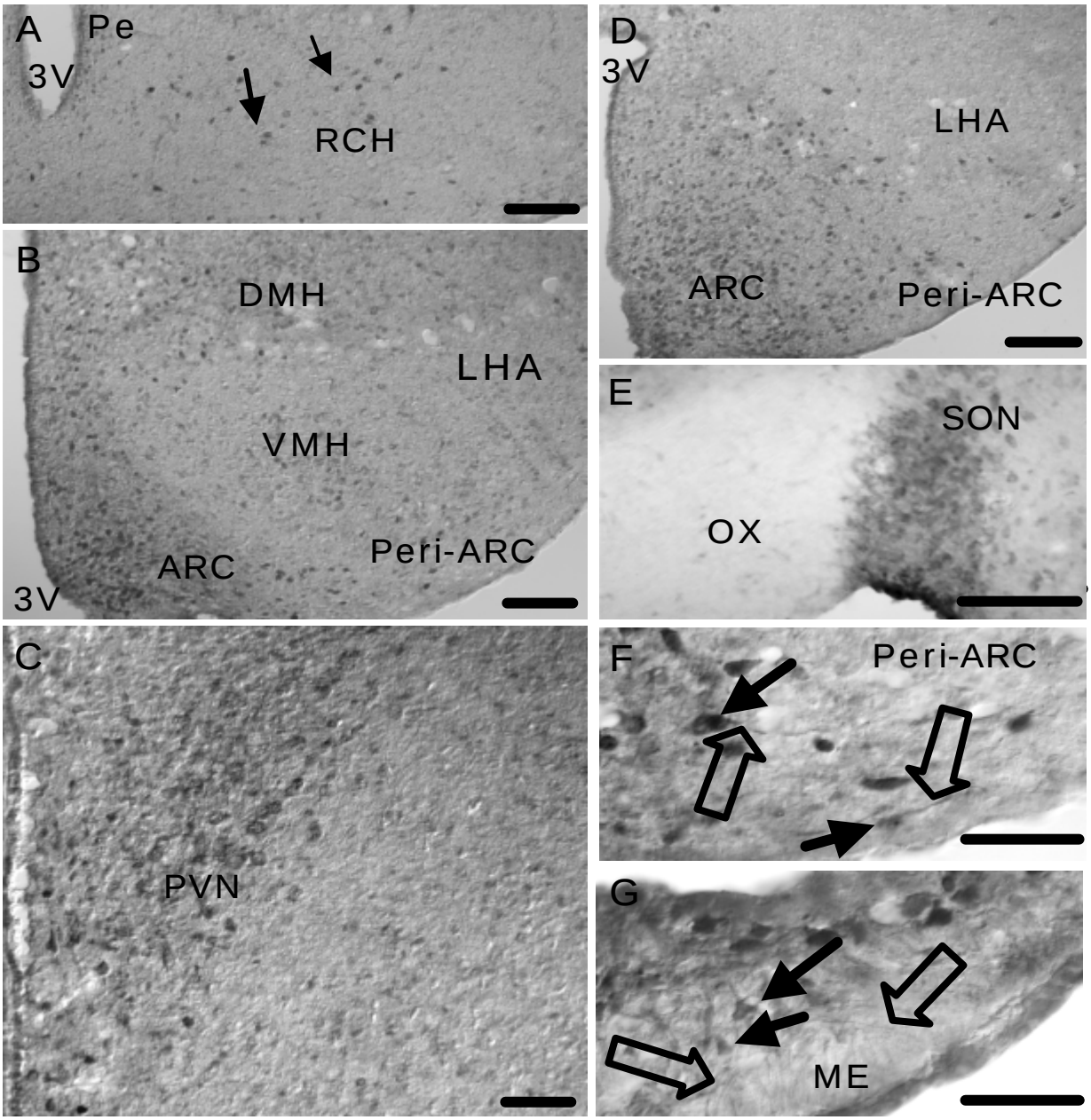

Figure 1. Photomicrographs showing STAT3-ir in several hypothalamic structures of Djungarian hamsters. STAT3-ir perikarya were present in RCH (arrows, A), ARC and peri-ARC (B and D), VMH (B), DMH (B), LHA (B and D), Pe (A), PVN (C) and SON (E). F) STAT3-ir was also found in fibers (open arrows) emerging from STAT3-ir cells (close arrows). G) Distribution of STAT3-ir perikarya within the internal zone (close arrows) and fibers immunostained with STAT3 (open arrows) within the external zone of ME is shown. DMH, hypothalamic dorsomedial nucleus. ARC, hypothalamic arcuate nucleus; DMH, hypothalamic dorsomedial nucleus; LHA; lateral hypothalamic nucleus, ME, median eminence; OX; optic chiasm, PVN, hypothalamic paraventricullar nucleus; Pe, periventricular hypothalamic nucleus; RCH, retrochiasmatic area; SON, supraoptic nucleus; VMH, hypothalamic ventromedial nucleus; 3V, third ventricle. Scale bar = 100 µm (A-E), 50 µm (F and G)

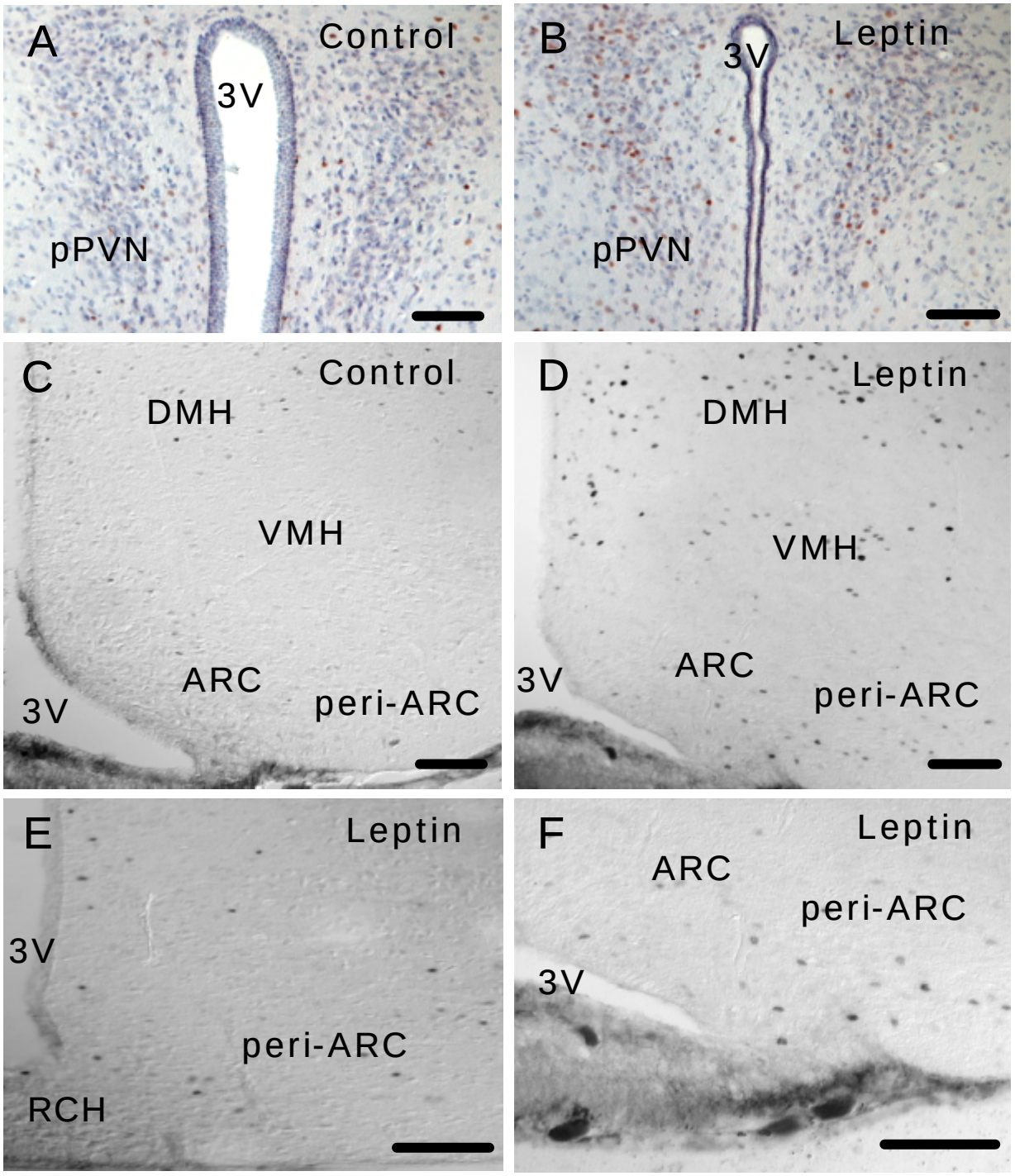

Figure 2. Fos-ir within the PVN, ARC, VMH, and DMH of fasted hamster after vehicle (control; A, C) and leptin treatment (B, D, E, F). Leptin treatment induced fos expression (red) within the pPVH (B). Leptin induced nuclei fos-ir in ventrolateral part of the ARC (peri-ARC; D), in rostral part of the ARC adjacent to the RCH (peri-ARC; E), dorsal part of the VMH (D) and in ventral part of the DMH (D). F) Larger magnification of ARC in D. Counterstaining of brain sections (PVN in A and B) with Hematoxylin (blue) revealed that only subpopulation of nuclei were fos positive (red). ARC, hypothalamic arcuate nucleus; DMH, hypothalamic dorsomedial nucleus; pPVN, parvocellular subdivision of hypothalamic paraventricular nucleus; VMH, hypothalamic ventromedial nucleus; 3V, third ventricle. Scale bar = 100 µm.

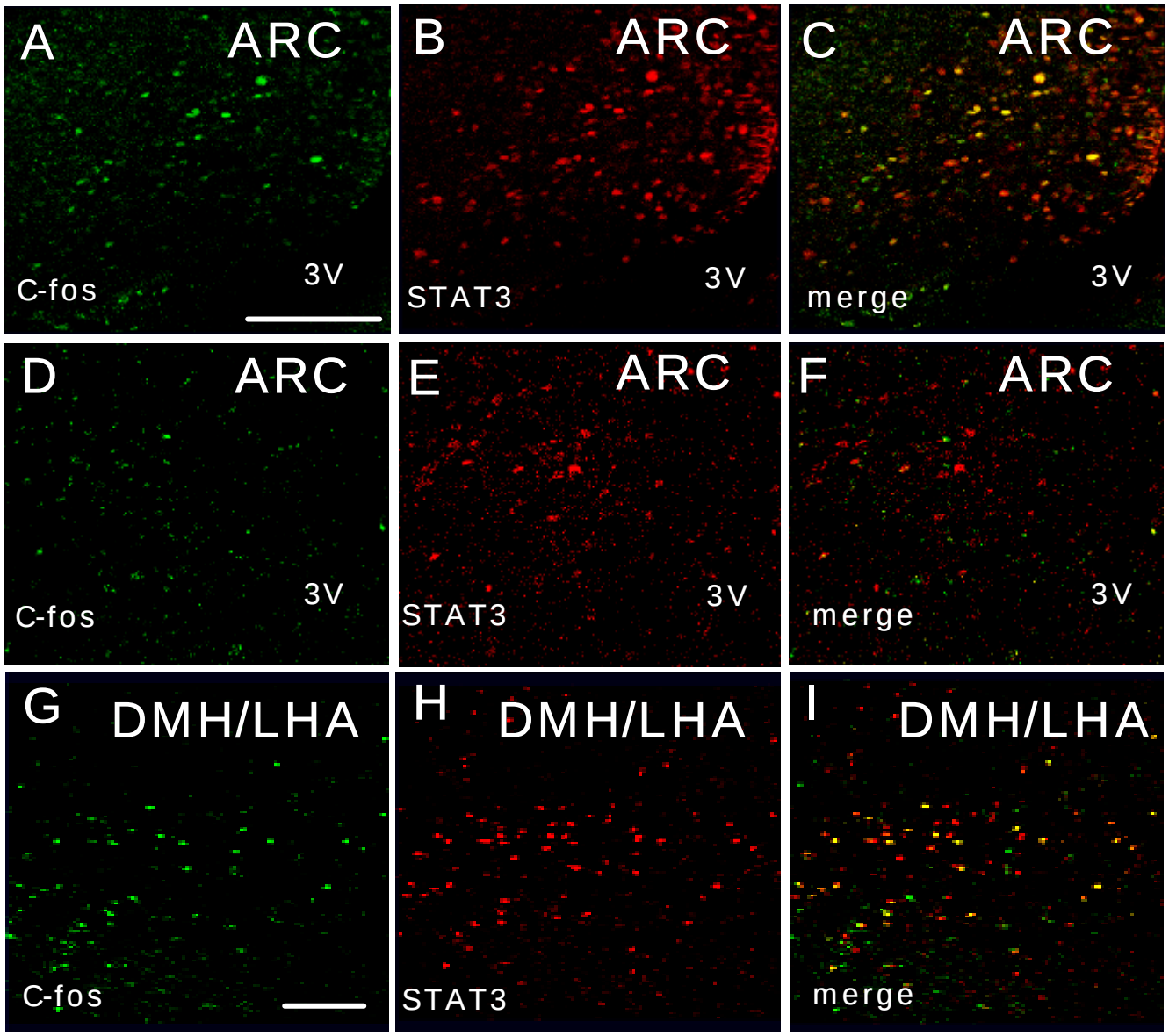

Figure 3. Immunofluorescence photomicrographs of fos- (green) and STAT3-ir (red) cells in brain sections of leptin- (A-C, G-I) and vehicle treated (D-F) fasted hamsters. Fos- (green) and STAT3-ir (red) in the ARC (A-F) and DMH/medial LHA (G-I) are shown. Only subpopulation of STAT3-immunostained cells showed double labeling with fos antibody (yellow). Also, more STAT3-ir cells than fos-ir nuclei could be detected by comparison of red with green immunofluorescence. ARC, arcuate nucleus; DMH, dorsomedial hypothalamic nucleus. LHA, lateral hypothalamic area; 3V, third ventricle. Scale bar in A is 100 µm for A-F, and in G is 100 µm for G-I.

Discussion

The aim of this study was to identify both the localization of STAT3 and the central pathway within the hypothalamus responsible for mediation of leptin action in the Djungarian hamster. The distribution of STAT3-ir appeared within several hypothalamic regions including the ARC, VMH, DMH, LHA and PVN. Within the CNS, the function of STAT3 as a mediator of leptin action is emphasized by studies showing a lack of STAT3 activation in db/db mice and increased STAT3 activity in wild-type and ob/ob mice following leptin treatment (Cheung et al. 1997;Vaisse et al. 1996). In addition, administration of leptin in rats increases STAT3 phosphorylation and its translocation within several hypothalamic nuclei (Hubschle et al. 2001;Munzberg et al. 2003). Thus, several studies have employed STAT3 to map a functional central pathway responsible for leptin action. In the present study, the distribution pattern of STAT3-ir was not affected by either fasting and/or leptin treatment. Since the antibody used in the present study recognizes STAT3-antigen independent of its phosphorylation state, a possible STAT3 phosphorylation and its nuclear translocation within the hypothalamic nuclei was not detectable. Further studies are therefore required to clarify whether leptin treatment induces STAT3 phosphorylation and/or nuclear translocation of phospho-STAT3 in the hamster. In general, the occurrence of STAT3-ir within the hypothalamus confirms this area as the principle site mediating the action of leptin in Djungarian hamsters as well as other rodents.

The present study demonstrated that exogenous leptin induced fos expression within a subpopulation of hypothalamic nuclei including ARC, VMH, DMH, LHA and PVN in fasted hamsters. Whereas only scattered fos-ir could be detected in *ad libitum* fed hamsters. In fasted hamsters, the increased sensitivity of cells responding to exogenous leptin could be due to increased OB-Rb expression following starvation (Mercer et al. 2000;Baskin et al. 1998;Baskin et al. 1999b;Baskin et al. 1999a;Becker et al. 1995;Weigle et al. 1997). In addition, SD decreases the level of OB-Rb mRNA and additional food restriction under SD causes elevation of OB-Rb mRNA levels (Mercer et al., 2000), which presumably could explain the stronger fos-immunostaining observed in the brain of fasted hamsters. Lack of fos-ir in *ad libitum*

fed hamsters in response to exogenous leptin could be due to the refractory period of resistance to leptin.

The present dual-labelling experiments demonstrated co-localization of fos-ir within only a subpopulation of STAT3-immunostained cells of ARC, VMH, DMH and PVN. In addition, more STAT3-immunostained cell bodies could be detected within the examined brain regions compared to fos-ir nuclei.

The ARC neurons contain both orexigenic and anorexigenic neuropeptides including NPY, AGRP, CART and alpha-MSH. Exogenous leptin increases CART and POMC mRNA and suppresses NPY and AGRP mRNA within the ARC (Mizuno and Mobbs 1999;Ahima and Hileman 2000;Mizuno et al. 1998;Mizuno and Mobbs 1999). Following leptin administration, Elias et al. (1999) (Elias et al. 1999) demonstrated that NPY cells in the ARC did not express fos but expressed SOCS-3 mRNA. Furthermore, Hakansson and Meister (1998) (Hakansson and Meister 1998) reported the presence of STAT3 in both NPY and POMC neurons of the ARC. In the present study, fos-ir in the ARC appeared mostly within the ventrolateral ARC and the peri-ARC, alongside CART and POMC containing neurons, indicating that these neurons are activated by leptin. The lack of fos-ir in the ventromedial part of the ARC suggests that this region is inhibited by leptin. These combined with our findings suggest that STAT3-ir cell bodies co-localising fos-ir may represent the anorexigenic neuropeptide producing cells and STAT3-ir cells without fos expression may represent the cells with orexigenic function.

In addition, fos-ir appeared within dorsomedial- and ventral parts of the VMH and DMH, respectively. Fos-ir appeared also in the medial part of the LHA. Experimental evidence has shown the importance of the DMH, VMH and LHA in regulating feeding and body weight. Leptin treatment of rats induces phosphorylation of STAT3 within these structures (Munzberg et al. 2003). In addition, DMH and LHA are known to house CART, MCH and orexin containing neurons involved in energy balance regulation. Leptin induces activity in these brain structures either directly or through projections from the ARC. The presence of fos- and STAT3-ir in these structures, however, suggests their involvement in mediating leptin action in the Djungarian hamster.

Leptin treatment induces fos-ir within a subpopulation of STAT3-iir cell bodies of the PVN. The PVN contains a subset of neurons projecting to the ME, brainstem, and spinal cord, and receives afferent fibres mostly from the ARC, DMH, and VMH, thus mediating the activities of parasympathetic, sympathetic and neuroendocrine pathways. The active role of PVN in regulation of food intake has been reported in animals with lesions in PVN (Shor-Posner et al. 1985). This finding is complimented by the identification of PVN neurons that not only contain anorexigenic neuropeptides, but also express OB-Rb mRNA and melanocortin-4-receptor, known as a target for alpha-MSH which inhibits food intake (Mercer et al. 2000;Mountjoy et al. 1994). Experimental evidence has demonstrated that leptin treatment causes activation of neurons in the PVN that project to parasympathetic and sympathetic neurons. Stimulation of PVN neurons containing anorexigenic neuropeptides by projections from different brain regions including the ARC and DMH, or directly by leptin may cause changes in feeding and neuroendocrine pathways. Fos-ir in subpopulations of the PVN immunostained with STAT3 indicates that this region is involved in mediating leptin action in the Djungarian hamster.

Using counter-staining, we demonstrated that only a subpopulation of hypothalamic cells was fos-ir. Whether observed fos-immunostaining is distributed in neuronal and/or non-neuronal cells is not answered in the present study.

In summary, the present observations suggest that the action of leptin in Djungarian hamsters is mediated within hypothalamic cell populations similar to that reported in other species. Also, the function of leptin in body weight regulation may be achieved by inducing activity in only a subpopulation of neurons and inhibition of other neurons.

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CHAPTER VI

GENERAL DISCUSSION

Neuroendocrine pathways underlying the regulation of seasonal body weight involve the hypothalamic neuropeptidergic system which responds both to the different state of energy balance and to changes in photoperiod (Mercer and Speakman 2001). Manipulation of energy balance induces a change in peripheral signals, such as leptin, that act in the hypothalamus to alter the activity of neuropeptides (Schwartz et al. 2000). Alternatively, changes in photoperiod result in pineal melatonin secretion that influences the hypothalamic neuropeptidergic system by which energy balance is controlled (Goldman and Darrow 1983;Morgan et al. 2003). Knowledge about neuropeptides and their distribution patterns is therefore of fundamental importance to provide a deeper understanding of neuroendocrine pathways involved in the regulation of seasonal body weight. Current knowledge about the hypothalamic neuropeptidergic system in hamsters is mainly based on studies investigating the effect of photoperiod and food restriction on the level of neuropeptides gene expression (Mercer et al. 2000;Reddy et al. 1999).

We therefore focused in the first experiment (chapter II) on the neuroanatomical localization of selected neuropeptides and their relationships in the Djungarian hamster brain. We investigated the distribution of CART-, MCH- and OXB peptides in selected brain regions harbouring neuroendocrine pathways which are involved in the control of food intake or implicated in circadian timing processes. The results of this study demonstrated that CART-, MCH- and OXB-ir cell bodies and fibers were present within hypothalamic structures that are implicated in the regulation of energy balance. The findings in the Djungarian hamsters indicate that the MCH-, CART- and OXB- neuronal system are strongly conserved between species suggesting an important role for these neuropeptides in the regulation of feeding. In addition, the wide distribution of MCH, OXB and CART fibers is indicative of their involvement in several physiological functions.

Fibers and terminals immunoreactive for CART, MCH and OXB were absent from the SCN, but present in the neuronal structures projecting to the SCN (Fig. 1). The interaction between the CART-MCH-OXB neuronal system and circadian timing

system may take place either in the IGL, raphe nuclei or peripheral structures of the SCN that project to the SCN (Fig. 1). This finding provides a basis for the CART-MCH-OXB neuronal system to indirectly influence the generation of circadian rhythmicity. The origin of CART-ir fibers within neuronal components of the circadian timing system is not known, since the distribution of CART-ir cell bodies in contrast to MCH- and OXB-ir is not restricted to the LHA.

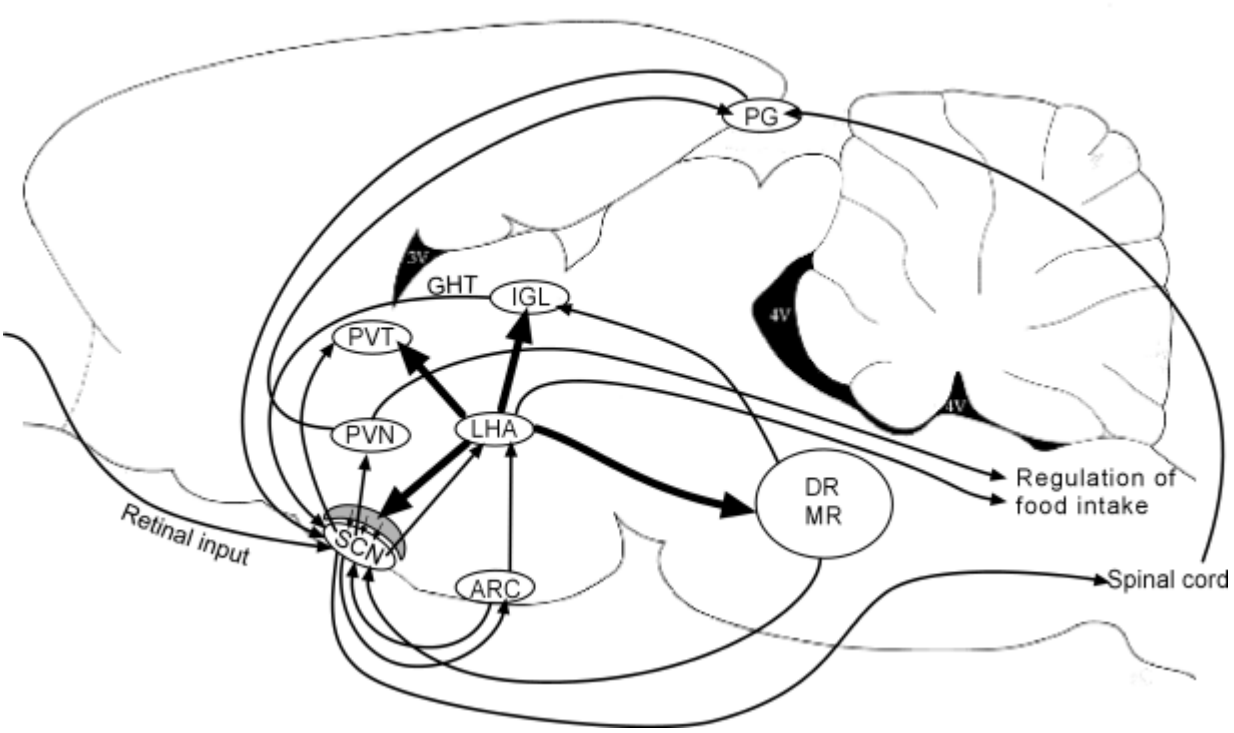

Figure 1. Diagram of neuronal connections between brain structures implicated in the regulation of energy balance and neuronal components of the circadian timing system (sagittal section of the rat brain). The shaded area above the SCN indicates the peripheral zone of the SCN. The SCN did not contain fibers and terminals immunoreactive for CART, MCH and OXB. In contrast, CART-, MCH- and OXB-ir fibers were present in the IGL, peripheral area of the SCN and raphe nuclei. In addition, fibers immunoreactive for these neuropeptides were present in the PVT. ARC, arcuate nucleus; DR, dorsal raphe nucleus; GHT, geniculohypothalamic tract; IGL, intergeniculate leaflet, LHA, lateral hypothalamic area; MR, median raphe nucleus; PG, pineal gland; PVN, paraventricular hypothalamic nucleus; PVT, paraventricular thalamic nucleus; SCN, suprachiasmatic nucleus.

Our experiments that focused on the relationship between CART, MCH and OXB revealed no co-localization of MCH- and OXB-ir, but demonstrated the co-existence of MCH- and CART-ir within several hypothalamic structures. These results provide

the first evidence of MCH-ir and CART-ir co-localization in seasonal animals. Whether such co-expression of two functionally distinct neuropeptides has a physiological function is not known, but CART may counteract the effect of MCH (Vrang et al. 1999a;Broberger 1999). In addition, close associations were detected between OXB-ir fibers and terminal boutons and MCH neurons. The presence of neuronal connections between orexins and MCH suggests that OXB may act as a putative neurotransmitter to regulate the function of MCH containing neurons. However, the functional relevance of these findings needs further investigation.

Based on our findings in chapter II, we suggest that selected neuropeptides can influence circadian timing processes through NPY neurons of the IGL that project to the SCN (Morin et al. 1992;Morin and Blanchard 1995;Smale et al. 1991). This suggestion was tested in chapter III. The results of this experiment revealed close associations between OXB-ir fibers and NPY-ir cell bodies and fibers within the IGL. An association was also found between OXB- and NPY-ir fibers in the peripheral part of the SCN. These findings represent a link between OXB neurons in the LHA and SCN (Fig. 1).

Furthermore, in chapter II, only a small number of CART-ir cell bodies were detected in the ARC of the Djungarian hamster compared to rats (Elias et al. 2001;Vrang et al. 1999b). We suggest that one possibility for the existence of only a small number of CART-ir cell bodies in the ARC could be due to the photoperiodic history of hamsters described in chapter II. The hypothesis suggesting that seasonal acclimation affects CART peptide expression was tested in chapter IV. Results of this study indicate that seasonal acclimation influences the number of CART-ir cells in the ARC. More CART-ir cells were present in seasonally acclimated hamsters. Further analysis on the regional distribution of CART-ir cells in the rostro-caudal extension revealed that the increase in cell numbers were restricted to the rostro to mid portion of the ARC, specifically the peri-ARC (Fig. 2). CART-ir cells in similar regions are known to mediate the action of leptin in rat (Elias et al. 1998b;Elias et al. 1999). In addition, food deprivation in seasonally acclimated hamsters caused reduction of CART-ir cell number measured in similar regions. Therefore, food status is part of the regulatory mechanism that induces a pronounced effect on CART-ir in hamsters. In part, this compensatory mechanism involves leptin, which is reduced due to the food

deprivation. Thus, it is likely that reduced numbers of CART-ir could be due to lower plasma levels of leptin.

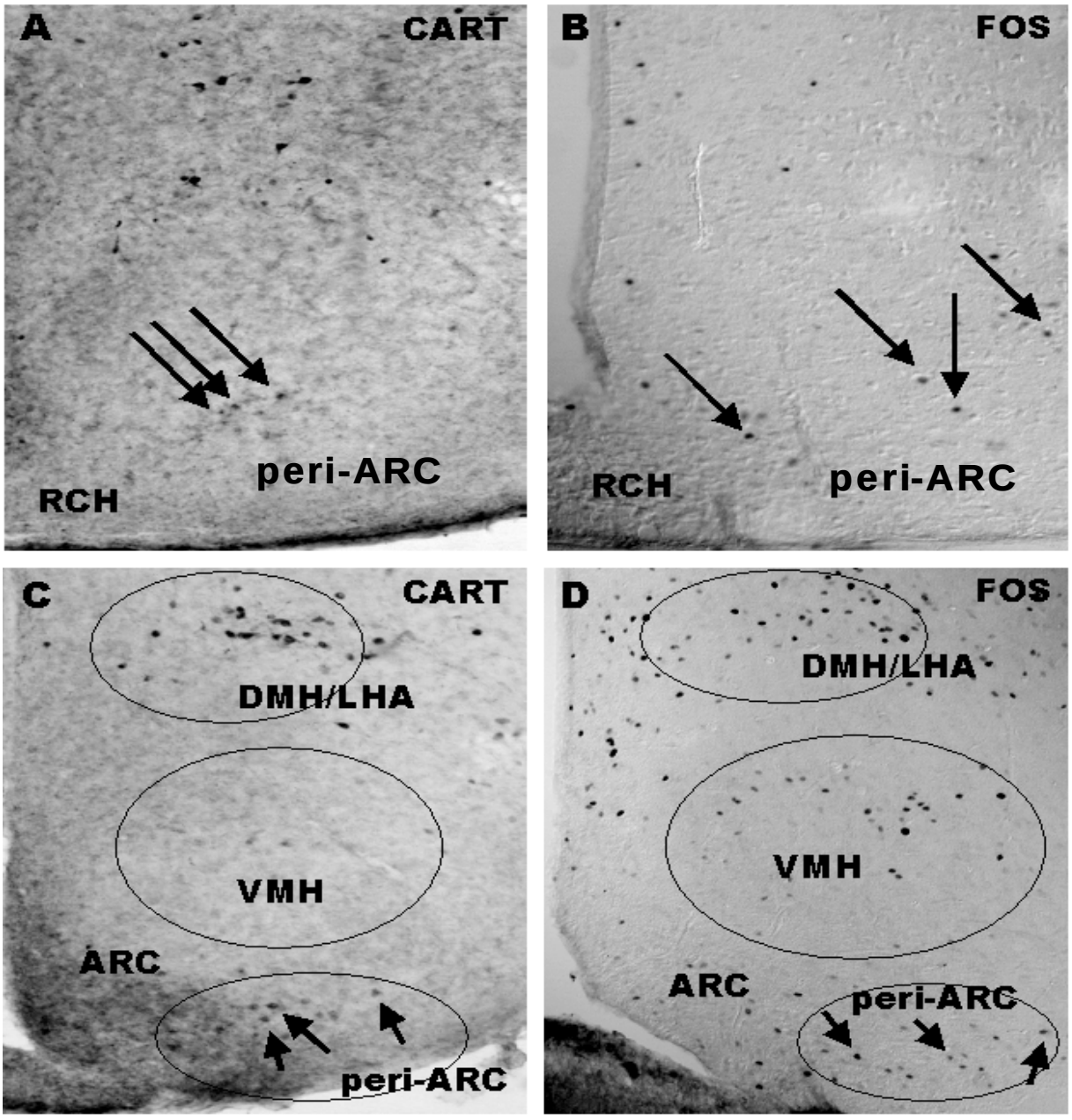

Figure 2. Comparison of brain sections immunostained with CART and fos antibodies. A and B compare CART- and fos-ir in rostral part of the ARC (peri-ARC) adjacent to the RCH, respectively. Images in C and D compare CART- and fos-ir in ventrolateral division of the ARC. Arrows show distribution of CART-ir cell bodies and fos-ir nuclei in the peri-ARC. A clear overlap between CART-ir cells and fos- ir nuclei can be observed in sections by comparing CART- with fos-ir. In the DMH/LHA, fos-ir overlap the distribution of CART-ir cells. ARC, arcuate nucleus; DMH, dorsomedial hypothalamic nucleus; LHA, lateral hypothalamic area; peri-ARC, peri arcuate nucleus; RCH, retrochiasmatic nucleus; VMH, ventrolateral hypothalamic nucleus. (A and C magnification x10) and (B and D magnification x20). Images are taken from chapter III and IV.

In response to SD, body fat content and serum leptin levels decreases in hamsters (Atcha et al. 2000;Klingenspor et al. 2000;Rousseau et al. 2002). Consequently, low leptin levels may reduce rather than increase CART activity in seasonally acclimated hamster, unless photoperiod modulates the sensitivity of hypothalamic neuroendocrine pathways to peripheral leptin action. Thus, photoperiod may act on similar hypothalamic structures involved in the mediation of leptin action. Furthermore, seasonal acclimation and fasting did not affect the number of CART-ir cells in the LHA. Together, it is likely that the CART neuronal system within the ARC provides a central anatomic substrate for integration of both body fat and photoperiodic information to regulate energy balance. This hypothesis provided the basis for the experiment described in chapter V.

In this experiment (chapter V), leptin treatment of hamsters induced cellular activity (fos-ir) within several hypothalamic areas including the ARC (peri-ARC), VMH, DMH, LHA and PVN. In addition, these hypothalamic regions also contained STAT3-ir cell bodies. Further, dual labeling revealed that fos-ir was only present within a subpopulation of STAT3-ir cells. However, it is not clear whether cellular activity in DMH, VMH, LHA and the PVN are due to direct effects of leptin on these nuclei, or through ARC projections to these areas. In addition, STAT3-ir appeared in both cytoplasm and nuclei independent of leptin treatment or fasting. There was no evidence indicating leptin had induced STAT3 translocation into nuclei as reported in the rat (Hubschle et al. 2001;Munzberg et al. 2003).

Leptin induced-cellular activity was detected in part of the ARC with increased numbers of CART (Fig. 2). This finding suggests that both seasonal and leptin regulation of energy balance involves a similar region and pathway in the ARC. Further studies are required to characterize leptin activated neurons in hamsters. However, it seems that our results on fos-ir expression concur with recent findings in the rat (Elias et al. 1998a;Elias et al. 1999;Elias et al. 2000), suggesting that activated neurons within the ARC may represent CART neurons. Interestingly, exogenous leptin induced cellular activity within DMH/LHA regions, whereas seasonal acclimation and food deprivation failed to alter the distribution of CART-ir cell bodies in similar regions (Fig. 2C and D). Similarly, neither the change in photoperiod nor food restriction have been shown to affect the level of CART-, MCH- and orexin mRNA expression in the DMH/LHA of hamsters (Mercer et al.

1995;Mercer et al. 1997;Reddy et al. 1999;Robson et al. 2002). In addition, leptin induced fos-ir in the PVN, where CRF mRNA expression is not affected by photoperiod (Mercer et al. 1995;Mercer et al. 1997).

Taken together, the CART neuronal system within the rostral and ventrolateral part of the ARC (peri-ARC; Fig. 2) provides a central anatomic substrate for integration of both body fat and photoperiodic information in the Djungarian hamster. CART containing neurons in these regions project to several areas including the spinal sympathetic preganglionic neurons, the PVN and the LHA. In the LHA, such projections may control neuronal activity of CART-, MCH- and OXB containing neurons that in addition to food intake regulation, indirectly influence circadian timing processes (Fig. 1). The link between the ARC-LHA and circadian timing system may create a route allowing both leptin induction of cellular activity in ARC-LHA neurons and photoperiod to influence the generation of circadian rhythmicity. The SCN in turn regulates neurosecretory functions of neurons in the ARC and the LHA resulting in circadian release of either orexigenic or anorexigenic signals. Coordinated interactions between components of neuroendocrine pathways and circadian timing system could contribute or control the effectiveness of the short photoperiod to trigger the sliding set-point decrease in body mass.

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Summary

The present dissertation describes the identification and characteristics of neuroendocrine pathways involved in the regulation of seasonal body weight cycles. In different investigations, we provide new and supportive information on the central hypothalamic pathways mediating the effect of photoperiod and leptin in a seasonal animal, the Djungarian hamster.

The first study (chapter II) focused on the distribution and characterisation of selected neuropeptides (CART, MCH, OXB) involved in the regulation of energy balance. There appears to be a neuroanatomical basis for CART-MCH-OXB neuronal system to indirectly influence the generation of circadian rhythmicity.

The second study (chapter III) focused on determining a possible interaction between neurons containing neuropeptides (OXB) and the main pathway, the geniculohypothalamic tract arising from NPY-containing neurons in the IGL. In the IGL, there was apposition of OXB-ir terminals on NPY-ir neurons, indicating the existence of a feedback input from neuropeptides to the circadian timing system.

The effect of seasonal acclimation on the distribution and expression of CART was investigated in chapter IV. Results of this study revealed increased numbers of CART within the rostral- and ventrolateral part of the ARC (peri-ARC). These regions are known to mediate the inhibitory effect of leptin on food intake in other species like rats and mice.

We therefore focused our study in chapter V to identify hypothalamic structures mediating the effect of leptin in Djungarian hamsters. Results of this study revealed that leptin induces cellular activity (induction of fos) within several hypothalamic structures including the rostral- and ventrolateral part of the ARC (peri-ARC).

Combined, these findings indicate that the ARC provides a central anatomic substrate for integration of both body fat and photoperiodic information in the Djungarian hamster.

The final part of the thesis comprises a general discussion of the obtained results and future directions.

Zusammenfassung

Die vorliegende Dissertation beschreibt die Identifizierung und Charakterisierung von neuroendokrinen Signalwegen, die in die saisonale Körpergewichtsregulation involviert sind. Die vorliegenden Untersuchungen liefern neue Befunde über die Bedeutung des zentralen hypothalamischen Signalweges, der die Interaktion von Photoperiode und Leptin auf die saisonale Anpassung des Dsungarischen Hamster vermittelt.

Die erste Studie (Kapitel 2) konzentriert sich auf die Verteilung und Charakterisierung von ausgewählten Neuropeptiden (CART, MCH, OXB), die in der Regulation der Energiebalance eine Rolle spielen. Es scheint eine neuroanatomische Grundlage für ein neuronales CART-MCH-OXB System zu geben, das indirekt die Erzeugung der ciradianen Rhythmik beeinflusst.

Die zweite Studie (Kapitel 3) beschäftigte sich mit einer möglichen Interaktion zwischen den neuropeptidhaltigen Neuronen (OXB) und dem Hauptsignalweg, dem geniculohypothalamischen Tractus. Dieser Hauptsignalweg gesteht aus NPY-enthaltenen Neuronen im IGL. OXB-ir Fasern innervieren dort die NPY Neuronen. Dies deutet auf die Existenz eines Rückkopplungsmechanismus von Neuropeptiden auf das circadiane Zeitgebersystem hin.

In Kaptitel IV wurde der Effekt der saisonalen Anpassung auf die Expression von CART untersucht. Die Ergebnisse dieser Studie offenbarten eine erhöhte Anzahl von CART ir Zellen innerhalb des rostralen und ventromedialen ARC. Diese Region spielt bei Tierarten wie Ratte und Maus eine wichtige Rolle bei der Vermittlung eines inhibitorischen Effektes von Leptin auf die Nahrungsaufnahme.

Deshalb haben wir uns im Kapitel V auf die Identifizierung der hypothalamischen Strukturen konzentriert, die den Effekt von Leptin im Dsungarischen Hamster vermitteln. Diese Studie ergab, dass Leptin die zelluläre Antwort (Induktion von fos) in mehreren hypothalmischen Strukturen induziert. Hierzu gehören auch die Strukturen der rostralen und ventrolateralen Region des ARC (peri-ARC).

Insgesamt deuten diese Befunde darauf hin, dass der ARC als zentrales anatomisches Integrationszentrum für Körperfett und photoperiodische Information, die Energiebilanz im Dsungarischen Hamster reguliert.

Der Schlussteil der Arbeit umfasst eine generelle Disksussion der Ergebnisse und einen Ausblick auf zukünftige mögliche Studien.

Acknowledgements

The studies in this dissertation have been performed at the Department of Animal Physiology, institute of Biology, Philipps University, Marburg, Germany.

I wish to thank Professor Gerhard Heldmaier for accepting me as a member to his research group (AG Heldmaier).

I am indebted to my supervisor Dr. Martin Klingenspor for his outstanding enthusiasm to this project, many good ideas, and for his always helpful advising comments, suggestions and support.

I wish to thank all the staff and students at the Department of Animal Physiology for fruitful discussions, help, advice and fun doing research. I would like to thank Alexander Tups and Michael Helwig for their support. Carola Meyer, Timo Kanzleiter, Cornelia Exner, Isabel Walter, Sigrid Stöhr, Mechthild Zissel, Gábor Szerencsi and Jan Rozman are thanked for their help.

Thanks are due to all the staff of Professor Dr. Uwe Homberg at the neurobiology I. I would like to extend my acknowledgments to Dr. Joachim Schachtner, Neurobiology I, for his invaluable suggestions. I would also like to thank Matthias Vömel and Wolf Hütteroth, neurobiology I, for their help.

I would like to thank Dr. Niels Einer-Jensen and Dr. Keith E. Baptiste for their support.

I am especially grateful to the Danish Research Agency and Deutsche Forschungsgemeinschaft (DFG, KL973-5) for their financial support to conduction of my studies.

I wish to thank my dear family for their support.

At last, but not at least, my dearest Heidi, Matin and Asal are thanked for their outstanding support, understanding, love and for being there. This work would not have been possible without them.

CURRICULUM VITAE

Reza M. H. Khorooshi
Date of birth: 03.051965
Place of birth: Mashad, Iran
Citizenship: Danish
Married, two children

Education

1995:	Bachelor of Science in molecular and cell biology Institute of Microbiology, University of Syddansk, University of Odense (OU), Denmark.
1998:	Master of Science in Biomedicine Institute of Medicine Biology (IMB), Dept. of Anatomy and neurobiology, and Institute of Pathology, University of Syddansk, University of Odense (OU), Denmark.
Sep. 2000:	Ph. D. Candidate Institute of Biology, Dept. of Animal Physiology, Philipps University, Marburg, Germany.

Erklärung

ich versichere, dass ich meine Dissertation

IDENTIFICATION AND CHARACTERIZATION OF NEUROENDOCRINE PATHWAYS INVOLVED IN THE REGULATION OF SEASONAL BODY WEIGHT CYCLES

selbstständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

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(Ort/Datum) (Mohammad H. Khorooshi)