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Neurochemical and functional characterisation of the melanin-concentrating hormone (MCH) system in the rat brain

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General abbreviations:

ACC	Acetyl-Coenzyme A
ACC2	Acetyl-Coenzyme A carboxylase type 2
ACRP-30	adipocyte complement related protein-30
ACTH	adrenocorticotropic hormone
aCSF	artificial cerebrospinal fluid
AgRP	Agouti related peptide
AIDS	aquired immunodeficiency syndrome
AL	*ad libitum* fed animals
AN	Anorexi nervosa
ANP	Atrio-natriuretic peptide
AROM	antisense-RNA-overlapping-MCH-gene
BAT	brown adipose tissue
BF	brightfield
BIA	Bioelectrical Impedance analysis
BMI	Body mass index
BMR	Basal metabolic rate
BSA	bovine serum albumine
BW	body weight
cAMP	cyclo-adenosin monophosphate
CART	cocaine- and amphetamine regulated transcript
CBR-1	Cannabinoid receptor subtype-1
CCK	Cholecystokinin
CDC	Center for disease control and prevention
cDNA	complementary desoxyribonucleic acid
CHO	chinese ovary hamster
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CRH	Corticotropin-realsing hormone
CT	Computed Tomography
DAPI	4`,6-diamidino-2-phenylindole dilactate
db/db	leptin receptor deficient mice
DGAT	diacylglycerol acetyltransferase
DIO	diet-induced obese
FAS	fatty acid synthase
FD	food deprived animals
FEO	food entrained oscillator
FR	food restricted animals
g	gram
GnRH	Gonadotropin releasing hormone
GLP	Glucagon like peptide
GPCR	G-protein coupled receptor
h	hour
HDL	high density lipids
HPLC	high performance liqid chromatography
11-ß HSD	11-ß hydroxysteroid-dehydrogenase
5-HT	5-Hydroxytryptamine (serotonin)
5-HT_{2c}	5-Hydroxytryptamine receptor subtype 2c
HRP	horse radish peroxidase
i.c.	intracerebral
i.c.v.	intracerebroventricular
i.e.	id est
IEG	immediate early gene
IgG,IgY	Immunoglobulin G, Immunoglobulin Y
i.n.	intranuclear
i.p.	intraperitoneal
IF	immunofluorescence
IR	immunoreactive, immunoreactivity
Kcal	kilocalorie
kg	kilogram
LHRH	luteinizing hormone releasing hormone
M	molar
MAP	mitogen activated protein
MC3,4	Melanocortin receptor subtypes 3,4
MCH	melanin concentrating hormone
mch1-r	MCHR-1 gene
MCHR-1	melanin concentrating hormone receptor subtype 1
MCHR-2	melanin concentrating hormone receptor subtype 2
MCH (-/-)	MCH deficient mice
MCHR-1 (-/-)	MCH deficient mice
MGOP	MCH-gene-overprinted-polypeptide
min	minute
ml	milliliter
mm	millimeter
µm	micrometer
MRI	Magnet resonance imaging
mRNA	messenger ribonucleic acid
α-MSH	alpha- melanocyte stimulating hormone
NaCl	sodium chloride
NDS	normal donkey serum
Neo/Neo	neonatal MCH deficient mice
NEAT	non-exercise activity thermogenesis
NEI	neuropeptide-glutaminc acid isoleucine
NGE	neuropeptide glycine-glutamic acid
NPY	neuropeptide Y
Ob-R	Leptin receptor
Ob-Rb	Leptin receptor subtype
ob/ob	leptin-deficient mice
OT,OXY	oxytocin
OxA	Orexin A
p.o.	per os (oral)
PBS	phosphate buffer saline
PET	Positron emission tomography
PFA	paraformaldehyde
PGC-1α	peroxisome proliferator-activated receptor γ
pmch	pro MCH gene
POMC	pro-opiomelanocortin
PP	Pancreatic polypeptide
PPARγ	peroxisome proliferator-activated receptor γ Co-activator I α
PPH	primary pulmonary hypertension
ppMCH	prepro Melanin-concentrating hormone
PTP-1B	protein-tyrosine phosphatase-1B
PVDF	Polyvinylidenfluorid
PYY(3-36)	Peptide YY (Fragment 3-36)
RFS	restricted feeding schedules
ROI	region of interest
RT	room temperature
s.c.	subcutaneous
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
sec	second
SNP	single nucleotide polymorphism
SST1-5	somatostatin receptor subtypes 1-5
TBS-T	Tris buffered solution with Triton X-100
TRH	Thyrotropin-releasing hormone
TRITC	tetramethylrhodamine isothiocyanate
TSA	tyramide signal amplification
TSH	thyreodea-stimulating hormone
US	Ultra Sound
USA	United States of America
VP	Vasopressin
w/v	weight/volume
WHO	World health organisation
WHR	Waist-to-hip circumference ratio
Y1,2,5-R	Neuropeptide Y receptor subtype 1,2,5
ZT	Zeitgeber time

Abbreviation of brain structures:

3V	third ventricle
4V	fourth ventricle
A	amygdala
AAV	anteroventral thalamic nucleus
aca	anterior commisure
ACB	nucleus accumbens
AcbC	Accumbens nucleus (core)
AcbSh	Accumbens nucleus (shell)
AD	anterodorsal thalamic nucleus
AHA	anterior hypothalamic area
AI	Agranular insula cortex
AON	anterior olfactory nucleus
APT	anterior pretectal nucleus
Arc/ARC	arcuate hypothalamic nucleus
BLA	Basolateral amydaloid nuclei
BNST	bed nucleus of the stria terminalis
CA1	CA 1 region of the hippocampus
CA2	CA 2 region of the hippocampus
CA3	CA 3 region of the hippocampus
Cbgr	granular cell layer of the cerebellar cortex
Ce	Central amygdaloid nuclei
CG	periaqueductal grey
CG/RSA,	
RSG	cingulate retrosplenial cortex
CIC	inferior colliculus
CL	centrolateral thalamic nucleus
CM	entralmedial thalamic nucleus
GP	Globus pallidus
CPu	Caudate Putamen
CTX	cortex
D3V	dorsal third ventricle
DEn	Dorsal endopiriform nucleus
DG	Dentate Gyrus
DMH	dorsomedial hypothalamus
DpMe	dorsal part of the medial amygdaloid nucleus
DTg	dorsal tegmental area
ec	external capsule
f	fornix
FR	frontal cortex
GI	granular insular cortex
HDB	nucleus horizontal limb diagonal band
HDB	nucleus of the horizontal limb of the diagonal band
Hi	hippocampus
IAM	interanterodorsal thalamic nuclei
IC	inferior colliculus
Icj	Islets of Calleja
IP	Interpeduncular nuclei
III	oculomotor nerve
IMD	intermediodorsal thalamic nucleus
Int	interpositus cerebellar nucleus
LC	locus coerulus
LH, LHA	lateral hypothalamus
LHB,	lateral habenular nucleus
LHBm,	lateral habenular nucleus (medial division)
LM	lateral mammillary nucleus
LSD	Lateral septal nucleus, dorsal part
LSV	Lateral septal nucleus, ventral part
MS	medial septal nucleus
7	facial nucleus
MD	mediodorsal thalamic nuclei
ME	median eminence
MHb	medial habenular nucleus
MM	medial mammillary nucleus
MPO	medial preoptic area
NAc	Nucleus accumbens
NLL	nucleus of the lateral lemniscus
NTS,Sol	nucleus of the solitary tract
3	occulomotor nucleus
OPT,oc,	
ocb	olivary complex
OT	olfactory tubercle
ox	optic chiasm
Par	Parietal cortex
PB	parabrachial nuclei
Pe	periventricular hypothalamic nucleus
PeF	perifornical nucleus
PF	parafascicular thalamic nucleus
PFA	perifornical area
PH	posterior hypothalamic area
Pir	Piriform Cortex
Pn	pontine reticular nucleus
Pr5	principle sensory trigeminal nucleus
PSV	principle sensory trigeminus nerve
PT	paratenial thalamic nucleus
PV	paraventricular thalamic nucleus
PVN	paraventricular hypothalamic nuclei
PVP	posterior paraventricular thalamic nucleus
R	Red nucleus
Re	reuniens thalamic nucleus
RMg, DR	Raphe nuclei
RT	reticular thalamic nucleus
S	Subiculum
SC	superior collicus
SCh	Suprachiasmatic nucleus
SI	substantia innominata
SNR	substantia nigra (pars reticulata)
SNC	substantia nigra (pars compacta)
SO	suprapotic nucleus
Sol	nucleus of the solitary tract
Sp	Spinal trigeminal nucleus
SPV	spinal tract of the trigeminus nerve
STh	subthalamic nucleus
SUB	subiculum
SuM	Supramammillary nucleus
TM, TMN	tuberomammillary nucleus
TT	Tenia Tecta
VE	vestibular nucleus
VL	ventrolateral thalamic nucleus
VLG,DLG	geniculate nuclei (ventrolateral/dorsolateral
VM	ventromedial thalamic nuclei
VMH	ventromedial hypothalamic nuclei
VMP	ventral posteromedial thalamic nucleus
VPL	ventral posterolateral thalamic nucleus
VTA	ventral tegmental area
ZI	Zona incerta

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Deutsche Zusammenfassung der Doktorarbeit

Neurochemische und funktionelle Charakterisierung des Melanin-konzentrierenden Hormon Systems im Rattenhirn

Die Regulation des Körpergewichts in einem physiologischen Rahmen setzt ein internes Energiegleichgewicht voraus und wird langfristig durch Abgleich von Nahrungsaufnahme einerseits und Energieverbrauch andererseits gewährleistet. Dieses Gleichgewicht ist bei massivem Übergewicht (Adipositas) oder chronischem Untergewicht (Kachexie) dauerhaft gestört.

Bei der Regulation des Energiegleichgewichts spielt der im Zwischenhirn gelegene Hypothalamus als Schaltstation eine wichtige Rolle. Hypothalamische Regelkreise gleichen sensorische, viszerale und humorale Signale miteinander ab und setzen sie in adäquates Verhalten (z.B. Nahrungsaufnahme) um. Innerhalb des Hypothalamus werden Hunger und Sättigung durch zentralnervöse Regulationssysteme kodiert. Dadurch stellt eine pharmakologische Inhibierung eines hunger-stimulierenden (orexigenen), hypothalamischen Regelkreises eine Möglichkeit dar, um Nahrungsaufnahme und Körpergewichts zu reduzieren.

Das im lateralen Hypothalamus gebildete Neuropeptid Melanin-konzentrierendes Hormon (MCH) ist ein solches orexigenes Signal. In unterschiedlichen Tiermodellen wurde gezeigt, dass MCH seine physiologischen Effekte auf das Energiegleichgewicht durch den funktionellen MCH Rezeptor Subtyp 1 (MCHR-1) vermittelt. Die Behandlung von Labornagern mit selektiv wirksamen MCHR-1 Antagonisten hat in verschiedenen Tiermodellen zu einer Verminderung der Nahrungsaufnahme und Körpergewichtsreduktion geführt (anorexigene Wirkung).

Das Ziel dieser Arbeit ist eine vertiefte Untersuchung des zentralen MCH Systems. Im ersten Teil der Arbeit werden MCHR-1 enthaltene Nervenzellen (Neurone) im Hypothalamus von Ratten immunhistochemisch identifiziert und neurochemisch charakterisiert. Dieser Teil der Arbeit soll mit Hilfe von Kolokalisationsstudien mögliche Interaktionen des MCH Systems mit anderen neuropeptidergen, hypothalamischen Systemen identifizieren.

Der zweite Teil der Arbeit befasst sich mit der Untersuchung von pharmakologischen Effekten bei MCH und MCHR-1 Antagonist behandelten Ratten auf Nahrungsaufnahme, Wasseraufnahme sowie Veränderung des Körpergewichts. Zentrale Regulationsmechanismen wurden durch den immunhistochemischen Nachweis des Transkriptionsfaktors und neuronalen Aktivierungsmarkers c-Fos im Rattenhirn ermittelt. Diese neuronalen Aktivierungsmuster

wurden mit solchen Mustern verglichen, die nach einem definierten physiologischen Stimulus (Fütterungsregime) mit derselben Methode aufgezeichnet wurden.

Erste Ergebnisse zeigten, dass der hier etablierte Antikörper gegen MCHR-1 spezifisch ist und MCHR-1 in mehreren hypothalamischen Kernarealen mit Hilfe dieses Antikörpers nachgewiesen werden konnte. So konnte im lateralen Hypothalamus eine Kolokalisation von MCHR-1 mit Orexin A nachgewiesen werden, im *arcuate* Nukleus des Hypothalamus, einem Kernareal, das eine bedeutende Funktion in der Integration von Hunger- und Sättigungssignalen hat, zeigten MCHR-1 positive Neurone eine Kolokalisation mit dem orexigenen Neuropeptid Y oder mit dem Adrenocorticotrophin Hormon, einem Marker für das anorexigen wirkende, zentrale Melanokortin System.
Der Paraventrikuläre Nukleus und der Supraoptische Nukleus des Hypothalamus spielen eine wichtige Rolle in neuroendokrinen Regulationen. Im paraventrikulären Hypothalamus konnte eine Kolokalisation von MCHR-1 mit den Neuropeptiden Vasopressin, Oxytocin und Corticotrophin-releasing Hormon festgestellt werden, außerdem konnte eine Kolokalisierung von MCHR-1 mit Vasopressin und Oxytocin im Supraoptischen Nukleus gezeigt werden. Zusätzlich konnte MCHR-1 immunhistochemisch auf Zellen der Adeno- und der Neurohypophyse nachgewiesen werden. Diese Ergebnisse lassen auf eine Interaktion von MCHR-1 im Hypothalamus nicht nur mit orexigenen (Orexin A und Neuropeptid Y) und anorexigenen (Adrenocorticotrophin Hormon) Signalen schließen, sondern weisen zusätzlich auf eine Rolle von MCHR-1 bei der Regulation des Wasserhaushalts (Vasopressin), der Fortpflanzung (Oxytocin) und bei Stress (Corticotrophin-releasing Hormon) hin.
Im zweiten Versuchsvorhaben führte die zentraler Gabe (intrazerebroventrikular) von MCH ins Rattengehirn zu einer akuten und signifikanten Steigerung der Futter- und Wasseraufnahme, es konnte jedoch kein spezifisches Aktivierungsmuster in hypothalamischen Kernarealen (Nuklei) definiert werden. Im Gegensatz dazu führte eine sub-chronische Gabe eines oral verfügbaren MCHR-1 Antagonisten in Ratten zu einer signifikanten Verminderung der Nahrungs-, Wasseraufnahme und des Körpergewichts. Bei qualitativer Analyse des immunhistochemischen Signals für c-Fos bei MCHR-1 Antagonist behandelten Ratten konnte eine spezifische Aktivierung im Paraventrikulären Hypothalamus, im Supraoptischen Nukleus und im Dorsomedialen Hypothalamus gezeigt werden. Diese Ergebnisse ließen sich durch automatisierte, software-unterstützte Quantifizierung des c-Fos Signals bestätigen und heben diese Hirnareale als mögliche neuroanatomische Substrate von MCHR-1 Antagonisten hervor.

Um eine mögliche neuronale Aktivierung des MCH Systems nach einem physiologischen Stimulus, hier Hunger oder Sättigung, zu untersuchen, wurden in einem weiteren Versuchsansatz Ratten in einem angepassten, neun Tage dauernden Fütterungsregime, täglich für nur drei Stunden Zugang zu Futter gewährt. Tiere, die am letzten Tag des Fütterungsregimes im 3 Stunden Zeitraum kein Futter bekamen und so als „Hunger-Stimulierte" definiert wurden, zeigten eine signifikante Induktion von c-Fos in unterschiedlichen hypothalamischen (*arcuate* Nukleus, Dorsomedial Hypothalamischen Nuklei, Lateral Hypothalamus) und extrahypothalamischen Hirnarealen (Nukleus Accumbens, Basolaterale Amygdala, Paraventriculärer Thalamischer Nukleus). Dieses Aktivierungsmuster unterschied sich von Ratten, die am letzten Tag des Fütterungsregims Futter erhalten hatten, den „gesättigte Tieren" (Aktivierung vor allem im supraoptischen Nukleus, im paraventrikulären Hypothalamus und Nukleus Tractus Solitarius), oder *ad libitum* gefütterten Kontrolltieren.

Um durch das Fütterungsregime aktivierte Neurone dem MCH System zuzuordnen, wurden immunhistochemische Kolokalisationsexperimente von c-Fos mit MCH beziehungsweise MCHR-1 spezifischen Antikörpern durchgeführt. Zwar konnte keine Kolokalisation von c-Fos mit MCH im lateralen Hypothalamus nachgewiesen werden, aber eine Vielzahl von durch Hunger oder Sättigung aktivierte, c-Fos positive Neurone zeigte MCHR-1 Immunoreaktivität. Zusammenfassend lässt sich daraus schließen, dass Nahrungskarenz differenziert unterschiedliche intra-hypothalamische und extra-hypothalamische Zielstrukturen aktiviert. Die funktionelle Rolle des MCHR-1 in solch aktivierten Neuronen bedarf weiterer Klärung.

Im abschließenden Teil der Arbeit wird eine mögliche Relevanz der hier beschriebenen Ergebnisse im Hinblick auf die Entwicklung von MCHR-1 Antagonisten und deren möglicher Einsatz bei Adipositas, diskutiert.

Abstract of the thesis

The central melanin-concentrating hormone (MCH) system has been intensively studied for its involvement in the regulation of feeding behaviour and body weight regulation. The importance of the neuropeptide MCH in the control of energy balance has been underlined by MCH knock out and Melanin-concentrating hormone receptor subtype 1 (MCHR-1) knock-out animals. The anorectic and anti-obesity effects of selective MCHR-1 antagonists have confirmed the notion that pharmacological blockade of MCHR-1 is a potential therapeutic approach for obesity.

First aim of this work is to study the neurochemical "equipment" of MCHR-1 immunoreactive neurons by double-labelling immunohistochemistry within the rat hypothalamus. Of special interest is the neuroanatomical identification of other hypothalamic neuropeptides that are co-distributed with MCHR-1.

A second part of this study deals with the examination of neuronal activation patterns after pharmacological or physiological, feeding-related stimuli and was introduced to further understand central regulatory mechanisms of the MCH system.

In the first part of work, I wanted to neurochemically characterize MCHR-1 immunoreactive neurons in the rat hypothalamus for colocalisation with neuropeptides of interest. Therefore I performed an immunohistochemical colocalisation study using a specific antibody against MCHR-1 in combination with antibodies against hypothalamic neuropeptides.

I showed that MCHR-1 immunoreactivity (IR) was co-localised with orexin A in the lateral hypothalamus, and with adrenocorticotropic hormone and neuropeptide Y in the arcuate nucleus. Additionally, MCHR-1 IR was co-localised with the neuropeptides vasopressin and oxytocin in magnocellular neurons of the supraoptic and paraventricular hypothalamic nucleus and corticotrophin releasing hormone in the parvocellular division of the paraventricular hypothalamic nucleus. Moreover, for the first time MCHR-1 immunoreactivity was found in both the adenohypophyseal and neurohypophyseal part of the rat pituitary.

These results provide the neurochemical basis for previously described potential physiological actions of MCH at its target receptor. In particular, the MCHR-1 may be involved not only in food intake regulation, but also in other physiological actions such as fluid regulation, reproduction and stress response, possibly through here examined neuropeptides.

Central activation patterns induced by pharmacological or physiological stimulation can be mapped using c-Fos immunohistochemistry. In the first experimental design, central

administration (icv) of MCH in the rat brain resulted in acute and significant increase of food and water intake, but this animal treatment did not induce a specific c-Fos induction pattern in hypothalamic nuclei. In contrast, sub-chronic application of MCHR-1 antagonist promoted a significant decrease in food- and water intake during an eight day treatment period.

A qualitative analysis of c-Fos immunohistochemistry of sections derived from MCHR-1 antagonist treated animals showed a specific neuronal activation in the paraventricular nucleus, the supraoptic nucleus and the dorsomedial hypothalamus. These results could be substantiated by quantitative evaluation of an automated, software-supported analysis of the c-Fos signal.

Additionally, I examined the activation pattern of rats in a restricted feeding schedule (RFS) to identify pathways involved in hunger and satiety. Animals were trained for 9 days to feed during a three hour period. On the last day, food restricted animals was also allowed to feed for the three hours, while food deprived (FD) animals did not receive food.

Mapping of neuronal activation showed a clear difference between stareved (FD) and satiated (FR) rats. FD animals showed significant induction of c-Fos in forebrain regions, several hypothalamic nuclei, amygdaloid thalamus and FR animals in the supraoptic nucleus and the paraventricular nucleus of the hypothalamus, and the nucleus of the solitary tract.

In the lateral hypothalamus of FD rats, c-Fos IR showed strong colocalisation for Orexin A, but no co-staining for MCH immunoreactivity. However, a large number of c-Fos IR neurons within activated regions of FD and FR animals was co-localised with MCHR-1 within selected regions. To conclude, the experimental set-up of scheduled feeding can be used to induce a specific hunger or satiety activation pattern within the rat brain. My results show a differential activation by hunger signals of MCH neurons and furthermore, demonstrates that MCHR-1 expressing neurons may be essential parts of downstream processing of physiological feeding/hunger stimuli.

In the final part of my work, the relevance of here presented studies is discussed with respect to possible introduction of MCHR-1 antagonists as drug candidates for the treatment of obesity.

1. General introduction

1.1. Energy homeostasis and the regulation of body weight

Homeostasis (greek: homeo, equal; -stasis, keep) can be viewed as one of the basic biological principles that keeps the body in constant internal environment. For example, energy homeostasis balances the energy stored in the body, mainly in the form of fat, in a physiological state (Kennedy, 1953). A number of theories have been discussed to describe the physiology behind homeostatic systems, among them the set point theory (Hardy, 1969 Bradley, 1978; Van Itallie et al., 1977). Although physiologists have introduced the set point theory to describe temperature regulation, the concept takes whole body functions into account including energy homeostasis (Cabanac, 1971, 2001, 2006). The set point theory suggests that body weight and body fat is controlled by an equivalent device named *ponderostat* residing in the brain. The ponderostat receives feedback input from the energy content in the form of body weight/body fat and generates an error signal, if this feedback signal is deviating from the set point (individual, physiological body weight/fat content). The error signal (change in body weight/body fat) is used to turn up or down any mechanisms to re-arrange the energy content of the body and as a consequence, bodies energy stores are metabolised or stored to keep bodies energy constant (= steady state).

In adult individuals, body weight and fat content is maintained at a relative stable level for long periods, which is necessary for key purposes such as survival or reproduction (Rohner-Jeanrennaud et al., 1999). The duty of energy homeostasis substantiates the ponderostat theory, but recent advances in the neurobiology of energy homeostasis imply additional arguments to the ponderostat theory. First, it now seems clear that not a single “ponderostat” regulates body weight and fat content, but an orchestra of numerous peripheral signals combined with several brain regulatory pathways have been described to inform the brain of bodies energy content. Second, these signals serve as orexigenic, anabolic (hunger-stimulating, body weight increasing, fat accumulating) factors, or anorexigenic, katabolic (hunger-inhibiting, body weight decreasing, fat metabolising) factors acting on brain and/or peripheral organs. Third, the sensible interaction of anabolic and katabolic pathways regulate bodies energy intake or energy expenditure and as a consequence, change the endocrine and metabolic state of the body. Fourth, these changes are sensed by peripheral organs that build up a feedback loop with the central nervous system (CNS) (Schwartz et al., 2000; 2006). A precise regulation of this complex mechanism of energy homeostasis seems essential since the knowledge, that changes in energy homeostasis are accompanied with different disease states.

On the one hand, starvation results in weight loss. In several disease states, cachexia (gr. Kachexi 'a; kako's bad; "e 'xis condition) induced by malnutrition and as a consequence, caloric deprivation is the major cause of weight loss and associated with increased mortality in pathological states (Morley et al., 2006). Examples of diseases that result in cachexia are tumor cachexia (von Meyenfeldt, 2005), the acquired immunodeficiency syndrome (AIDS)- cachexia (Faintuch et al., 2006) and anorexia nervosa (AN) (Mitchell et al., 2006). Although intense weight loss is based on slightly different mechanisms in these disease states, cachexia manifests clinically with excessive weight loss in the setting of ongoing disease, usually with disproportionate muscle wasting (Agiles et al., 2006).

On the other hand, massive overweight, termed obesity, can be defined as a chronic disorder with a long-term excess of energy intake over energy expenditure that results in massive white adipose tissue accumulation (Spiegelman and Flier, 2001). Although obesity has been partly simplified to the fact that obese patients eat more than their physiological needs and prefer high caloric food combined with reduced physical activity patterns (Bray GA, 2006), a number of additional environmental or genetic factors contribute to the pathogenesis of obesity (Fig. 1). However, the magnitude of influence of both causes are under current discussion (Malecka-Tendera and Mazur, 2006) and current obesity research points out, that understanding of the complexity of the disease helps to change a simplified stigma of this disease (Friedman, 2004; Volkow and Wise, 2005). Without doubt, obesity and its associated disorders, are the mayor health problem of people in the western, industrialized countries.

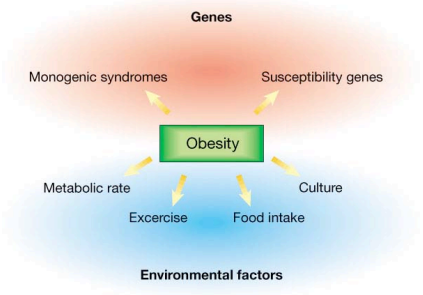


Fig.1. A number of factors influence obesity. Environmental factors in our society seem to play a prominent role, but also the genetic background impinge on the development of obesity (From Kopelman et al., 2000).

1.2. Components of energy homeostasis: energy intake and energy expenditure

In order to keep bodies energy content stable, energy intake in the form of food calories must fit with daily energy expenditure. Findings from studies of nutritional research provide evidence that the energy density (kcal energy/gram (g) ingested food) and the portion size (g food) are important determinants of energy intake (Kral and Rolls, 2004). Energy density of foods, as opposed to their sugar or fat content, is said to be a key determinant of energy intakes.

Recent laboratory studies have shown that, under free access (*ad libitum*), subjects consume a constant weight or volume of food, so that their energy intakes depend on the energy density of the diet. In most of the snacks we consume (such as chocolate bars and potato chips), a high energy density equals high palatability and vice versa (Drewnoski, 1999).

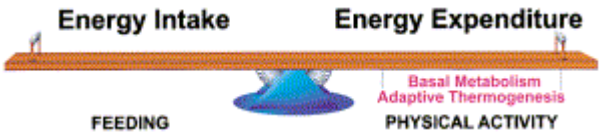


Fig. 2. Key component of the energy balance system. Obesity develops only if energy intake, in the form of food, chronically exceeds total body expenditure. Energy expenditure includes basal metabolic rate (BMR), adaptive thermogenesis and physical activity. Thermogenesis can be separated into exercise-related activity thermogenesis and non-exercise activity thermogenesis (From Spiegelman and Flier, 2001).

There are three principal components of human energy expenditure (Fig. 2). The basal metabolic rate (BMR) is the energy expended when an individual is laying at complete rest, in the morning, after sleep, in the post-absorptive state. BMR accounts for approximately 60% of total daily energy expenditure and is predicted by lean body mass within and across species (Ford, 1984; Deriaz et al., 1992). Thermic effect of food, also called adaptive thermogenesis, is the increase in energy expenditure associated with the digestion, absorption and storage of food, and accounts for approximately 10–15% of the total daily energy (Reed and Hill, 1996). Activity thermogenesis can be separated into two components: exercise-related activity thermogenesis and 'non-exercise activity thermogenesis' (NEAT). While for the vast majority of the populations of developed countries exercise-related activity (physical activity) thermogenesis is negligible, NEAT is the predominant component of activity thermogenesis and is the energy expenditure associated with all the activities undertaken by the human population as vibrant independent beings (Levine, 2003).

The concept of energy homeostasis implies two prerequisites in case of body weight regulation: If humans ingest low energy density foods that provide fewer calories per eating bout, they should -in theory- loose weight by reduced energy intakes. In contrast, high caloric intake combined with energy storage will increase body weight. With respect to energy expenditure, body weight will increase if energy intake exceeds total energy expenditure and decrease, if energy intake is lower than the amount of burned energy.

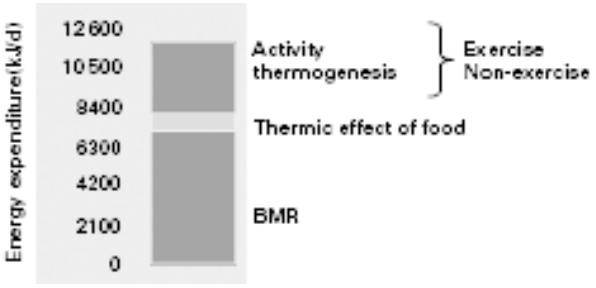


Fig. 3. Components of energy expenditure in sedentary adults (BMR, basal metabolic rate) (from Levine, 2003)

1.3. Body weight, energy storage and states of disturbed energy homeostasis

Most commonly, underweight normal weight, overweight and obesity are classified in adults by anatomical parameters, above all the body mass index (BMI), which is calculated by the body weight in kilogram (kg) divided by the square height in meter (kg/m^2) (WHO, 2000) (see also table 1). Because in adolescence body weight is highly age- and sex-specific dependent, children`s body weight is categorized in monograms of body mass indices, for the USA worked out by the Centers for Disease Control and Prevention (CDC) (Daniels et al., 2005). Also for Germany, there exists a percentile categorisation based on epidemiological data of school children (Toschke et al., 2006).

Other parameters are more specific for a categorisation of obese patients. Bodies fat distribution can be easily assessed using anthropometric measurements such as skinfolds thickness and waist-to-hip circumference ratio (WHR). Although both parameters are simple and convenient for epidemiological studies and provide useful estimation of the proportion of abdominal or upper-body fat, they do not distinguish between accumulation of deep abdominal (visceral) fat and subcutaneous abdominal fat (Björntorp, 1984). More precisely, imaging techniques, particularly computed tomography (CT) and magnetic resonance imaging (MRI), clearly distinguishes fat from other tissues and allow the measurement of visceral and subcutaneous abdominal fat (Märin et al., 1992). Supplementary, ultrasound (US) can determine subcutaneous and intra-abdominal thickness and the intra-individual reproducibility of US measurements was very high both for intraabdominal and subcutaneous thickness as well as for interoperators (Armellini et al., 1993; Tornaghi et al., 1994). Furthermore, the composition of the body (mostly water, muscle and fat) can be measured by bioelectric impedance analysis (BIA) (Roubenoff, 1996). Since an inconstant correlation between BMI and metabolic disturbances have been found, the distribution of body fat appears to be an important

factor, as alterations in the endocrine, metabolic, cardiovascular status of obese patients suggest (Kannel et al., 1985; Larsson, 1991).

In humans, two possibilities for energy expenditure measurements are currently used: energy expenditure can be monitored using doubly labelled water (Prentice et al., 1996) or by indirect calorimetric measurements (Segal, 1987).

Parameter	Characteristic changes in obese individuals
	Classification (WHO)
BMI (in kg/m^2)	25-29.9 overweight
	30-39.9 obesity
	>40.0 morbid obesity
Body composition	abdominal/subcutaneous fat
	WHR: men > 94 cm, Women > 88 cm
Clinical symptoms	hypoactivity
	menses irregularities, chronic oligo-anovulation and infertility in adulthood
	hypertension
Laboratory diagnosis	Increased fasting glucose value, hyperinsulinemia, increased lipid parameters (triglycerides, cholesterol, high density lipoprotein)
	hyperleptinemia

Table 1. Comparison of several anthropometric and clinical parameters in obese and anorectic patients. (data are summarized from the review edited by Hebebrand et al., 2006).

In modern times, the excess in adipose tissue as fuel storage is associated with a disease state, but it is important to remember that what is now considered "pathophysiology" was probably advantageous when food was less available and a high level of energy expenditure in the form of physical activity was a way of life (Eckel, 2003). The problem of obesity is not the massive overweight *per se*, but the obesity-associated endocrine and metabolic disturbance, a pathological state called the metabolic syndrome (Lamounier-Zepter et al., 2006; Alberti et al., 2006; Grundy, 2006). Obesity is now accepted as a mayor risk factor for multiple morbidities ranging from non insulin-dependent (diabetes type 2) diabetes, hypertension, peripheral and cardiovascular disease, stroke, various musculo-skeletal disorders, sleep apnoe, and certain forms of cancer (Kopelman, 2000). Additionally, massive overweight affects the pathogenesis of other diseases, to include cholelithiasis (Maclure, 1998), osteoarthritis (Felson, 1988), infertility (Grodstein, 1994), stroke (Rexrode, 1998), cutaneous infections (Scheinfeld, 2004), and wound healing deficiencies (Gottschlich, 1993).

1.4. Obesity: epidemic disease of "first world"countries?

The prevalence of obesity has increased dramatically in the recent years such that it is now recognized as a serious medical problem, particularly in the western world (Cummings, 2003; Tataranni, 2003). In the USA, it is estimated that more than 30 and 65% of the american adults are overweight or obese, respectively (Flegal, 2002). In parallel, in the USA the prevalence of childhood overweight tripled between 1980 and 2000 (Ogden et al., 2002). Trends for an increase in obesity are also rising considerably amongst children and adolescents, which is worrying because poor eating habbits are often established during childhood (Daniels et al., 2005). Today, epidemiological data concerning the prevalence of obesity among children in the European Community shows that Europe follows the American trend and makes it a future problem for our health system (Janssen et al., 2005). These observations brings the problem of obesity and the pathophysiology behind the disease in a focus of current research (Spiegel and Nabel, 2006).

1.5. Biological mechanisms of food intake and body weight regulation

1.5.1. Peripheral information inform us about the energy content of the body

The set-point theory suggests that body weight is regulated at a predetermined, or preferred, level by a feedback control mechanism from peripheral signals to the "ponderostat" in the central nervous system (CNS). The ponderostat theory assumes that following food ingestion, physiological changes in the periphery induce termination of the meal, defined as short-term satiety (Berthoud, 2002). Gibbs and Smith proposed that signals generated during a meal (termed satiety factors), including peptides secreted from the gastrointestinal tract, provide information to the brain that inhibit feeding and lead to meal termination (Gibbs and Smith, 1973). Ingestive behaviour comprise the presence of food, stimulation by sensory cues, the directed drive towards food intake (anticipatory behaviour), initiation of the meal, consummation and finally, the termination of the meal that results with satiation (Watts, 2000). When naturally satiated, rats stop eating, grooming and explore for a short time, and then rest or sleep (Antin et al., 1975). Which physiological consequences are associated with satiety and which peripheral factors complex regulation of are involved in the regulation of ingestive behaviour? Today, numerous factors have been described to be involved in the neural control of ingestive behaviour. These signals can be subdivided into short-term satiety signals that terminate a meal or long-term satiation signals that regulate hunger until the start of the next meal (Blundell, 1979).

Within this regulatory processes, a number of circulating signals of different origin communicate with the brain through neural, metabolic, or endocrine pathways (Berthoud, 2002). Signals regarding sensory cues of ingested food such as smell, sight and odour are important information for the choice of food (Gibson, 2006) and mechanical signals indicating the stretch of the stomach (gastric tension) (Grill and Norgren, 1978). Numerous short –term, meal-related, gastrointestinal peptides are responsible for the regulation of satiety (Luckman, 2003). The most studied peptides released from the gut are cholecystokinin (CCK), pancreatic polypeptide (PP), peptide YY fragment (3-36) (PYY_{3-36}) and glucagon-like peptide-1 (GLP) (reviewed by Huda et al., 2006; Strader and Woods, 2005). These gut peptides act locally within the gut or through the vagus nerve to increase satiety and signals a stop of ingestion or can act directly via their receptor located in the brain (Chaudri et al., 2006). Thus, they influence food intake to maintain the appropriate meal size in order to regulate daily energy intake. There is evidence that dietary nutrients, as well as circulating and even intracellular nutrients, can affect the expression of peptides involved in eating behaviour, shunting fuels from short-term availability to long-term storage (Obici and Rossetti, 2003; Seeley and Woods, 2005). Especially macronutrients including carbohydrates and lipids of a diet we ingest have effects not only on peripheral metabolism, and release of hormones but also on the brain site to influence when to stop eating or what to next (Wise, 2002). In addition, hormones like ghrelin, insulin and leptin act in the brain as hunger or satiety signals (Schwartz, 2006) (see table 2).

Peripheral, hunger signals	**peripheral, satiety/satiation signals**
ghrelin (X/A-like cells intestine)	PYY(3-36) (L cells, intestine)
	GLP-1 (L cells, intestine)
	oxyntomodulin (L cells, intestine)
	amylin (L cells, intestine)
	CCK (I cells, intestine)
	insulin (ß-cells, pancreas)
	leptin (adipose tissue)

Table 2. Peripheral signals that promote hunger or satiety/satiation within the brain and their origin (derived from Strader and Woods, 2005; Huda, 2006).

Circulating ghrelin is released from the stomach and is the only endogeneous hormone that has been shown to induce hunger and increase food intake (Kojima et al., 1999; Laferrere et al., 2005). In contrast, insulin from the pancreas and the adipocyte-derived leptin are hormons

capable to reduce hunger and promote satiation (Wang and Leibowitz, 1997). Increased blood-glucose promotes insulin release from pancreatic ß-cell, that reduces not only bood glucose levels, but also act as a signal in the brain to reduce food intake (Niswender, 2003). More than 50 years ago, Kennedy proposed, that inhibitory signals generated in proportion to body fat stores act in the brain to reduce food intake (Kennedy, 1953). Leptin (greek leptos, thin) seemed to fit as an adipose tissue-brain feedback mechanism and serves as a long-term satiety signal which is produced in proportion to the fat stores. Adequate leptin levels communicate the repletion of bodies energy stores to the CNS in order to suppress food intake and permit energy expenditure (Jequier et al., 2002; Bates et al., 2003). Leptin, insulin, ghrelin and PYY $_{(3-36)}$ are transported through the bloodstream and exert their orexigenic (hunger-stimulating) or anorexigenic (satiety/satiety promoting) actions in several brain regions, above all in the hypothalamus, that serves as an integrator for feeding-related signals (Fig. 4).

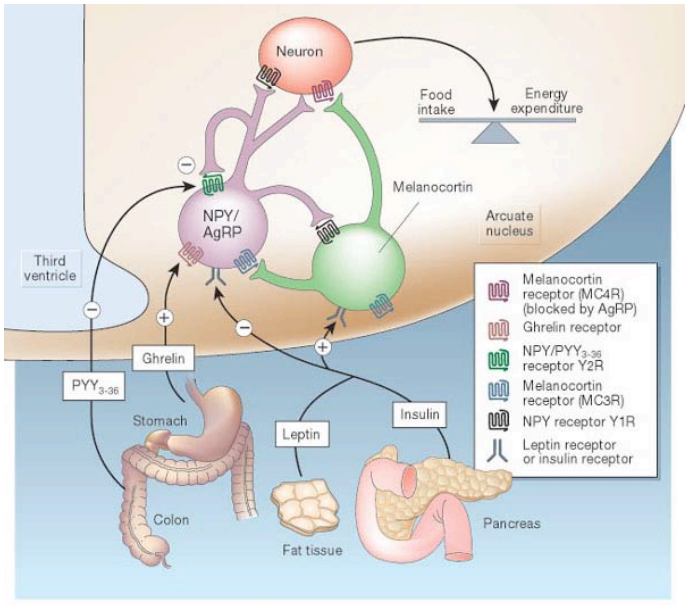


Fig. 4. Peripheral hormones act as hunger or satiety factors in the brain. The gastric hormone ghrelin stimulates appetite by activating *arcuate* neurons expressing the neuropeptides NPY/AgRP. PYY_{3-36} from the colon inhibits these neurons and thereby decreases appetite for up to 12 hours. Leptin and insulin by either inhibition orexigenic neuronal NPY/AgRP pathways or activation the anorexigenic melanocortin pathway via another subset of neurons (top) (from Schwartz and Morton, 2000). A detailed description of hypothalamic orexigenic and anorexigenic pathways are described in the following part.

Abbreviations: peptide YY, PYY_{3-36}; NPY, neuropeptide Y; Y1R/Y2R, neuropeptide Y receptor subtype 1,2; /PYY_{3-36} receptor; MC3R/MC3R, melanocortin receptors; AgRP, agouti related peptide.

1.5.2. Integration of peripheral signals in the central nervous system

Since the discovery of leptin by Zhang (1994), a growing number of centrally acting neuromodulators and their receptors are known to orchestra food intake regulation and energy homeostasis in the brain (for review see Elmquist et al., 1998; Schwartz et al., 2000; Broberger , 2005; Leibowitz and Wortley, 2004).

For example, leptin is released from adipose tissue in proportion to the bodies fat content and released to the blood circulation. From there leptin is transported in the bloodstream to the brain and is capable to pass the blood brain barrier such as in the median eminence, a part of the circumventricular organs. Leptin bind at its functional leptin receptor Ob-R, which is expressed in several hypothalamic regions (Münzberg et al., 2005). Similar mechanisms have been found for ghrelin, insulin and PYY_{3-36} (Schwartz and Morton, 2000; see also Fig. 4). Thus, brain nuclei located within the hypothalamus have been proposed to act as a *relay station* not only for endocrine factors but sensory, metabolic and neural information pass hypothalamic regions and enter neuronal pathways (Elmquist, 1998).

One important hypothalamic structure for the control of energy hoemostasis is the *arcuate* nucleus (Arc) of the hypothalamus located in direct proximity of the third ventricle and the ME. Arcuate neurons have been observed as leptin-sensitive because they express the leptin receptor Ob-R and have been characterized as "first order neurons" within a neuronal feeding network (Elias et al., 1998). Arcuate neurons can be subdivided into two neuronal populations: While one neuronal subpopulation express the orexigenic neuropeptides Neuropeptide Y (NPY) and agouti related peptide (AgRP) and are inhibited by leptin, the other neuronal subpopulation express the anorexigenic prohormone proopiomelanocortin (POMC; cleavage products are the melanocortins) and the neuropeptide cocaine- and amphetamine-regulated transcript (CART) that are enhanced by leptin (Broberger et al., 1998; Elmquist et al., 2001) (Fig. 4, 5).

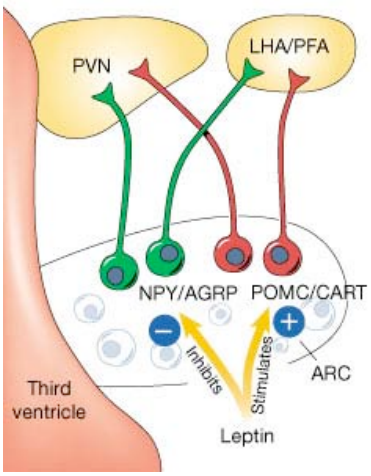


Fig. 5. Populations of "first-order" NPY/AgRP (green) and POMC/CART (red) neurons in the arcuate nucleus (ARC) are regulated by leptin and project to the PVN, the LHA and PFA, which are locations of "second-order" hypothalamic neuropeptide neurons involved in the regulation of food intake and energy homeostasis (from Schwartz et al., 2000).

AgRP, Agouti related peptide; ARC, arcuate nucleus; CART, cocaine and amphetamine regulated peptide; LHA, lateral hypothalamic area; NPY, Neuropeptide Y; POMC, Proopiomelanocortin; PVN, paraventricular hypothalamic nucleus; PFA, perfornical area.

Axons of NPY/AgRP and POMC/CART neurons have been found to innervate for example the lateral hypothalamic neurons (LHA) and neurons located in the paraventricular nucleus of the hypothalamus (PVN). These neurons serve as second order expressing the orexigenic neuropeptides Melanin-concentrating hormone (MCH) and Orexin A (OxA) (Broberger et al., 1998; Elias et al., 1998, 1999) and express the neuropeptides corticotropin releasing hormone (CRH), thyrotrophin-releasing hormone (TRH), oxytocin (OXY) and vasopressin (VP) (Sawchenko et al., 1998) (Fig. 6).

LHA and PVN neurotransmitter systems have been examined as mediators of regulatory pathways and relay afferent information from the Arc for a complex adaptive responses required for energy homeostasis (Schwartz et al., 2002). This process is mediated via three mechanisms: effect on behaviour, including feeding and physical activity, effects on autonomic nervous system activity through sympathetic and parasympathetic preganglionic neurons, that regulate energy expenditure and aspects of metabolism mediated by the hypothalamic-pituitary-axis (Spiegelman and Flier, 2001; Elmquist et al., 1998).

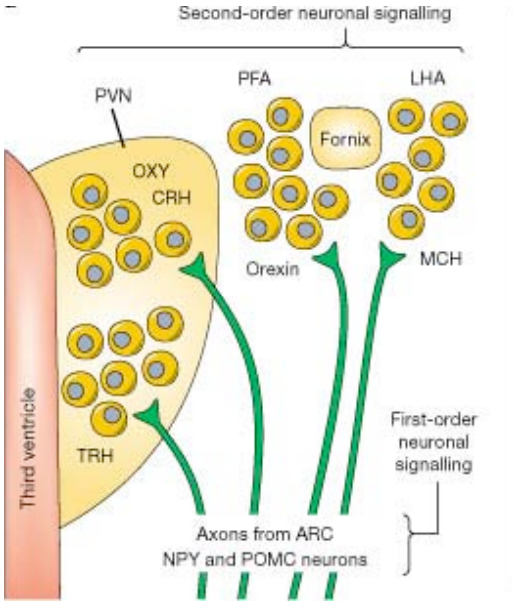


Fig. 6. Diagram showing second order , feeding-related neurons located in the PFA/LHA or PVN of the hypothalamus. PFA/LHA neurons express the neuropeptides orexin (Orexin A and orexin B) or MCH, neurons of the PVN express TRH, CRH and OXY in the PVN (not shown VP) (from Schwartz et al., 2000).

CRH, corticotrophin-releasing hormone; MCH, Melanin-concentrating hormone; NPY, neuropeptide Y; OXY, oxytocin;

PFA perifornical area, POMC, proopiomelanocortin; TRH, thyrotrophin-releasing hormone.

The schematic presentation of the hypothalamic feeding circuitry seems to be quite simple, but besides the aforementioned neuropeptidergic systems, a large number of other neuropeptides and non peptide neurotransmitters (such as the monoamines dopamine and serotonin) have been shown to modify feeding behaviour (Meguid et al., 2000). These systems use parallel or diverse orexigenic and anorexigenic signalling pathways within the brain and orchestra integrative feeding behaviour (table 3). To note, hypothalamic neuropeptides are dynamic in

their expression and are shifted by circadian rhythm, circulating factors, metabolites and the level of stored energy (Leibowitz and Wortley, 2004).

orexigenic neuroactive substances	anorexigenic neuroactive substances
neuropeptide Y (a)	α-melanocyte stimulating hormone (e)
agouti related peptide (b)	cocaine-and amphetamine- regulated transcript (f)
melanin concentrating hormone (a)	Corticotrophin-releasing factor (g)
orexin A (a)	serotonin (5-hydroxytryptamine) (h)
galanin (c)	
galanin like peptide (d)	

Table 3. Selected central-acting neuroactive substances that influence feeding behaviour within the hypothalamus.
Cited literature (a-h) are referred to *in vivo* experiments in rodents that provoked acute effects on feeding (orexigenic effects) or inhibition of feeding (anorexigenic effects).

Additionally, in higher species, food intake has an additional hedonic character and we are motivated to eat more, if food has a rewarding nature such as a dinner with delicious flavour (Mela et al., 2001; Saper et al., 2002). Food visualisation, tasting, olfaction and other sensory compounds such as texture build up the "rewarding properties" of food, which eventually influence our food preference and the amount of energy we ingest (Levine et al., 2003; Kringelbach, 2004). Aspects of "Wanting and liking" give food similar characteristics of a drug with possible problems concerning therapeutic side effects such as addiction (Volkow and Wise, 2005).

1.5.3. Effector pathways that regulate food intake and energy expenditure

Brain lesioning and stimulation experiments in laboratory animals performed more than five decades ago led to the proposal of a dual centre hypothesis (Brobeck, 1946; Anand and Brobeck, 1951). Discrete lesioning of hypothalamic nuclei involved in the central control of energy balance, resulted in the observation that the ventromedial hypothalamic nucleus functions as the "satiety centre" and the lateral hypothalamus represented a "feeding centre" (Bernardis and Bellinger, 1993; Storlin and Alberts, 1972). Although the hypothesis of these "feeding-related brain centres" has been replaced to the notion of discrete neuronal pathways that integrate feeding related information in a more complex neuronal network, the PVN and the LHA of the hypothalamus contain critical cell groups that have been described to regulate many aspects of energy balance (Watts, 2000). Based on morphological and physiological

analysis, both brain regions have been reviewed for their integrative roles in energy homeostasis (Kalra et al., 1999).

A number of studies have shown that central administration of neuropeptides induce feeding, while others inhibit feeding (table 3), giving further evidence for effects of these neuropeptides on feeding behaviour. All of the here mentioned neuroactive substances and their receptors have been defined to have orexigenic or anorexigenic properties and have been shown to be involved in the hypothalamic pathways underlying the endocrine, autonomic, and behavioural control of feeding (Kalra et al., 1999; Elmquist, 2001).

Additionally, recent evidence indicate that spontaneous physical activity and thermogenesis (NEAT) may be regulated by these brain systems. Especially the lateral hypothalamus derived neuropeptides, Orexin A and MCH are supposed to act directly on the regulation of energy expenditure by decreasing thermogenesis (Kotz, 2006; Shi, 2004). Energy expenditure is also affected by the neuroendocrine effector pathways downstream the PVN by modulation of CRH release (activation of the hypothalamic-pituitary-adrenal axis) (Koob and Heinrichs, 1999) or TRH release (hypothalamic-pituitary-thyroid axis) (Lechan, 1993).

1.5.4. Animal models of obesity and their relation to the human problem

The identification and analysis of monogenetic mutant mice by the method of forward genetics yielded in a number of mutant animals that lack hormones, neuropeptides or their receptors (Barsh and Schwartz, 2002; Imai and Ahima, 2005). Furthermore, polygenic rodent models which differ in body weight, fat content and susceptibility to diabetes, hyperlipidemia and other metabolic abnormalities compared to its strains, have provided new insights into the interplay between genetics and the environment (Barsh et al., 2000).

The history of the anorexigenic hormone leptin may reflect difficulties in obesity research. Leptin was discovered and the fully chemical characterised by Zhang et al., 1994. Obese, leptin-deficient ($Lep^{ob/ob}$) mice showed hyperphagia, morbid obesity, insulin resistence, steatosis and a number of neuroendocrine deficits (Ahima and Flier, 2000; O`Rahilly, 2003). Importantly, ablation of the functional leptin receptor Ob-Rb ($Lepr^{db/db}$) produces the same obese phenotype (Chen et al., 1996), but these effects have been restricted to central Ob-Rb deficiency (Cohen et al., 2005). Central and peripheral leptin administration reverses these effects (Pelleymounter et al., 1995). Since the discovery of leptin, it was hoped that exogeneous leptin might induce satiety and weight loss in obese humans (Friedman, 1998; Tartaglia et al., 1997). Unfortunately, a clinical trial study in humans have demonstrated that a leptin-based obesity regimen only results in limited weight loss (Heymsfield et al., 1999), but in the rare

cases of monogenetic leptin deficiency, treatment with recombinant leptin has virtually normalized body weight in heavily obese children (Farooqi et al., 2002). Although leptin treatment reduces appetite and promotes weight loss in obese humans, the scale of the body weight loss has been modest and disappointing (Manzoros et al., 2000). Indeed, obese individuals have elevated leptin levels as a consequence of their body fat, but they do not adequate respond to these increased leptin levels with reduced body weight, a phenomenon also called hyperleptinemia and leptin resistence (Frederich et al., 1995; Bell et al., 2005).

Consistently with leptin, in obese individuals insulin levels are elevated by fasting and the insulin response is exaggerated to an oral glucose load (Koltermann et al., 1980). Moreover, fasting ghrelin levels are reduced in obese subjects and diet-induced weight loss raises ghrelin levels (Tschöp et al., 2001), physiological reactions following the "obesigenic" environment.

As a consequence, the susceptibility to obesity is determined to a significant extent by genetic factors, but a favorable environment is necessary for its phenotypic expression (Ravussin and Brochard, 2000).

1.6. Pharmacological therapies for obesity -potentials and pitfalls

However, the discovery of leptin and its role in the regulation of energy homeostasis opened the field for other pharmacotherapeutic option to treat obesity. In general, an anti-obesity-therapy should promote a decrease of fat deposits, improve metabolic parameters that coincident with the metabolic syndrome and decrease body weight for a long period of time. An obesity prevention and treatment program is recommended to consist of a multitude of techniques including cognitive-behavioural aspects, nutritional therapy combined with physical activity (www.deutsche adipositasgesellschaft.de). It may be unrealistic that enhancing self-regulatory skills will be sufficient to overcome the combined influence of our appetitive predispositions and the obesigenic environment (Lowe, 2003). In addition, the lost weight after a hard diet has been shown to be disappointing, when obese patients fall in a rebound pattern in the follow-up time and re-gain weight (Lowe, 2003). In most cases of morbid obesity (BMI above 40 kg/m^2), self-regulatory effords such as dieting may not be efficient enough, and therefore supportive therapeutic tools are needed.

One option to support weight loss and to ameliorate the consequences of rebound effects may involve the use of a pharmacotherapy of obesity. In response to the pronounced trend of increasing obesity among the population of the developed world, the pharmaceutical industry has embarked upon various strategies for drug intervention, primarily targeting reduction of energy intake (Chaki, 2001; Cowley et al., 2002; Weigle et al., 2003; Cheetham et al., 2004). A

summary of possible peripheral and central targets using different mechanisms to govern energy hoemostasis are illustrated in figure 7.

Among peripheral targets for a possible pharmacotherapy of obesity, animal experiments have shown that the skeletal muscle is involved in adaptive thermogenesis (increase in energy expenditure) (Dullo, 2006) and genetically modified mice that lack enzymatic protein-tyrosine phosphatase-1B (PTP-1B) have shown insulin sensitivity resistence of obesity (Elchebly et al., 1999). Thyroid hormone analoga and 11-ß hydroxysteroid-dehydrogenase (11-ß HSD) inhibitors increase hepatic cholesterol/ insulin sensitivity reinforcers for metabolic benefits (Grover et al., 2003; Andrews et al., 2003). Intervention on effector pathways that involve gastrointestinal hormones such as ghrelin, PYY_{3-36} and CCK release aim to promote satiety through peripheral and central mechanisms. The pancreatic lipase inhibitor orlistat inhibit absorption of lipids and reduces energy intake (see below). The main organ for energy storage is the adipose tissue and a therapy should burn down fat reservoirs, a possible mechanism involve modulation of adipose tissue-derived secretory factors called adipokines (Hutley and Prins, 2005). The adipocyte enzymes diacylglycerol acetyltransferase (DGAT), Acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) have been determined to regulate lipid metabolism, adipogenesis and additionally, may serve as satiety signals in the brain, as proved for leptin (Münzberg et al., 2005).

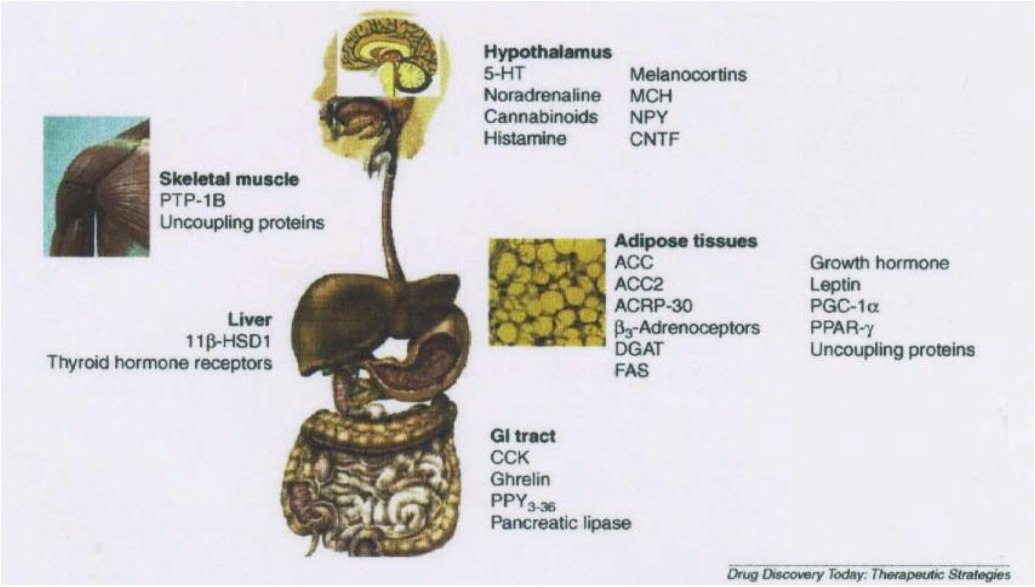


Fig. 7. Central and peripheral molecular targets for novel anti-obesity targets (from Cheetham et al., 2004). For abbreviations see list at page 3.

Most promising agents that act on the level of the hypothalamus contribute to the central inhibition of food intake with the possible side effect activating downstream energy expenditure pathways. Examples for pharmacological targets of current interest involve selective agonists to the serotonin (5-hydroxytryptamine, 5-HT) receptor subype 2c ($5HT_{2c}$) and other monoamine systems (noradrenaline, dopamine) or the Melanocortin pathway (MC4 receptor agonist). Another option for the central inhibition of hunger signals involve YR-1/YR-5 blockade or inactivation of the endocannabinoid receptor pathway (CBR-1). These molecular drug targets of orexigenic and anorexigenic central pathways belong to a large degree to the superfamily G protein coupled receptors (GPCR`s) (Schiöth, 2006).

However, the use of a drug in obesity therapy involves a risk-benefit assessment. Some obesity drugs that have been introduced on the market have shown an unfavorable risk/efficacy ratio (Greenway and Caruso, 2005). Some of the medications used for weight loss in the management of obesity have been associated with unacceptable morbidity and mortality. Safety concerns have led to the withdrawal of aminorex (amphetamine like compound), followed by the fenfluramines (selective serotonin reuptake inhibitor, SSRI) 1997, and phenylpropanolamine (norephedrine) in the year 2000. Aminorex was associated with an increased prevalence of primary pulmonary hypertension (PPH), fenfluramines with an increased prevalence of PPH and valvulopathy, and phenylpropanolamine with an increased risk of haemorrhagic stroke (Ioannides-Demos et al., 2006).

Three drugs acting on different mechanism are currently on the market for the indication of obesity. Orlistat (marketed as Xenical®), Sibutramine (trade name Meridia® in the USA, Reductil® in Europe) and Rimonabant (SR141716, trade name Acomplia®) are the only currently approved medications for the pharmacotherapeutic management of obesity. Orlistat is a reversible inhibitor of pancreatic and gastric lipases and acts peripherally on the gut lumen resulting that about 30% of dietary fat is not absorbed and passes into the stool in the form of triglycerides (Sjostrom et al., 1998). Sibutramine (noradrenaline and serotonine reuptake inhibitor) causes an increase of central anorexigenic serotonin and noradrenaline monoamines. The most controversial aspect of Sibutramine has been its effect on the increase of diastolic blood pressure (Jordan et al., 2005). A meta analysis of the randomized, double-blind clinical trials of obesity medications lasting longer than a year summarized that Orlistat gave a 2.9% greater weight loss than placebo and Sibutramine gave a 4.6% greater weight loss than placebo (Padwal et al., 2003), which can be interpreted as a mild efficacy. Although the benefit-risk profiles of Sibutramine and Orlistat appear positive, Sibutramine continues to be monitored because of long-term safety concerns.

Furthermore, hope has been attracted to Rimonabant, which acts as a selective antagonist of the CBR-1 (Pagotto et al., 2003; Smith and Fathi, 2005). Although the weight loss has been monitored to be effective comparable to Sibutramine, Rimonabant has additional beneficial effects on triglycerides, high density lipoprotein, and insulin sensitivity (Carai, 2006). However, prominent adverse effects involved nausea, diarrhoea, dizziness, anxiety and depression symptoms (Gelfand and Cannon, 2006).

A number of anti-obesity drugs are currently undergoing *clinical* development. These include: (1) centrally-acting drugs, such as the noradrenergic and dopaminergic reuptake inhibitors radafaxine and the selective serotonin 5-HT_{2c} agonist APD-356; (2) drugs that target peripheral episodic satiety signals, such as glucagon-like peptide-1 (exenatide, exenatide-LAR and liraglutide), peptide YY (intranasal PYY_{3-36} and AC-162325) and amylin (pramlintide); (3) drugs that block fat absorption, such as the novel lipase inhibitors cetilistat and GT-389255; and (4) a human growth hormone fragment (AOD-9604) that increases adipose tissue breakdown (Halford et al., 2006).

Because of the most potent stimulatory actions of orexigenic pathways within the CNS, amongst other orexigenic systems, blockade of the central NPY-, Orexin- or MCH-system may also serve as potential targets for a pharmacological intervention. A number of recently published reviews have pushed the central MCH system as an interesting target for the treatment of obesity (Handlon et al., 2006; Rokosz, 2006; Hervieu, 2006).

1.7. Involvement of the Melanin-concentrating hormone (MCH) system in the regulation of energy hoemostasis

1.7.1. MCH is an orexigenic neuropeptide in the brain

The mammalian MCH gene encodes the precursor of the 165 amino acid prepro MCH (ppMCH) that reveals several putative neuropeptides including MCH, neuropeptide-glutaminc acid isoleucine (NEI), neuropeptide glycine-glutamic acid (NGE) (Nahon et al., 1989; Presse et al., 1990) and an alternative spliced product termed MCH-gene-overprinted-polypeptide (MGOP) (Toumaniantz et al., 1996) (Fig. 8).

These mature peptides were processed by proteolytic cleavage of th enzyme proconvertase (Maulon-Ferraille et al., 2002; Viale et al., 1997). (Fig. 9). A natural antisense transcription mechanism give rise to the antisense-RNA-overlapping-MCH-gene (AROM) and nontranslated antisense RNAs, that may affect ppMCH gene activity (Hervieu et al., 2003).

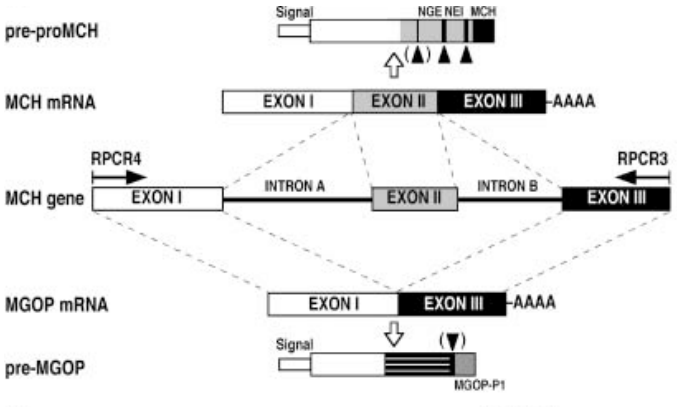


Fig. 8. Expression of the MCH gene results in Mammalian ppMCH and its peptide products. Hatched lines indicate that the N-terminal part of the large MCH-containing form identified at the periphery was not yet established. (from Maulon-Feraille, et al., 2002).

MCH, Melanin concentrating hormone, MGOP, MCH-gene-overprinted-polypeptide ; NEI, neuropeptide-glutaminc acid isoleucine, NGE, neuropeptide glycine-glutamic acid, ppMCH, prepro MCH.

The most studied cleavage product of ppMCH is the cyclic, 19-amino acid neuropeptide MCH that was first isolated from fish teleost pituitaries where it is involved in pigmental control (Baker et al., 1994; Ludwig et al., 1998). In the fish, MCH functionally antagonises the pigmentation effects of α-MSH (Kawauchi et al., 1983). In rodents, MCH was first isolated from rat hypothalami (Nahon et al., 1994; Grifond and Baker, 2002).

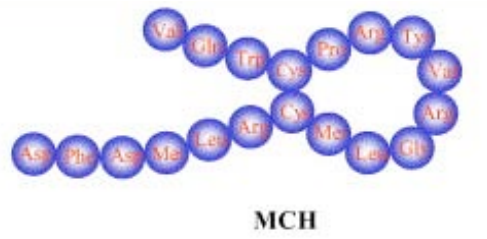


Fig. 9. Chemical structure of MCH. The cyclic, 19 amino acid containing neuropeptide contains a disulfide bridge (from Shi, 2004). For abbreviations of amino acids see textbooks of biochemistry such as Löffler/Pedrides.

In the rat, MCH is synthetised mainly in the lateral hypothalamic area (LHA) and perifornical area, but also in a large number of other hypothalamic nuclei, such as the tuberomammillary nucleus (TMN), posterior nucleus and the zona incerta (Takahashi et al., 1995; Sawchenko et al., 1998). MCH nerve fibers are broadly distributed in the rat brain and could be detected by methods of immunohistochemistry in the neocortex, allocortex, hippocampus, basal ganglia and brainstem (Bittencourt et al., 1992). In accordance to the distribution of MCH in the rat brain, the MCH gene is strongly expressed in the human hypothalamus and the most prominent cluster of MCH cells runs along the entire rostrocaudal extension of the fornix (Viale et al., 1997; Boutin et al., 2002; Unmehopa et al., 2005).

MCH gene expression is up-regulated after acute and chronic food deprivation (Presse et al., 1992; Bertile et al., 2003) and re-feeding of rodents restored MCH mRNA levels (Herve et al., 1997; Tritos et al., 2001). Furthermore, hypothalamic MCH is induced by cold exposure and participates in the process that allows for efficient use of energy for heat production during thermogenic adaptation to cold (Peirera-Da Silva et al., 2003).

Genetic ablation of the MCH gene, MCH (-/-) results in reduced body weight and leanness due to hyperphagia and an inappropriately increased metabolic rate, despite their reduced amounts of both leptin and arcuate nucleus POMC mRNA (Shimada et al., 1998). Mice with selective loss of MCH neurons developed reduced body weight, body length, fat mass, lean mass, and leptin levels. These mice are deficient in both leptin and MCH with improved obesity, diabetes, hepatic steatosis, indications suggesting that MCH neurons are important mediators of the response to leptin deficiency (Alon et al., 2006).

In contrast, transgenic mice with chronic overexpression of MCH eat more of a high-fat diet and had a higher body weight, increased leptin levels and insulin resistence, an obese phenotype as also found in leptin-deficient *ob/ob* mice (Ludwig et al., 1998; Segal-Lieberman et al., 2003). Additionally, mice overexpressing the gene encoding MCH are susceptible to obesity and insulin resistence (Qu et al., 1996; Boutin et al., 2002). Finally, mRNA levels of MCH increased in genetic obesity models such as leptin deficient *ob/ob* mice, leptin receptor deficient (*db/db)* mice and Zucker fatty rats (Hamada et al., 2000; Mizuno et al., 1998; Stricker-Kongrad et al., 2001).

MCH influences energy homeostasis not only by orexigenic effects on feeding behaviour, but also on energy expenditure through changes in locomotor activity and thermogenesis. MCH (-/-) mice showed an altered lipid metabolism, increased oxygen consumption and increased locomotor activity (Shimada et al., 1998). Consistently, double MCH (-/-) and *ob/ob* (-/-) mice were still hyperphagic, but animals were hyperactive with both increased resting energy expenditure and locomotor activity (Segal-Lieberman et al., 2003). Finally, one research group underlined these results, since MCH mutant mice attenuated their weight gain due to increased energy expenditure and enhanced locomotor activity, the latter being even more pronounced when animals were fed on a high caloric diet (Kokkotou et al., 2005).

In another rodent model of altered nutritional status, the diet-induced obesity (DIO) rats, MCH levels were increased and positively regulated by leptin and insulin (Elliott et al., 2004).

Acute central administration of MCH results in spontaneous feeding (Rossi et al., 1997; Edwards et al., 1999; Chaffer et al., 2000; Sahu, 2002). In addition, chronic infusion of MCH significantly increased food intake, body weight, white adipose tissue mass and liver mass in *ad libitum* fed mice and produced an obese phenotype in moderately high–fat diet (Gomori et al., 2003; Ito et al., 2003). MCH injected into the brain ventricles had no effect on spontaneous locomotor behaviour *per se* during either the light- or the dark cyle (Gomori et al., 2003; Georgescu et al., 2005). However, chronic infusion of MCH reduced rectal temperatures and expression of key molecules involved in thermogenesis and fatty acid oxidation such as uncoupling protein-1, acyl-CoA oxidase, and carnitine palmitoyltransferase were reduced in the brown adipose tissue (Ito et al., 2003).

These animal studies potentially underline that MCH is one of the candidate neuropeptides that influence energy homeostasis by increasing feeding and reducing energy expenditure.

1.7.2. Besides energy hoemostasis, MCH is involved in the regulation of several other behavioural and peripheral functions

After the discovery, that MCH does not only induce skin pigmentation in the teleost fish but is also involved in complex behaviour such as food intake regulation in mammals, a number of additional central and peripheral effects have been supposed (Fig. 10).

MCH expressing neurons located in the LHA have extensive projections within the brain regions associated to complex behavioural responses such as the ventral tegmental area (VTA), the nucleus accumbens (NAc) and amygdaloid nuclei (A) (Bittencourt et al., 1992). These regions have been extensively reviewed for their importance in complex behaviour such as reward, emotion and motivation (Saper et al., 2002; Kishi et al., 2005). The NAc is a ventral striatal nucleus with a critical role in both appetitive and consummatory phases of behaviour (Berridge and Robinson, 1998). Interestingly, the VTA-NAc-A brain circuitry is functionally connected to the mesolimbic dopaminergic system, which is itself most often associated with the rewarding effects of food and drugs of abuse (Nestler and Carlezon, 2006).

In addition, there is a direct link between the ventral tegmental area (VTA), the nucleus accumbens (NAc) and the amygdala (A) in addictive, anxiety and depressive behaviour (Di Leone et al., 2003; Hervieu et al., 2006). In number of behavioural studies that are used to study anxiety and depressive behaviour in rodent, central administration of MCH evoked anxiety-like properties for MCH (Monzon and De Barioglio, 1999; Kela et al., 2003).

Additionally, MCH has been reported to affect other brain activities, such as learning abilities and memory retention in rats (Monzon et al., 1999; Varas et al., 2002), which give MCH attitudes to important social behaviour.

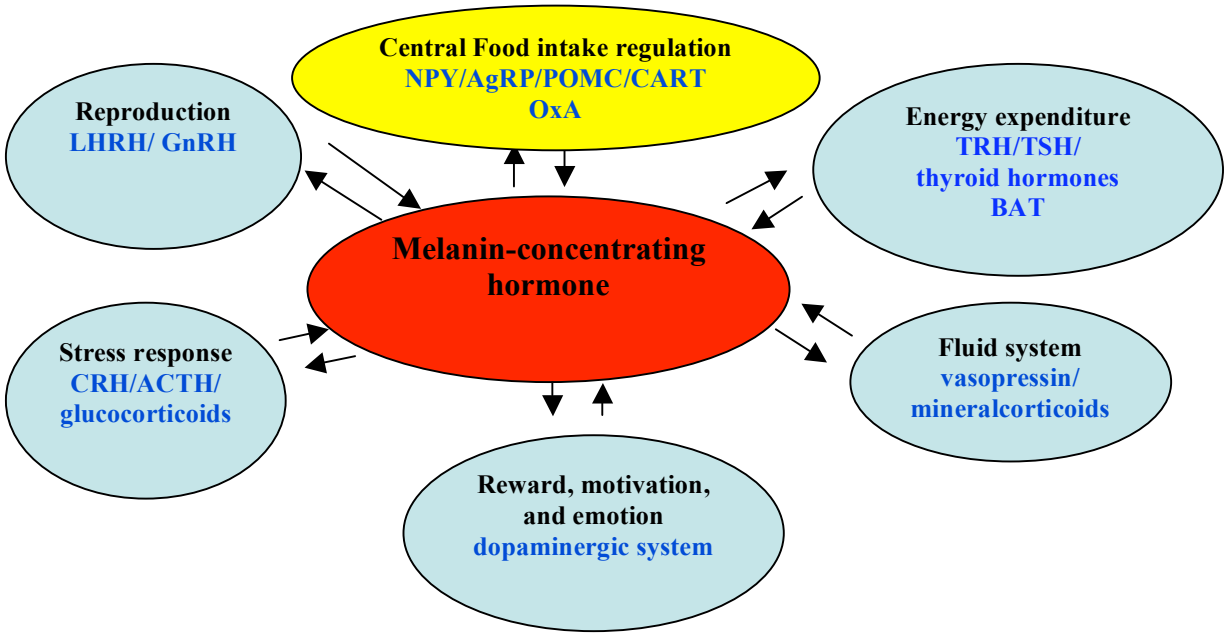


Fig. 10. Possible interaction of the MCH system on central and peripheral effector systems. Notable, MCH has been discussed for its potential actions in the regulation of energy homeostasis affecting both food intake and energy expenditure. In addition, MCH have been described to influence fluid hoemostasis, reproduction, stress response, and behavioural aspects such as rewarding and emotion. These effector systems have been connected to specific neuroactive systems.

Abbreviations: LHRH, Lutein-releasing hormone; GnRH, Gonadotrophin-releasing hormone; NPY, Neuropeptide Y; AgRP, Agouti-related peptide; POMC, proopiomelanocortin, CART, cocaine-and-amphetamine-regulated transcript; TRH, tyreotrophin-realeasing hormone, TSH, thyroidea-stimulating hormone; BAT, brown adipose tissue; CRH, corticotrophin-realsing hormone; ACTH, adrenocorticotrophic hormone.

MCH acts as a neuropeptide and neuromodulator in the brain, other tissues expressing the MCH gene are the pituitary, parts of the enertic nervous system, the reproductive tract, the immune system and the skin (Breton et al., 1993; Hervieu et al., 1995; Viale et al., 1997). The observation that MCH was not found in the blood plasma of human and rat (Takahashi et al., 1995) supports the idea that MCH exerts its peripheral actions in an autocrine/paracrine manner, or indirectly, via modulation of the hypothalamic-pituitary axis (Shi, 2004). Since the discovery, that MCH is involved in energy homeostasis in mammals, MCH has been attracted to a number of other peripheral effector pathways, including fluid regulation, reproduction and stress (Shi, 2004). An earlier study demonstrated, that an osmotic stimulus caused a marked increase in MCH-like immunoreactivity (IR) in the lateral hypothalamus and the

neurointermediate lobe (Zamir et al., 1986). Further evidence for an involvement of MCH on water intake were given by the observation that MCH immunoreactivity was similar distributed within the lamina propria on both duodenal and colonic levels, as vasopressinergic (VP) and atrio-natriuretic factor (ANP) expressing cells (Hervieu et al., 1996). Subsequently, central administration of MCH has shown dipsogenic effects, this could be demonstrated to be been independent from the orexigenic effects of MCH-induced feeding (Clegg et al., 2003; Morens et al., 2005).

Anatomical studies defined MCH-immunoreactive fiber projections came in close apposition with Gonadotrophin-releasing hormone (GnRH) cell bodies throughout the preoptic area and anterior hypothalamic area in the rat, evidence a possible direct neuroanatomical pathway by which MCH may play a role in the regulation of GnRH neuronal function (Williamson-Hughes et al., 2005). In rats that were steroid-primed to generate a surge-like release of LH, MCH administration into the zona incerta blocked this rise in LH release: no such effect occurred when MCH was injected into the preoptic area (Murray et al., 2006).

A state of stress is associated with various external and internal challenges to the body and brain, usually termed stressors (Koob and Heinrichs, 1999). The effects of MCH on the hypothalamic-pituitary-adrenal ("stress") axis has been discussed extensively by others, but the mode of action is still discussed controversely (Hervieu, 2003). MCH has been shown to be partly responsible for CRH and ACTH release in conscious rats (Jezova et al., 1992), but MCH`s effects on ACTH have been found to depend on the circadian rhythm (Bluet-Pajot et al., 1995). Additionally, central administration of MCH activates neurons located in the PVN of the hypothalamus and the locus coerulus, main brain regions of CRH expression (Parkes et al., 1992; Koob, 1999).

1.7.3. The MCH receptor subtype 1 (MCHR-1) is responsible for MCH`s central effects

The biological function of MCH is mediated by at least two MCH receptor subtypes. Primarily, MCH-R1 was identified as a somatostatin like receptor, because this receptor shows a high sequence homology with the somatostatin receptors (SST1-SST5) (Kolakowski et al., 1996; Mori et al., 2001). At present, two MCH receptors have been identified, characterised and named Melanin-concentrating hormone receptor subtype 1 (MCHR-1) (Saito et al., 1999; Chambers et al., 1999) and Melanin-concentrating hormone receptor subtype 2 (MCHR-2) (Sailer et al., 2001; Hill et al., 2001). Both receptor subtypes belong to the superfamily of GPCRs (Vitale et al., 2003).

Structurally, both MCH receptors fall into the rhodopsin-like or adrenergic-receptor-like family of GPCRs and share a common core domain constisting of seven transmembrane helices connected by 3 intracellular and 3 extracellular loops, with the N- and C-terminal domains protruding, respectively, in the extracellular or intracellular sides of the membrane. Common features of GPCRs and mutational analysis of MCHR-1 and MCHR-2 have revealed that the extracellular regions and the transmembrane domains contribute to the formation of the ligand binding site, whereas the intracellular loops interact with G proteins, as well as with other regulatory proteins (Bockaert and Pin, 1999; Bednarek et al., 2001).

With the aid of molecular simulations, the seven helix bundle of MCH receptors have been identified as essential for binding of MCH or synthetic ligands such as MCH receptor antagonists (Fig.11).

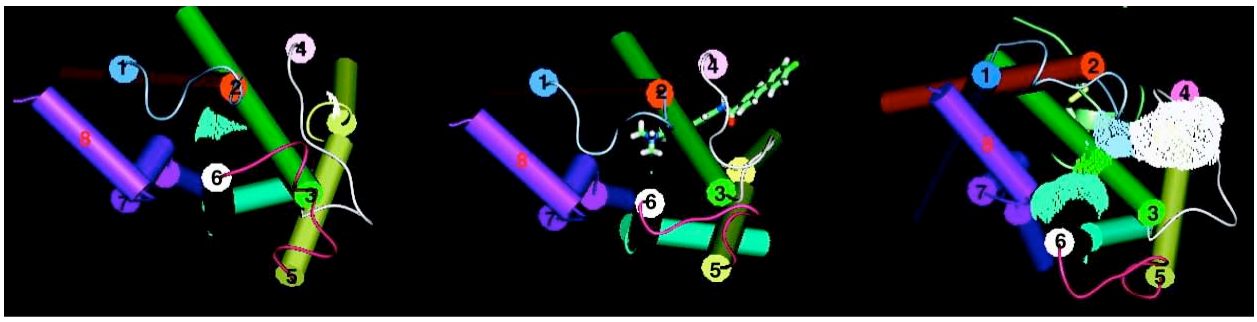


Fig. 11. Schematic representation of structures of the empty (left), MCHR-1 antagonist-bound (T-226296) (middle), and MCH-bound (right) forms of MCHR-1 Helices 1, 2, 3, 4, 5, 6, and 7 are, respectively, colored blue, orange, green, pink, yellow, cyan, and violet, whereas the intracellular loops 1, 2, and 3 are colored in gray, white, and purple, respectively. The 7-helix bundle (represented by cylinders) and the 3 intracellular loops are shown from the intracellular side in a direction perpendicular to the membrane surface (From Vitale et al., 2004).

While the MCHR-1 receptor subtype was isolated from several different species including rodents and humans (Chambers et al., 1999, Lembo et al., 1999; Saito et al., 1999), MCHR-2 was found only in primates, dogs and ferrets, but not in rodents (Mori et al., 2001).

In order to characterise the biochemical properties of both receptor subtypes, numerous studies have used cloning and expression strategies to generate stably transfected MCH receptor expressing cell lines. In addition, a number of mammalian cell lines have been identified which endogenously express MCHR-1 in "natural" cellular conditions (Eberle et al., 2004). Using these tools, MCHR-1 has been shown to intracellular couple to G_i, G_o and G_q proteins (Hawes et al., 2000). Results of *in vitro* studies clearly identified MCH as the endogeneous ligand that binds with high affinity to both receptor subtypes. Thus, functional activity of MCHR-1 can be measured by reduction of c-AMP, increase of inositol phosphate, increase of intracellular

calcium mobilisation or stimulation of mitogen-activated protein (MAP) kinase activity (Pissios et al., 2003). In contrast, MCHR-2 exhibits multiple exons and the receptor was shown to couple exclusively to G_q (Wang et al., 2001; Rodriguez et al., 2001). To note, none of the alternate peptides produced from the *pmch* gene, NEI, NEG,MGOP or AROM, are capable of eliciting a functional response from either MCHR-1 (Saito et al., 1999) or MCHR-2 (Sailer et al., 2001; Hill et al., 2001). To date, the MCHR-1 is the only functional receptor for MCH-mediated activities in rodents (Tan et al., 2002).

1.7.4. The wide distribution of MCHR-1 in the rodent brain reflects MCH´s various central functions

A number of studies have evaluated a widespread distribution of MCHR-1 mRNA and protein within the rodent brain. *In situ* hybridisation studies found high levels of expression of MCHR mRNA in most anatomical areas implicated in the control of olfaction (olfactory bulb/tubercle, piriform cortex). Labelling was also detected in the hippocampal formation, basolateral amygdala, nucleus accumbens, hypothalamus and brainstem (Saito et al., 1999; Hervieu et al., 2000). The distribution pattern of MCHR-1 give rise that MCHR-1 exerts MCH`s central actions through a number of neuronal systems.

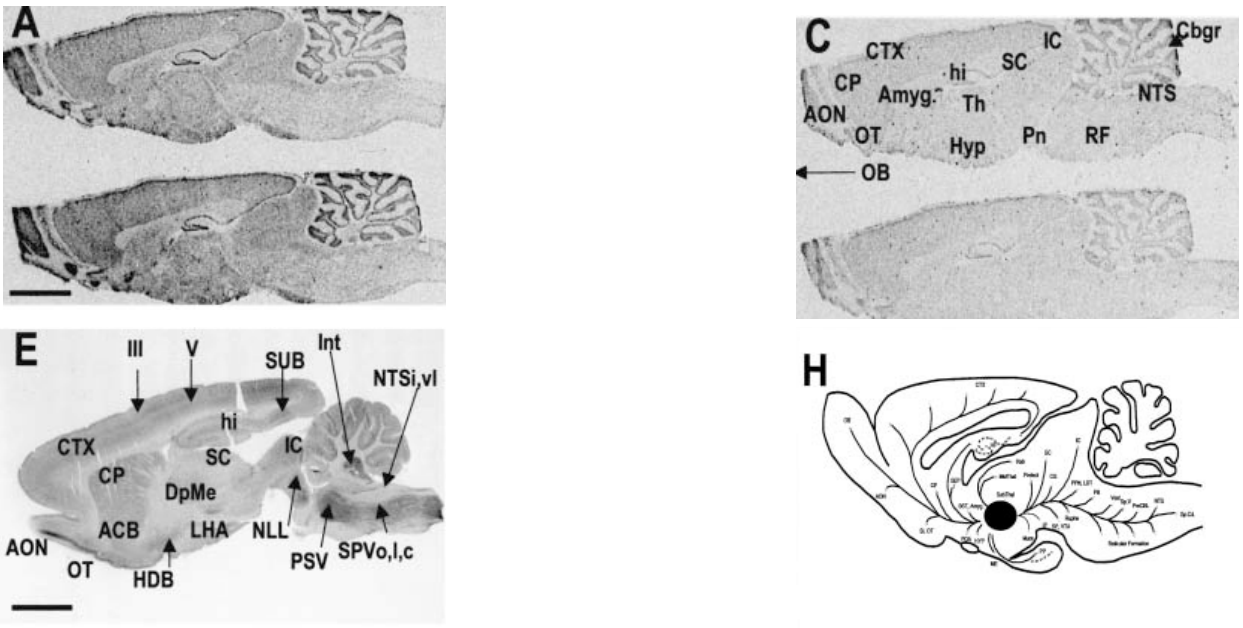


Fig. 12. mRNA and protein detection of MCHR-1 within the rat brain. **(A)** Strong mRNA labelling was observed in the cortex (CTX), anterior olfactory nucleus (AON) and olfactory tubercle (OT), caudate-putamen (CP), nucleus accumbens (ACB), amygdala (Amyg.), hippocampal formation (hi), thalamus (Th), hypothalamus including lateral hypothalamic area (Hyp/LHA) and midbrain areas including nucleus of the solitary tract (NTS) and cerebellum (Cbgr). **(C)** Excess of unlabelled oligoprobes diminished signal intensity. **(E)** Immunohistochemical analysis on rat brain sagittal sections detected a similar protein distribution of MCHR-1. **(H)** Overlapping of MCH- and MCHR-1 IR is shown by a schematic representation of the MCH projections through the rat brain. (from Hervieu et al., 2000, for other abbreviations see abbreviation list at page 4).

Additionally, the distribution pattern of MCHR-1 overlaps to a high degree with the location of MCH-immunoreactive fibers (Bittencourt et al., 1992).
In most regions, there was a good correspondence between MCHR mRNA and protein distribution (Hervieu et al., 2000) (Fig. 12).

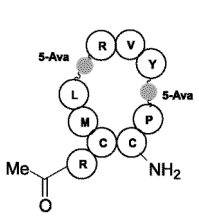
1.7.5. Animal models for the study of MCHR-1 behavioural and physiological effects

Numerous animal models, including MCHR-1 knock out models and rodent models that reflect an obese phenotype support the idea that MCHR-1 is responsible for MCH actions on energy homeostasis.
Mice with deficiency to MCHR-1 (-/-) have normal body weights but reduced body fat mass as a consequence of hyperactivity and altered metabolism, but they are hyperphagic when maintained on a normal laboratory chow. Importantly, chronic central influsions did not induce hyperphagia and mild obesity in MCHR-1 (-/-), compared to their wild-type littermates (Marsh et al., 2002). Male and female MCHR-1 (-/-) mice that were fed with a high-fat diet results keep lean body mass constant and animals resistent to diet-induced obesity and resistence to diet-induced obesity (Chen et al., 2002). If the phenotype of *ob/ob* mice are compared to mice lacking both MCHR-1 and leptin (double null MCHR-1 *(-/-)*, *ob/ob)*, despite beeing severely obese, in these animals insulin levels, locomotor activity, body temperatore, body fat and lean body mass are affected (Bjursell et al., 2006).
Male MCHR1(-/-) mice were also demonstrated to have a significantly increased heart rate, locomotor activity and core body temperature during the dark phase, possibly through activation of autonomous pathways (Astrand et al., 2004). MCHR-1 (-/-) mice exhibit significantly higher levels of locomotor activity elicited by the novelty of the test chambers and higher susceptibility for cocaine, which may indicate a higher sensitivity to unconditioned stimuli (Tyhon et al., 2006). Interestingly, in another transgenic mice model that lacks MCHR-1, the Neo/Neo mice, behavioural testings have shown impaired cognitive properties (Adamantidis et al., 2005).
It is worth to note, that genetic studies in humans have found several single nucleotide polymorphisms (SNPs) in the MCHR-1 gene, but only one mutation appeared to cosegregate with the obesity trait (Bell et al., 2005). However, analysis of single nucleotide polymorphism in the MCHR-1 gene in obese german children only found rare associations of these mutations to juvenile obesity indicating a particular genetic and environmental background for the onset of the disease (Wermter et al., 2005).

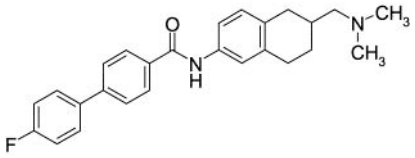
In accordance to the observation of increased MCH expression in animal models with an obese phenotype, hypothalamic expression of MCHR-1 is significantly elevated and plasma insulin und leptin concentration correlate for MCHR-1 mRNA levels in DIO rats (Elliott et al., 2004). Moreover, these animals showed higher exercise examined by voluntary wheel runnning (Zhou et al., 2005).

1.7.6. Development of MCHR-1 antagonists for the treatment of obesity

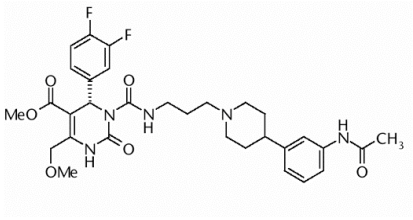
High throughput screening of different GPCR-directed libraries identified several lead compounds as MCHR-1 antagonists (Handlon and Zhou, 2006; Rokosz and Hobbs, 2006). Structure-activity relationships were used to optimize these small molecules with additional favorable physicochemical properties (Clark et al., 2004). In the recent years, a number of these MCHR-1 antagonists have been characterised for their selectivity and potency to inhibit MCH-induced intracellular signalling *in vitro* and consequently, for their potency to reduce food intake and body weight *in vivo*, such as the compound compound B/compound 30 (Bednarek et al., 2002; Shearman et al., 2003), T-226296 (Takekawa et al., 2002), SNAP-7941 (Borowsky et al., 2002), ATC0175 (Kanuma et al., 2005) or GW803430 (Carpenter et al., 2006) (Fig. 13). All these MCHR-1 antagonist have been pharmacologically profiled to inhibit MCH binding on its MCHR-1 receptor *in vitro*.



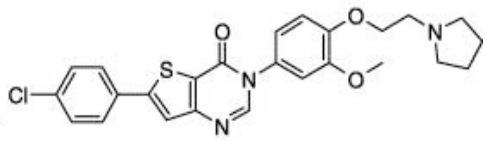
compound 30/
compound B



T-226296



SNAP-7941



GW803430

Fig. 13. Chemical structures of MCHR-1 antagonists that have been functionally charcterised in vitro and in vivo (Bednarek et al., 2002; Shearman et al., 2002; Takekawa et al., 2002; Borowsky et al., 2002; Carpenter et al., 2006).

Except for the MCHR-1 antagonist compound B/compound 30, a cyclic peptide which is the only MCHR-1 antagonist suitable for intracerebral (ic) injections, MCHR-1 antagonist under current examination can be chemically defined as non-peptidyl substances belonging to benzamidazole-based derivatives such as the naphtalenes/piperidine (T-226296, SNAP-7941), quinazoline (ATC0175) (Palani et al., 2005) and thienopyrimidinones (SB-282254, GW803430) (Witty et al., 2006). These compounds have been reported to be orally available and efficacious to reduce feeding, but a detailed description of other MCHR-1 antagonists has been disclosed (Arienzo et al., 2006; Palani et al., 2005; Mc Briar et al., 2005, 2006). Although the structure of the yet published MCHR-1 antagonist represent a high diversity, the recurring structural motive of orally available compounds is a characteristic central amide residue with an amino group at the one end of the molecule and a lipophilic moiety at the other end (Handlon and Zhou, 2006). This structural feature may be necessary for CNS penetration and consequently, for bioavailability following oral administration.

1.7.7. Possible therapeutic indications for MCHR-1 antagonists: obesity and mood disorders

MCHR-1 antagonist`s behavioural actions have been studied *in vivo* in different animal models. In detail, intracerebroventricular (i.c.v.) administration of MCH induces feeding that could be significantly inhibited when the cyclic MCHR-1 antagonist compound B is co-administered (Shearman et al., 2003). Anorexigenic acute effects have also been detected with the MCHR-1 antagonist compound 30 (i.c.v.) in Wistar rats (Morens et al., 2005). Also peripheral, oral (p.o.) (T-226296) or intraperitoneal (ip) (SNAP-7941) administration potently inhibited food intake (Takekawa et al., 2002; Borowsky et al., 2002). Subchronic application of T-226296 (po) significantly reduced body weight and food intake in DIO rats, but had no effects on locomotor activity (Kowalski et al., 2004). Similar observations have been described for SNAP-7941, that dose-dependent suppressed acute normal as well as high palatable food and decreased body weight in DIO rats (Borowsky et al., 2002). Chronic administration of the MCHR-1 antagonist SNAP-7941 has been shown to be more effective on body weight and food intake than the 5-HT reuptake inhibitor D-fenfluramine (Borowsky et al., 2002).

A detailed characterisation of one MCHR-1 antagonist, GW803430, was described with a high selectivity profile for MCHR-1 *in vitro*, good bioavailability and potent anorexigenic effects when given (p.o., subchronically) in DIO AKR/J mice (Carpenter et al., 2006). A direct comparison of GW803430 (3mg/kg, p.o.) with sibutramine on weight loss in DIO AKR/J mice has resulted in 13% weight loss over the course of the study, whereas sibutramine at the same dose gave only a 3% weight loss (Handlon et al., 2004). Finally, chronic administration of

another orally available MCH-R1 antagonist was shown to inhibit food intake dose dependent and stronger than sibutramine (Kym et al., 2005).
To note, chronic treatment of MCHR-1 antagonists resulted not only in reduction of food intake, but also in altered adiposity and energy expenditure (Borowsky et al., 2002).

Some of the studied MCHR-1 antagonist indicated effects on anxiety and depressive behaviour in different rodent models. Moreover, SNAP-7941 and GW803430 exhibited antidepressant and anxiolytic effects in anxiolytic behavioural testings (Borowsky et al., 2002, 2003; Smith et al., 2006), but the results have been discussed controversely (Basso et al., 2006). Interestingly, direct delivery (intrastriatal) of the MCHR-1 receptor antagonist compound 30 blocked feeding and produced an antidepressant-like effect in the forced swim test, whereas intranuclear injection of MCH into the nucleus accumbens shell (AcbSh) had opposite effect, which points on the striatum as an important brain site for MCHR-1 anorexigenic and anxiolytic actions (Bednarek et al., 2002). More recent behavioural studies on another MCHR-1 antagonist ATC0175 confirmed a modulatory actions of MCHR-1 on anxiolytic and antidepressive activity (Chaki e al., 2005).
However, non of the MCHR-1 antagonists have obtained clinical validation for the therapeutic treatment of obesity or mood-related disorders such as depression or anxiety disorders. Thus it may be of interest to further understand the molecular mechanisms of the MCH system to identify its mode of action on food intake, water intake regulation and body weight control . With respect to these physiological consequences, possible side effects of MCHR-1 antagonists may be of relevance for a detailed study.

1.8. Aim of this work

MCH exerts several physiological functions through its functional receptor subtype MCHR-1, but the molecular mediators of these regulatory mechanisms are still not known. A first ambition of my work is to neurochemically define MCHR-1 immunoreactive neurons for their colocalisation with selective neuroactive substances within the rat hypothalamus. Therefore I used an established MCHR-1 antibody that has been described by us as a neuroanatomical tool to perform colocalisation studies for MCHR-1 with hypothalamic neuropeptides that are especially known for their role in the regulation of food intake, water intake, reproduction and stress within the rat hypothalamus.
The main part of the my work deals with the examination of the neuronal activation patterns after stimulation or inhibition of the central MCH system. Therefore I introduce various feeding-related stimuli to rats *in vivo*: Pharmacological stimuli include central administration of

MCH (i.c.v.) or in contrast, I sub-chronically apply orally available MCHR-1 antagonists (GW803430, compound B4) to inactivate MCHR-1 signalling. Effects of antagonist treatments on food intake, water intake and body weight change is measured. Neuronal activation patterns were mapped using immunohistochemical detection of the neuronal activation marker c-Fos within the rat brain of treated animals. Results of the c-Fos immunopositive neurons were assessed in selected hypothalamic regions by qualitative and quantitative image analysis.

In order to identify parallel or distinct neuronal activation patterns between a pharmacological or a physiological stimulus, I introduce an adjusted scheduled feeding paradigm (restricted feeding schedule, RFS) as a physiological stimulus to induce orexigenic neuronal pathways within the rat CNS. Quantitative analysis of c-Fos immunoreactivity in food-restricted and food-deprived animals give further information of common and diverse activation within brain regions of interest. The aim of the last step of work address the question if "hunger-activated" or "satiety-activated" neurons by RFS treated animals produce the neuropeptide MCH, or if they are MCH-sensitive indicated by MCHR-1 expression. This question is methodically elaborated by an immunohistochemical colocalisation study for c-Fos with MCH or MCHR-1, respectively.

Above all, my work may help to explain the neurobiology of the central MCH system and its involvement in the regulation of energy homeostasis in mammals.

2. Neurochemical characterisation of MCHR-1 immunoreactive neurons within the rat hypothalamus

2.1. Introduction

MCH neurons are specifically distributed around the fornix, mostly within the LH of the hypothalamus. The LH is known to govern many functions, such as feeding, blood pressure, neuroendocrine axis, thermoregulation, sleep-waking cycle, emotion, sensorimotor integration and reward processes (Bernadis and Bellinger 1996). Anatomically, the LH is interconnected with other hypothalamic and extra-hypothalamic nuclei through extensive reciprocal projections. The distribution of MCH containing nerve terminals coincides with the expression pattern of MCHR-1 on both the level of messenger RNA and protein (Bittencourt et al. 1992; Hervieu, 2000). Within the rat brain, high levels of MCHR-1 mRNA are widely distributed in various brain regions including limbic and olfactory structures, neocortex, hypothalamic, intralaminar thalamic nuclei, specific brainstem nuclei, throughout the reticular formation and in the spinal cord (Lembo et al., 1999; Hervieu, 2000). The overall distribution pattern of MCHR-1 is compatible with it having functions in ingestion, generalized arousal, sensimotor

integration and autonomic control (Lembo et al., 1999). In a detailed mapping study, MCHR-1 gene expression largely overlapped with MCHR1 immunoreactivity in the rat brain (Hervieu, 2000). MCHR-1 immunoreactivity was found in the *arcuate* nucleus (Arc), lateral hypothalamus (LH), paraventricular hypothalamic nucleus (PVN) and the supraoptic nucleus (SO).

Along with MCH, other neuropeptidergic systems have been involved as hypothalamic regulators of energy homeostasis (Williams, 2006). For example, the orexigenic neuropeptide Orexin A is differentially expressed in another subset of neurons within the LH (Kotz, 2006; De Lecea, 1998; Broberger, 1998). OxA expressing neurons send extensive projections throughout the nervous system, including monosynaptic projection to several regions of the cerebral cortex, limbic system and the brainstem (Peyron et al., 1998) and the functional OxA receptor subtype Ox-R1, has been identified as a key mediator of OxA`s physiological properties and has been found to be widely distributed throughout the rat brain hypothalamic regions (Sakurai, 1999, Hervieu, 2001; 2000).

Dense reciprocal innervations from the LH with other hypothalamic regions including the the Arc and the PVN (Broberger et al 1998; Elias et al., 1998). Neurons located in the Arc have been defined as primary targets of a number of peripheral signals such as leptin, insulin and ghrelin (Hillebrand et al., 2002; Leibowitz and Wortley 2004). In one subpopulation, Arc neurons have been shown to express the orexigenic neuropeptides NPY and AgrP, in another subpopulation of Arc neurons, the precursor protein POMC has been shown to be coexpressed with CART, neuropeptides with anorexigenic properties. POMC is enzymatically cleaved into the melanocortins ACTH, α-MSH and ß-endorphin (Pritchard al., 2002). POMC derived peptides act as natural ligands for the Melanocortin receptor subtype 4 (MC4-R) (Mountjoy et al., 1994), are endogenously antagonised by AgrP (Ollmann et al., 1997) and appears to be a key regulatory element of energy homeostasis (Cone, 1999). In conclusion, Arc neurons can be viewed as important substrates for feeding behaviour.

Afferent inputs have been also traced from the LH to the PVN (Simerly, 1995). Based on morphological and functional criteria, the PVN can be further subdivided into parvocellular neurons that express corticotrophin-releasing hormone (CRH) or thyroid-releasing hormone (TRH) or magnocellular neurons that express the neuropeptides vasopressin (VP) or oxytocin (OT). CRH has been extensively studied as a key mediator in stress response and mediates its effects on the hypothalamic-pituitary-adrenal axis (Koob, 1999; Flier 1995), whereas VP was been reviewed for its role in fluid homeostasis and OT for its involvement in reproduction. Magnocellular neurons are transported via the pituitary stalk to the posterior pituitary lobe,

were they are released into the blood circulation (De Wardener, 2001). Furthermore, magnocellular neurons have also been found in the supraoptic nucleus (SO) that has also been shown to exhibit OT or VP immunoreactivity (Yamada et al., 2004).

The observed anatomical distribution of MCH fibres and MCHR-1 immunoreactivity, suppose that the MCH system interacts with other neuropeptides on the level of the hypothalamus. In order to examine the neurochemical phenotype of neurons expressing MCHR-1, I established immunohistochemistry for MCHR-1 on rat brain cryosections with an antibody against MCHR-1, that has been previously described by us to specifically detect MCHR-1. Using this antibody, I performed an immunohistochemical colocalisation study for MCHR-1 with selected hypothalamic neuropeptides. Furthermore, the presence of MCHR-1 immunoreactivity on the neurohypophysis and adenohypophysis of the rat pituitary was examined.

2.2. Material and Methods

2.2.1. Material

2.2.1.1.Chemicals

- 0.1% Eosin; Doenitz BioLab, 86156 Augsburg, Germany
- DPBS without Ca and Mg (10x); Cambrex Bio Science, B-4800 Verviers, Belgium
- DPX Mountant for histology; Sigma-Aldrich Chemie GmbH, 89555 Steinheim, Germany
- Ethanol, absolut; Sigma-Aldrich Laborchemikalien GmbH, 30926 Seelze, Germany
- IgG free Bovine Serum Albumine; Dianova, 20148 Hamburg, Germany
- Ímmersol; Immersion Oil, Carl Zeiss AG, 73447 Oberkochen, Germany
- Mayers Hämalaun Lösung, Merck KGaA, 64271 Darmstadt, Germany
- Mounting Medium Vectashield (H-1000); Vector Laboratories,Burlinghame, CA, USA
- Normal Donkey Serum, Dianova, 20148 Hamburg, Germany
- Triton X-100, ICN Biochemicals GmbH, 37269 Eschwege, Germany
- UltraCruz Mounting Medium with DAPI, Santa Cruz Biotechnology, Inc. Santa Cruz, CA 95060 USA

2.2.1.2. Solutions and buffers

The pre-incubation and incubation solution consisted of

0.1 M PBS

0.3 % Triton

2 % BSA

dilute 10x PBS buffer (1:100) with demineralised water, add 0.3 % Triton X-100 with a shortened pipette in a volume of 200 ml, dissolved while stirring. For the colocalisation study MCHR-1, the solution was modified and 0.1 Triton/1% NDS was used.

2.2.1.3. Drugs

- Ketavet, Wirkstoff Ketaminhydrochlorid, 100 mg/ml, Pharmacia GmbH, 91058 Erlangen, Germany
- Rompun 2%, Wirkstoff Xylazinhydrochlorid, Bayer Vital GmbH, 51368 Leverkusen, Germany

2.2.1.4. Kits

For the immunohistochemical detection of MCHR-1, chemicals of the TSA kit (NEL-700, Perkin Elmer Life-Sciences) was used.

2.2.1.5. Antibodies

2.2.1.5.1. Primary antibodies

Antibody	Art. no.	Source	Final dilution
Sheep anti NPY	H-049-03	Phoenixpeptide,	1:500
Guinea pig undiluted antiserum anti–ACTH (1-39)	T-5024	Peninsula Laboratories, San Carlos, CA 94070, USA	1:2000
Affinity purfied polyclonal chicken anti–CART	AB5340P	Chemicon	ND
Affinity purified rabbit anti-AgrP	Ab-6446	Biotrend, Köln, Germany	ND
affinity purified polyclonal goat anti-OxA (C-19)	sc-8070	Santa Cruz Biotechnology, Inc	1:2000
Rabbit polyclonal antiserum to MCH		Calbiochem	1:1000
Guinea Pig Anti-Arg8-Vasopressin Serum	T-5007	Peninsula Laboratories, San Carlos, CA 94070, USA	1:4000
Guinea Pig Anti CRF Serum	C-6002	Peninsula Laboratories, San Carlos, CA 94070, USA	1:8000
undiluted antiserum guinea pig anti Oxytocin	T-5021	Calbiochem	1:2000
Rabbit anti Tyrosine hydroxylase	Ab-152	Peninsula Laboratories, San Carlos, CA 94070, USA	1:1000

Table 4: Primary antibodies used for immunohistochemical procedures. ND, not determined.

Characterisation of the immunogenic peptide for the generation of MCHR-1 antibody

For the generation of the MCHR-1 antibody, rabbits were immunised using the hexadecapeptide H-SNAQTADEERTESKGT-OH. Animals blood serum samples were

supplied by the Eurogentec EGT Group, 4102 Seraing, Begium. The specificity of the MCHR-1 antibody was verified by radioimmunoassay. Upon arrival, the crude antiserum containing the antibody was stored at –80 ^{0}C.

For on column purification, the immunogenic peptide was re-synthetised (purity 98.3%) by Biotrend Chemikalien GmbH, Köln, Germany.

2.2.1.5.2. Secondary antibodies

Antibody	Art. no.	Source	Final dilution
Biotinylated anti rabbit IgG	BA-1000	Biozol Diagnostica Vertrieb GmbH, 85380 Eching, Germany	1:1000
Cy3-conjugated affinity purified goat anti-rabbit IgG	111-166-003	Dianova, 20148 Hamburg, Germany	1:400
Cy3-conjugated affinity purified donkey anti goat IgG	705-165-147	Dianova, 20148 Hamburg, Germany	1:400
Cy3-conjugated affinity purified donkey anti sheep IgG	713-165-003	Dianova, 20148 Hamburg, Germany	1:400

Table 5: Secondary antibodies used for immunohistochemical procedures

Working dilutions for all primary and secondary antibodies have been optimized in a suitable dilution range.

2.2.1.6. Characterisation of the animals

All studies were performed using adult male HanWistar rats weighing 250-300g were purchased from Charles River, Kissleg, Germany and handled in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Animals were kept in fixed conditions in the animal facility of Boehringer-Ingelheim Pharma GmbH & Co KG, Department of Metabolic Diseases, Biberach/Riss, Germany) with a 12h light-dark-cycle, food and water *ad libitum* and constant temperature. For colocalisation studies of MCHR-1 with the orexigenic neuropeptides, rats were fasted overnight.

2.2.1.7. Additional material

- Superfrost Plus Microscopical slides 25x75x1.0mm; Menzel GmbH & Co KG, 38116 Braunschweig, Germany
- ImmEdge Pen, Vector Laboratories, Inc. Burlinghame, USA

2.2.1.8. Technical equiment

- IKA-Schüttler MTS, Staufen, Germany
- Pumpe Typ MV-GE, 50W, 230V, Ismatec Dosiertechnik, 8152 Glattbrugg-Zürich, Switzerland
- Cryostat HM 74, Leica, Nussloch, Germany
- Horizontal shaker Heidolph Polymax 1040
- Waage Sartorius 210D, Sartorius AG, Göttingen, Germany

The complete optical apparatus was purchased from the Carl Zeiss AG, Göttingen, Germany. The fluorescence microscope consists of

- Axioplan 2 imaging microscope
- Filtersatz 43, Cy3, Excitation BP 545/ 25, Beamsplitter FT 570, Emission BP 605/ 70
- Filtersatz 10, Cy2, Excitation BP 450-490, Beamsplitter FT 510, Emission BP 515-565
- Filtersatz 49, DAPI, Excitation G365, Beamsplitter FT 395, Emission BP 445/50
- Objective Plan Neofluar 5x/0,15
- Objective Apochromat 10x/0,45
- Objective Plan-Apochromat 20x/0,75
- Objective Plan-Apochromat 63x/1,40 Oil
- Schiebersystem Apotome with Apotome Transmissionsgitter PL for x5, x10, x20 objectives, or Apotome Transmissionsgitter PH for the x63 objective
- Mikroskopie-Kamera AxioCam MRm Rev.2 (D)

2.2.2. Methods

2.2.2.1. Tissue preparation

Six male HanWistar rats (300g BW) were anaesthetized with Ketaminehydrochlorid (100 mg/kg BW) and xylazine hydrochlorid 10 mg/kg BW prior to transcardial perfusion with 0.9% NaCl followed by 4% paraformaldehyde in 0.1M phosphate-buffered saline. From each animal, Brains and pituitaries were removed, postfixed over night at 4 ^{0}C in the same solution and cryoprotected with a 30% sucrose solution in 0.1M PBS until the brains sunk to the bottom of the plastic vial. Following shock-freezing in -60 ^{0}C cold petrolether, brain tissue blocks were stored in -80 ^{0}C until further processing.

2.2.2.2. Characterisation of the MCHR-1 antibody

(A detailed description of this part of this work has been published in the diploma thesis of Daniela Friebe, Fachhochschule Sachsen-Anhalt, Köthen, Germany.)

Rabbits were immunized with an immunogenic peptide corresponding to the MCHR-1 sequence previously used (Hervieu et al., 2000). Obtained crude antisera were affinity purified using the re-synthetised immunogenic peptide bound to a sulfolink matrix covalently linked to the N-terminal cystein extended peptide. Purification of the antibody was done in accordance to the SulfoLink kits description.

Briefly**,** for dot blot analysis, a PVDF membrane was equilibrated in methanol (10 sec) and washed in H_2O and TBS-T buffer. Spots of the immunogenic peptide were applied to the membrane, dried and pre-incubated in TBS-T buffer with 5% skim milk powder. The membrane was incubated with the primary MCHR-1 antibody (1:250, diluted in 3% skim milk powder, TBS-T, 6h, 4^0C). The membrane was washed and incubated with the secondary donkey anti rabbit HRP conjugated antibody (1:5,000 diluted in 3% skim milk powder, TBS-T, 1h, RT, shaker). After washing steps in TBS-T buffer, dots were visualized using the ECL Plus Western Blotting Detection Reagent.

Wild type or human *mch-r1* cDNA transfected CHO cells were used for western blot analysis and immunocytochemistry. Crude cell extracts of five billion cells were homogenised, heat denatured and protein concentration was measured by Bradford assay. For western blot analysis, 10 μg of cell extracts were resolved by SDS-PAGE on a 10% BIS-TRIS gel, and transferred onto a nitrocellulose membrane, which was cut in stripes that represent *mch-r1* CHO cells or wild type CHO cells, respectively. All stripes were incubated according to the protocol of the dot blot experiment. As negative control, the antiserum was pre-absorbed with a ten-fold excess of the immunogenic MCHR-1 peptide (over night, 4 ^{0}C).

For immunocytochemistry, wild type or human *mch-r1* cDNA transfected CHO cells were grown on cell culture slides. Cells were fixed with 2% PFA (30 min, RT) washed in 1 M PBS and blocked with a solution of 10% NDS, 1 M PBS, 0.1% Triton X-100 (1h, RT). Then cells were incubated with the MCHR-1 antibody (1:250, diluted in 2% NDS, 1M PBS, 1h, RT). After washing, cells were incubated with a Cyanin 3 conjugated, anti rabbit antibody (1:500, diluted in 1 M PBS, 2% NDS, 1h, RT), washed in 1M PBS , counterstained with 0.1 μg/ml DAPI and mounted with 10% glycerol in 1 M PBS. Wild type cells and pre-absorption with the immunogenic peptide served as negative control experiments.

2.2.2.3. Single-labelling immunohistochemistry for MCHR-1 on rat hypothalamic cryosections

Thirty μm coronal cryosections of the rat hypothalamus were cut on a cryostat according to the anatomical atlas (Paxinos and Watson, 1989). Tissue sections were collected in 0.1M PBS and

processed after the free-floating method. All necessary washing steps were performed with 0.1M PBS, 3x10 min at room temperature on a horizontal shaker. After initial washing steps, sections were placed in a pre-incubation solution containing 2% NDS, 1% BSA, 0.1% Triton X-100 in 0.1M PBS (1 h, RT).

After a second blocking step with a biotin blocking solution supplied by the TSA kit, sections were incubated with the first primary antibody against MCHR-1 (diluted 1:100 in the pre-incubation solution) for 48h at 4 ^{0}C on a horizontal shaker. Subsequently, sections were washed and incubated with a biotinylated goat anti-rabbit antibody (1:1000, diluted in 2% NDS 0.1M PBS) for 45 min diluted 1:400 in 2% NDS, 0.1M PBS). After washing, sections were incubated in a streptavidin- Cyanin 2 fluorophore (diluted 1:1000 in 0.1M PBS).

2.2.2.4. Single-labelling immunohistochemistry for neuroactive substances in the rat hypothalamus

In order to identify the neuroanatomical distribution of the neuroactive substances within hypothalamic regions, single labelling immunohistochemistry for each investigated antibody was established. 30 μm cryosections corresponding to the rat hypothalamus were preincubated in 2% NDS, 2% BSA, 0.1% Triton X100 in 0.1M PBS (1 h, RT). Subsequently, one set of sections comprising the Arc and LH were incubated with an ascending series of sheep polyclonal antiserum to neuropeptide Y (NPY) (1:250-1:2000), guinea pig polyclonal antiserum to adrenocorticotrophin (ACTH) (1:500-1:2000), chicken polyclonal antiserum to cocaine-amphetamine regulated transcript (CART) (1:1600), guinea pig anti vasopressin (VP) (1:1000-1:8000), guinea pig antiserum to corticotrophin-releasing factor (CRF) (1:8000-1:8000) and guinea pig antiserum to oxytocin (1:2000), or goat antiserum to Orexin A (OxA) (1:2000), rabbit anti MCH serum (1:1000), rabbit antibody to tyrosine hydroxylase (1:1000).

Primary antibodies were diluted in a solution of 0.1M PBS, 2% BSA and incubated for 1h at RT on the horizontal shaker. For visualisation of neuropeptides, suitable Cyanin 3 conjugated secondary antibodies were used (see table 7). Finally, sections were washed, mounted onto gelatine-coated object slides and coverslipped with aequeous mounting medium.

2.2.2.5. Colocalisation studies for MCHR-1 with neuropeptides in the rat hypothalamus

Thirty μm coronal cryosections of the rat hypothalamus that were defined by the anatomical atlas and were cut on a cryostat (Paxinos and Watson, 1989). All sections were collected in 0.1M PBS and processed after the free-floating method. All necessary washing steps were performed with 0.1M PBS, 3x10 min at room temperature on a horizontal shaker. After initial

washing steps, sections were placed in a pre-incubation solution containing 2% NDS, 1% BSA, 0.1% Triton X-100 in 0.1M PBS (1 h, RT). After a second blocking step with a biotin blocking solution supplied by the TSA kit, sections were incubated with the first primary antibody (diluted 1:100 in the preincubation solution) for 48h at 4 ^{0}C on a horizontal shaker. Subsequently, sections were washed and incubated with a biotinylated goat anti-rabbit antibody (1:1000, diluted in 2% NDS 0.1M PBS) for 45 min. After washing, sections were incubated in a streptavidin- Cyanin 2 fluorophore (diluted 1:1000 in 0.1M PBS, 1h/RT/dark). Finally, sections were washed extensively in 0.1 M PBS and further processing were performed in the dark.

Hypothalamic sections were separated according to the brain level of interest. For OxA, ACTH and NPY, sections comprised the Arc and the LH (from bregma level –2.8 mm to bregma level –3.3 mm), for the detection of VP, OT or CRF, sections were used that comprised the the SO and the PVN (from bregma level –1.3 mm to bregma level –1.8 mm). Based on single-labelling results, antibodies were used according to their optimal working conditions and according to single labelling results, optimized on dilutions described below. After the immunohistochemical detection of MCHR-1, double labelling was performed using a goat antiserum to OxA (1:2000), guinea pig polyclonal antiserum to ACTH (1:2000), sheep polyclonal antiserum to NPY (1:500), guinea pig anti VP (1:4000), guinea pig antiserum to OT (1: 1000) guinea pig antiserum to CRF (1:4000). These antibodies were diluted in 2% BSA, 0.1N PBS and incubated for 1h/RT on the horizontal shaker. For visualisation of neuropeptides, suitable Cyanin 3 conjugated secondary antibodies were used (see table). Finally, sections were washed, mounted onto gelatine-coated object slides and coverslipped with a DAPI containing mounting medium for nuclear staining.

2.2.2.6. Single-labelling immunohistochemistry for MCHR-1 on rat pituitary cryosections

Seven μm thick coronal sections were cut on the cryostat from the dorsal to the ventral dimension of the rat pituitary. Sections were thaw-mounted in a series of one to four onto Superfrost plus slides and air-dried for 30 min at RT. One set of sections were used for the immunohistochemical detection of MCHR1, two sets were processed for negative controls (pre-absorption with the immunogenic peptide and one negative control without primary antibody, see also experiments for the establishment of the MCHR-1 antibody). Additionally, one set of sections was stained with the histological Hematoxylin& Eosin (H&E) staining.

For immunohistochemistry of MCHR-1, procedures were done as described above, but sections were processed directly on objective slides. Briefly, sections were washed twice in 0.1 M PBS

(5 min), preincubated and blocked with TNB buffer. Sections were then incubated over night with the first antibody (diluted 1:100 in preincubation solution at 4 ^{0}C on the horizontal shaker). Subsequently, sections were washed and incubated with the biotinylated goat anti-rabbit antibody (1:1000, 45 min diluted in 2% NDS, 0.1 M PBS) followed by streptavidin-Cyanin 2 fluorophore (diluted 1:1000 in 0.1M PBS, 1h/RT/dark).

Negative control sections were either incubated with the described MCHR-1 antibody that was preincubated with a tenfold excess of the immunogenic peptide for 4h at RT or replacement of the primary antibody was replaced with NDS.

Histological staining with H&E were performed after standardized histology protocols. Briefly, sections were washed twice in 0.1 M PBS, and stained with a 10% Hematoxylin solution, diluted in 0.1 M PBS. Then, section were washed again and counterstained with an 1% solution of Eosin, followed by dehydration with an ascending series of ethanol (50%, 70%, 95%, 100%). After the last step, sections were air dried under the hood and mounted with DPX Mounting Medium.

2.2.2.7. Image acquisition

Sections were visualized and images were obtained using the axioplan 2 microscope with a x10 objective and for the higher magnification, a x63 objective was taken. For the anatomical identification of pituitary , an overview photograph was taken with a x2.5 objective.

2.5. Results

2.3.1. Characterisation of the MCHR-1

The here introduced affinity purified polyclonal antibody to MCHR-1. This antibody was shown to specifically recognize the C-terminal located peptide sequence, if the immunogenic peptide is spotted onto a nitrocellulose membrane (Fig. 14 A). In order to test the specificity of the MCHR-1 antibody, we performed western blotting and immunocytochemistry based on stably transfected hMCHR-1 in CHO cells. Western blotting of crude membrane prepararions revealed a specific band of expected size (60 kDa), but failed to detect this band after the pre-absorption of the antibody and with the immunogenic peptide nor on untransfected CHO cells (Fig. 14 B).

Immunocytochemistry of the same cell line showed a membrane-associated staining of hMCHR-1 CHO cells, whereas corresponding controls. Preabsorption as well as untransfected cells) showed no specific labelling (Fig. 14C-F).

Finally, I determined, if the antibody is suitable to detect MCHR-1 on rat brain cryosections. Early studies conducted by others have detected strongest MCHR-1 expression in the nucleus accumbens of the rat brain by methods of in situ hybridisation and immunohistochemistry (Hervieu et al., 2000). Therefore I used this area of the brain as positive control for the immunohistochemical detection of MCHR-1. I could demonstrate a specific and strong MCHR-1 IR in the nucleus accumbens shell and to a lesser extend in the core division of the accumbens. Pre-absorption of the primary antibody and omission of the first antibody revealed no signal (Fig. 14 G-J). The subcellular distribution of the signal was restricted to the cellular membrane or the somata (Fig. 14 J).

2.3.2. MCHR-1 immunoreactivity is present in the rat hypothalamus

Besides strong immunoreactivity for MCHR-1 in the nucleus accumbens core and shell, other forebrain structures have been shown to express MCHR-1, but to a lesser extend (e.g. caudate putamen and medial septal nucleus). Strong labelling for MCHR-1 was shown in many other brain regions, including olfactory nuclei, cerebral cortex structures, the hippocampal formation (especially CA3 neurons of the hippocampus), several thalamic and amygdaloid structures and to a lesser extend, midbrain and hindbrain structures. The observed distribution pattern of MCHR-1 was in full agreement with the detailed mapping study published by Hervieu et al., 2000 (see also Figs. 37, 38 in this work).

Within the hypothalamus, many MCHR-1 immunoreactive cell bodies were demonstrated in the supraoptic nucleus (SO, Fig.38 A), the magnocellular and parvovellular proportion of the paraventricular hypothalamic nucleus (PVN, Fig.38 E) as well as in the dorsomedial hypothalamic nucleus (Fig. 37E). MCHR-1 IR was seen to a lesser extend in the suprachiasmatic nucleus (SCh, data not shown), the ventromedial hypothalamic nucleus (VMH) (data not shown) the lateral hypothalamic area including the fornix (Fig. 37 I) and the tuberomammillary nuclei (data not shown).

Fig. 14. Examination of the specificity of the rabbit polyclonal MCHR-1 antibody.

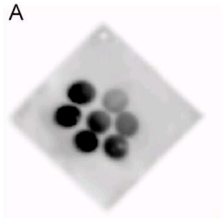
Dot blot of the immunogenic peptide is detected by the *in house* polyclonal rabbit anti MCHR-1 antibody (A). MCHR-1 was identified as a specific band (middle lane)in cellular extracts derived from stably transfected hMCHR-1 CHO cells by SDS-PAGE and immunoblotting (B). Pre-absorption with the immunogenic peptide (left lane) or untransfected, wild-type cells (right lane) were used as negative controls.

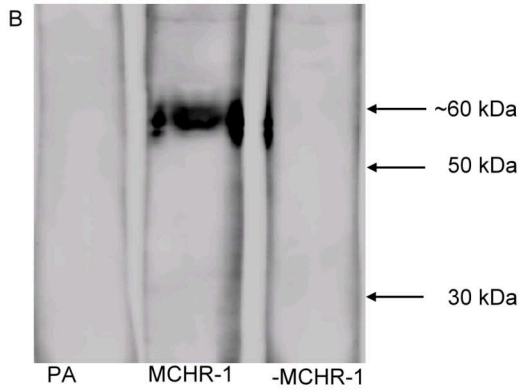
Photomicrographs of immunocytochemistry of transfected hMCHR-1 CHO cells show specific staining for MCHR-1 (D). Under a x63 magnification, cells show a with a characteristic membrane-associated staining (white arrow, (F)). Negative controls involved pre-absorption (C) or untransfected CHO cells (E). Cells were counterstained with DAPI. Scale bar (E) ,100 μm; (F), 20 μm.

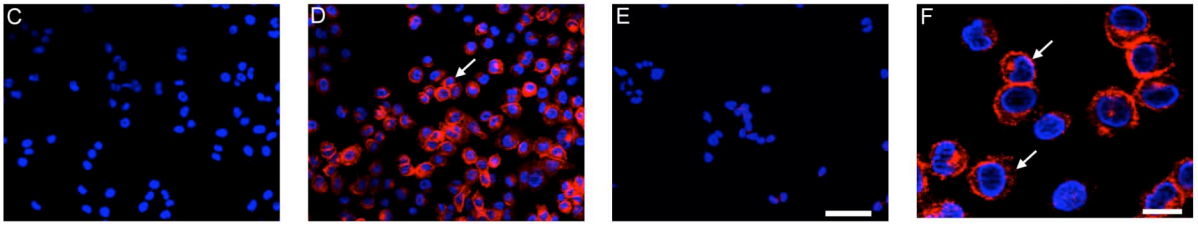
Photomicrographs of immunohistochemistry of MCHR-1 on rat brain cryosections representing the nucleus accumbens. Strong MCHR-1 labelling was found in the nucleus accumbens shell division (H). Under a x63 magnification, neurons showed membrane-associated staining (white arrow, J). Cells were counterstained with DAPI. Negative controls involved pre-absorption (G) or omission of the primary antibody (I).

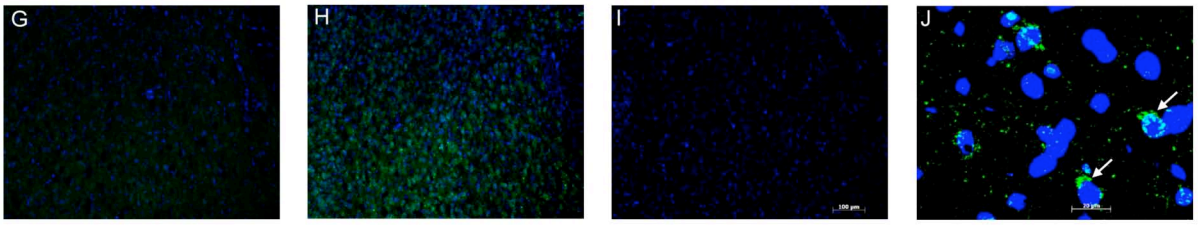
Scale bar (I) ,100 μm; (J), 20 μm.

CHO Chinese ovary hamster
SDS-PAGE sodium dodecyl sulphate polyacrylamide gelelectophoresis
hMCHR-1 human Melanin concentrating hormone
DAPI 4`,6-Diamidino-2-phenylindol Dihydrochloride
PA Pre-absorption
kDa kilo Dalton


A


B
~60 kDa
50 kDa
30 kDa
PA
MCHR-1
-MCHR-1


C
D
E
F


G
H
I
J

2.3.3. Single-labelling immunohistochemistry for neuroactive substances in the rat hypothalamus

In order to establish antibodies against neuropeptides for a consecutive combination with the polyclonal anti rabbit MCHR-1 antibody, I initially performed immunohistochemistry for a number of neuroactive substances of interest within the rat hypothalamus. To avoid possible cross-reactivities between fluorophore coupled secondary antibodies, most of the here described antibodies chosen to be raised in other species than rabbit. On rat brain cryosections, in the lateral hypothalamus, the perifornical area, and to a lesser extend, in the dorsal area of the hypothalamus, the neuropeptides MCH (Fig. 15A) and OxA (Fig. 15C) were detected. Both neuropeptides were shown to be highly abundant within these areas, and the antisera used for both neuropeptides proved effective staining not only in cell bodies, but also in fibres and axons (Fig. 15B,D). MCH and OxA immunoreactive neuronal projections could be detected throughout the rat brain including intrahypotahalamic and extrahypothalamic brain regions (data not shown). With this respect, the fibre intensity was found to be intense on OxA immunostained sections than for MCH and the subcellular resolution for the detection of OxA shows a clear axonal transport of OxA immunoreactive vesicles (Fig. 15D).

Within the arcuate nucleus of the hypothalamus, an immunohistochemical detection for the feeding-related neuropeptides ACTH (as a marker for POMC), CART, and NPY, AgrP was performed. Using the here described antibodies, from these neuropeptides only ACTH and NPY showed a specific staining pattern within the arcuate nucleus, with some rare immunoreactive material stained for in the anterior-lateral proportion of the Arc for ACTH (Fig. 15E) or in the more medio-ventral part of the Arc for NPY (Fig. 15G).

Under a higher magnification (x63), ACTH-IR was found on neuronal boutons that were visualized to be abundant not only in the Arc (Fig. 15F) but also within several hypothalamic nuclei including the LHA, DMH and PVN. However, only casual ACTH immunopositive neurons were detected in the ventrolateral proportion of the Arc (data not shown).

In contrast, there was a distinct but small population of NPY-IR neurons stained in the more ventrally located arcuate nucleus, and neurons that exhibit NPY-IR demonstrate both cytoplasmatic or bouton-associated staining (Fig.15H).

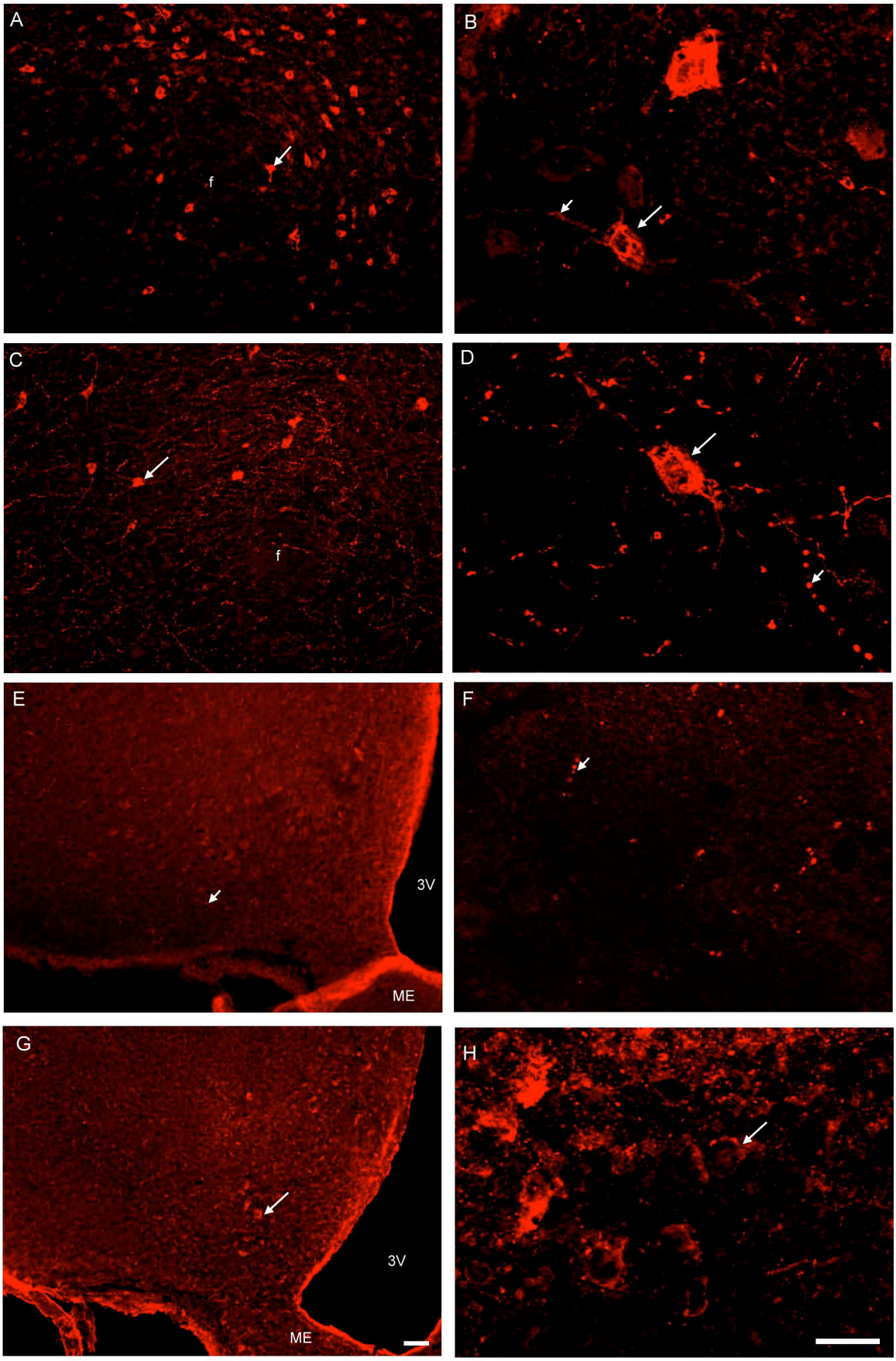
However, although several experimental conditions including primary and secondary antibody dilution and incubation times were changes, the AgrP and the CART antibodies failed to work on brain cryosections, and for the detection of ACTH or NPY, a weak to moderate staining background was still present after the immunohistochemical procedures.

Fig. 15.

Low and high magnification of single-labelling immunohistochemistry for Melanin-concentrating hormone (MCH) (A,B) or Orexin A (OxA) (C,D) in the lateral hypothalamus, Adrenocorticotropic hormone (ACTH) (E,F) or Neuropeptide Y (NPY) (G,H) in the arcute nucleus of the rat hypothalamus. Images were obtained by fluorescence microscopy using polyclonal rat antiserum to MCH, polyclonal goat antiserum to Orexin A, polyclonal guinea pig antiserum to ACTH and sheep antiserum to NPY. Long arrows indicate immunopositive cell bodies for the neuropeptides, whereas short arrows indicate immunopositive boutons or dendrites within the same hypothalamic regions.

Scale bar (G) 100 μm, (H) 20 μm

f fornix; 3V third ventricle, ME median eminence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Within the PVN, neurons that exhibit VP immunoreactivity was detected in the ventral and ventrolateral magnocellular division of the nucleus (Fig. 16A). Staining revealed a strong and intense labelling for vasopressin in the cytoplasm of these neurons and VP-IR fibres are abundant within the PVN (Fig. 16B). The same distribution pattern was seen for oxytocin immunoreactive neurons that were shown as a discrete cluster of neurons in the PVN (Fig. 16 C, D). Vasopressinergic as well as oxytocinergic varicose axons seem to project from the PVN to more ventral located brain regions (Fig. 16 A,D). For example, on the same bregma level (-1.4 mm) immunoreactive fibres and terminals for VP and OT were visualized in the SO (see also Fig. 19 A,E).

Within the PVN, CRF immunoreactive neurons were detected in the PVN, but the distribution of CRF-IR neurons was defined more posteriomedial in the parvocellular, medial subdivision of the PVN (Fig. 16 E,F). In contrast to VP and OT, CRF immunoreactivity was absent in the SO (data not shown). Finally, strong and potent staining was found for tyrosine hydroxylase in the periventricular nucleus of the medial hypothalamus (Fig. 16 G,H).

2.3.4. Colocalisation studies of MCHR-1 with neuroactive substances in the rat hypothalamus

After profiling of antibodies directed against neuroactive substances, colocalisation experiments of MCH-R1 with OxA, ACTH, NPY, VP, OT and CRF were performed by double-labelling immunohistochemistry, detection of fluorophore-coupled secondary antibodies were done by fluorescence microscopy. In the rat hypothalamus, the anatomical distribution of immunoreactive material for all of these neuropeptides showed strong co-distribution with immunohistochemical localisation of MCHR-1.

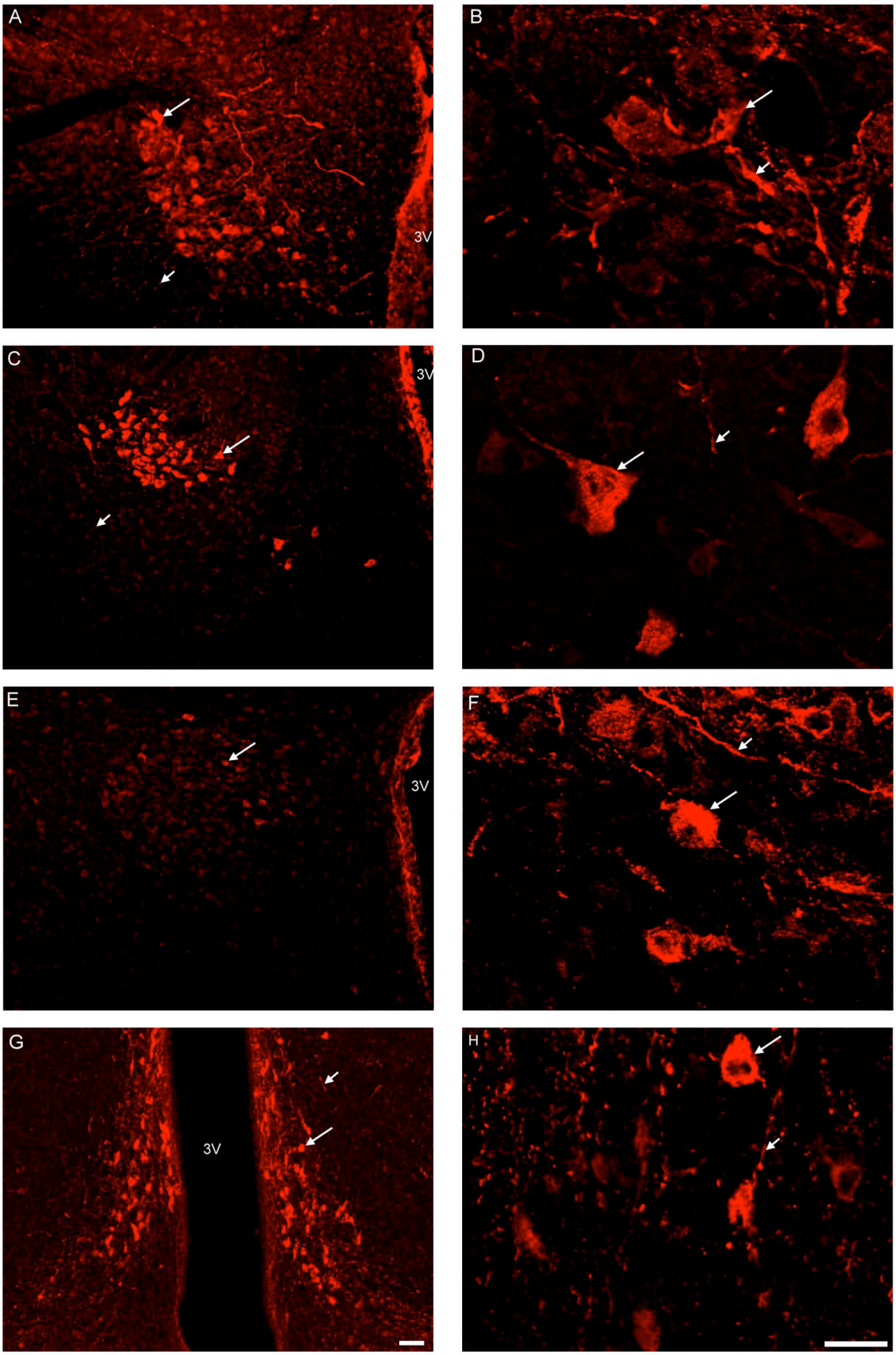
In the LHA, a vast majority of neurons that exhibit cytoplasmatic OxA-IR was also shown to present MCHR-1 immunoreactivity (Fig. 17 A-F). Within the arcuate nucleus, only single neurons exhibit ACTH-IR terminals that colocalize with selective MCHR-1 immunoreactivity (Fig. 17 G-L). Furthermore, in the ventromedial arcuate nucleus, NPY immunoreactive neurons with a specific cytoplasmatic and terminal staining was demonstrated to possess membrane associated MCHR-1 staining (Fig. 17 M-R).

Fig. 16.

Low and high magnification of single-labelling immunohistochemistry for Vasopressin (VP) (A,B) Oxytocin (OT) (C,D) or corticotropin releasing factor (CRF) (E,F) in the paraventricular nucleus or Tyrosine hydroxylase (G,H) in the periventricular nucleus of the rat hypothalamus. Images were obtained by fluorescence microscopy using polyclonal guinea pig antiserum to VP, OT or CRF and rabbit antiserum to TH. Long arrows indicate immunopositive cell bodies for the neuroactive substances VP, OT, CRF or TH, whereas short arrows indicate immunopositive boutons or dendrites with in the same hypothalamic regions.

Scale bar (G) 100 μm, (H) 20 μm

3V third ventricle,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Further analysis of brain sections that contain the PVN of the hypothalamus showed a strong degree of colocalisation for immunolabelled VP neurons with MCHR-1 neurons. Of these, the major part of VP neurons shown cytoplasmatic VP staining that co-localize with cytoplasmatic or membrane associated MCHR-1 staining (Fig. 18 F). Although there was a large amount of cytoplasmatic stained OT-IR neurons contain MCHR-1, there were also some single labelled MCHR-1 neurons (Fig.18 G-L). The high degree of colocalisation was strengthened by the observation, that a large number of MCHR-1 IR neurons are visualized in direct contact with VP immunoreactive (Fig 18 A-D) or OT immunoreactive terminals (Fig. 18 E-F). However, some MCHR-1 neurons were also detected to be VP or negative, which gives a credit to the specificity of the immunostaining.

In addition, within the parvocellular division of the PVN, a large extent of CRF containing neurons also showed MCHR-1 immunoreactivity (Fig. 18 M-R).

In addition, in the SO nucleus of the hypothalamus, vasopressinergic and oxytocinergic immunoreactivity was highly present, but subcellular restricted to neuronal terminals (Fig. 19A,B). The distribution pattern of VP and OT converged to a high degree with the observed MCHR-1 IR (Fig. 19B,F). In fact, vasopressinergic and oxytocinergic nerve terminals were defined to end in direct proximity to MCHR-1 IR neurons within the SO (Fig.19 D,H)

2.3.5. Distribution of MCHR-1 immunoreactivity in the rat pituitary

To date, the immunohistochemical distribution of MCHR-1 on the rat pituitary has not been examined. Based on the immunohistochemical detection of MCHR-1 on rat brain cryosections, I used a similar approach to perform single-labelling immunohistochemistry for MCHR-1 on rat pituitary cryosections. In accordance to the experimental design forebrain sections (see Fig. 16 G-J), incubation of pituitary sections with MCHR-1 revealed a specific staining pattern of MCHR-1 immunopositive cells to be highly abundant in the pars distalis and to a lesser extend, in the pars neuralis of the pituitary (Fig. 20). Under a higher magnification, MCHR-1 was subcellular defined as a specific, membrane-associated staining in both the pars distalis (Fig. 20 C,E) and pars neuralis (Fig. 20 C,F). Pre-absorption of the antibody with the immunogenic peptide (Fig 20B) or omission of the first antibody (Fig. 20D) revealed only rare unspecific staining.

Fig. 17.

Low and high magnification of fluorescence photomicrographs of representative sections after direct double-labelling combining the polyclonal antiserum to MCHR-1 with the goat polyclonal Orexin A antibody in the lateral hypothalamus (A-F), or MCHR-1 with guinea pig antiserum to ACTH (G-L) or sheep polyclonal NPY antibody (M-R) in the arcuate nucleus of the rat hypothalamus. Overlay of images show double-labelled neurons for OxA (artifical red, long arrow) with MCHR-1 (artificial green, short arrow) (C, F) in the lateral hypothalamus. ACTH (I, L) or NPY (O,R) immunopositive boutons appear to co-distribute with MCHR-1 on arcuate neurons. MCHR-1 was visualized with streptavidin Cyanin 2 (artificial green, short arrow), immunopositive material for neuropeptides are visualized by the appropiate cyanin 3 antibody (artifical red, long arrows). Neuronal nuclei were counterstained with DAPI.

f, fornix; 3V, third ventricle, ME, median eminence

Scale bar (G) 100 μm, (H) 20 μm

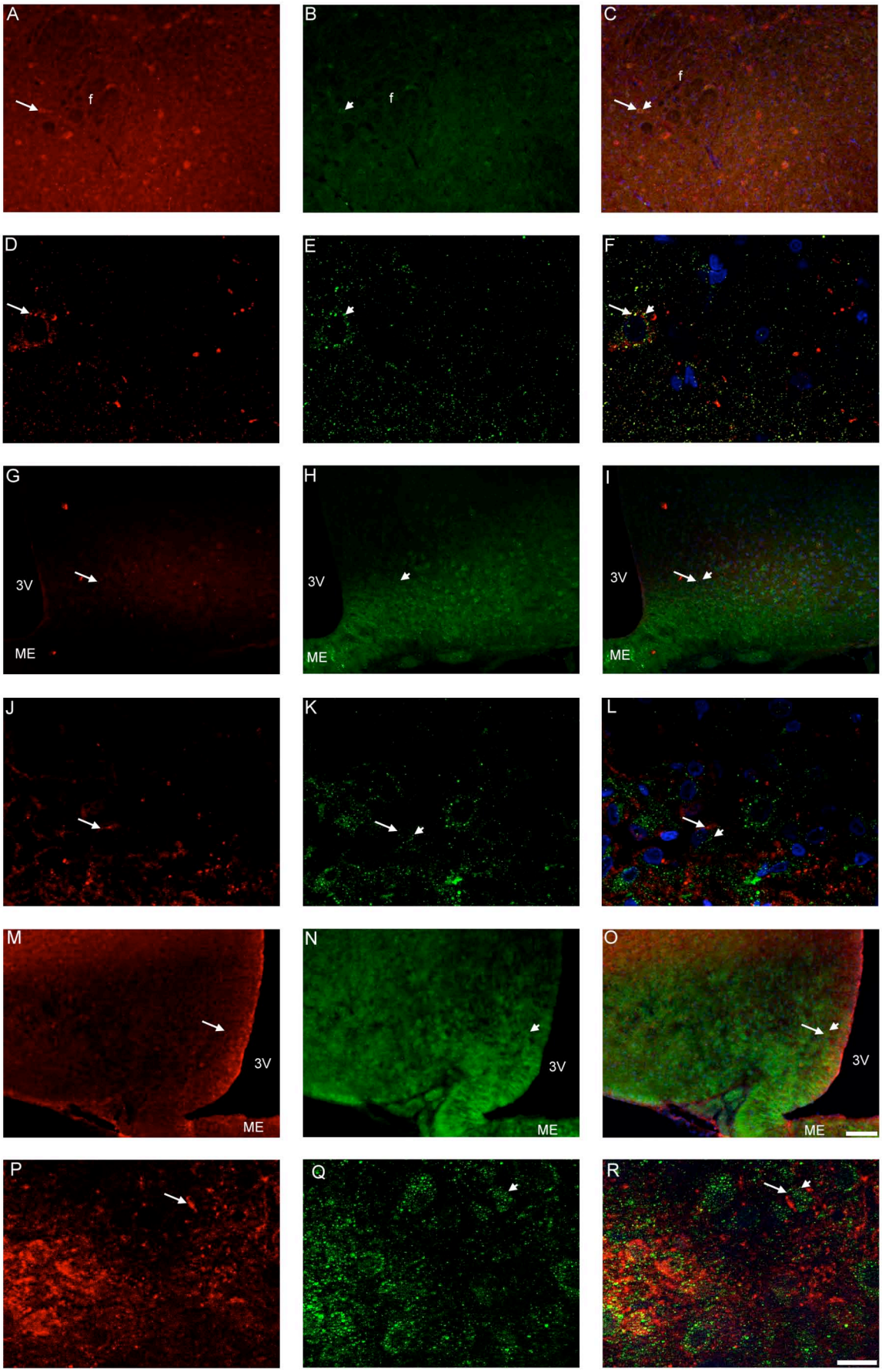
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Fig. 18.

Low and high magnification of fluorescence photomicrographs of representative sections after direct double-labelling combining polyclonal antiserum to MCHR-1 with polyclonal guinea pig antibodies against vasopressin (VP) (A-F), Oxytocin (OT) (G-L) or corticotropin releasing factor (CRF) (M-R) in the paraventricular hypothalamic nucleus of the rat brain. Overlay of images show double-labelled neurons for VP with (C, F) OT (I, L) or CRF (O,R). MCHR-1 are visualized with streptavidin Cyanin 2 (artificial green, short arrow), immunopositive material for neuropeptides are visualized by the appropiate cyanin 3 antibody (artifical red, long arrows). Neuronal nuclei were counterstained with DAPI.

f fornix; 3V, third ventricle

Scale bar (G) 100 μm, (H) 20 μm

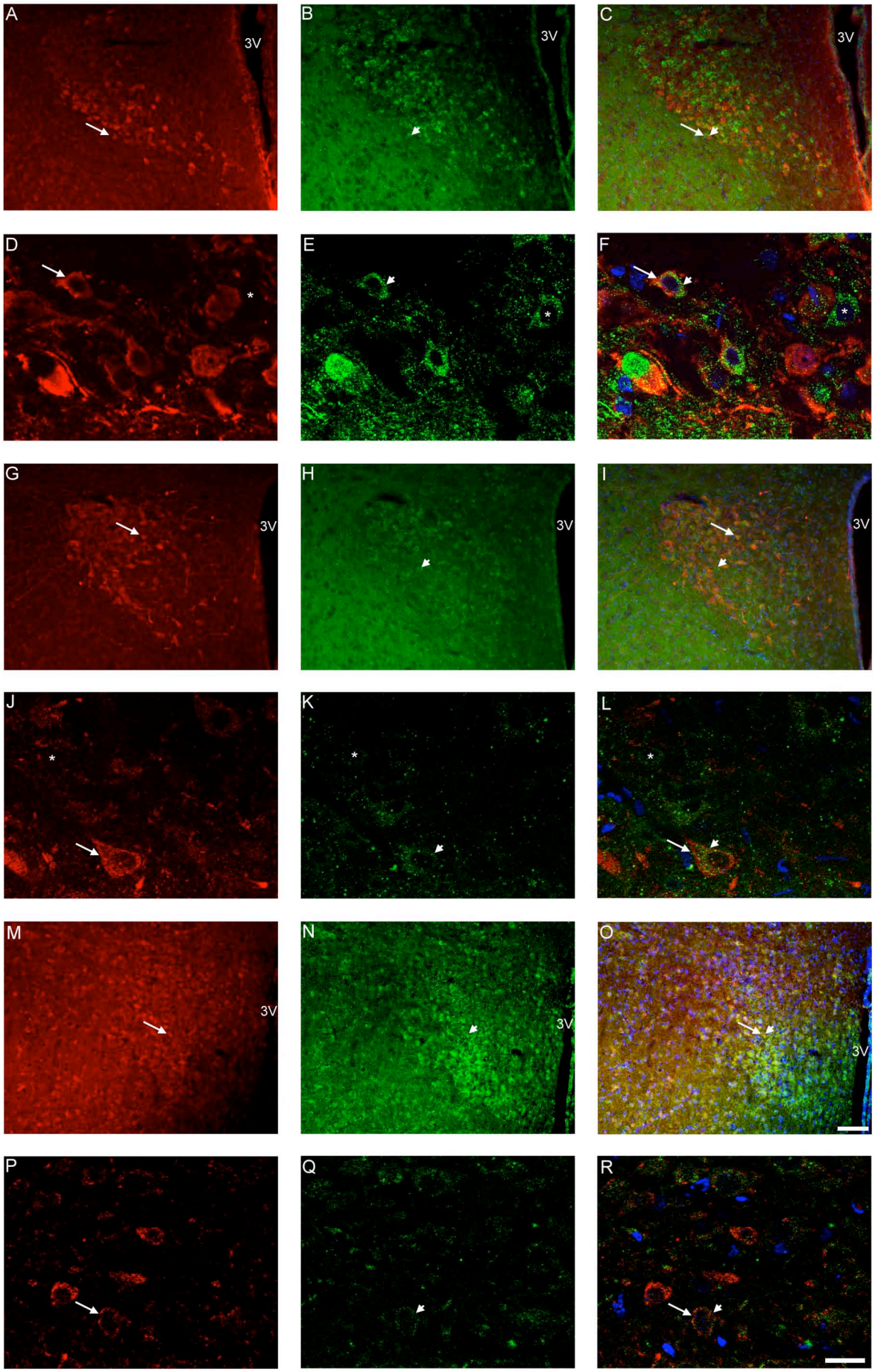
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Fig. 19.

Low and high magnification of fluorescence photomicrographs of representative sections after direct double-labelling combining polyclonal antiserum to MCHR-1 with the guinea pig anti vasopressin (VP) (A-D) or guinea pig anti Oxytocin (OT) (E-H) antibody in the supraoptic nucleus of the rat brain. Overlay of images show double-labelled neurons for VP or (C, F) OT (I, L) (both artifical red) with MCHR-1 (artificial green). Subcellular distribution of the neuropeptides was detected as VP (D) or OT (H) immunopositive fibers and boutons, (short arrows), on the same neurons MCHR-1 staining was found to be cytoplasmatic or membrane-associated (long arrows). Neuronal nuclei were counterstained with DAPI.

ox optic chiasm

Scale bar (G) 100 μm, (H) 20 μm

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Fig. 20.

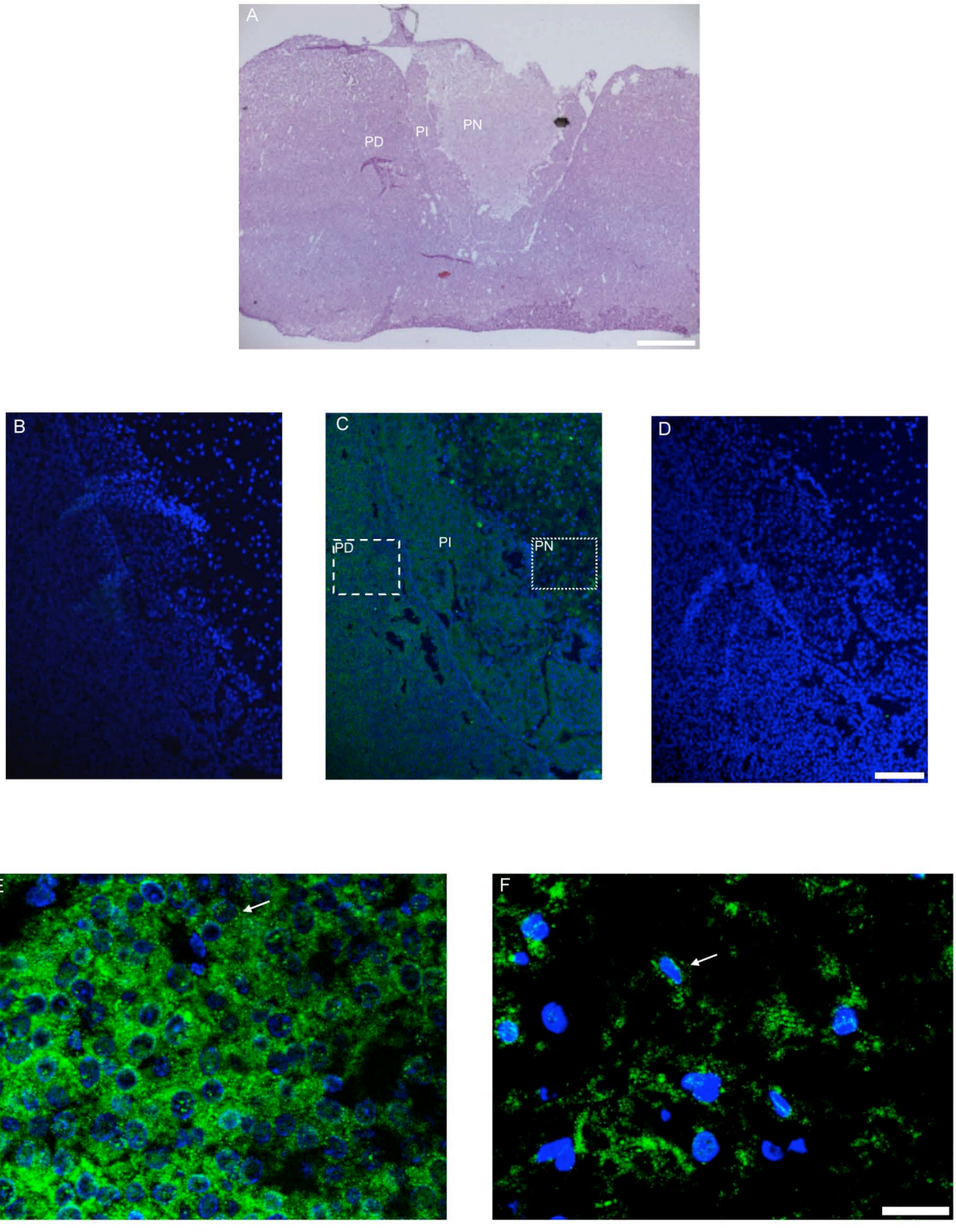
MCH-R1 immunoreactivity in the rat pituitary.

Histological staining of a hematoxylin-eosin processed coronal section of the rat pituitary (A). The different compartments of the pituitary, pars distalis (PD), pars intermediate (PI) and pars nervalis (PN) are defined.

Representative photomicrographs after immunohistochemistry on rat pituitary sections using the polyclonal MCHR-1 antibody.

Low (x5) and high magnification (x63) show intense MCHR-1 immunopositive staining in the PD (C,E) and only weak to moderate staining in the PN (C,F), depicted by white arrow. On subsequent pituitary sections, Incubation of subsequent pituitary sections with the MCHR-1 antibody preabsorbed with the corresponding peptide (B) or omission of the MCHR-1 antibody (D) both result in no staining.

Scale bar (A) 500 μm; (D) 100 μm; (F) 20 μm.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2.4. Discussion

The purpose of the here presented study was to further define the neurochemical phenotype of MCHR-1 immunoreactive neurons within the rat hypothalamus. Single labelling experiments with the herein used antibodies to MCHR-1 and MCH , detected a high abundance of the MCHR-1 signal within the PVN and SO and moderate to weak MCHR-1 immunoreactivity (IR) within several hypothalamic regions including the LH and the Arc. These results are in full agreement with the described distribution pattern of MCHR-1 IR by others that used the same immunogenic peptide sequence for the generation of a polyclonal antibody to MCHR-1 (Hervieu et al., 2000).

Additonally, the widespread distribution of MCH IR fibres and boutons is consistent with others (Bittencourt JC et al., 1992). Double-labelling immunohistochemistry combined with fluorescence microscopy for MCHR-1 with hypothalamic neuropeptides revealed a high degree of colocalisation of MCHR-1 with OxA in the LHA, with NPY and ACTH in the Arc and with VP, OT and CRF in the PVN of the hypothalamus. These hypothalamic nuclei have been discussed for their essential roles in feeding and drinking behaviour, stress response and endocrine, autonomous hypothalamic output systems (Kalra et al., 1999; Leibowitz and Wortley, 2004; Wilding, 2002).

2.4.1. Possible involvement of MCHR-1 on other feeding-related pathways

Numerous studies that have focused on the orexigenic properties of MCH (Rossi et al., 1997, Edwards et al., 2001). Given the fact, that the functional MCHR-1 has been shown to be expressed in a number of hypothalamic regions including the LH, Arc, PVN and SO, it seems feasible that MCHR-1 may be not only a key mediator of MCH`s orexigenic actions, but corresponds to other physiological actions besides feeding (Shi, 2004; Hervieu, 2006).

The LHA is known to regulate many different functions, such as feeding, blood pressure, fluid regulation, neuroendocrine axis, thermoregulation, sleep-wake cycle, emotion, sensorimotor integration and reward aspects (Berthoud, 2002). In the LHA, neurons that express OxA have been shown to express the MCHR-1, which give rise to the assumption that the orexigenic MCH system directly interacts with the central OxA system on the level of the lateral hypothalamus (Elias et al., 1998; Kotz, 2006; Nahon, 2006). Although MCH and OxA are expressed in different neurons of the LHA (Guan et al., 2002) there is a great overlap of MCH/OxA IR and MCHR-1/OxR-1 IR (Bittencourt et al., 1992; Peyron et al., 1998; Hervieu et al., 2000; 2001). Electrophysiological studies showed reciprocal connections between MCH and OxA neurons in the LHA (Gao et al., 2000) and the orexin receptor subtype 1 (OxR-1) was

found to be present on LH MCH neurons (Bäckberg et al., 2002). Thus, within the LH, MCH and OxA neurons communicate with each other to generate a common hypothalamic output signal that contribute to energy homeostasis (Elmquist 2001; Williams et al., 2001). Furthermore, functionally overlapping pathways (e.g feeding behaviour and energy expenditure), but also distinct pathways within the CNS (such as sleep-wake-cycle) may be characteristically for each of the neuropeptides (Harthoorn et al., 2005).

Neurons located in the LHA build up a network of monosynaptic connections to other intrahypothalamic and extrahypothalamic brain sites (Bernardis and Bellinger, 1996). For example, LH neurons are strongly interconnected with the Arc, an important hypothalamic region for the regulation of energy homeostasis (Elmquist et al., 1998; Woods et al., 1998; Schwartz et al., 2000). The abundance of Arc projections to MCH neurons in the LH supports the hypothesis that the MCH system acts downstream of Arc neurons expressing the neuropeptides NPY/AgrP or POMC/CART (Elias et al., 1999). However, I was not able to detect the neuropeptides AgRP or CART using the here investigated antibodies, but it was possible to identify NPY-IR in the more ventromedial extension of the Arc and ACTH-IR more lateral distributed. Although labelling of MCHR-1 was only moderate within Arc neurons, NPY-IR terminals and boutons were found to exhibit MCHR-1 IR and ACTH immunoreactive terminals are in direct proximity to MCHR-1 neurons. Thus it could be argued, that MCH sensitive neurons contact both NPY and ACTH expressing neurons to build up a reciprocal network between LH situated MCH neurons and Arc neurons. The NPY receptor subtype NPY R1 and NPY R5 are both expressed in the LHA also the Melanocortin receptor MC-4 has been defined on MCH-IR neurons within the LH (Haarthorn et al., 2005). Interestingly, MCH was reported to stimulate the release of both orexigenic NPY and AgrP, but inhibit the release of α-MSH and CART from hypothalamic explants (Abbott et al., 2003). Although I could not detect any colocalisation for MCHR-1 with CART or AgrP, it seems likely that these neuropeptides are colocalised with MCHR-1, since both neuropeptides were shown to colocalise with arcuate NPY-IR (Broberger et al., 1998) or POMC-IR (Elias et al., 2001), respectively.

2.4.2. Possible influence of MCHR-1 on the neuroendocrine effector pathways on the level of the hypothalamus

The PVN is a hypothalamic region which is known to express a number of potent neuropeptides with specific physiological functions. In the PVN, I detected MCHR-1 IR on the complete anterior to posterior extension. Double-labelling studies of hypothalamic sections

revealed a clear co-distribution of MCHR-1 on vasopressinergic and oxytocinergic neurons in the PVN and SO.

The hypothalamic neuropeptide VP is known to be involved in the regulation of central and peripheral effector pathways VP is released from hypothalamic PVN and SO neurons by ascending osmotic (osmolality) and nonosmotic (vomiting and baroreceptors pathways from the brainstem (Poulain and Wakerley, 1982). Within the CNS, VP acts as a neuromodulator/neurotransmitter regulating several functions, including neuroendocrine reactivity, thermoregulation and autonomous functions (Gutkowska et al., 1997; Ring, 2005). Early, it has been reported, that pituitary extracts were shown to decrease urinary output, increase the density of the urine and reduced thirst (Starling and Verney, 1924). In addition, effects of VP release from the posterior pituitary primary include endocrine effects such as vasoconstriction, glycogen metabolism and antidiuresis (Goldsmith, 1987). The central VP system is directly connected to angiotensin, catecholaminergic and cholinergic pathways (De Wardener, 2001). Thus, vasopressin has been observed to promote hypertension when administered centrally (Pittman et al., 1982;Porter et al., 1986).

Interestingly, others have observed dipsogenic effects of MCH in the rat after central administration, which is in agreement with my observations (see second part of this work) and this increase in water intake was independent for its orexigenic effects (Clegg et al., 2002). Central diuretic and natriuretic actions of MCH have been also determined in the sheep (Parkes, 1996). Consequently, it remains to elucidated, if MCH interacts with the central VP system, possibly through with mediation of inhibitory effects through MCHR-1. This issue could be addressed by *in vitro* experiments that examine effects of MCH or MCHR-1 antagonists hypothalamic explants, or posterior pituitary preparations. Moreover, it would be interesting to elaborate effects of the MCH system in an animal model of dehydration-induced anorexia (Watts et al., 1999) or in an animal model representing vasopressin deficiency, such as the congenital diabetes insipidus rat. Finally, MCH and MCHR-1 has been shown to be present on peripheral sites such as the kidneys or the intestine (Hervieu et al., 1996; Saito et al., 1999; Kokkotou et al., 2001), which may account not only for central diuretic effects of MCH on water homeostasis, but also peripheral effect fluid regulating hormones.

In a number of the MCHR-1 IR located in the PVN and SO, MCHR-1 protein expressing neurons co-contained OT-IR. OT is known to be involved in parturition and lactation, but essential physiological responses of the body for reproduction. The central oxytocin system acts as a neuromodulator in different behaviours. Peripheral OT has been defined as a potent stimulator of erectile function in male and stimulation of uterine smooth muscle contraction

during labor and milk ejection during lactation (Kiss and Mikkelsen, 2005). Multiple lines of evidence have shown that OT inhibits ingestion and thus works as an anorexigenic neuropeptide (Verbalis et al., 1995). MCH-IR was significantly increased in the LH of in lactating rats, thus MCH be a possible mediator of orexigenic effects during lactation (Sun et al., 2004). Possible effects of the MCH system on lactation, reproduction and energy homeostasis and the involvement of MCHR-1 on OT physiological functions could be investigated using a rodent lactation model (Smith and Grove, 2002).

Possibly, a number of I have identified strong MCHR-1 IR on PVN neurons and of these, a high number was shown to exert CRF immunoreactivity. CRF has not only been shown to be an essential mediator for the central stress system, but has also been discussed for its involvement in feeding behaviour (Krahn et al., 1988). The influence of the central MCH system on stress response has been reviewed recently (Hervieu et al., 2003, 2006). A number of studies identified an interaction between the hypothalamic-pituitary-adrenal axis and the MCH system. For example, dexamethasone stimulate the synthesis of MCH mRNA *in vitro* and pro-MCH derived peptides hypothalamic cell cultures, but CRH suppress the release of MCH release (Parkes et al., 1992). Administration of MCH on hypothalamic explants were found increase CRH release (Kennedy et al., 2003). Central MCH administration had stimulatory effects on ACTH release and peripheral administration of MCH dose dependently increase plasma corticosterone levels (Jezova et al., 1992; Bluet-Pajot et al., 1995; Ashmeade et al., 2000; Kennedy et al., 2003). However, it remains open on which level of the stress axis MCH mediates its stimulatory actions, not only on hypothalamic sites, but possibly on peripheral sites including the pituitary, where MCHR-1 expression has been observed on rat and human tissue (Saito et al., 1999, Hill et al., 2001). Furthermore, MCH and CRH may synergistically interact in terms of stress induced inhibition of food intake, as shown for CRF receptor antagonists (Koob and Heinrichs 1999; Sekino et al., 2004; Carlini et al., 2006). And finally, the MCH and the CRH system have been discussed for their role in anxiety and depressive behaviour (Hervieu, 2006; Nielsen, 2006), and it may be of further interest if and how both systems modulate these complex behaviours.

2.4.3. Possible influence of MCHR-1 on the pituitary system

The detection of MCHR-1 IR within the posterior and anterior pituitary supports the idea of modulatory effects of MCH on the neuroendocrine axis. Others have reported the presence of MCHR-1 expression on pituitary tissue (Saito et al., 1999; Hill et al., 2001). Additionally MCH was shown to be partly released into the pituitary stalk suggesting direct influence on the

neuro- and adenohypophysis (Zamir et al. 1986). Besides the release of hypothalamic derived VP and OT, the pars distalis of the anterior pituitary (adenohypophysis) is reponsible for the release of growth hormone, prolactin and thyroid-stimulating hormone, follicle-stimulating hormone, luteinizing hormone, adrenocorticotropic hormone and Melanocyte-stimulating hormone (Dorton, 2005). The presence of MCHR-1 on the rat pituitary supports the idea of direct regulatory actions of MCH on the release of these pituitary hormones. As pituitary-derived hormones are involved in the regulation of numerous endocrine pathways including growth, reproduction, energy expenditure, and stress, further analysis of MCH`actions on the release of pituitary hormones are needed. In conclusion, the present study shows that MCHR-1 immunoreactivity is colocalized with hypothalamic neuropeptides that have been determined for their role in feeding behaviour and body weight regulation.

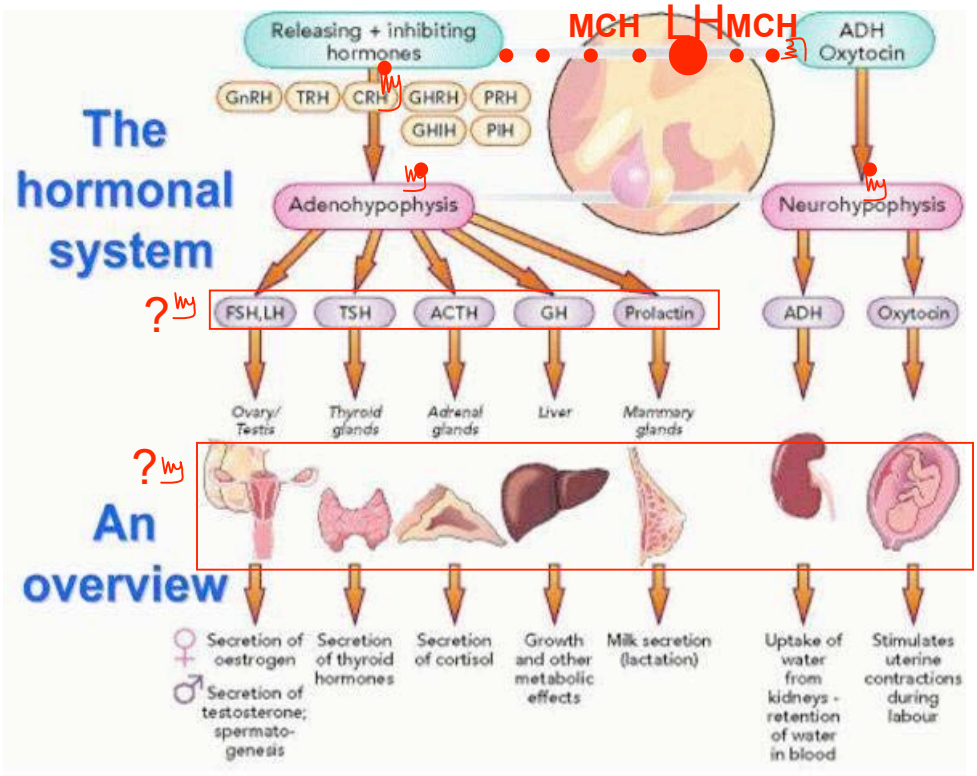

Fig. 21. Possible effects of MCH on the hypothalamic-pituitary-axis.
MCH (red dots) is produced in the lateral hypothalamus (LH) and released from nerve terminals. MCH binds to its functional receptor MCHR-1. MCHR-1 is detected on paraventricular hypothalamic neurons that express Vasopressin (VP), also named antidiuretic hormone (ADH) and Oxytocin. ADH and oxytocin are released from the neurohypohysis that also express MCHR-1, indications for a possible influence of MCH on water homeostasis and reproduction. MCHR-1 is also expressed on PVN neurons that produce corticotrophin-releasing factor (CRH). CRH exerts its actions on adenohypophysis cells that produce Adrenocorticotropic hormone (ACTH), which stimulates the release of cortisol from the adrenals. Strong MCHR-1 expression within the PVN and SO of the hypothalamus and on the adenohypophysis may indicate MCH`s influence on the hypothalamic-pituitary-ovary-axis, hypothalamic-pituitary-thyroid axis or hypothalamic-pituitary-growth-axis. These hormonal systems influence reproduction, energy expenditure and growth.
Abbreviations: ACTH, adrenocorticotropic hormone; ADH, antidiuretic hormone; GnRH, gonadotropin-releasing hormone; GHIH, growth hormone inhibiting hormone; GHRH, growth hormone releasing hormone; GH, growth hormone; FSH, follicle stimulating hormone; LH, Lateral hypothalamus, PRH, prolactin releasing hormone; PRI, prolactin inhibiting hormone; MCH, Melanin-concentrating hormone, TRH, thyreotropin releasing hormone, TSH, thyroidea-stimulating hormone.(modified from a presentation of the Ferring Arzneimittel GmbH, Kiel, Germany)

Furthermore, MCHR-1 immunoreactivity is also colocalized on neuropeptides of the neuroendocrine axis, has hypothalamic feeding circuitries among neuropeptides in the hypothalamus, which gives a morphological basis for the involvement of the central MCH system on fluid regulation, reproductive behaviour and stress response. Further in vivo studies are necessary to provide more insights of the interaction of MCHR-1 with here examined neuropeptidergic systems. Additionally, the hypothalamic and pituitary explant models suggest that other neuromodulatory systems are modulated by MCHR-1. Among them, the regulation of reproduction (hypothalamic-pituitary gonadal axis (Luteinizing hormone release) (Murray et al., 2000; 2006; Williamson-Hughes et al., 2005) and the hypothalamic pituitary- thyroid axis (TRH, tyrotropin-releasing hormone) (Kennedy et al., 2000). Therefore, the MCH system may serve as an important link between bodies energy depletion and these hormonal systems.

3. Neuronal activation patterns after a feeding-related stimulus within the rat brain

3.1. Introduction: The use of c-Fos immunohistochemistry as a biomarker to detect neuronal activation within the MCH system

A number of molecular imaging methods have been introduced to visually represent physiological, pathophysiological and pharmacological processes at the cellular and molecular level. This include Magnet Resonance Imaging (MRI), Positron Emission Tomography (PET), near-infrared optical imaging, scintigraphy and autoradigraphy (Heckl et al., 2004). Today, MRI and PET have been successfully introduced as brain imaging techniques in clinical neurology. These methods have also been used to identify neuroanatomical and functional correlates of hunger and satiation (Del Parigi et al., 2002) and furthermore, to detect differences in the local neuronal activity between normal-weighted humans, obese humans and patients that suffer from eating disorders (Gautier et al., 2000; Wang et al., 2001; Del Parigi et al., 2002; Frank et al., 2004).

A number of additional powerful functional neuroanatomical tool have been developed in molecular biology to study structure-function relationships on the molecular level (Konkle and Bielajew, 2004). Among them, the stereotypic inducibility of c-fos proto-oncogene rendered the cellular immediate-early gene (IEG) to be the most widely used functional anatomical mapping tool to identify cells and extended circuitries that became activated in response to various stimuli (Kovacs, 1998). The rapid and transient induction of c-Fos in response to diverse physiological and pharmacological stimuli is induced after various intracellular signalling cascades (Sheng et al., 1990; Hoffmann et al., 1993). Thus, examination of the c-Fos

expression pattern can be utilized to produce high resolution maps of cellular activation in the CNS on both mRNA and protein level (Herrera et al., 1996; Herdegen et al., 1998).
Based on visualisation of c-Fos induction, various physiological and pharmacological stimuli have been studied. Examination of c-Fos immunohistochemistry was done in animals models of obesity such as the DIO mice (Lin and Huang, 1999) and in obese fa/fa Zucker rats during food deprivation (Timofeeva and Richard, 2001). Numerous studies have identified c-Fos immunoreactivity in brain regions after adminstration of feeding-related hormones such as leptin (Hübschle et al., 2001), ghrelin (Hewson and Dickson, 2000) or orexigenic and anorexigenic hypothalamic neuropeptides such as α-MSH (Olszewksi et al., 2001 or CRH (Bittencourt et al., 2000). Additionally, pharmacological administration of centrally acting anti-obesity drugs such as the CBR-1 antagonist SR 141716 or the SSRI Fluoxetine have resulted in a specific c-Fos expression pattern (Singh et al., 2004; Rowland et al., 2000).
Among physiological stimuli, animals that are entrained by restricted feeding schedules (RFS) as a potent physiological stimulus display daily rhythms of food anticipatory activity and entrainment of physiological parameters (Mistelberger, 1992; Escobar et al., 1998; Angelles-Castellanos et al., 2006; Diaz-Munoz et al., 2000). The introduction of the RFS paradigm combined with c-Fos immunohistochemistry was used in order to identify neuronal substrates within the rat hypothalamus that are involved in food entrainment (Angelles-Castellanos et al., 2004; Gooley et al., 2006). Rats that underwent a RFS were examined for the neuronal activation patterns within brain regions that are involved in the generation of anticipatory behaviour (Inzunza et al., 2000; Chaillou et al., 2000) and reward (Mendoza et al., 2005).

Although several behavioural and pharmacological models underlined the effects of the MCH system on feeding and body weight regulation, the mechanism of action of the MCH system on food intake regulation *per se* and feeding-related circuitries, such as water intake regulation, reward, motivation, stress, neuroendocrine and autonomic pathways remains to be determined.

The here presented second part of my work investigate the mechanism of action after pharmacological or physiological activation of neurons within the MCH system.
In experiment 1, after central administration of MCH in rats, I monitored acute effects of MCH on food and water intake followed by post mortem analysis of c-Fos immunohistochemistry within the brain.
In experiment 2, rats were treated for eight days with the orally available MCHR-1 antagonist compound B4 and changes in body weight, food intake and water intake were monitored. After

the last animal treatment, c-Fos immunohistochemistry was performed on sections representing the rat hypothalamus.

The purpose of the last part of this work was to identify neuronal activation patterns after a potent physiological, feeding related-stimulus. Therefore I introduced a RFS adjusted to c-Fos induction and I examine, if this RFS is capable to activate neuronal pathways after fasting (food-deprivation) or re-feeding (food-restriction) by a detailed qualitative and quantitative analysis of c-Fos immunohistochemstry of brains derived from RFS treated rats. Moreover, I define, if activated neurons are part of the central MCH system using specific antibodies to MCH or MCHR-1.

The overall aim of this part of work is to identify common or overlap neuronal activation patterns after physiological or pharmacological stimulation and additionally, after pharmacological inhibition of the central MCH system. The experimental background of the animal treatment and its possible effects on neuronal activation is illustrated in the scheme presented in Figure 22.

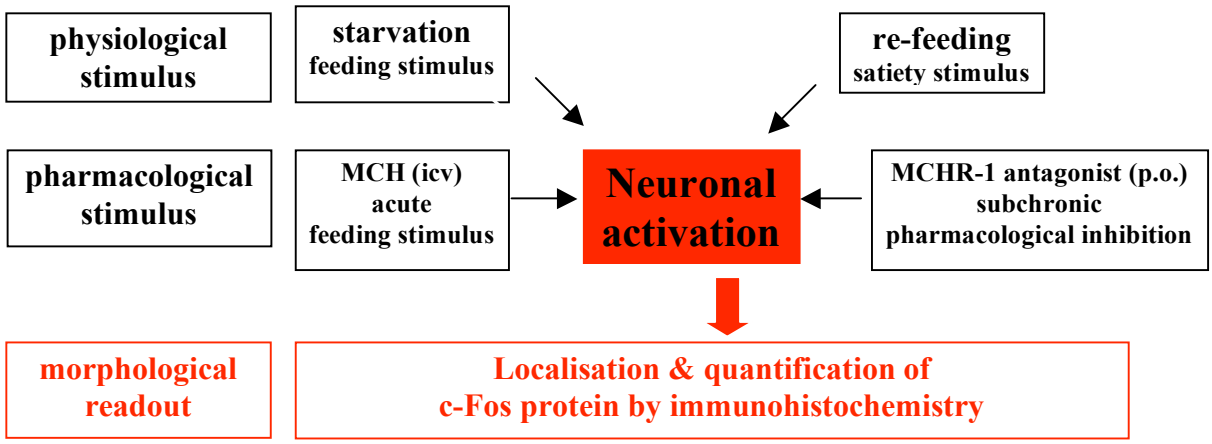

Fig. 22. Workflow for the detection of neuronal activation after activation or inhibition of MCH-related pathways within the rat brain. The restricted feeding schedule (RFS) was introduced as a physiological stimulus to challenge the animals by a potent physiological, orexigenic stimulus. Food deprivation (starvation) was defined as an orexigenic stimulus, whereas re-feeding was used as a satiety stimulus.
An acute, orexigenic pharmacological stimulus of the MCH system was introduced by icv administration of human MCH. Sub-chronic, oral administration of MCHR-1 antagonist aimed to inhibit MCH signalling in the rat brain as an anorexigenic stimulus. Neuronal activation patterns were examined after each mode of treatment using c-Fos immunohistochemistry on rat brain cryosections. icv, intracerebroventricular, po, per os (oral administration).

3.2. Material and Methods

3.2.1. Material

3.2.1.1. Chemicals, solutions and buffers

3.2.1.1.1. Chemicals

- artificial Cerebrospinal Fluid (aCSF); Harvard Inc., Holliston, MA, USA

- 10x Dulbecco`s phosphate buffered saline; Camprex, B-4800, Verviers, Belgium
- Bovine Serum Albumine (BSA), Fraction V; Serva Electrophoresis, Heidelberg, Germany
- BSA (IgG free), protease free; Dianova, Hamburg, Germany
- Cyanin 2 conjugated streptavidin; Dianova, Hamburg, Germany
- di Natriumhydrogenphosphate (Na_2HPO_4) 2 H_2O; Merck KGaA, Darmstadt, Germany
- Ethylene glycol; achieved in house, Boehringer-Ingelheim GmbH, Biberach/Riss
- Gelatine; Merck KGaA, Darmstadt, Germany
- Glycerol formal; Fluka Chemie AG, 9471-Buchs, Switzerland
- human Melanin-concentrating hormone (hMCH); Bachem CH- 4416 Bubendorf
- Immunogenic peptide Biotrend (5mg/ml, purity > 95%), Biotrend Chemicalien GmbH, Köln, Germany
- Mounting Medium; Vectashield (H-1000);Vector Laboratories,Burlinghame, CA, USA
- Natrium di-Hydrogen phosphate (NaH_2PO_4) H_2O; Merck KGaA, Darmstadt, Germany
- Natrosol (Hydroxyethylcell Natrosol 250 HX); Herkules GmbH Düsseldorf
- Normal Donkey Serum (NDS); Dianova, Hamburg, Germany
- Paraformaldehyde; Sigma-Aldrich GmbH, Steinheim, Germany
- Polyvinylpyrrolidone PVP-40; Sigma-Aldrich GmbH, Steinheim, Germany
- Potassium chromic (III) sulfate decahydrate; Fluka Chemika, CH-9471 Buchs
- Sodium azide; Sigma Chemicals, Inc. , St. Louis, USA
- Sodium chloride; Merck KGaA, Darmstadt, Germany
- Sucrose (D+ Saccharose); Carl Roth GmbH , Karlsruhe, Germany
- Tissue Tek O.C.T. Compound; Sakura, 2380 AA Zoeterwoude, The Netherlands
- Tris Hydrochloride; Promega Corp., Madison WI, USA
- Triton X-100; ICN Biomedicals Inc., Ohio, USA
- UltraCruz Mounting Medium with DAPI (SC-24941); Santa Cruz Biotechnology, Inc. Santa Cruz, CA 95060 USA

3.2.1.1.2. Preparation of solutions and buffers

Preparation of TNB blocking buffer

volume of demineralised water: 250 ml

0.15 M NaCl

0,1 M Tris HCl

ad 250 ml destilled water and adjust to pH 7.5

heat until 60 ^{0}C while stirring, then slowly add blocking reagent, 0.5% TNB Blocking reagent (supplied by the TSA kit) to the buffer. Filter while warm after TNB blocking reagent is dissolved completely. Storage at –20 ^{0}C.

Primary antibody incubation solution

0.1 M PBS

0.3 % Triton

2 % BSA

dilute 10 M PBS buffer (1:100) with demineralised water, add 0.3% Triton with a shortened pipette in a volume of 200 ml, dissolve while stirring.

For the colocalisation study MCHR-1/c-Fos, the solution was modified and 0.1 Triton/1% NDS was used.

Cryoprotectant solution

30 % sucrose in 0.1 M PBS

0.03 % Sodium Azide

Store at 4 ^{0}C.

Antifreezing Cryoprotectant solution

500 ml 0.1 M Sodium phosphate buffer (pH 7.2)

1.59 g NaH_2PO_4

5.47 g Na_2HPO_4

500 ml dH_2O

300g sucrose

10 g polyvinylpyrrolidone

300 ml ethylene glycol

Adjust volume to 1000 ml with distilled water

Preparation of 0.5% Natrosol

10 g Natrosol, were slowly dissolved in 2 liter aqua bidest (1h, shaker, 70 ^{0}C), followed by 2h at 4^{0}C. Solution can be stored at 4 ^{0}C for up to two weeks.

Preparation of human MCH solution for icv injection

314 µl aCSF were added to one vial consisting 1mg of lyophylised 78.6% hMCH. 10 µl aliquots in final dilution of 5 µg/2 µl hMCH were stored at -20 ^{0}C. Before injection procedure, one aliquot was equilibrated to room temperature.

3.2.1.2. Preparation of gelatine-coated sections

Conventional microscopical glas-slides were transferred to plastic racks each suitable for 25 slides, in a clean plastic buckets and washed as follows: detergent with hot tab water, hot tab water, 80 % ethanol in de-mineralized water, then 4x demineralised water. The gelatine solution was prepared with:

0.1 g gelatine

0.01 g chromic potassium sulfate

in 200 ml destilled water.

Water was heated until 60 ^{0}C, then gelatine and chromic potassium sulphate were added until completely dissoved (approx. 2h) while stirring. The warm solution was filtered, racks containing slides were shortly subbed (3 x 5 sec) in a container containing the warm gelatine solution, drained off on paper towels and dried over night in an oven (60 ^{0}C).

3.2.1.3. Antibodies

3.2.1.3.1. Primary antibodies

- polyclonal rabbit anti c-Fos antiserum (Ab-5); EMD Biosciences, Inc. La Jolla, CA, USA
- affinity purified rabbit anti c-Fos antibody, tetramethylrhodamine isothiocyanate (TRITC) coupled; (sc-52 TRITC), Santa Cruz Biotechnology, Inc. Santa Cruz, USA
- polyclonal rabbit anti MCH serum; Phoenix Pharmaceuticals Inc., USA
- affinity purified polyclonal goat anti OxA; Santa Cruz Biotechnology Inc., Santa Cruz, USA
- affinity purified goat anti rabbit antibody, unlabelled; Dianova, Hamburg, Germany
- For the identification of MCHR-1, the above introduced rabbit anti MCHR-1 was used.

3.2.1.3.2. Secondary antibodies

- Goat anti rabbit secondary antibody, biotinylated (BA-1000); Biozol Diagnostica, Eching, Germany
- Cyanin 3 conjugated, affinity purified goat anti rabbit IgG; Dianova, Hamburg, Germany
- Cyanin 3 conjugated, affinity purified donkey anti goat IgG; Dianova, Hamburg, Germany
- donkey anti rabbit IgG, biotinylated; Santa Cruz Biotechnology, Inc., Santa Cruz, USA

3.2.1.4. Kits

- Tyramide Signal Amplification System (NEL700); Perkin Life Sciences, Boston USA

3.2.1.5. Drugs and MCHR-1 antagonists

3.2.1.5.1. Drugs

- Ketavet, Ketaminhydrochlorid, 100 mg/ml; Pharmacia GmbH, Erlangen, Germany
- Rompun 2 %, Xylazinhydrochlorid; Bayer Vital GmbH, Leverkusen, Germany
- Narcoren®; Merial GmbH, Hallbergmoos, Germany

3.2.1.5.2. MCHR-1 antagonists

- The chemical structure of MCHR-1 antagonist GW 803430 and its synthesis has been published by:
  The Metabolic and Viral Centre of Excellence for Drug Discovery, GlaxoSmithKline, Research Triangle Park, USA (see also Carpenter et al., 2005)
  This compound was re-synthesized as a solid formulation by the Department of Chemistry, Boehringer-Ingelheim GmbH & Co KG,
  Biberach/Riss, German.
- The in house MCHR-1 antagonist B4 was synthesized in a solid formulation by the Department of Chemistry, Boehringer-Ingelheim GmbH & Co KG, Biberach/Riss, Germany.

3.2.1.6. Characterisation of animals and housing conditions

For central administration of human MCH, eight male CrlGlxBrlHan:Wistar rats (initial body weight, BW approx. 300 g) were used.

For the sub-chronic administration of MCHR-1 antagonists, female HanWistar rats (HsdBrlHan:Wist, Exbreeder, initial BW 240-300 g) were used.

For RFS, male HanWistar rats with an initial BW ranging between 230 – 250 g were used.

All animals used in this study were purchased from Charles River, Kisslegg, Germany. Animals were housed at the animal house in an environmentally controlled facility at, Boehringer Ingelheim Pharma GmbH Co. KG, Biberach/Riss, Germany. Animals were maintained in transparent acryl cages with a light-dark cycle 12:12 (lights on at 6am = Zeitgeber time 0 (ZT 0), with regulated temperature 22 +/- 1 ^{0}C, 56% air humidity. Animals had free access to tap water, and regular laboratory rat diet (Maus/Ratte Haltung “GLP” Vitamin fortified, Provimi Kliba SA, Kaiseraugst, Switzerland) *ad libitum*, unless otherwise stated.

Here presented animal experiments were performed according to the policies of the German Animal Protection Law, issue from 25th, May 1998, and were approved by the Regierungspräsidium Tübingen, Referat 35, Germany.

3.2.1.7. Technical material

- Drinking/Feeding Monitoring system; TSE GmbH, 61350 Bad Homburg, Germany
  (for further information concerning the cage system see also www.TSE-Systems.com)
  The automated feeding system measures changes of food intake (pellets) and water intake each 30 sec.
- Cryostat HM 74; Leica GmbH, Nussloch, Germany
- IKA-Schüttler; MTS, Staufen, Germany
- Pumpe Typ MV-GE, 50W, 230V; Ismatec Dosiertechnik, 8152 Glattbrugg-Zürich, Switzerland
- Plastik Flasche für Kleintierhaltung, Biberach, Riss

3.2.1.8. Software

For imaging analysis:

- HALCON software; MVTec software GmbH, München, Germany

Imaging processing:

- PhotoImpact XL, version 2003, Ulead Systems, Inc. USA

Microscopical software:

- Axiovision Rel.4.3 software; Carl Zeiss AG, Göttingen, Germany

Statistical analysis:

- Software packages SAS Version 8.2; SAS Institute Inc., North Carolina, USA
- S-PLUS® 6.1; Insightful Corp., Seattle, Washington, USA

3.2.2. Methods

3.2.2.1. Animal treatments

3.2.2.1.1. Intracerebroventricular administration of human MCH

A permanent injection apparatus was positioned into the third ventricle (3V) of the rat brain. Surgery of the animals were done in assistestence with Dr. Isabel Schwienbacher, Department of Metabolic Diseases, Boehringer-Ingelheim Pharma GmbH & Co KG.

Prior to surgery, rats were anaesthetised with an i.p. injection of ketamine (100 mg/kg BW)/xylazine (100 mg/kg BW). Animal's dorsal head were shaved and disinfected with 70% ethanol. Using ear bars, the head of the rat was positioned in the stereotaxic instrument and animal's eyes were covered with Bepanthen ointment to protect them from drying out. For the cannula implantation, the stereotaxic coordinates for the 3V were defined as 2.2 mm caudal to bregma point and 7.5 mm ventral to sinus, with lambda and bregma at the same vertical

coordinates (Paxinos and Watson, 1986). For the correct position of the cannula, the cannula was fixed at the stereotaxic apparatus and moved to the defined coordinates. One hole was drilled in the scull for the cannula and the cannula was inserted into 3V. Three additional holes were drilled, screws were inserted into the prepared holes and the cannula was fixed with dental cement attached to the screws. Animals were left in the stereotaxic apparatus until the cement was solidified. To protect the animal from infection, the opening of the cannula was sealed with a self-constructed stillet prepared from the cannula.

During the recovery period of five days after surgery, animals were left in their home cages in groups of four in acryl cages with free access to food and water. In order to minimize stress, animals were accustomed to the acute icv injection with two separate test injections of artificial cerebrospinal fluid (aCSF) within a time period of two weeks prior to MCH injection.

For the injection procedure, the protection stillet was removed, the penetrability of the inserted cannula was checked and the cannula was attached to the PP30 tubing. A total volume of 2 µl aCSF was conducted into conscious animals with a 10 µl Hamilton syringe. The complete injection volume was injected slowly into the third ventricle (time of duration: 1min/injection, time of injection start of the first animal, 11 am (zeitgeber time , ZT 5).

On the day of MCH administration, animals were randomly assigned into two groups (n=4/group). For each treated animal, one cage was prepared: a defined amount of food pellets were pre-weighted and each cage was equipped with a plastic bottle filled with tap water intake. Following a restricted injection schedule, a single bolus of MCH or aCSF was injected into 3V as described above. After each injection procedure, animals were positioned in the prepared plastic cages and 2h food intake and water intake were measured. Two hours after the injection procedure (ZT 7), animals were deeply anaesthetised by an intraperitoneal (ip) injection of a ketamine-xylazine solution at the dose of ketamine hydrochlorid 100 mg/kg BW and xylazine hydrochlorid 10 mg/kg BW and transcardially perfused by an automated perfusion pump with 100 ml ice-cold 0.9% NaCl, followed by 250 ml ice-cold 4% PFA in 0.1 M PBS.

3.2.2.1.2. Sub-chronic treatment with MCHR-1 antagonists

Six days before the start of experiment, animals were kept individually in conventional cages that were directly attached to an automated high throughput cage system. At 3pm (ZT 9) of each day, animals were removed from their cages, animal`s body weight was monitored and animals were applied a volume of 10 ml/kg BW vehicle (= 0.5% Natrosol) *per os* (po). This procedures was performed to minimize stress during experimental procedure. After each

treatment, animals were re-located into their cages in automated feeding system that continuously (each 30 sec) calculates pellet intake and water intake.

During the subsequent eight days of drug treatment, animals had free access to normal laboratory chow and drinking water. On day seven (day 0 of experiment), animals were randomized according to their body weight into three groups (n=6) and at ZT 9, one group was orally applied a bolus of 10 ml volume of 10 mg/kg GW803430, 25 mg/kg compound B4, or 0.5% Natrosol (control group). Animals were treated as described for the seven consecutive days. After the last drug treatment of animals (ZT 9, day eight), two animals per group were sacrified for the evaluation of drug pharmacokinetics using high performance liquid chromatography (HPLC). Other animals of each group were again transferred to their feeding system cages. At day nine of the experiment, at 8 am (ZT 2), the remaining animals were deeply anesthetised by inhalation of Narcorene. Two animals per group were killed by cerebral dislocation for hypothalamic RNA extraction and two animals were perfused as described above for single labelling immunohistochemistry for c-Fos.

3.2.2.1.3. Animal treatment during the restricted feeding schedules (RFS)

During the complete animal experiment, rats were kept in individual cages and food intake and body weight was monitored daily. After acclimatisation of animals to the environmental conditions, animals were randomly assigned into three experimental groups: an *ad libitum* fed group (AL), a food restricted (FR) or a food deprived (FD) group. While the AL group had free access to food during the feeding regimen, FR and FD animals were maintained under a restricted feeding regimen for nine days with laboratory chow available only for 3h per day (noon to 3pm, ZT 6-ZT 9). On day 10 of the experiment, FR rats had again access to food during ZT 6-ZT 9 as trained, in contrast FD rats were deprived of food during this period (Fig. 23).

After ZT 9, all animals were deeply anesthetized with an intraperitoneal injection of a ketamine-xylazine solution and transcardially perfused as described above by an automated perfusion pump with 180 ml 0.9% ice-cold NaCl followed by 250 ml ice cold 4% PFA in 0.1 M PBS for single labelling immunohistochemistry for c-Fos.

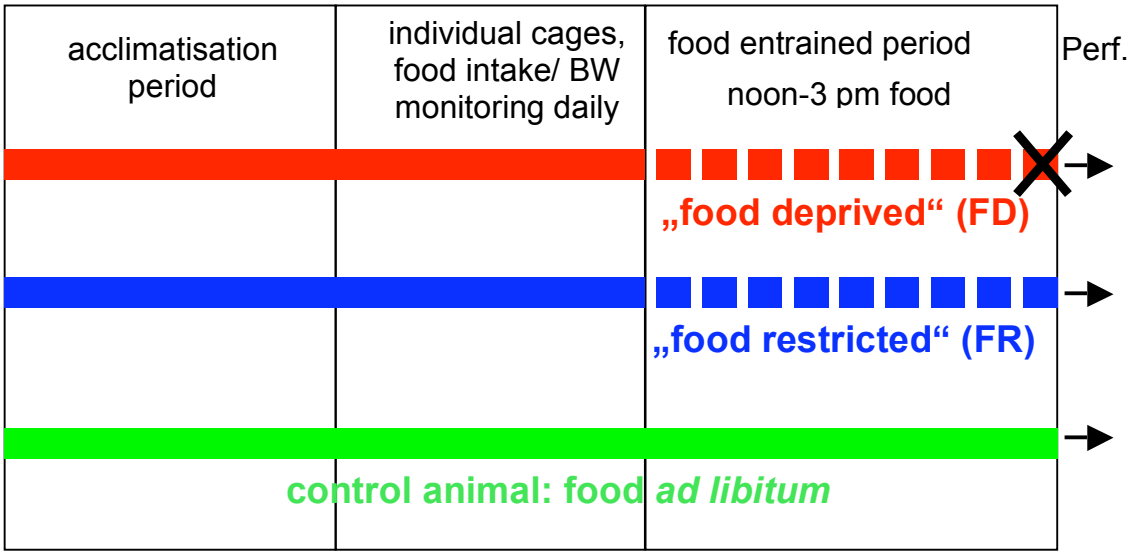

Fig. 23. Flow diagram that illustrates the different experimental periods for rats that underwent the restricted feeding paradigm. After acclimatisation of animals to the animal facility conditions (acclimatisation period, approx. 5 days), animals were housed in individual cages, where food intake and body weight (BW) were monitored daily.
On day 0 of restricted feeding schedule (RFS) animals were divided into three groups: the food-deprived (FD, red) and the food-restricted (FR, blue) animals (n=6) received only 3h in the middle of the light cycle access to food, ad libitum fed animals (control animal, green) received 24h food access.
On the last day of the experiment (day 9), FD rats were deprived of food, controversely FR received food as trained before. Following RFS, animal were time-dependent transcardially perfused (perf., black arrows) for c-Fos immunohistochemistry.

3.2.2.2. Immunohistochemical studies using c-Fos on RFS treated animals

3.2.2.2.1. Single-labelling immunohistochemistry for c-Fos after animal treatment

Following perfusion after each animal treatment, the brains was rapidly dissected and post-fixed in the same fixative overnight. Then, tissue was cryoprotected in 30% w/v sucrose, 0.1% w/v sodium azide in 0.1 M PBS, snap frozen in petrolether (-70 ^{0}C) and stored at -80 ^{0}C until further processing. An the day of sectioning of brains, brains were kept in the cryostat for approximately 1h to reach a constant tissue temperature of –20 ^{0}C for the sectioning process. until they -30 µm coronal cryosections of the rat brain were cut on a cryostat.

From MCH treated and MCHR-1 treated animals, each second tissue section comprising the dimension of the hypothalamus (from bregma level –1.3 mm to bregma level –4.56 mm according to the rat brain atlas from Paxinos and Watson, 1998) was collected in icecold 0.1 M PBS for direct immunohistochemical procedures using the free-floating method.

Brains derived from the scheduled feeding experiment were separated into two series of sections. Every first section that contain the AcbC/AcbSh (from bregma 1.0mm to -1.2mm), SCh/SO (from -1.3 mm to -1.4 mm), PVN (from -1.8 mm to -2.12 mm), Arc/DMH/LHA/BLA/LHBm/PVP (from -3.14 mm to -3.3 mm) and Sol (from -12.68 mm to

-13.3 mm) was collected in 0.1 M PBS, every second section of the entire hypothalamus (from bregma level -1.3 mm to -4.56 mm) was collected in antifreezing cryoprotectant and stored at – 20 ^{0}C for colocalisation studies. Remaining sections, representing all other bregma levels of the rat brain were collected in 0.1 M PBS and processed for mapping of c-Fos immunoreactivity.

Immunohistochemistry for c-Fos was performed using the free-floating method combined with an adjusted tyramide signal amplification protocol. At all washing steps, sections were rinsed in self-prepared plastic buckets with a bottom-placed nylon net in 0.1 M PBS (3x 10 min, gentle agitation). Then sections were blocked in 10% BSA (0.1 M PBS with 0.3% Triton X-100, 1h, RT), following an additional blocking step in TNB buffer (RT, 30 min), as suggested for the use of the tyramide signal amplification system (TSA). The primary antibody directed against c-Fos (Ab-5) (1:25,000 diluted in 0.1 M PBS, 2% BSA, 0.3% Triton X-100) was incubated for 48h at 4 ^{0}C with gentle agitation, followed by washing steps (3x 10 min, gentle agitation).

The tyramide signal amplification protocol was continued according to the kit description but using a 0.1 M PBS buffer and not the suggested borate buffer. Incubation steps comprised incubation with the goat anti rabbit biotinylated antibody (BA-1000) (1:200, diluted in 0.1M PBS, 2% BSA, 45 min), streptavidin horse-radish peroxidase (1:100 in 0.1 M PBS, 30 min, RT) and tyramide reagent (1:100 in tyramide diluent, 10 min, RT, both supplied by the TSA kit), each followed by washing steps. Visualization of c-Fos was performed by a final incubation step with Cyanin 2 conjugated streptavidin (1:400 in 0.1 M PBS, 45 min, dark). After each incubation step, sections were rinsed in a large volume of 0.1 M PBS (3x 10 min, gentle agitation). After the last washing steps, free-floating sections were mounted onto gelatine-coated object slides and cover-slipped with aqueous mounting medium.

Detection of all single- and double-labelling experiments were conducted by an Axioplan Imaging Microscope attached with the Apotome Schiebersystem. For all images taken with a higher magnification (x63), the Apotome Schiebersystem was used to focus at a single optical plane of interest. Images were obtained by a AxioCam MRm Rev.2 (D) camera, using the Axiovision Rel.4.3 software.

3.2.2.2.2. Quantitative analysis of c-Fos IR neurons in the hypothalamus of the rat after MCHR-1 antagonist treatment

For the evaluation of quantification, all stained sections were involved in the analysis. For each section, the hypothalamic region of interest was identified by the anatomical atlas and demarcated on both hemispheres. Depending on the expansion of the defined bregma level for

each brain region, a number of 35-50 regions of interest were involved in the calculation. All six animals, two animals per treatment group were involved. As the total number for each brain region is dependent on the area of the region of interest, the number of c-Fos IR cells were related to the area of interest.

Immunofluorescence (IF) and brightfield (BF) images were taken from each brain region of interest (ROI) for both hemispheres. Images were taken with the monochrome CCD camera using a constant exposure time. Overlay images of IF and BF photomicrographs were used to create a mask that corresponds to the anatomical shape of the ROI. For the automated, software-supported c-Fos quantification within the ROI, a counting threshold was established based on the consistent and measurable immunoreactive cell nuclei. This counting threshold was based on the staining density, the target size and target shape, defined by a the diameter of round-shaped objects. All post-production image processing and layout were conducted using the HALCON software. The settings for the analysis of immunohistochemical data were adjusted with the help of Dr. Gerald Birk, Department of Pulmonary Diseases, Boehringer-Ingelheim Pharma GmbH & Co KG, Biberach/Riss.

3.2.2.2.3. Quantitative analysis of c-Fos IR neurons in the rat brain of RFS treated animals

Serial section that were processed for the quantification were analysed under the Axioplan 2 Fluorescence Microscope using a 5x objective and the integrated filter set for Cyanin 2. For each brain section, the bregma level was defined by morphological criteria under the brightfield (BF) using the anatomical atlas (Paxinos and Watson, 1998).

Sections derived from all animals that were defined within the following brain regions were applied to an the quantification: AcbC/AcbSh (from bregma 1.00 to 1.20 mm), SO/ SCh (from bregma -1.30 to -1.40 mm), PVN (from bregma -1.80 to -2.40 mm), Arc/DMH/ LH/BLA/LHBm/PVP (from bregma –3.14 to -3.30 mm) and Sol (from bregma –12.80 mm to -13.24 mm). The evaluated software solution that was used for the quantification of c-Fos IR after MCHR-1 treatment was used on photomicrographs derived from RFS treated rats.

3.2.2.2.4. Statistical analysis of c-Fos immunohistochemistry of RFS rats

Quantification of c-Fos revealed a differential pattern of c-Fos IR between FD, FR and AL fed animals. For statistical analysis of these results, single values of section were summarized to the median value per animal and brain region. For this summary measure the number of observations, the median, minimum and maximum value were calculated per treatment group. To investigate the influence of the different feeding schedules on the fraction of c-Fos positive

cells a Kruscal Wallis test was used for every brain region separately. If the Kruscal Wallis test shows a significant influence of feeding schedules ($p<0.05$), two sided Wilcoxon rank sum tests were applied for all pairwise comparisons of the three groups FD, FR and AL. A $p<0.05$ value provides an indication of a difference between the groups.

Concerning body weight and food intake measurements, secondary endpoints of body weight change *versus* baseline and food intake was compared by a two-factorial analysis of variance for repeated measurements including the factors group (AL, FR, FD) and time point (for body weight change versus baseline and food intake, respectively) and their interaction term as fix and the factor animal as random factor. For the covariance matrix, a block diagonal structure with a first-order autoregressive structure (AR(1)) of blocks was used and estimated for each group separately. A significant p value for the interaction term ($p<0.05$) indicates a difference in the time course for the different groups. Taking the error term of the analysis of variance as an estimate for the variation t-tests followed for the pairwise group comparisons for each timepoint. No correction for multiple testing was performed and raw p values were reported for descriptive purposes only. A p-value <0.05 provides an indication of a difference between the groups. The statistical evaluation was prepared using the software packages SAS Version 8.2 and S-PLUS® 6.1. Analysis of statistical results were elaborated in cooperation with Dr. Carina Ittrich and Dr. Kryzkalla, Medical Data Services Group, Boehringer-Ingelheim Pharma GmbH& Co.KG, Biberach/Riss.

3.2.2.3. Colocalisation studies

3.2.2.3.1. Colocalisation studies for c-Fos with MCH or OxA in the LH of RFS rats

Since the MCH antibody was raised in the same species as the c-Fos antibody (rabbit), this colocalisation study was based on the consecutive detection of the first antigen using the high sensitive tyramide signal amplification, followed by a conventional immunohistochemical detection of MCH, a method described by Shindler and Roth (1996).

Briefly, for colocalisation of c-Fos with MCH or OxA, sections were removed from cryoprotectant solution, washed in 0.1M PBS (3x 10 min, gentle agitation) and the described c-Fos protocol was performed as described above. After the last washing step, remaining anti rabbit binding sites were blocked by incubation with a goat anti rabbit unlabelled antibody (1:1000 diluted in 2% BSA, 0.1 M PBS, 45 min, RT), rinsed with 0.1 M PBS (3x 10 min, gentle agitation) and incubated with the rabbit anti MCH serum (1:1,000) or an affinity purified polyclonal goat anti-OxA (1:2,000), both diluted in 2% BSA in 0.1 M PBS at 4 ^{0}C over night. On the following day, sections were washed and incubated with an Cyanin 3 conjugated,

affinity purified goat anti rabbit IgG or the Cyanin 3 conjugated, affinity purified donkey anti goat IgG (1:400, both diluted in 0.1M PBS, 1h, RT). Finally, sections were rinsed, mounted onto gelatine coated slides and cover-slipped with fluorescence aqueous mounting medium or a mounting medium that contain the nuclear stain 4`,6-diamidino-2-phenylindole dilactate (DAPI).

3.2.2.3.2. Colocalisation studies for MCHR-1 with c-Fos within the rat brain of RFS treated rats

For colocalisation of MCHR-1 with c-Fos, MCHR-1 immunohistochemistry was processed first, followed by the detection of c-Fos. Brain sections derived from scheduled fed and control animals were used to study a possible colocalisation of MCHR-1 with c-Fos immunoreactivity. Because both MCHR-1 and the c-Fos antibody are raised in the same species, an additional c-Fos antibody that is directly conjugated with tetramethylrhodamine isothiocyanate (TRITC) had to be introduced. Although this antibody reacts with a different epitop of c-Fos as the Ab-5 antibody, initial experiments manifested that the SC-52 TRITC conjugated antibody is capable to detect c-Fos IR in the same areas as described in the here presented c-Fos mapping study.

30 μm consecutive brain sections brain sections were removed from the antifreezing solution and after extensive washing steps in 0.1M PBS, sections were placed in a pre-incubation solution (2% NDS, 1% BSA, 0.1% Triton X-100, 0.1 M PBS, 1h, RT, shaker). After a blocking step with the TNB blocking solution supplied by the TSA kit, sections were incubated with the affinity purified MCHR-1 antibody (diluted 1:100 in the pre-incubation solution, 48 h, 4 ^{0}C). Subsequently, sections were washed and incubated with a biotinylated donkey anti rabbit IgG (1:400 in 2% NDS, 0.1 M PBS, 45 min, RT). After washing, sections were incubated in streptavidin Cyanin 2 (1:800, diluted in 0.1 M PBS), washed and incubated with the affinity purified rabbit anti c-Fos antibody (sc-52 TRITC conjugated) (1:500, diluted in 0.1 M PBS, 2% BSA, 0.1% Triton X-100, over night, 4 ^{0}C, dark). Finally, sections were washed in 0.1 M PBS, mounted onto gelatine-coated object slides and cover-slipped with the DAPI containing mounting medium.

3.3. Results

3.3.1. Effects of animal treatments on food intake, water intake and body weight

3.3.1.1. Effects of central MCH on acute food- and water intake

After central administration of MCH at ZT 6, two hour food intake and body weight were monitored, then animals were anaesthetised and transcardially perfused. MCH significantly stimulate food intake directly after injection procedure compared to aCSF, that was used as

control (Fig. 24 A,B). Results from the feeding analysis in response to icv administration of MCH or aCSF, respectively are summarized in table 6.

MCH treated animals ingested a mean amount of standard lab chow of 2.60g ± 0.15g compared to 0.85g ± 0.17g of aCSF treated animals (Fig. 24 A). In addition, following central MCH injection, water intake was significantly elevated (2.68g ± 0.33g), compared to control animals (1.55g ± 0.21g) (Fig. 24 B.)

	MCH treatment (icv)		vehicle treatment (aCSF, icv)	
animal	food intake [g/2h]	water intake [g/2h]	food intake [g/2h]	water intake [g/2h]
1	1.9	3.9	0.3	0.6
2	2.5	3.7	0.4	1.3
3	2.6	1.3	1.8	2.6
4	3.4	1.8	0.9	1.7
mean	**2.60**	**2.68**	**0.85**	**1.55**
SD	**0,62**	**1,32**	**0.69**	**0.83**

Table 6. Effect of central MCH administration on 2h food intake and water intake.

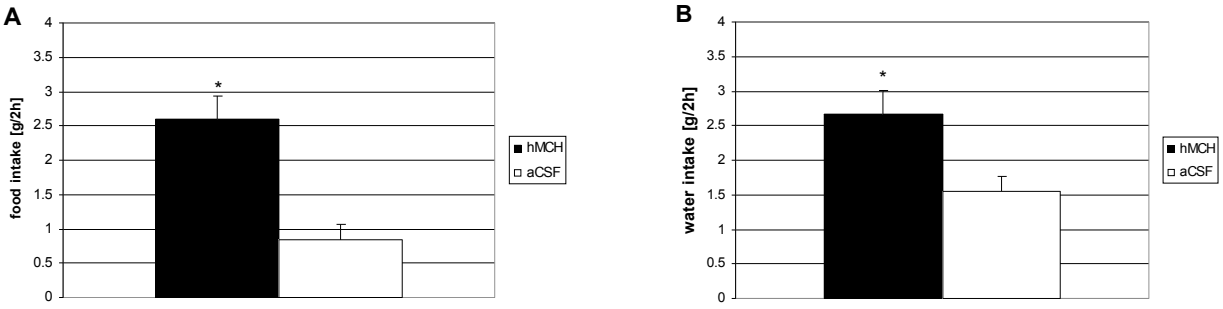

Fig. 24. Effect of human MCH on spontaneous food intake (A) and water intake (B). MCH was injected into the third ventricle in the middle of the light phase. Food intake and water intake were measured 2h after injection. Asterisk indicates $P<0.05$. Statistical significance was determined by Student`s t-test.

3.3.1.2. Effects of sub-chronic MCHR-1 treatment on body weight change, food intake and water intake

During the eight day experiment, MCHR-1 antagonists (GW803430, 10mg/kg BW or compound B4, 25mg/kg BW) was administered once daily *po* at ZT 9 to female HanWistar rats. During the treatment period, body weight, food intake and water intake was monitored

daily. Administration of both MCHR-1 antagonist, GW 803430 or compound B4, respectively result in a significant reduction of body weight during the eight day treatment period.
Total body weight is summarized in table 5. MCHR-1 treated animals change their body weight from the start of the experiment from day 0, 276.86g ± 7.3g to day 8, 266.73 ± 10.24g (-3.7%) in GW803430 treated rats and MCHR-1 antagonist effects on body weight was even stronger in compound B4 treated animals changing from day 0 267.37g ± 5.79g to day 8 246.02 ± 5.64g (-10.9%). In the same period of time, control animals did not change their body weight (day 0 276.33 ± 11.01g to day 8 276.03g ± 8.03g (+0.15%) (table 7, Fig. 25 A).

Oral administration of GW803430 or compound B4 to rats for 8 consecutive days produced a reduction in cumulative food intake of standard laboratory chow (GW803430, 10mg/kg BW, 119.65 ± -4.31 g/kg BW (-11% compared to vehicle); compound B4 85.17 ± 7.74 g/kg BW(-37% compared to vehicle treatment)) *versus* control animals cumulatively ingested a food intake of 134.46 ± 8.16 g/kg BW. Daily monitoring of food intake showed that GW803430 showed a lower effect on the reduction of laboratory chow ranging from 45.92 g/kg BW (day 2) to 61.45 g/kg BW (day 5), with a significant ($p<0.05$) reduction of 24h food intake only at day 2 and day 8. In comparison to GW803430, compound B4 significant ($p<0.05$ at days 2,3,4,5,6,8,) reduced daily food intake ranging from 32.51 g/kg BW/24h at day 2 to 48.14 g/kg/BW at day 8) (Fig. 25 B).

A differential effect of the MCHR-1 antagonists can be observed on water intake. While GW803430 did not show any effects on cumulative water intake (136.02 ± 20.23 g/kg BW) compared to control animals (133.18 ± 11.55) (+2% compared to vehicle) and no significant change of daily water intake during the days of experiments. In contrast, compound B4 showed a strong effect on cumulative water intake (69.05 ± 9.08g) compared to the control group (-48% compared to vehicle). These effects could also been seen in daily water intake of and a significant reduction in at all time points ($p<0.05$) (Fig. 25 C).

	GW803430 10 mg/kg BW	compound B4 25 mg/kg BW	vehicle
Changes in BW [g BW] Day 0	276.86 ± 7.30	267.37 ± 5.79	276.33 ± 11.01
Day 8	266.72 ± 10.24	246.02 ± 5.64	276.02 ± 8.03
Cumulative food intake [g]	119.65 ± 4.31	85.17 ± 7.74	134.46 ± 8.16
Cumulative water intake [g]	136.02 ± 20.23	69.05 ± 9.08	133.18 ± 11.55

Table 7: Effects of subchronic MCHR-1 antagonist treatment (GW803430 or compound B4) on body weight change, cumulative food intake and cumulative water intake.

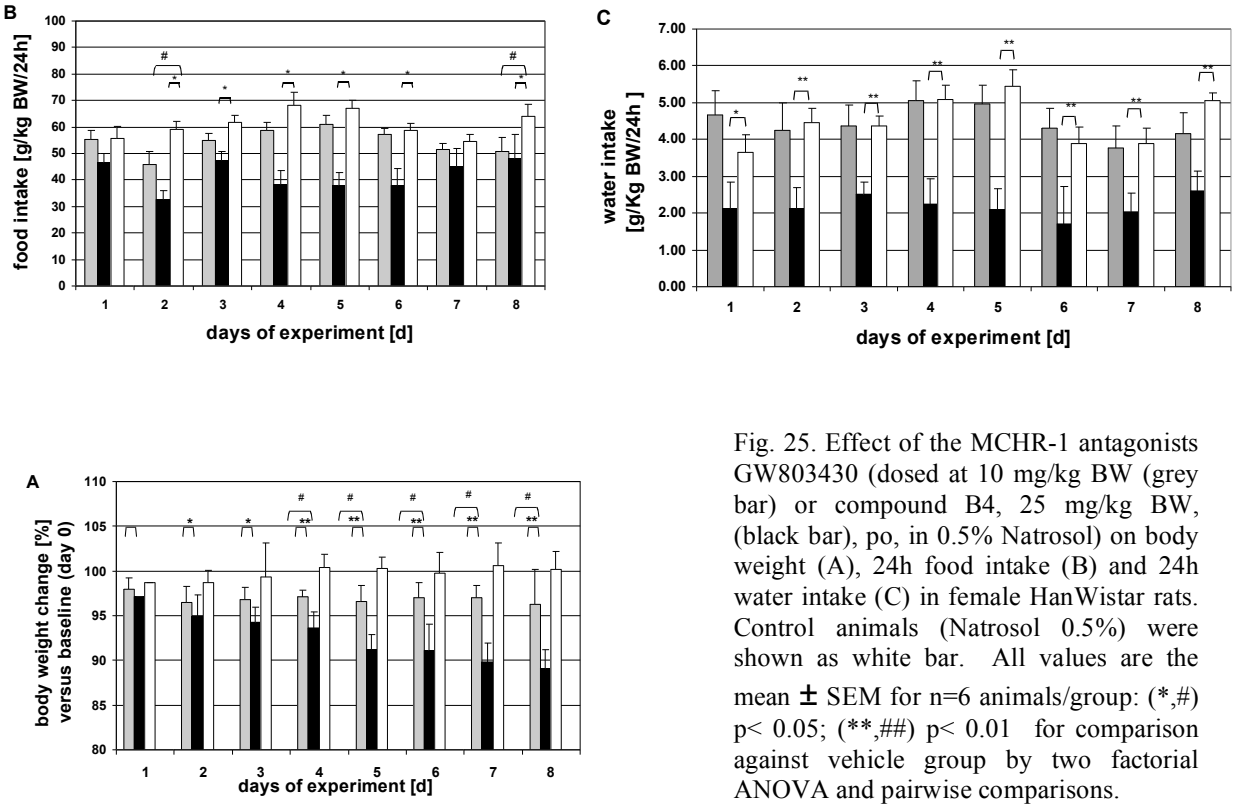

Fig. 25. Effect of the MCHR-1 antagonists GW803430 (dosed at 10 mg/kg BW (grey bar) or compound B4, 25 mg/kg BW, (black bar), po, in 0.5% Natrosol) on body weight (A), 24h food intake (B) and 24h water intake (C) in female HanWistar rats. Control animals (Natrosol 0.5%) were shown as white bar. All values are the mean ± SEM for n=6 animals/group: (*,#) $p< 0.05$; (**,##) $p< 0.01$ for comparison against vehicle group by two factorial ANOVA and pairwise comparisons.

3.3.1.3. Effects of restricted feeding schedule on body weight change and food intake

From day 2 of the scheduled feeding paradigm, scheduled fed animals showed a significant difference in the development of body weight (pairwise comparison FD, FR animals *versus* AL animals, $p<0.05$) and food intake compared to AL fed animals (Fig. 26). Animals that underwent the restricted feeding paradigm lost a significant amount of their initial body weight until experiment termination (day 9) (mean FD -34.62 g/-9.1 %; mean FR -24.68 g/-6.41 %), while AL fed animals gain body weight during the same time period (mean +27.05 g/ +9.5%). In scheduled fed animals, the decline in body weight was more pronounced in the first half of the scheduled feeding paradigm (from day 1 to day 4), then body weight stabilized until the last day of the paradigm (Fig. 26A).

In parallel, animals changed their feeding behaviour during scheduled feeding which was reflected in changes of the amount of food intake during the three hour period of food access

(Fig. 26B). Starting with over night fasting, FD animals ingested on day one of scheduled feeding 22.55 g/kg BW/3h and FR animals 21.88 g/kg BW/3h, respectively. Until day eight of scheduled feeding, food intake was progressively increased in scheduled fed animals (day eight FD 53.33 kg/BW/3h; FR 56.31 kg/BW/3h).

In comparison, during the three hour period, AL animals ingested an amount of chow between 6.30 g/kg BW/3h (day 1) and 1.49 g/kg BW/3h (day 8) during the same time period of the day and the total amount of chow intake per 24h was between 82.97 and 80.38 g/kg BW, which reflects a daily amount of approximately 22 g standard chow. On the day of experiment termination (day 9), FR animals ingested 58.34g/kg/BW between ZT6 and ZT9, which makes an absolute chow intake of 14.34 g, while FD animals were left without food. Statistical analysis of variance (ANOVA) for repeated measurements indicated significant effects ($p<0.05$) of the FD and FR animals *versus* AL animals at all time points.

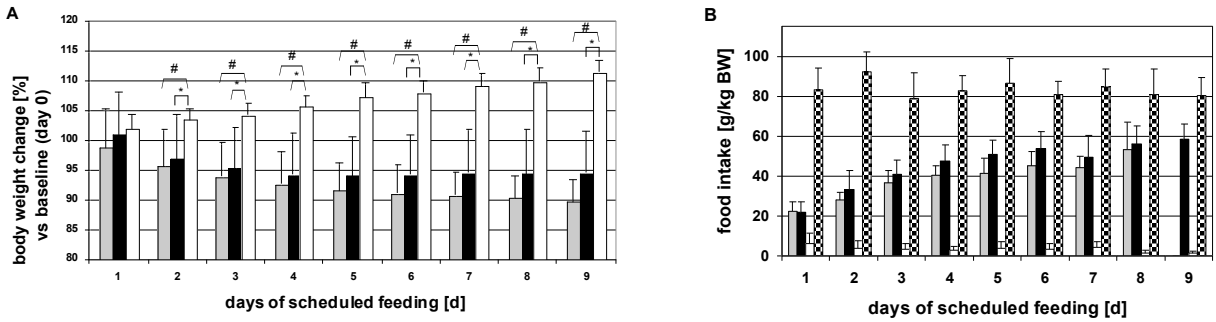

Fig. 26. Daily change in body weight and food intake during nine day scheduled feeding paradigm. (A) Body weight change of animals that had food access for only three hours per day (FR group, grey bar and FD, black bar), compared with *ad libitum* (AL) fed animals (white bar). FR and FD group treatment differ only on day nine of scheduled feeding. Significant difference between AL group and FD, FR groups obtained by two factorial ANOVA and pairwise comparisons. ($p<0.05$). * for the FD group and # FR group.
(B) Analysis during 3 hours of scheduled feeding (grey bars FD group, black bar FR group, white bar AL group/3h; chequered AL group 24h). To note, on day nine of scheduled feeding (experiment termination) FD were left without food, FR received 3h food as trained.

3.3.2. Analysis of neuronal activation patterns after animal treatment using c-Fos immunohistochemistry

3.3.2.1. Results of immunohistochemistry for c-Fos within the rat hypothalamus after acute, central MCH administration

To examine, whether central administration results in changes of neuronal activity in the rat hypothalamus, I examined qualitative differences in the expression of c-Fos immunoreactivity (IR) in coronal rat brain sections derived from MCH treated animals. Compared to rats that treated with aCSF as controls, no difference could be visible within the rat hypothalamus. Results for c-Fos immunohistochemistry on rat brain sections after MCH treatment are presented in representative sections showing the Arc (Fig. 27A), DMH (Fig. 27C), PVN (Fig. 27E) and SO (Fig. 27G) versus matched sections derived from control animals that show the Arc (Fig. 27B), DMH (Fig. 27C), PVN (Fig. 27E) and SO (Fig. 27G). Because some animals showed an intra-individual difference in the activation pattern in one region of both hemispheres, only a total number of two animals per group were extensively studied for c-Fos immunohistochemistry.

Within these animals, no differences in c-Fos IR was detected in cortical regions of both hemispheres such as the retrosplenial cortex (Fig.27 I,J). The correct position of the cannula was verified by histological examination of the cannula track on sections that were defined as the bregma level used for surgical implantation (bregma .-2.6 mm, Fig. 27 K,L).

3.3.2.2. Results of immunohistochemistry for c-Fos within the rat hypothalamus after sub-chronic MCHR-1 treatment

3.3.2.2.1. Distribution of c-Fos immunoreactive neurons within the rat brain of MCHR-1 treated animals

After day eight of sub-chronic treatment with MCHR-1, two animals per treatment group were transcardially perfused and subjected for the identification of changes in the neuronal activation pattern between different treatment groups. Within brain sections of treated animals, a qualitative examination of c-Fos immunohistochemistry was performed in sections that represent the rat hypothalamus. Photomicrographs were then subjected for a automated, software-supported, quantitative analysis within selected hypothalamic regions.

Fig. 27.

Typical photomicrographs of c-Fos immunoreactivity (c-Fos IR) within selected brain areas two hours after intracerebroventricular administration of human MCH (5 μg/2 μl) into the third ventricle. Sections are derived from representative animals that were acutely treated with either

hMCH (A,C,E,G,I,K) or vehicle (B,D,F,H,J,L). Selected regions shown are the arcuate nucleus (A,B), the dorsomedial hypothalamic nucleus (C,D), the paraventricular hypothalamus (E,F) or the supraoptic nucleus (G,H). For the identification of possible unilateral activation patterns, c-Fos immunohistochemistry was compared between both hemispheres of a single section (I, J). Cannula position was verified by histological examination of brain sections representing the corresponding bregma level (K, L).

Scale bar 100 μm.

Schematic drawings have been modified from: The Rat Brain in Stereotaxic Coordinates, Fourth Edition, Paxinos G and Watson C, 1998.

Abbreviations:

3V, third ventricle; AAV, anterior amygdaloid area, ventral part; AHP anterior hypothalamic area, posterior part; ArcD arcuate nucleus, dorsal part; ArcL arcuate nucleus, lateral part ; ArcM arcuate nucleus, medial part; DMD dorsomedial hypothalamic nucleus, dorsal part; *EL*, ependymal layer of the third ventricle; LOT, nucleus of the lateral olfactory tract; M1, primary motor cortex; M2 secondary motor cortex; MCPO, magnocellular preoptic nucleus; ME Median Eminence; MeAD, medial amygdaloid nucleus, anterodorsal part; MEE medial eminence, external layer ; MEI medial eminence, internal layer; ox, optic chiasm; PaLM paraventricular hypothalamic nucleus, lateral magnocellular part; PaMP paraventricular hypothalamic nucleus, medial parvocellular part; PaPo paraventricular hypothalamic nucleus, posterior part; PaR pararubral nucleus; PaV paraventricular hypothalamic nucleus, ventral part; Pe periventricular hypothalamic nucleus; RSA, retrosplenial agranular cortex; RSGb, retrosplenial granular b cortex; S1HL primary somatosensory cortex, hindlimb region; SO supraoptic nucleus; SPa subparaventricular zone of the hypothalamus; TC tuber cinereum area; VMHC ventromedial hypothalamic nucleus, central part; VMHDM ventromedial hypothalamic nucleus, dorsomedial part; ZI, zona incerta.

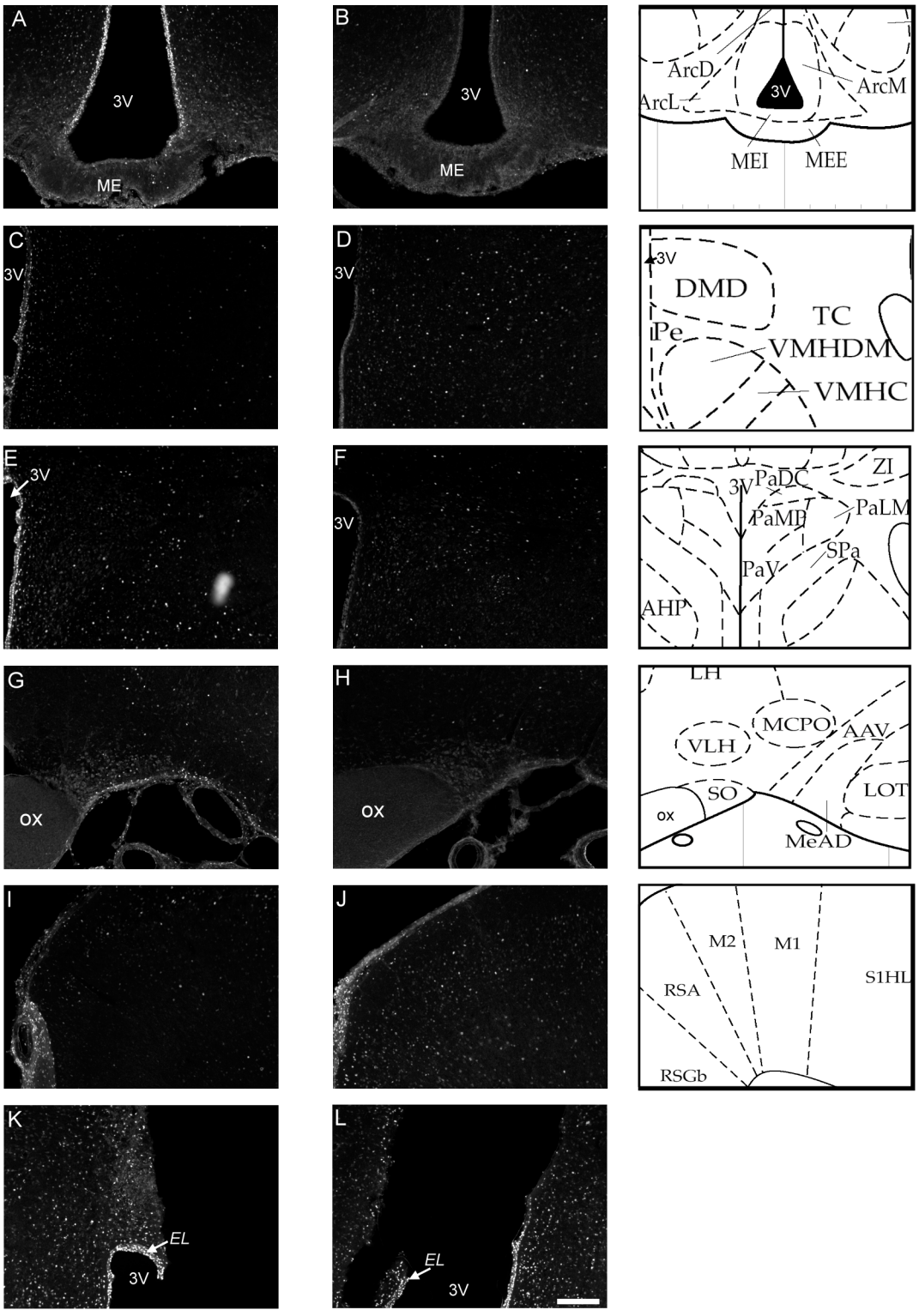
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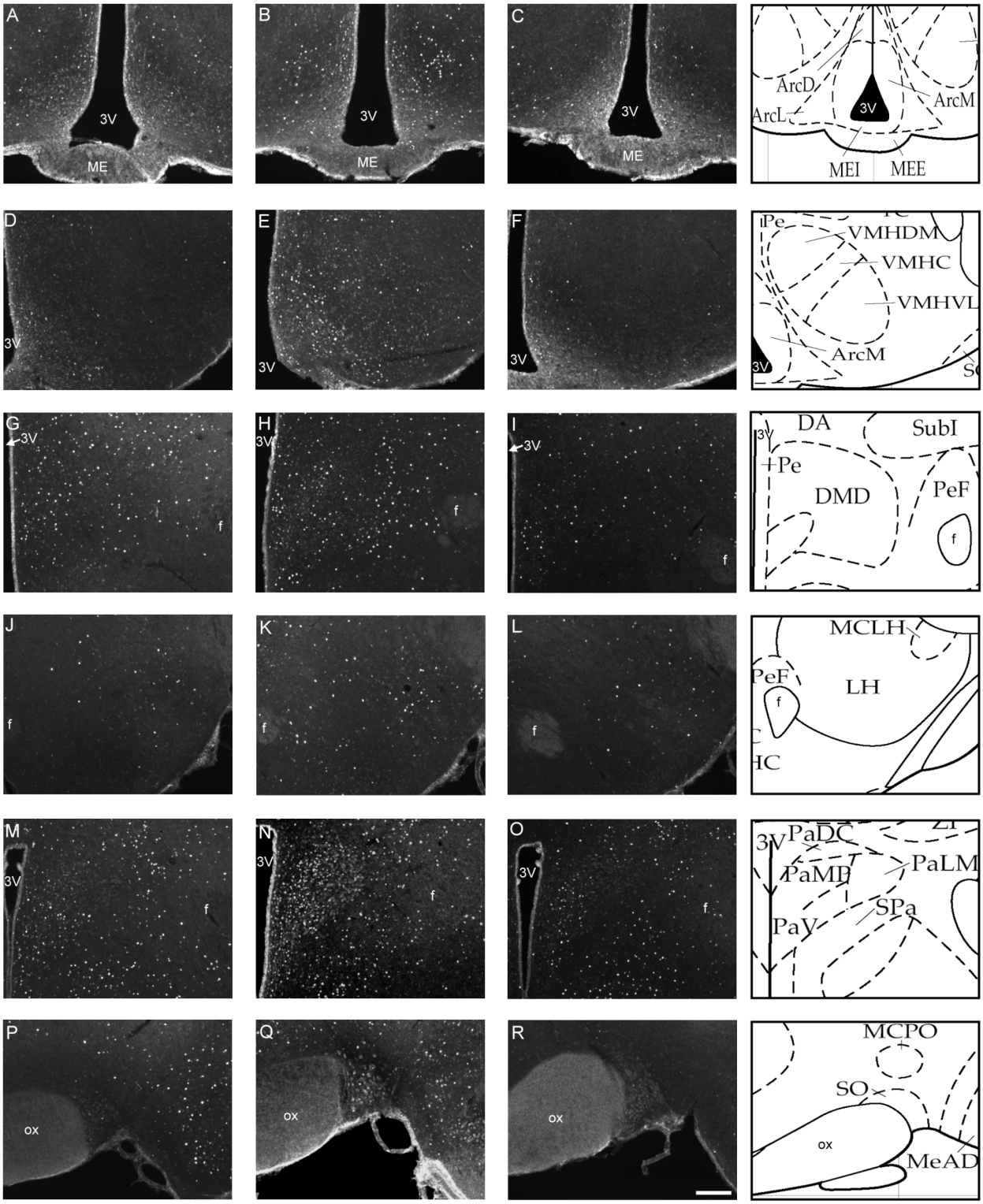
Fig. 28.

Representative photomicrographs of c-Fos immunoreactivity (c-Fos IR) within the rat hypothalamus after sub-chronic MCHR-1 antagonist treatment. On the day following the last oral administration of GW803430 (10 mg/kg), compound B4 (25 mg/kg) or vehicle (0.5 % Natrosol), rats were transcardially perfused and immunohistochemistry for c-Fos was performed on brain sections derived from two animals per group. Fos-IR is shown after GW803430 (A,D,G,J,M,P; animal 1), compound B4 (B,E,H,K,N,Q; animal 2) or vehicle (C,F,I,L,O,R; animal 1) treatment in the arcuate nucleus (A-C), the ventromedial hypothalamic nucleus (D-E), the dorsomedial hypothalamic nucleus (G-I), the lateral hypothalamic area (J-L), the paraventricular hypothalamic nucleus (M-O) or the supraoptic nucleus (P-R).

Scale bar 100 μm.

Schematic drawings have been modified from: The Rat Brain in stereotaxic coordinates, Fourth Edition, Paxinos G and Watson C, 1998.

ArcD arcuate nucleus, dorsal part; ArcL arcuate nucleus, lateral part ; ArcM arcuate nucleus, medial part; DA dorsal hypothalamic area; DMD dorsomedial hypothalamic nucleus, dorsal part; f fornix; LH lateral hypothalamic area; LOT, nucleus of the lateral olfactory tract; M1, primary motor cortex; M2 secondary motor cortex; MCLH magnocellular nucleus of the lateral hypothalamus; MCPO magnocellular preoptic nucleus; ME Median Eminence; MeAD, medial amygdaloid nucleus, anterodorsal part; MEE medial eminence, external layer ; MEI medial eminence, internal layer; ox, optic chiasm; PaLM paraventricular hypothalamic nucleus, lateral magnocellular part; PaMP paraventricular hypothalamic nucleus, medial parvocellular part; PaPo paraventricular hypothalamic nucleus, posterior part; PaR pararubral nucleus; PaV paraventricular hypothalamic nucleus, ventral part; Pe periventricular hypothalamic nucleus; PeF perifornical nucleusRSA, retrosplenial agranular cortex; RSGb, retrosplenial granular b cortex; S1HL primary somatosensory cortex, hindlimb region; SO supraoptic nucleus; SPa subparaventricular zone of the hypothalamus; SubI; TC tuber cinereum area; VMHC ventromedial hypothalamic nucleus, central part; VMHDM ventromedial hypothalamic nucleus, dorsomedial part; VMHVL ventromedial hypothalamic nucleus, ventrolateral part; ZI zona incerta.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Typical photomicrographs of c-Fos IR in GW803430, compound B4 and vehicle treated animals were illustrated in Figure 28. Compared visual analysis of stained sections derived from GW803430 and compound B4 treated animals showed only little differences within hypothalamic nuclei. Within the complete extension of the Arc, modest c-Fos expression could be detected within both GW803430 and compound B4 treated animals, and the expression density does not differ from control animals (Fig. 28A-C). In other medial hypothalamic nuclei including the VMH (Fig. 28D-F), DMH (Fig. 28G-I) and LHA (Fig. 28J-L), there were apparently more c-Fos IR cells in compound B4 treated animals compared to both GW803430 and control animals. In the PVN and SO, c-Fos IR was altered in compound B4 treated animals, and the intensity was markedly stronger, and c-Fos IR was weak in the PVN after GW803430 or vehicle treatment. In these experiment, c-Fos IR was weak to absent in the SO of the hypothalamus.

3.3.2.2.2. Evaluation of quantitative analysis of c-Fos immunohistochemistry within the rat hypothalamus after MCHR-1 antagonist treatment

Results of the analysis of c-Fos quantification was given for each animal are summarized in table 6.

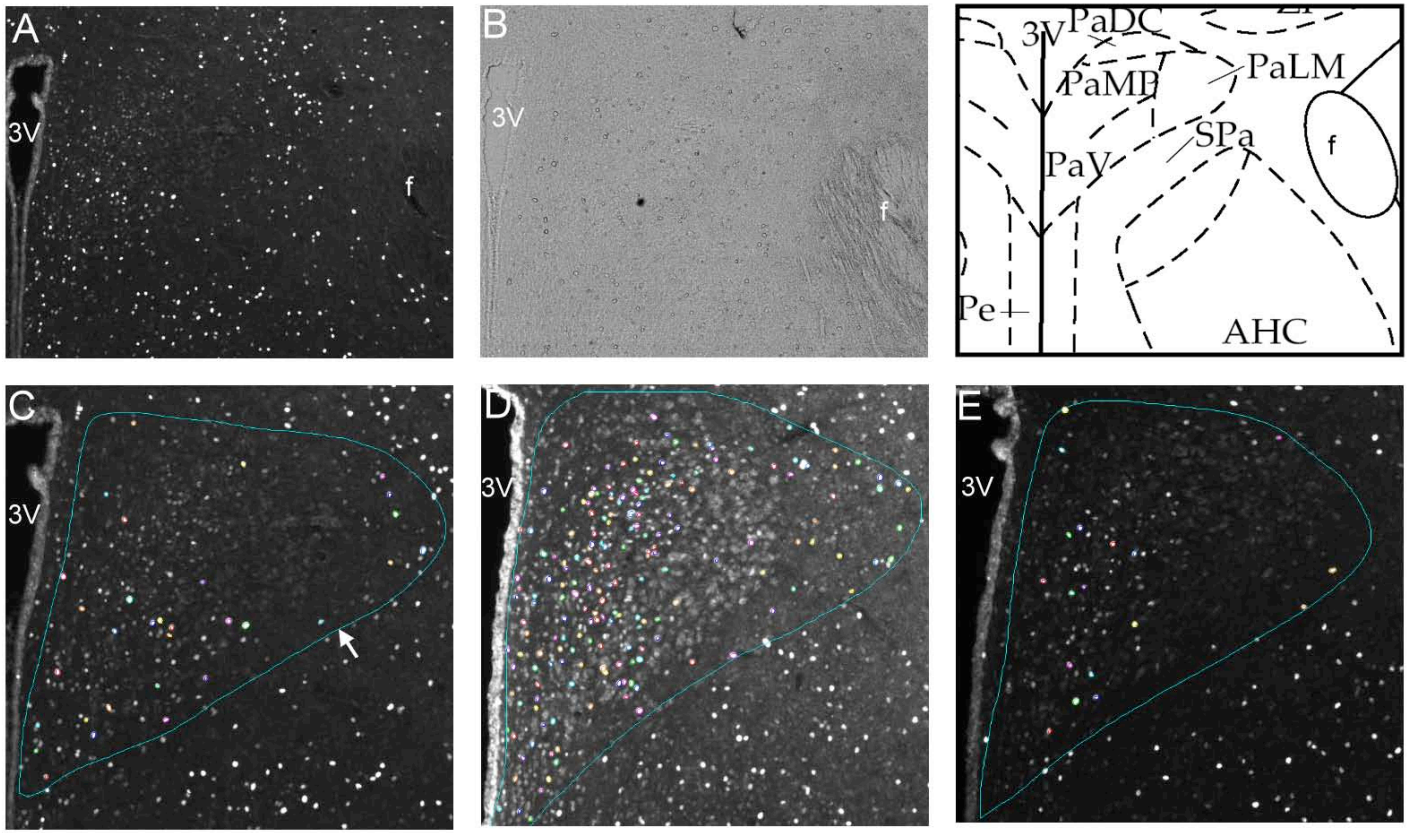
Neuronal activation results in expression of c-Fos, which was detected by an adjusted method of immunohistochemistry combined with fluorescence microscopy. The quantification of this signal is based on the identical and simultaneous histological treatment to obtain a valid quantitative comparison between different serial sections derived from different animals, were treated in parallel in reaction trays. Stimulus-induced neurons show a clear, round-shaped signal. Evaluation of a automated, software-supported quantification of c-Fos IR cells involved a number of photomicrograph processing and are illustrated in Fig. 29.

Using the automated, software-supported quantification, there was a trend of increased c-Fos IR detectable within the ARC, DMH, PVN and the SO of compound B4 compared to GW803430 and vehicle treated animals (Fig. 22). Although this study was restricted to two animals per treatment group, I could detect a differential c-Fos expression pattern for at least one compound B4 treated animal in comparison to the GW803430 or vehicle treated animals.

Fig. 29.

Evaluation of automated, software-supported quantification for c-Fos immunoreactivity (c-Fos IR) exemplified for the paraventricular hypothalamic nucleus (PVN). From each stained brain section, the region of interest (ROI) was photographed as a fluorescence image (A) and a brightfield image (B) by the fluorescence microscope using constant exposure time. For the quantitative analysis of c-Fos IR within each image, an overlay of the fluorescence image with the artificially coloured brightfield image was used to create a mask that corresponds to the shape of the ROI (blue lining, depicted by white arrow (C). An adjusted software solution (Halcon,. MVTec) counts all circular objects of a defined diameter and brightness. Photomicrographs derived from c-Fos stained brain cryosection after GW803430 (C), compound B4 (D) or vehicle (E) treatment of rats.

3V third ventricle; AHC anterior hypothalamic area, central part; f fornix; PaDC paraventricular hypothalamic nucleus, dorsal cap; PaLM paraventricular hypothalamic nucleus, lateral magnocellular part; PaMP paraventricular hypothalamic nucleus, medial parvocellular part; PaV paraventricular hypothalamic nucleus, ventral part; Pe periventricular hypothalamic nucleus; SPa subparaventricular zone of the hypothalamus.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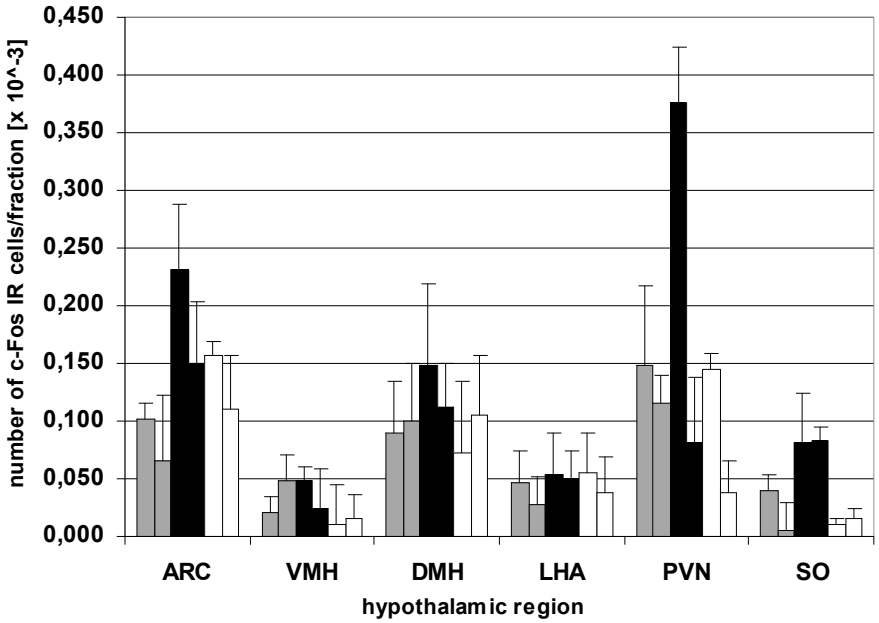


Fig. 30. Automated, software-supported, quantification of c-Fos immunoreactive neurons within hypothalamic region of GW803430 (grey bars), compound B4 (black bars) or vehicle (white bars) treated animals (n=2). Each bar represents one animal (first bar of each group animal 1, second bar animal two respectively), showing the mean +/- SEM number of c-Fos immunoreactive cells per selected area of the region. ARC, arcuate nucleus, DMH, dorsomedial hypothalamus, LH, lateral hypothalamus, VMH, ventromedial hypothalamic area, PVN, paraventricular hypothalamic nucleus, SO, supraoptic nucleus.

Although the number of c-Fos IR cells per region was stronger in one compound B4 treated animal (animal 1) than in the second animal treated with compound B4 (animal 2), both compound B4 treated animals showed an increase of c-Fos IR cells within the Arc, DMH and SO of the hypothalamus. Especially, within the PVN and the SO, the number of c-Fos IR cells were greater in one compound B4 treated animal (animal 1) than in GW803430 and control treated animals.

Strongest c-Fos expression was found in the PVN of compound B4 treated animal (animal 1), but within the second compound B4 treated animal (animal 2), c-Fos IR was only moderate compared to both GW803430 and vehicle treated animals. Between GW803430 animals and vehicle treated animals, there was no quantitative difference detectable within all examined hypothalamic regions. However, there was a high variance between the results of c-Fos counts within each treatment group and within each brain region (table 8).

Brain region	GW 803430 (1)	GW 803430 (2)	Compound B4 (1)	Compound B4 (2)	Vehicle (1)	Vehicle (2)
ARC	0.102±0.013	0.065±0.014	0.231±0.046	0.150±0.027	0.156±0.070	0.111±0.014
VMH	0.021±0.014	0.048±0.024	0.049±0.050	0.024±0.023	0.010±0.023	0.016±0.024
DMH	0.089±0.046	0.100±0.012	0.149±0.069	0.112±0.037	0.073±0.050	0.105±0.043
LHA	0.047±0.027	0.028±0.035	0.053±0.037	0.051±0.024	0.056±0.056	0.038±0.013
PVN	0.148±0.070	0.116±0.035	0.375±0.061	0.081±0.034	0.144±0.014	0.038±0.006
SO	0.040±0.014	0.006±0.020	0.081±0.051	0.083±0.031	0.01±0.027	0.016±0.008

Table 8. Effects of sub-chronic treatment with GW803430, compound B4 or vehicle on c-Fos immunoreactivity within selected hypothalamic regions. Results are given as mean number of c-Fos IR cells per fraction size*1000 ± SE counts for each animal (n=2).

3.3.2.3. Results of immunohistochemistry for c-Fos after restricted feeding schedule

3.3.2.3.1. Distribution of c-Fos immunoreactive neurons within the rat brain of RFS animals

In order to detect differences in the activation pattern between FD and FR, I mapped c-Fos immunoreactivity within the complete anterior to posterior extension of the rat brain (Table 9).

Brain regions	**Intensity of c-Fos IR**		
	FD (food deprived)	**FR (food restricted)**	**AL (*ad libitum*)**
Olfactory bulbs	++	++	++
Telencephon			
Olfactory system			
Dorsal endopiriform nucleus (DEn)	+	+	+
Olfactory nuclei (AOE, AOL)	++	++	+/-
Islands of Calleja (ICj)	+++	++	++
Piriform cortex (Pir)	+++	++	+
Tenia Tecta (TT)	++	+	+/-
Neocortex			
Agranular insular cortex (AI)	+	+/-	+
Frontal cortex (Fr)	+	+	+
Granular insular cortex (GI)	+	+	+
Parietal cortex (Par)	+	++	+
Metacortex			
Cingulate/retrosplenial cortex (Cg/RSA,RSG)	++	+/-	+
Basal ganglia			
Caudate putamen (CPu)	+/-	+/-	+/-
Globus pallidus (GP)	+/-	+/-	+/-
Hippocampal formation			
CA1 region (CA1)	-	-	-
CA2 region (CA2)	-	-	-
CA3 region (CA3)	+	+/-	+
Dentate gyrus (DG)	+	+	+
Subiculum (S)	-	-	-
Amydala			
Central amydaloid nuclei (Ce)	+	+/-	+/-
Basolateral amygdaloid nuclei (BLA)	++	+	+
Substantia innominata (SI)	-	-	-
Septal and basal magnocellular nuclei			
Accumbens nucleus (core) (AcbC)	++	++	+
Accumbens nucleus (shell) (AcbSh)	+++	+	+/-
Bed Nucleus of the stria terminalis (BNST)	+	+	+
Lateral septal nucleus, dorsal part (LSD)	-	+	-
Lateral septal nucleus, ventral part (LSV)	++	+	-

Medial septal nucleus (MS)	-	-	-
Nucleus of the horizontal limb of the diagonal band (HDB)	+/-	+	+/-
Diencephalon			
Thalamus			
Anterodorsal thalamic nucleus (AD)	-	-	-
Anteroventral thalamic nucleus (AAV)	+	+	+
Centrolateral thalamic nucleus (CL)	+/-	+/-	+/-
Central medial thalamic nucleus (CM)	++	+	+/-
Mediodorsal thalamic nuclei (MD)	++	+	+
Geniculate nuclei (VLG, DLG)	++	++	++
Interanterodorsal thalamic nuclei (IAM)	+	+	+/-
Intermediodorsal thalamic nucleus (IMD)	++	+	+/-
Lateral habenular nucleus (LHb)	+++	+	+/-
Medial habenular nucleus (MHb)	+	-	-
Parafascicular thalamic nucleus (PF)	-	-	-
Paratenial thalamic nucleus (PT)	-	-	-
Paraventricular thalamic nucleus (PV)	+++	+	+
Reticular thalamic nucleus (RT)	++	+	+
Reuniens thalamic nucleus (Re)	++	+	+/-
Ventral posterolateral thalamic nucleus (VPL)	++	++	++
Ventral posteromedial thalamic nucleus (VMP)	-	-	-
Ventrolateral thalamic nucleus (VL)	-	-	-
Ventromedial thalamic nuclei (VM)	++	++	++
Subthalamic nucleus (STh)	++	++	+
Zona incerta (ZI)	++	+/-	+/-
Hypothalamus			
Anterior hypothalamic area (AHA)	++	+	+/-
Arcuate hypothalamic nucleus (Arc)	+++	++	+/-
Dorsomedial hypothalamic nuclei (DMH)	++	++	+
Lateral hypothalamic area (LH)	++	+	+/-
Perifornical nucleus (PeF)	++	+	-
Lateral mammillary nucleus (LM)	++	+	-
Medial mammillary nucleus (MM)	-	-	-
Tubero mammillary nucleus (TM)	++	+	-
Supramammillary nucleus (SuM)	+	++	+
Medial preoptic area (MPO)	+	+	+/-
Paraventricular hypothalamic nuclei (PVN)	+	+++	+
Periventricular hypothalamic nucleus (Pe)	+	+	+
Posterior hypothalamic area (PH)	+/-	+/-	+/-
Supraoptic nucleus (SO)	+	+++	+/-
Suprachiasmatic nucleus (SCh)	++	++	+
Ventromedial hypothalamic nuclei(VMH)	-	-	-
Mesencephalon			
Anterior pretectal nucleus (APT)	+/-	-	-
Dorsal tegmental area (DTg)	++	+/-	-
Inferior colliculus (CIC)	++	+/-	-
Superior colliculis (SC)	+	++	-
Interpeduncular nuclei (IP)	+	+/-	+/-
Occulomotor nucleus (3)	-	-	-
Periaqueductal grey (CG)	+/++	-	-
Principal sensory trigeminal nucleus (Pr5)	+	-	-
Raphe nuclei (RMg, DR)	+	+	+/-
Substantia nigra (SNR/SNC/SNL)	-	-	-
Red nucleus (R)	-	-	-
Ventral tegmental area (VTA)	+/-	+/-	+/-
Rhombencephalon			
Cochlear nucleus complex	-	-	-
Facial nucleus (7)	+/-	-	-
Parabrachial nuclei (PB)	-	+	-
Locus coeruleus (LC)	-	-	-
Nucleus of the solitary tract (Sol)	+/-	+++	-
Olivary complex (OPT, oc, ocb)	+/-	+/++	+/++
Pontine reticular nucleus (Pn)	+++	+	++
Spinal trigeminal nucleus (Sp)	+/-	+/-	-
Vestibular nucleus (Ve)	++	+/-	-
Cerebellum			
Cerebellar cortex	-	-	-
Deep cerebellan nuclei	-	-	-

Table 9
Relative amount of c-Fos IR of scheduled fed animals within the rat brain. Sections that represent all brain levels were investigated from three animals per group.

Only weak c-Fos IR could be detected within most of the brain regions of AL animals, compared to scheduled fed animals. In FD and FR animals, histological localisation of forebrain structures showed strong to moderate c-Fos IR in the tenia tecta, the islets of Calleja and along the complete extension of the piriform cortex. Within neocortical and metacortical areas, only weak to moderate c-Fos labelling was observed, which did not differ in the intensity between FD, FR and AL animals.

In all groups, within basal ganglia nuclei such as the caudate putamen and the globus pallidus only single cells were found to express c-Fos. High c-Fos induction was visible in FD and FR animals within the core division of the nucleus accumbens (Fig. 30A-C, bregma level +1.2 mm), and this expression pattern was even stronger in the shell division of the nucleus accumbens of FD animals (Fig. 30D-E, bregma level +1.2 mm). No c-Fos staining was detected in the entorhinal area and the different layers of the hippocampal formation, except for the CA 3 cells of the hippocampus and the dentate gyrus, where weak c-Fos IR was detected in all animal groups.

Within the amygdaloid nuclei, there were moderate staining in the basolateral and lateral nuclei of the amygdala, and there were some c-Fos IR neurons also detected in the central amygdala of FD animals, however, c-Fos IR was clearly weaker in FR animals (Fig 30G-I, bregma level –3.3 mm). Within the septal nuclei, especially the lateral septal nucleus of FD animals showed moderate density of c-Fos IR.

Most of the thalamic structures contained high levels of c-Fos signal in both scheduled fed groups, with a more densely level of c-Fos expression in the central medial thalamic nuclei, the mediodorsal and intermediodorsal thalamic nucleus, ventromedial and ventrolateral thalamic nuclei of FD animals. Amongst thalamic structures, the posterior part of the paraventricular thalamic nucleus of FD animals was visible as strongly immunostained for c-Fos (Fig. 30J-L, bregma level -3.3 mm). Additionally, the adjacent medial part of the lateral habenular nucleus showed immense c-Fos IR within sections of FD animals (Fig. 30J-L, bregma level -3.3 mm). Strong to moderate c-Fos labelling could be recorded specifically in the subthalamic nucleus and the more ventrally located thalamic nuclei of FD animals. Also within the zona incerta located at the dorsal border of the posterior hypothalamus showed strong to moderate c-Fos labelling in sections derived from FD animals.

Within hypothalamic structures, a diverse pattern of c-Fos IR could be detected between FD and FR animals. Strongest expression of c-Fos was detected in the SCh of both FD and FR animals (Fig. 31A-C, bregma level –1.3mm). On the same brain level, the anterior hypothamic

nucleus was markedly induced in FD and FR animals. The medial part of the SO (Fig. 31P-R, bregma level –1.3mm) and its reticular fraction were exclusively c-Fos IR in FR animals. This was also the case for both the parvocellular and magnocellular division of the PVN (Fig. 31M-O, bregma level –1.8 mm) were heavily immunostained in FR animals, however some of the FD animals also showed specific c-Fos staining in the PVN. In contrast, the Arc (ventromedial part) (Fig. 31D-F, bregma level –3.14) and the complete rostral to dorsal extension of the LHA (Fig 31J-L, bregma level –3.13) obtained strong immunoreactivity in FD rats. Both experimental groups exhibit dense to moderate labelling in the difuse and compact subdivision of the DMH (Fig. 31G-I, bregma –3.3 mm). Rare c-Fos immunostaining could be detected within the ventromedial hypothalamic nucleus of all rat brains. In the more dorsal part of the hypothalamus, FD animals showed moderate labelling in the lateral and tuberomammillary nuclei and within the other nuclei of the mammillary complex sparce immunostaining was observed.

In most of the nuclei located in the mesencephalon, only sparce labelling could be detected, except for the dorsal tegmental nucleus, the inferior colliculus and the periaqueductal grey in FD animals and the superior colliculus in FR animals, respectively. Within the substantia nigra, no c-Fos IR was detected. Rhombencephalon structures generally showed weak to moderate c-Fos staining in the olivary complex, heavy staining was observed in the pontine reticular nucleus of FD animals. Some c-Fos IR cells could also be identified in the parabrachial nucleus of FR rats. Strongest immunostaining was present on the commisural part, the central and the medial divisions of the Sol on sections derived from FR animals (Fig. 30M-O, bregma level –13.30 mm) and in the adjacent area postrema of FR animals, dense labelling was observed. On the same bregma level, within the Sol of FD animals, c-Fos IR was only sparce or absent.

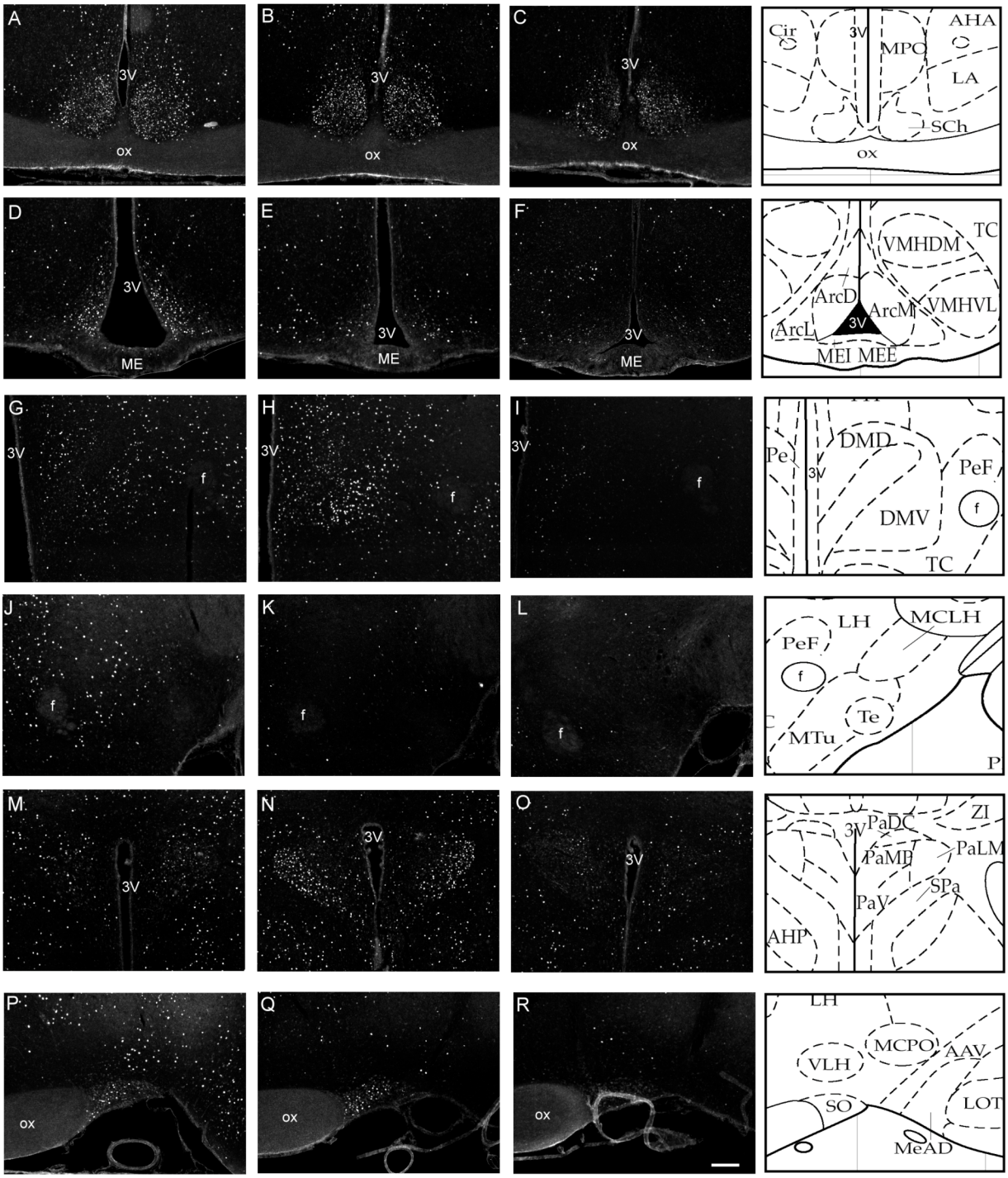
Fig. 31.

Panel of representative photomicrographs of c-Fos immunoreactivity within different hypothalamic nuclei of scheduled fed, food-deprived (A,D,G,J,M,P) or food-restricted (B,E,H,K,N,Q) rats compared to *ad libitum* (C,F,I,L,O,R) fed rats. Brain sections were matched for their bregma levels and show Fos-labelling in the suprachiasmatic nucleus (A-C), arcuate nucleus (D-F), dorsomedial hypothalamic nucleus (G-I), lateral hypothalamic area (J-L), paraventricular hypothalamic nucleus (M-O) and supraoptic nucleus (P-R).

Scale bar 200 μm.

Schematic drawings have been modified from: The Rat Brain in stereotaxic coordinates, Fourth Edition, Paxinos G and Watson C, 1998.

3V, third ventricle; AAV, anterior amygdaloid area, ventral part; AHA anterior hypothalamic area, anterior partAHP anterior hypothalamic area, posterior part; ArcD arcuate nucleus, dorsal part; ArcL arcuate nucleus, lateral part ; ArcM arcuate nucleus, medial part; Cir circular nucleus; DMD dorsomedial hypothalamic nucleus, dorsal part; DMV dorsomedial hypothalamic nucleus, ventral partEL, ependymal layer. f fornix; LA lateroanterior hypothalamic nucleus LH lateral hypothalamic area; LOT, nucleus of the lateral olfactory tract; MCLH magnocellular nucleus of the lateral hypothalamus; MCPO, magnocellular preoptic nucleus; ME Median Eminence; MeAD, medial amygdaloid nucleus, anterodorsal part; MEE medial eminence, external layer ; MEI medial eminence, internal layer; MPO medial preoptic nucleus; MTu medial tuberal nucleus; ox, optic chiasm; PaDC paraventricular hypothalamic nucleus, dorsal cap; PaLM paraventricular hypothalamic nucleus, lateral magnocellular part; PaMP paraventricular hypothalamic nucleus, medial parvocellular part; PaPo paraventricular hypothalamic nucleus, posterior part; PaR pararubral nucleus; PaV paraventricular hypothalamic nucleus, ventral part; Pe periventricular hypothalamic nucleus; PeF perifornical nucleusSCh suprachiasmatic nucleus; SO supraoptic nucleus; SPa subparaventricular zone of the hypothalamus; TC tuber cinereum area; Te terete hypothalamic nucleus; VLH ventrolateral hypothalamic nucleusVMHC ventromedial hypothalamic nucleus, central part; VMHDM ventromedial hypothalamic nucleus, dorsomedial part; VMHVL ventromedial hypothalamic nucleus, dorsomedial part; ZI zona incerta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ZI
3V
PaDC
PaLM
PaMP
SPa
PaV
AHP
P
ox
Q
ox
R
ox
LH
MCPO
AAV
VLH
SO
LOT
MeAD

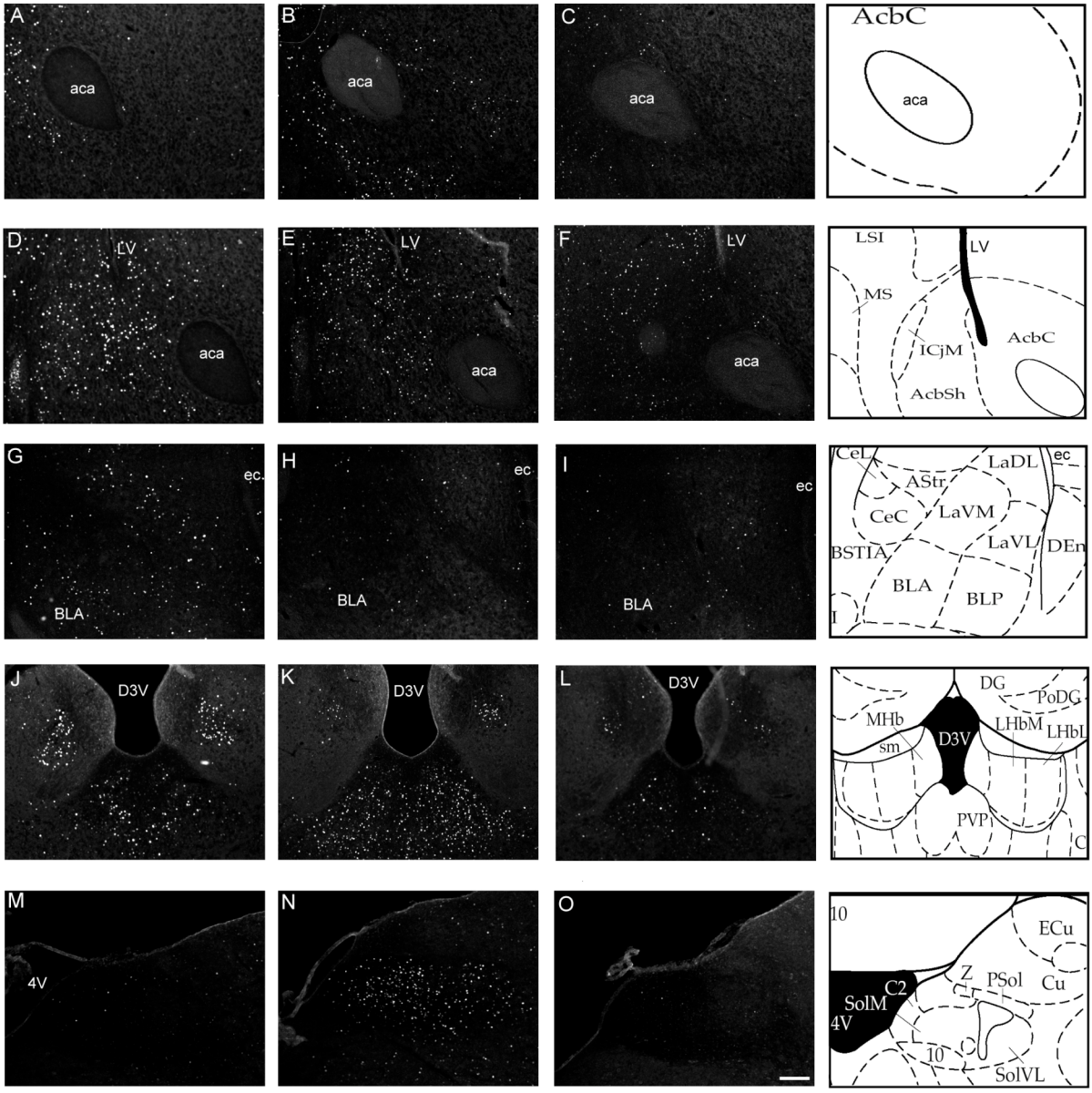
Fig. 32.

Panel of photomicrographs of c-Fos immunoreactivity in extrahypothalamic regions of the rat brain nuclei derived from scheduled fed food-deprived (A,D,G,J,M) or food-restricted (B,E,H,K,N) rats compared to *ad libitum* (C,F,I,L,O) fed rats. Brain sections were matched for their bregma levels and show Fos-labelling in the accumbens nucleus, core (A-C), accumbens nucleus, shell (D-F), basolateral amygdaloid nucleus (G-I), lateral habenular nucleus/posterior paraventricular thalamic nucleus (J-L), and nucleus of the solitary tract (M-O).

Scale bar 200 μm.

Schematic drawings have been modified from: The Rat Brain in stereotaxic coordinates, Fourth Edition, Paxinos G and Watson C, 1998.

10 dorsal motor nucleus of vagus; 4V 4th ventricle; aca anterior commissure, anterior part; AcbC accumbens nucleus, core; AcbSh accumbens nucleus, shell; AStr amygdalostriatal transition area; BLA basolateral amygdaloid nucleus, anterior part; BLP basolateral amygdaloid nucleus, posterior part; C2 C2 adrenaline cells; CeC central amygdaloid nucleus, capsular part; CeL central amygdaloid nucleus, lateral division; Cu cuneate nucleus; DEn dorsal endopiriform nucleus; DG dentate gyrus; ec external capsule; ECu external cuneate nucleus; ICjM islands of Calleja, major island; LaDL lateral amygdaloid nucleus, dorsolateral part; LaVL lateral amygdaloid nucleus, ventrolateral part; LaVM lateral amygdaloid nucleus, ventromedial part; LHbL lateral habenular nucleus, lateral part; LHbM lateral habenular nucleus, medial part; LSI lateral septal nucleus, intermediate part; LV lateral ventricle; MHb medial habenular nucleus; MS medial septal nucleus; PoDG polymorph layer of the dentate gyrus; Psol parasolitary nucleus; PVP paraventricular thalamic nucleus, posterior part; sm stria medullaris of the thalamus; SolM nucleus of the solitary tract, medial part; SolVL nucleus of the solitary tract, ventrolateral part; Z nucleus Z


A
aca
B
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C
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AcbC
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D
LV
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AcbC
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BLA
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LaDL
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AStr
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LaVM
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D3V
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D3V
DG
PoDG
MHb
LHbM
LHbL
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D3V
PVP
C
M
4V
N
O
10
ECu
Z
PSol
Cu
C2
SolM
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SolVL

3.3.2.3.2. Quantitative statistical analysis for c-Fos immunohistochemistry within selected brain regions of RFS treated rats

Results of the differential mapping study of c-Fos staining within the rat brain of all animal groups were used for a further quantitative analysis of c-Fos immunoreactivity within selected regions of interest. For this purpose, sections derived from all animals (n = 6 per group) were involved, that contain intrahypothalamic (SCh, Arc, DMH, LHA, SO, PVN) and extra-hypothalamic brain structures (AcbC, AcbSh, BLA, LHb, PVP, Sol). These sections were introduced to a software-supported, automated counting procedure of c-Fos immunoreactivity. Within two adjacent bregma levels, 10-15 sections per animal were used and photomicrographs were taken from each selected brain region on both hemispheres. Results of statistical analysis of each brain region are given as the mean number of cells in relation to the ROI, summarized as a panel of diagrams in Figure 33**.**

Within the hypothalamus, FD animals showed a remarkable increase in c-Fos IR in all investigated hypothalamic nuclei, although it was only significant in the SCh, Arc, DMH and LHA compared to the AL group. Within the same regions, FR animals showed significant effects ($p<0.05$) of scheduled feeding on c-Fos induction in the DMH, LHA, PVN and SO compared to AL animals, but with a non statistical difference in the SCh and in the Arc. A pairwise comparisons of the FD with the FR rats showed that c-Fos IR cell counts were significantly higher ($p<0.05$) in the AcbSh, the BLA and the LHBm of FD animals, and for FR rats, the SO and the Sol were significantly c-Fos IR.

3.3.2.4. Biochemical characterisation of c-Fos immunoreactive neurons within the rat brain of RFS rats

3.3.2.4.1. Results of immunohistochemical colocalisation studies for c-Fos with the neuropeptides MCH or OxA in the rat lateral hypothalamus

One series of sections that derived from FD and FR animals were additionally examined for a possible colocalisation of the orexigenic neuropeptides MCH or OxA. For this experiment, we used sections derived from both experimental groups that comprise the LHA (bregma Level -3.14 mm to -3.3 mm). Brain regions located around the fornix, defined as the perifornical area (PeF) and the lateral hypothalamus (LH) showed a remarkable c-Fos induction in FD animals (Fig. 34 A, Fig. 35 A). Within the LH, neurons stained for MCH were found around the fornix, and staining was more intensive in FD animals than in FR animals (Fig. 34B; Fig. 35B).

Fig. 33.

Statistical analysis of automated, software-supported quantification of c-Fos immunopositive cells of scheduled fed, food-deprived (FD, red) or scheduled fed, food-restricted (FR, blue) compared to *ad libitum* (AL, green) fed animals within selected hypothalamic and extra-hypothalamic brain regions.

Fos immunoreactivity was automatically counted within each region of interest that was defined according to the anatomical atlas. Results are calculated as cells per area of the region (fraction), and illustrated as plots of median value per animal within the brain region, horizontal lines indicate median value per group.

AcbC accumbens nucleus, core; AcbSh accumbens nucleus, shell; Arc arcuate nucleus; BLA basolateral amygdaloid nucleus; DMH dorsomedial hypothalamic nucleus; LH lateral hypothalamic area; LHbM lateral habenular nucleus, medial; PVN paraventricular hypothalamic nucleus; PVP paraventricular thalamic nucleus, posterior part; SCh suprachiasmatic nucleus; SO supraoptic nucleus; Sol nucleus of the solitary tract.

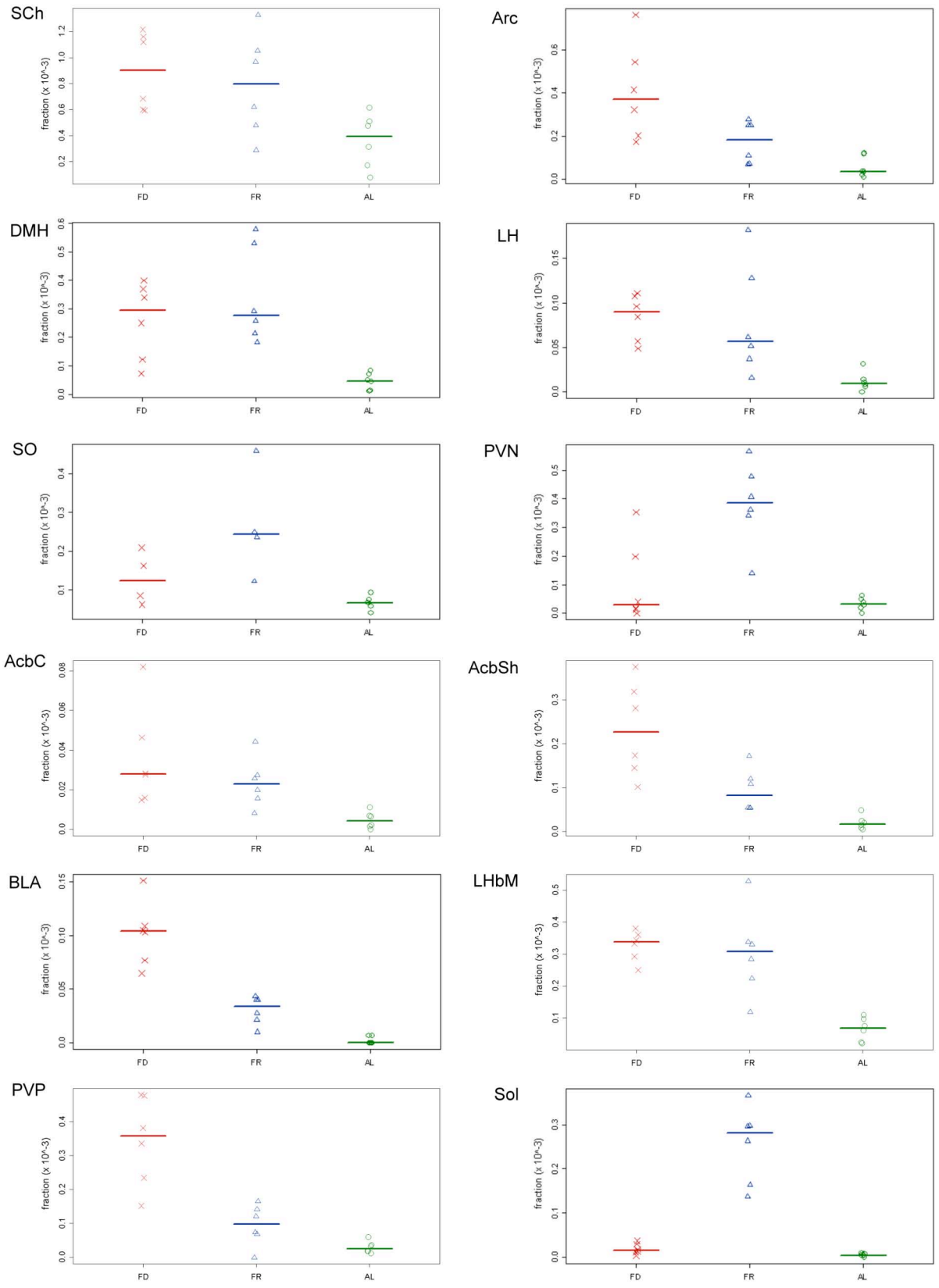

SCh
fraction (x 10^-3)
FD
FR
AL
Arc
DMH
LH
SO
PVN
AcbC
AcbSh
BLA
LHbM
PVP
Sol

Fig. 34.

Immunofluorescence photomicrographs of dual-labelled neurons immunostained for c-Fos (artificial green, A,E) and Melanin-concentrating hormone (MCH) (artificial red, B,F) in the rat lateral hypothalamic area of scheduled fed animals. Photomicrographs were taken from cryosections derived from a representative food-deprived animal (A,B,C) or food-restricted animal (E,F,G). Long arrows indicate stained nuclei c-Fos immunoreactivity, short arrows indicate cytoplasmatic MCH staining. Under a higher magnification (x63), no colocalisation was detected for both food-deprived animal (D) or food-restricted animal (H). Scale bar (G) 20 μm; (H) 100 μm.

f fornix

MCH Melanin-concentrating hormone

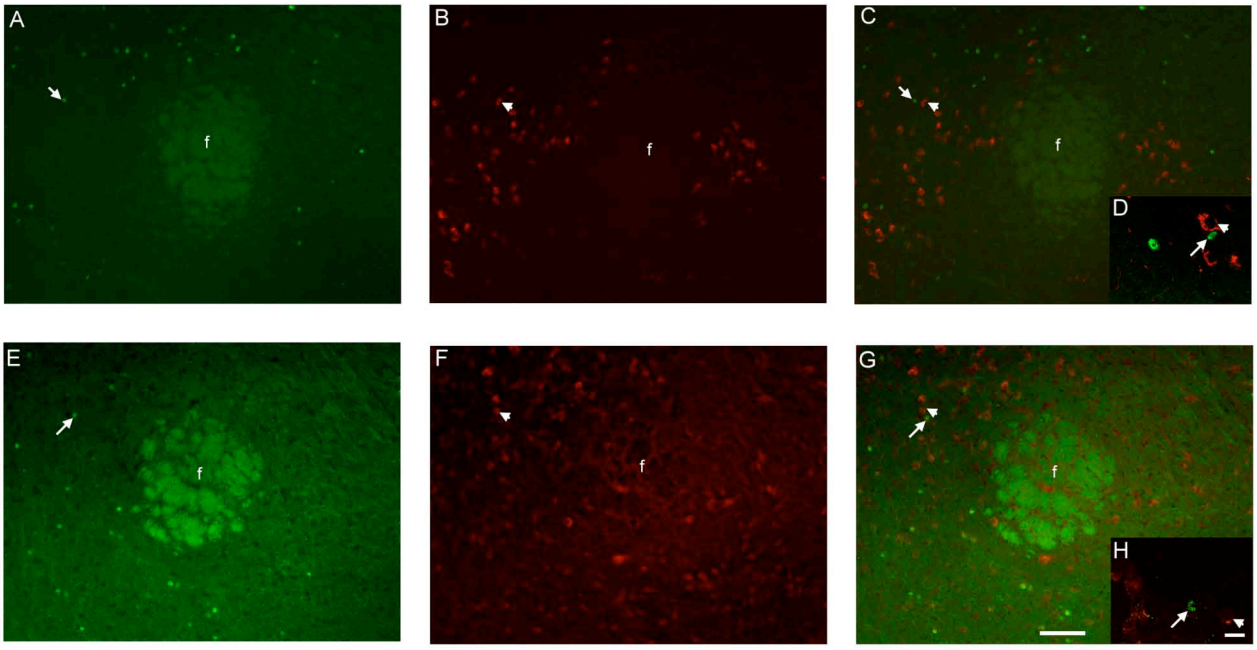
Fig. 35.

Immunofluorescence photomicrographs of dual-labelled neurons immunostained for c-Fos (artificial green, A,E) and Orexin A (OxA) (artificial red, B,F) in the rat lateral hypothalamic area from scheduled fed animals. Photomicrographs were taken from cryosections derived from a representative food-deprived animal (A,B,C) or food-restricted animal (E,F,G). Long arrows indicate stained immunoreactive nuclei for c-Fos, short arrows indicate cytoplasmatic staining for OxA. Strong colocalisation for c-Fos with OxA was found in food deprived animals (C), which was verified under a higher magnification (x63) (D), but only rare colocalisation for c-Fos with OxA was detected in sections derived from FR animals (G, H).

Scale bar (G) 20 μm; (H) 100 μm.

f fornix

OxA Orexin A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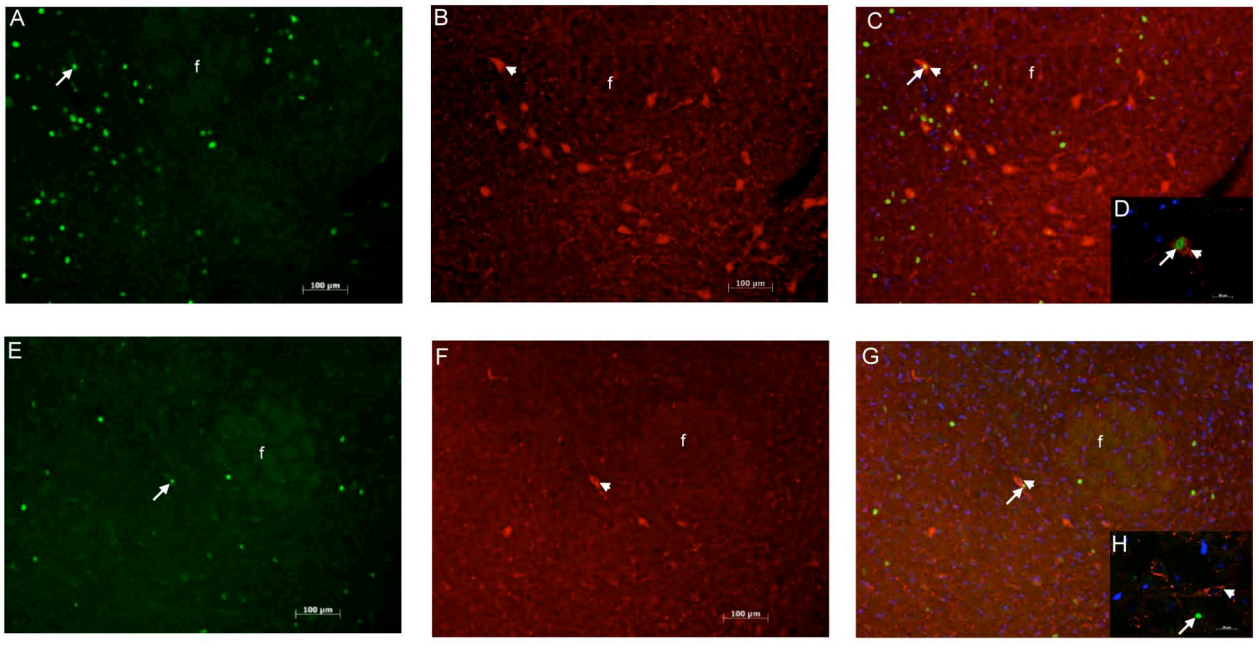

A
B
C
D
E
F
G
H
f
100 µm

However, the distribution patterns of c-Fos IR did not converge with MCH neurons in the FD state and those neurons that are in the vicinity of MCH-IR neurons did not show any colocalisation with c-Fos (Fig. 34C,D). Furthermore, no colocalisation was found for the rare neurons that express c-Fos or MCH in FR animals (Fig. 34G,H).

The immunohistochemical density of OxA neurons in FD animals was more pronounced compared to the FR animals and converged especially within the PeF with the c-Fos expression pattern (Fig. 35B,F). A great number of neurons that showed nuclear c-Fos IR also exhibit OxA IR within their somata (approximately 70%) within FD animals (Fig. 35C,D). In contrast, none of the c-Fos labelled cells colocalize OxA IR in FR rats (Fig. 35G,H).

3.3.2.4.2. Characterisation of the c-Fos antibody sc-52 TRITC used for colocalisation of MCHR-1 with c-Fos

In order to examine, if neurons that have been shown to be activated after scheduled feeding (FD or FR animals) express MCHR-1, I planned to perform a colocalisation study combining MCHR-1 immunohistochemistry with c-Fos immunohistochemistry. Unfortunately, both antibodies herein used have been raised in rabbits, thus I needed to modulate again the immunohistochemical procedure. In order to avoid false-positive results, I introduced an additional c-Fos antibody (c-Fos antibody sc-52-TRITC coupled). Although this antibody is directed against a different epitope and the immunohistochemical signal is not amplified by additional amplification steps, the antibody was capable to detect c-Fos IR cells in the Arc of FD animals or in the Sol of FR animals (Fig 36). Both brain regions have been shown to specifically express c-Fos IR after scheduled feeding (see also Fig. 31 or Fig. 32, respectively). Although the comparable intensity of the c-Fos signal was weaker using the SC-52 TRITC compared to the TSA amplification procedure used for the quantitative analysis of scheduled fed animals, the SC-52 TRITC was found to be a suitable tool to detect c-Fos on rat cryosections.

3.3.2.4.3. Colocalisation for MCHR-1 with c-Fos immunoreactivity in the rat brain of of RFS treated rats

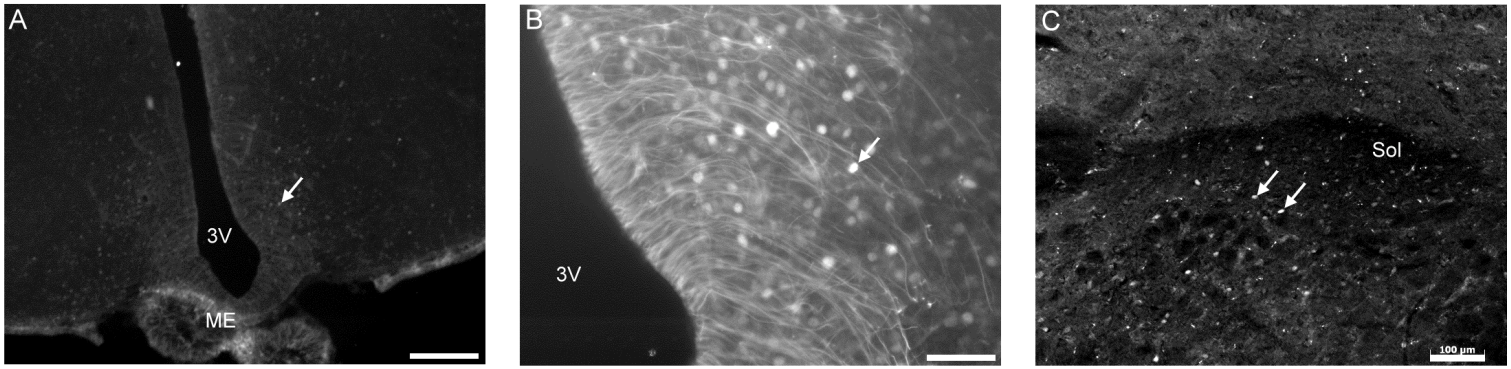
Immunohistochemical analysis of MCHR-1 IR within the rat brain revealed a similar distribution pattern as described by Hervieu (2000). Results from quantitative PCR using MCHR-1 specific primers setting within hole hypothalami, showed no difference in the expression of MCHR-1 in FD and FR animals (data not shown).

Fig. 36.

Evaluation of immunohistochemistry for c-Fos on rat brain cryosections for the fluorophore coupled c-Fos primary antibody SC-52 TRITC. Photomicrograph was taken from sections that were derived from a scheduled fed, food deprived animal, representing neuronal activation in the arcuate nucleus (A,B) or from a scheduled fed, food restricted animal showing the nucleus tractus solitarius. White arrows indicate nuclear stained c-Fos labelled neurons. The c-Fos antibody SC-52 TRITC was found to detect c-Fos immunoreactivity within activated brain regions of scheduled fed animals and was used for subsequent colocalisation studies with the MCHR-1 antibody.

Scale bar (A) ,200 μm; (B), 20 μm; (C) ,100 μm.

3V third ventricle; ME Median Eminence; Sol nucleus tractus solitarii.


A
3V
ME
B
3V
C
Sol
100 µm

Strongest MCHR-1 immunoreactivity was detected within several hypothalamic regions, including the magnocellular and parvocellular part of the PVN (Fig. 38 D), the SO (Fig. 38 F), and DMH (Fig. 37 E), modest MCHR-1 positive cells were found in the LHA (Fig. 37 E), and to a lesser extend, MCHR-1 was visualised in the ventromedial portion of the Arc and the VMH (Fig. 37 A). Additionally, we observed strong IR in the amygdaloid nuclei and several thalamic nuclei, including the BLA, central amygdala (Fig. 37 Q) and PVP (Fig. 37 U). Weak to no staining was observed in the Sol (Fig. 38 J).
For the colocalisation of MCHR-1 with c-Fos, a number of 3-5 sections per ROI of each FD or FR rat origin was chosen. Special interest was focused on those areas of the brain, that were found to be specifically activated after scheduled feeding in the FD animals. Under a higher magnification, subcellular localisation of the immunoreactive material for MCHR-1 showed again a membrane-associated or cytoplasmatic staining, as shown in many MCHR-1 IR regions, such as the nucleus accumbens shell (Fig. 37 P). However, some cells showed a covering membrane staining, while others showed a more defined punctuate staining of cell bodies examplied for MCHR-1 IR in the BLA (Fig. 37 T). Staining of c-Fos IR neurons were restricted to the nucleus, which is known to be characteristic for c-Fos.

Within the hypothalamus, those neurons that were defined to be activated within FD animals, a large number exhibit MCHR-1 immunoreactivity. Of those, a large number of c-Fos IR cells co-express MCHR-1 and this observation was generally the case for all of the observed brain regions. In detail, c-Fos/MCHR-1 IR neurons were found to be abundant in the ventrolateral and the ventromedial extension of the Arc (Fig. 37 A-D), in the diffuse and the central parts of the DMH (Fig. 37 E-H), in the lateral hypothalamus (Fig. 37 I-L). In the nucleus accumbens, c-Fos/MCHR-1 IR could be detected in the shell division, and to a lesser extend, to the nucleus accumbens core (Fig. 37 M-P).
In the BLA, the relative expression of MCHR-1 was weak and there exist an additional subsets of neurons that exclusively express c-FOS or MCHR-1 (Fig. 37 Q-T). Finally, strong immunoreactivity for MCHR-1 could be observed within several thalamic structures, and a large number of MCHR-1/c-Fos IR could be observed within the posterior part of the PVP (Fig. 37 U-X). In summary, neuronal activatition induced by food deprivation could be defined in a large number of MCHR-1 expressing neurons.

Fig. 37.

Images of sections derived from a food-deprived scheduled fed animal after double-labelling combining MCHR-1 immunoreactivity (artificial green) with c-Fos immunoreactivity (artificial red) in the arcuate nucleus (A-D), dorsomedial hypothalamic nucleus (E-H), lateral hypothalamic area (I-L), accumbens nucleus, shell and core (M-P), basolateral amygdaloid nucleus (Q-T), and paraventricular thalamic nucleus, posterior part (U-X). Long, thick arrows indicate c-Fos neurons, long, thin arrows indicate single MCHR-1 labelled neurons and short thin arrows highlight co-localized MCHR-1/c-Fos immunoreactive neurons.

Cells were counterstained for DAPI.

Scale bar (W), 100 μm; (X), 20 μm.

3V third ventricle; f fornix; aca anterior commissure, anterior; ec, external capsule; D3V dorsal third ventricle

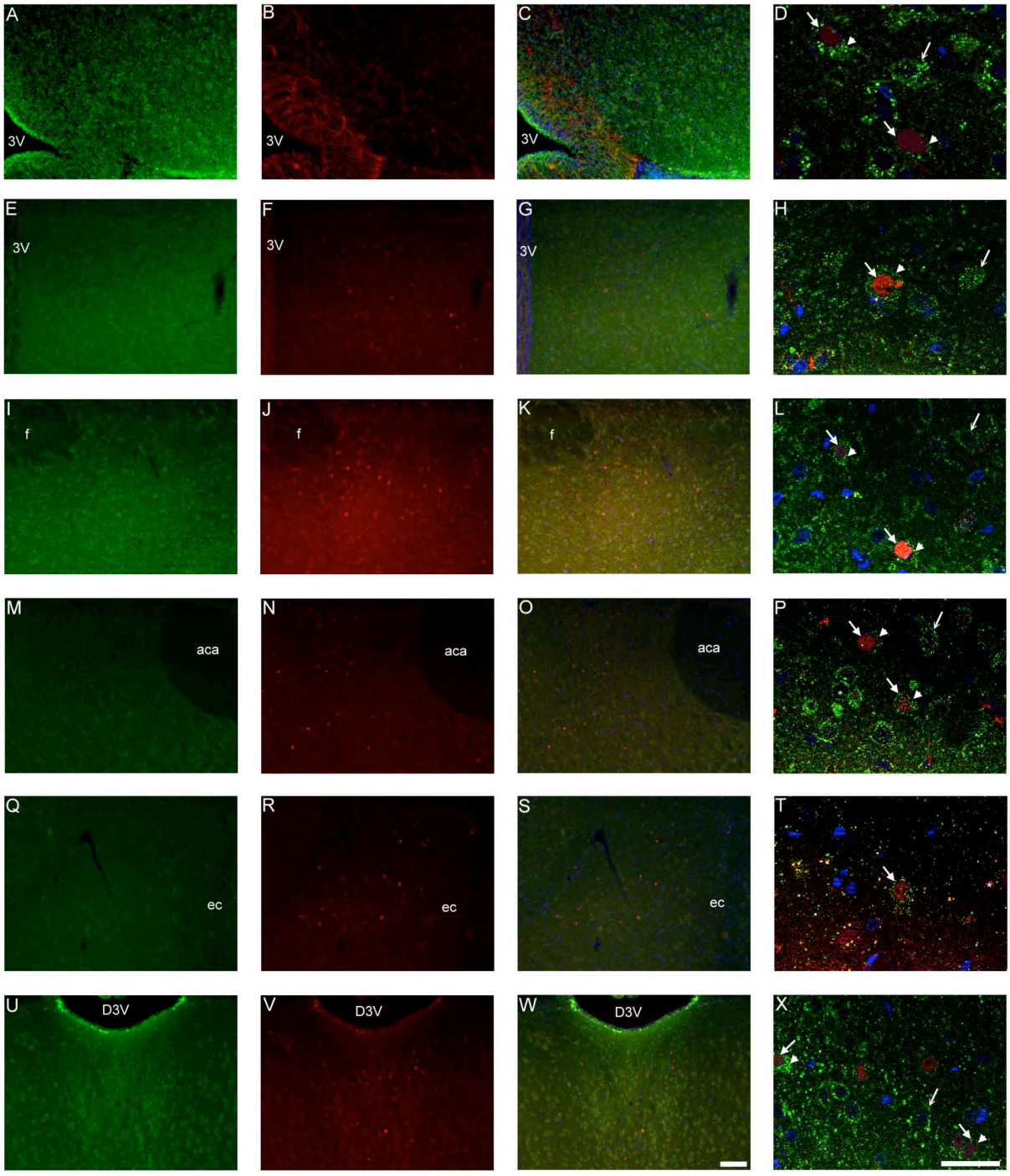

A
3V
B
3V
C
3V
D
E
3V
F
3V
G
3V
H
I
f
J
f
K
f
L
M
aca
N
aca
O
aca
P
Q
ec
R
ec
S
ec
T
U
D3V
V
D3V
W
D3V
X

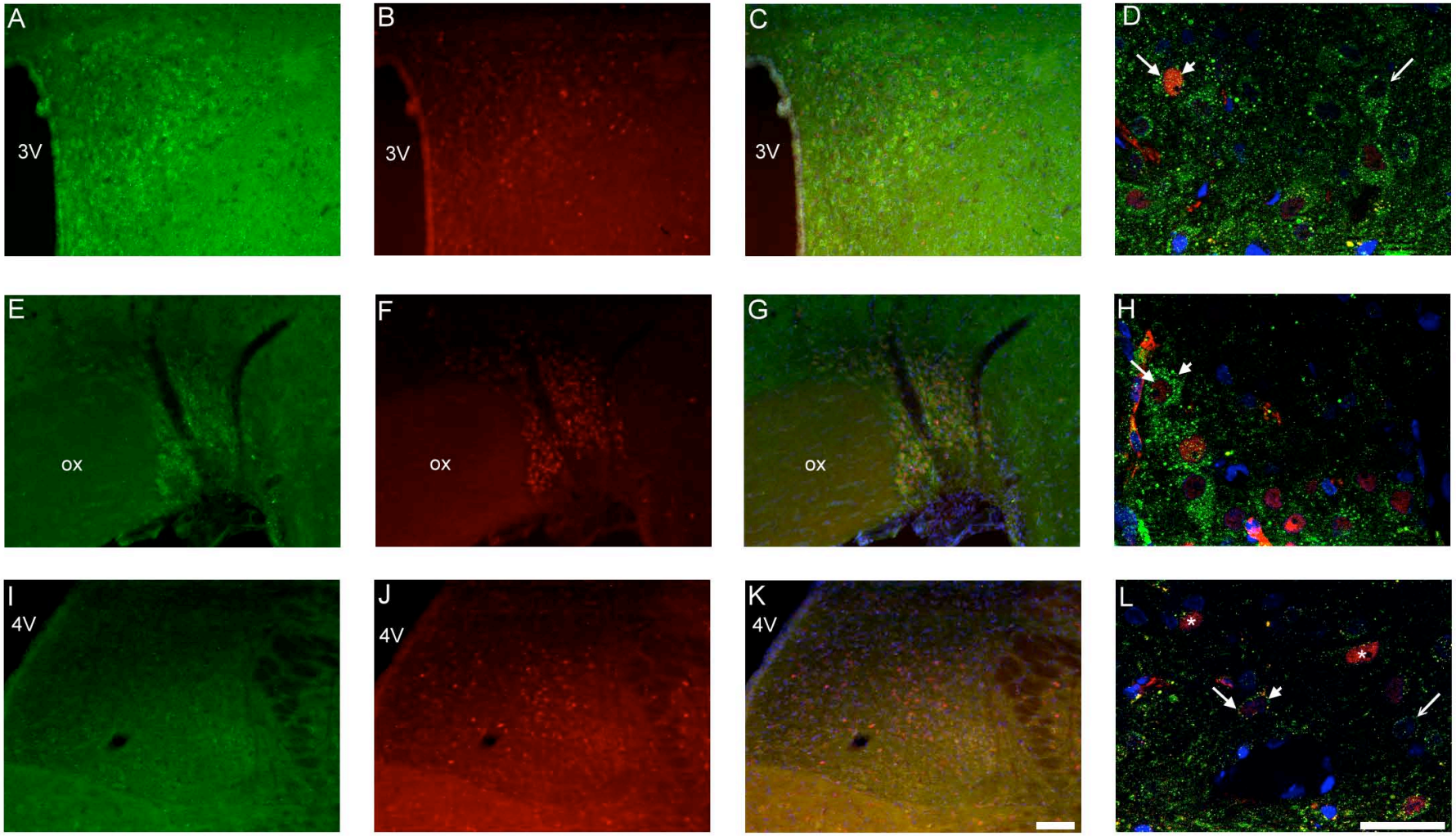
Fig. 38.

Colocalisation study for MCHR-1 immunoreactivity (IR) with c-Fos IR within selected brain regions of food-restricted, scheduled fed animals.

Images of sections derived from a food-restricted animal after double-labelling combining MCHR-1 immunoreactivity (artificial green) with c-Fos immunoreactivity (artificial red) in the paraventricular hypothalamic nucleus (A-D), supraoptic nucleus (E-H) and nucleus of the solitary tract (I-L). Long, white arrows indicate c-Fos neurons, long, thin arrows indicate single MCHR-1 labelled neurons and short thin arrows highlight co-localized MCHR-1/c-Fos immunoreactive neurons. Asteriks indicate single c-Fos IR neurons within the nucleus of the solitary tract.

Scale bar (K), 100 μm; (L), 20 μm.

3V, third ventricle; ox, optic chiasm; 4V, fourth ventricle.


A
3V
B
3V
C
3V
D
E
ox
F
ox
G
ox
H
I
4V
J
4V
K
4V
L
*
*

Interestingly, there is also a large co-distribution of c-Fos/MCHR-1 in the ventral paraventricular nucleus of the hypothalamus of FR rats (Fig. 38 A-D). Apart from these observations, strongest colocalisation of MCHR-1 and c-Fos was detected in the SO nucleus of the hypothalamus (Fig 38, I-L). In contrast, in the Sol, where strong c-Fos induction could be demonstrated, only weak MCHR-1/c-Fos IR coexist, but there was a great number of c-Fos expressing neurons that showed no MCHR-1 signal.

3.4. Discussion

The purpose of this part of work was to functionally examine the central MCH system by visualization of neuronal activation after a feeding-related stimulus. Among these stimuli, I induced central orexigenic pathways by pharmacological administration of MCH (icv) or by potent stimulation using the restricted feeding paradigm. Furthermore, I pharmacologically blocked the central MCH system by subchronic treatment of MCHR-1 antagonist. Each experimental set-up involved monitoring of its physiological effects and a detailed examination of neuronal activation patterns by immunohistochemical detection of the immediate early gene product c-Fos.

3.4.1. Acute central MCH administration potently increases food and water intake, but did not induce c-Fos expression within hypothalamic nuclei

In the present study, central administration of MCH results in acute and profound effects on two hour food intake. Consistently, a number of *in vivo* studies determined MCH`s orexigenic actions. Central, acute administration of has been shown to MCH increases food intake in rats (Rossi et al., 1997; Sahu, 2000; Chaffer et al., 2000) and chronic infusion of MCH into the third ventricle (3V) of MCH leads to hyperphagia and obesity (Ito et al., 2003). This hyperphagic effect of MCH is still present in satiated rats when MCH is injected into the brain (Della-Zuana et al., 2002). Consequently, MCH is a strong orexigenic stimulus, with a comparable potency on feeding as NPY and OxA (Edwards et al., 2000). Moreover, feeding could be also induced if MCH is infused into the brain both in the light and dark phases of the day of the circadian cycle (Rossi and Bloom, 1997). Additionally, MCH has been shown to mediate its effects on feeding on specific hypothalamic sites. After intranuclear injections of MCH into the Arc, PVN and DMH of the hypothalamus, a feeding response was observed (Abbott et al., 2003).

Additionally, our results further support a dipsogenic, central effect of MCH on water intake. Following icv administration, measurement of 2h water intake has shown a strong response of MCH on water intake. This observation is in line with a recent study, that icv MCH (3V) elicits an increase in water intake, which was shown to be independent from the orexigenic actions of MCH on food intake (Clegg et al., 2002). It may be of interest, if MCH is upregulated in a model of dehydration-induced anorexia. In this model, chronic food restriction results in upregulation of NPY and POMC in the arcuate nucleus, whereas food and water deprivation results in increase of CRH and NT in the LHA of the hypothalamus (Watts et al., 1999), and MCH may be also increased in this animal model.

An extensive examination of MCH treated animals for their changes in the magnitude or distribution pattern of c-Fos IR neurons compared to aCSF-treated animals, did not show any differences in c-Fos IR within the examined hypothalamic nuclei, namely the Arc, DMH, LH, VMH, PVN and SO. These results are surprising, because I clearly demonstrated a significant orexigenic and dipsogenic effect of MCH compared to control animals, which should also result in a specific neuronal activation pattern. A number of other studies have sucessfully introduced c-Fos IHC to identify neuronal activation patterns after central orexigenic or anorexigenic neuropeptide administration within the rat brain. For example, central NPY administration induced 2h-feeding and results in a specific increase in c-Fos IR in the DMH, SO and PVN of the rat hypothalamus (Yokosuka et al., 2001). Additionally, OxA induces c-Fos expression in the rat hypothalamus. In another study, the consummatory behaviour of the anorexigenic POMC derivative α-MSH was examined after its central administration, and c-Fos was specifically induced after meal termination (Olszewski et al., 2001).

For the interpretation of my results, a number of technical and functional considerations need to be discussed. Observed behavioural effect on ingestion after MCH treatment verified the activity of the substance, the correct position of the cannula and the right administration procedure to MCH`s site of action. Additionally, the 2h time-schedule for animal`s perfusion was chosen because the specific expression of c-Fos follows after a discrete stimulus within a specific time course within the brain with a maximum peak of 30-60 min for mRNA expression and 1-3 hours for protein expression (Kovacs et al., 1998; Morgan et al., 1991).

The detection of unspecific c-Fos expression indicates that also the immunohistochemical procedure did not limited the detection of the c-Fos profile. As a consequence of these arguments, there may be some functional effects responsible for the inability to detect MCH induced effects on c-Fos expression.

One possible explanation for the inability to detect a specific c-Fos IR expression pattern within the rat hypothalamus may be because of functional aspects of MCH. Possibly MCH is not able to induce especially c-Fos. Assuming that MCH exerts its central actions through MCHR-1, this receptor subtypes couples to multiple G Proteins and to activate several intracellular signalling cascades including activation of the c-AMP pathway, MAP kinase activation and the activation of Calcium release through phospholipase C (Hawes et al., 2000; Pissios et al., 2003; Eberle et al., 2004). All these signalling cascades have been described to induce activation of c-Fos expression (Kovacs, 1998; Chawla, 2002). To my knowledge, no study detected induction of c-Fos after central MCH administration, thus it may be possible that c-Fos is not a valid biomarker for neuronal activation. This question could be addressed by detection of a differential expression of c-Fos protein western blotting analysis based on protein lysates of the rat hypothalamus, or in addition, other possible molecular markers that are involved in MCH`s observed intracellular signalling cascades such as the cAMP response element binding element (CREB) or ERK/P-ERK. Other relevant biomarker for the detection of neuronal activation in the CNS after central MCH administration may be Egr-1. Egr-1 has been described as a transcription factor that belongs to the zinc finger proteins (Gass et al., 1992).

Finally, others have used a viral approach coupled to the detection of green fluorescent protein (GFP) for the examination of MCH`s effect on cellular physiology in hypothalamic slices (van den Pol et al., 2004). Theoretically, the use of MCH-GFP transgenic combined with IHC for GFP seems to be an interesting model to study MCH`s central effects *in vivo.*

3.4.2. The MCHR-1 antagonist suppress food intake and water intake, reduces body weight and results in neuronal activation of specific hypothalamic regions

Subchronic treatment of normal rats with the MCHR-1 antagonist compound B4 results in significant reduction of chow intake (-37%), body weight loss (-11%) and a reduction of drinking water (-48%) compared to control animals. My results characterises compound B4 as a potent anorexigenic substance, and together with its strong effects on reduction of body weight, this compound class is orally available and effective in promoting body weight change. Consistently with the pharmacological profiles of numerous other MCHR-1 antagonists, pharmacological inhibition of MCHR-1 seems to fit as a mechanism for the treatment of obesity. Others have shown that chronic administration of selective, orally available MCHR-1 antagonists reduce body weight in animal models of obesity, as shown for GW 803430 in AKR mice (Carpenter et al., 2006) or for SNAP-7941 in DIO rats (Borowsky et al., 2002). For

SNAP-7941, body weight reduction was also due to a decrease in food intake (Borowsky et al., 2002). It should be noted, that compound B4 has been demonstrated to activate mechanisms of energy expenditure, which may account for the higher efficacy of body weight loss in compound B4 treated animals compared to GW 803430 treatment.

Although food intake is directly correlated with the intake of fluid, it should be noted that compound B4 strongly reduces water intake during the treatment period. Only one study that centrally administered the water-soluble MCHR-1 antagonist compound B30/compound B (Bednarek et al., 2002; Shearman et al., 2003) showed a decrease in MCH-induced water intake in rats (Morens et al., 2005). Further studies are needed to characterise to which extend the orexigenic effects of the MCH system and adipsogenic effects of MCHR-1 antagonist are dependent on their influence on fluid intake. This question can be addressed in animal models of dehydration-induced anorexia (Watts et al., 2000) or the identification of MCHR-1 antagonist effects on fluid regulation pathways such as the vasopressinergic/angiotensin system.

It still remains open, which central mechanism are responsible for the anorexigenic actions of MCHR-1 antagonists. Because hypothalamic nuclei have been discussed for their significance to integrate information originated from several CNS pathways (Berthoud, 2002; Schwartz et al., 2006), I examined the neuroanatomical substrates after MCHR-1 antagonist treatment, using automated-software supported quantification of c-Fos IHC in hypothalamic nuclei.

This experiment was aimed to detect differences in the c-Fos IR pattern especially after compound B4 treatment and its observed anorexigenic and adipsogenic actions. For at least compound B4 treated animal, I detected an increase in c-Fos IR in the Arc and the DMH of the hypothalamus. Both regions have been shown to be activated by central leptin administration (Hübschle et al., 2000) and express the functional leptin receptor Ob-Rb (Elias et al. 2000). Additionally, my results were in agreement with those of others, who have shown a moderate to high expression of MCHR-1 in these regions (Hervieu et al., 2001).

Interstingly, one animal showed a strong increase of c-Fos IR in the PVN of the hypothalamus, and both animals showed strong c-Fos in the SO after compound B4 treatment. Both nuclei have been discussed for their role in the regulation of the neuroendocrine (hypothalamic-pituitary axis, that regulates numerous important physiological functions including stress, growth, reproduction and fluid regulation (Elmquist, 2001). The PVN and the SO have been especially implicated in body fluid regulation and show c-Fos IR during dehydration (Rowland et al., 1998).

To note, unfortunately this experiment is restricted to the small number of animals involved in the c-Fos study and it remains open my results are statistically confirmed with more animals per group. Additionally, the identification of c-Fos has been used in numerous studies to idenfy changes in expression after an acute stimulus rather than a chronic stimulus. Because the main goal of MCHR-1 antagonist treatment was aimed at the in vivo pharmacology of sub-chronic MCHR-1 antagonist treatment, the here used experimental setting does not fit optimal to the time- resolved expression c-Fos after acute stimulation. However, pharmacokinetic evaluation of compound B4 brain levels after administration have been measured to be in an effective range also on the day after administration (personal communication Marcus Schindler). In conclusion, the treatment modality may account for the observed interindividual variances of the results and overall, the modest expression level of c-Fos among all animals (compared to RFS paradigm).

It remains open, which mediators of MCHR-1 signalling are involved within the observed regions. Additionally, c-Fos IHC is used as a biomarker for neuronal activation and we here applied an antagonist which induces a behavioural response (inhibition of food and water intake) but inhibits MCH signalling. As a consequence, other hypothalamic systems may be activated following inhibition of the MCH system, such as the orexigenic NPY and OxA or the anorexigenic POMC peptides in the Arc and DMH. Additional studies may also determine the effects of MCHR-1 antagonist treatment on the anorexignic stress neuropeptides CRF and VP that are known to be expressed in the rat PVN and especially VP, for its crucial role in fluid regulation.

3.4.3. RFS can be used as a potent, orexigenic stimulus to activate orexigenic pathways within the rat brain

In the present work, I wanted to introduce a potent orexigenic response to activate orexigenic neurons within the rat brain. Others have used a long period of food deprivation (24h) to induce c-Fos expression in hypothalamic nuclei (Hewson and Dickson, 2000; Lin and Huang, 1999). In my initial experiments, fasting over night results in increased c-Fos expression in the Arc, DMH and PVN, but the interindividual difference was shown to be high and as a consequence, manually counts of c-Fos IR neurons failed to give a clear difference between overnight fasted and AL fed rats.

In order to discriminate RFS induced neurons that hunger-related and those that are satiety-related, we defined the scheduled fed animals into two subgroups on the day of experimental determination: the FD animals were omitted to food on the last day of scheduled feeding which

served as a strong orexigenic stimulus, while the FR animals were refed during the same period. The great quantity of chow ingested by FR animals should provide a potent short therm satiety signal.

In order to use c-Fos IR as a tool to detect RFS-induced neuronal activation, I needed to adjust some experimental conditions to the immunohistochemical analysis. Expression of c-Fos follows after a discrete stimulus within a specific time course within the brain with a maximum peak of 30-60 min for mRNA expression and 1-3 hours for protein expression (Kovacs et al., 1998; Morgan et al., 1991). However, our initial feeding experiments have shown that food intake and body weight did not stabilize within a daily two hour period of food access (data not shown). Moreover, this time-span of daily food access within a ten-day-period caused an unspecific increase of c-Fos expression in scheduled-fed animals without differences between both RFS groups. This may be due to the fact, that food deprivation could be used as a stressor and activator of neuronal activation *per se* (Chaillou et al., 2000; Bittencourt et al., 2000; Cullinan et al., 1995). Given the fact that diet restriction is one of the strongest physiological stimuli that may induce several effector systems, I wanted to introduce the RFS as a strong, well-defined challenge for the animal, but I also want to take into account, that the RFS animals can accommodate their physiology and behaviour to the katabolic experimental background. Therefore, I kept animals under a daily RFS of 3h food access within the middle of the light cycle, a time point used where animals are normally in the resting period and as a consequence, basal c-Fos expression is observed to be minimal (Grassi-Zucchoni et al., 1993).

My results demonstrate, that the here described RFS rapidly induced reduction of body weight during the first half of RFS (day 1-5), but body weight stabilized within the onset of the RFS. This steady state may be partly due to the entrained feeding conditions because animals respond to the restricted feeding conditions by an increased food intake during the 3h period of food access during the treatment peroid. However, other explanations imply an altered fat metabolism (Mistlberger et al., 1994; Rashotte et al., 1995) or by an adjusted energy expenditure (Stephan, 1986; Alvarenga, 2005).

A number of studies used similar RFS to study different behavioural aspects of food entrainment. For example, rats that underwent a restricted feeding show differences in locomotor activity and feeding behaviour (Stephan 1994; Mistlberger, 1994). These fundamental observations raised the idea to use the experimental setting of RFS for the examination of feeding- related aspects. Above all, the combination of RFS with downstream detection of neuronal activation by c-Fos IHC has been shown to identify brain nuclei activated after food-entrainment (Mendoza et al., 2004; Angelles-Castellanos et al., 2004). A number of

studies used the detection of c-Fos IHC to examine neuroanatomical correlates of feeding-related anticipatory behaviour (Nakahara et al., 2004) circadian rhythm (Angelles-Castellanos et al., 2006; Gooley et al., 2006) or effects of palatable food (a potent rewarding effect of food) on anticipatory activity in rats that were fed under restricted conditions. Furthermore, others used colocalisation studies to identfy the nature of c-Fos IR neurons after RFS (Inzunza et al., 2000; Meynard et al., 2005; Johnstone et al., 2006).

Basically, I wanted to use the RFS to introduce a strong physiological stimulus for activation of orexigenic pathways within the rat brain. Observed changes of daily food intake and body weight during the here established RFS support the idea, that this experimental setting is suitable to induce an orexigenic stimulus after an initial entrainment period. Especially those animals that were refed on the last day of the experiment (FR animals) showed a strong drive to feed. Therefore, it is arguable, that I in fact activated orexigenic pathways in animals that were deprived of food on the last day of the experiment (FD animals).

However, other common behavioural parameters in animals of food restriction may be of relevance within both FD and FR animals. For example, others have demonstrated that RFS entrained animals showed anticipatory behaviour measurable by increased locomotor activity prior to the presence of food (Kas et al., 2004; Mendoza et al., 2005; Maynard et al., 2005). The anticipatory behavior in expectance of the meal appears to the specific c-Fos expression pattern of hypothalamic nuclei. I detected an increase of c-Fos expression in the FD animals in the Arc, DMH and LH of the hypothalamus. These results are in line with the observations, that fasting in RFS showed an increase of c-Fos IR before and after a meal (Angelles-Castellenos et al., 2004) that showed a time-dependent change in fasting, fed, and refed animals (Angelles-Castellanos et al., 2004). In the same study, the authors showed a markedly increase in c-Fos IR in the PVN in the re-fed group, which corresponds to the increase in c-Fos IR in the my FR group.

Results of the here described c-Fos pattern largely overlap with another RFS study, that detected specific c-Fos expressio only I hypothalamic regions, but also in extrahypothalamic regions such as the LHB, PVP and Sol (Johnstone et al., 2006). This study also include the SO as satiety-related regions, with activation observed under refed conditions. Keeping in mind that the here investigated three hour period from presence of food to the perfusion it cannot be excluded that I also detected activated neurons induced by anticipatory behaviour. However, a more precise discrimination between feeding behaviour and neuronal activation patterns within the three hour in FD and FR animals may be subjected by automated feeding-cages (Inoue et al., 2004) or perfusion following a time course (Angelles-Castellanos et al., 2004).

3.4.4. Hunger activates specific brain sites feeding- from anatomical studies to possible functional effects

Mapping of c-Fos IHC in the here presented results showed a differential expression pattern throughout the olfactory system (olfactory nuclei, piriform cortex) within several cortical areas (such as insular cortex), in the basal ganglia (except for parts of the substantia nigra), thalamic and amygdaloid structures, hypothalamic nuclei and to a lesser extend, midbrain and brainstem nuclei. A number of brain regions and their connectivities have been identified to be responsible for their significance in different aspects of feeding (Berthoud, 2002; Saper et al., 2002; Kishi et al., 2005). After the RFS, I detected a specific neuronal activation pattern in hypothalamic and extrahypothalamic regions. A comparison of the here identified neuronal activation pattern with the literature give further indications of brain circuitries related to the RFS stimulus.

3.4.4.1. The suprachiasmatic nucleus and the dorsomedial hypothalamus- a possible food oscillator pathway

In RFS induced an increase in c-Fos expression in the SCh of both FR and FD animals. In addition, the DMH was found to be strongly activated in FD and FR animals, and not only in FD animals, but also in FR rats (data not shown), strong colocalisation for c-Fos with MCHR-1 could be detected neurons. A possible role for the DMH as an integrator of circadian cues in feeding behaviour was given by anatomical and functional studies. Anatomically, anterograde tracing studies demonstrated reciprocal connection of the DMH to the LHA and a more intense projection aimed at parvocellular paraventricular nucleus (Ter Horst et al., 1987, Dai et al., 1998). The DMH was shown to be interconnected with the SCh, which implies a crucial role of DMH neurons in the regulation of circadian rhythm (Watts et al., 1987). Further neurochemical incidents for the involvement of the DMH in hypothalamic feeding circuitries is that DMH neurons are sensitive to leptin (Elmquist et al., 1998; Elias et al., 2000) and express neuropeptide receptors for NPY (Y-R1) and OxA (OX-R1) (Kishi et al., 2005; Hervieu et al., 2001).

Although I could detect c-Fos IR in the SCh of both FD and FR rats, recent studies support the idea that the DMH contains a food-entrainable oscillator (FEO) that is independent of the suprachiasmatic nucleus. Our results of enhanced c-Fos expression in the DMH of both FD and FR rats, demonstrates a general activation profile within this region. This is in full agreement with increased c-Fos-IR before and after meal time in the DMH (Angelles-Castellanos et al., 2005). Period genes have been found that the compact part of the DMH demonstrates a robust

oscillation of mPer expression only under restricted feeding (Mieda et al., 2006). Excitotoxic lesions of the DMH reduce circadian rhythms of wakefulness, feeding, locomotor activity, serum corticosteroid levels and the DMH sends mainly GABAergic projection to the wake-promoting lateral hypothalamic area, including orexin neurons. (Chou et al., 2003). Anterograde labeling from the DMH resulted in moderate to dense labelling of varicose fibres in the orexin field and the tuberomammillary nucleus suggesting that DMH is one of the possible relay nuclei for indirect SCN projections not only to sleep-promoting preoptic nuclei, but also to wake-regulatory cell groups throughout the brain (Deurvailher and Semba, 2005). However, during food restriction, unlesioned rats showed a marked preprandial rise in locomotor activity, body temperature and wakefulness, and these responses were blocked by cell-specific lesions in the DMH (Gooley, et al., 2006). Conversely, DMH ablation was associated with a significant attenuation of light-dark-entrained activity rhythms and a single 3-h daily RFS showed, that all DMH and intact rats exhibited significant food anticipatory behaviour which would further suggest, that the DMH is not the only site of oscillatory or entrainment pathways but it may participate on the output side of this circadian function. (Landry et al., 2006). However, the study of Gooley et al., 2006 suggest a relationship between the SCh and the DMH in feeding-oscillatory mechanism (Herzog and Muglia, 2006).
In conclusion, my data suggest, that activation of DMH neurons in both FD and FR animals indicate an involvement of the DMH in the regulation of feeding anticipatory behaviour.

3.4.4.2. The Arc nucleus and the lateral hypothalamus, key nuclei for hypothalamic feeding-related pathways

These regions have been discussed for their involvement in sensory, motivational, neuroendocrine, and autonomic pathways of feeding circuitries (Elmquist et al., 2001). Chronic food restriction causes c-Fos induction in the LH (Carr et al., 1998), which possibly accounts for the anticipation of food access (Angelles-Castellanos et al., 2004; Meynard et al., 2005;). I could demonstrate, that within scheduled fed rats, FD animals showed a clear neuronal activation within the Arc and the LH. Both regions have been shown to be directly related to feeding behaviour.
For a further definition of the neurochemical phenotype of those neurons that are activated by RFS, we used the immunohistochemical detection of MCH or Orexin A as orexigenic biomarkers within the rat LHA. In our experimental design, FD animals and not FR animals were found to activate OxA IR neurons, but not MCH expressing neurons. These results were contradicting, because both neuropeptides have been shown to be potent orexigenic signals (Qu

et al., 1996; Sakurai et al., 1998). One explanation for this observation could be that reciprocal, inhibitory GABAergic connections have been detected between OxA and MCH expressing neurons of the LHA (Guan et al., 2002) and consequently, activated OxA neurons inhibit MCH expression or vise versa. MCH inhibited the synaptic activity of both glutamatergic and GABAergic LH neurons (Gao et al., 2001), which may deregulate OxA neurons. Another interpretation could be, that MCH expressing neurons use other signallling molecules besides c-Fos activation. LHA neurons are known to originate the orexigenic neuropeptides MCH and OxA and immunoreactivity for both neuropeptides is increased after acute food restriction (Haarthorn et al., 2005). Other studies that introduced a similar RFS in rats also showed that MCH IR neurons do not express c-Fos IR (Meynard et al., 2005). Recent studies that administered MCH in the sheep brain showed feeding as a physiological response and indicated a subset of neurons within the LHA that coexpress c-Fos with MCH IR (Whitlock et al., 2005). In addition, intrastriatal administration of the GABA agonist muscimol initiated feeding and strong c-Fos expression in orexin neurons, but not in MCH expressing neurons (Zheng et al., 2002). Furthermore, our observation that hunger (FD) increases c-Fos activation specifically in OxA neurons, may suggest that their exist distinct, potentially parallel feeding-related pathways involving OxA and MCH.

Also within the Arc and DMH, neurons exhibit moderate to strong MCHR-1/c-Fos IR, thus may be proposed that these brain regions are direct MCH target structures that interfere with other intrahypothalamic structures that orchestra energy homeostasis.

Interestingly, most of MCHR-1 IR structures have been also immunostained for the Orexin A receptor Ox-R1, including several limbic structures (Hervieu et al., 2001; Backberg et al., 2002; Suzuki et al., 2002). A number of studies determined the effects of leptin-, insulin- and ghrelin-responsive neurons on Arc neurons (Münzberg et al., 2005; Niswender, 2003; Kojima et al., 1999; Luckman, 1999). Administration of leptin, insulin or ghrelin is capable to induce c-Fos expression within the Arc (Hübschle et al., 2000; Niimi et al., 1995; Hewson et al., 2000). Blood-borne leptin concentration and both MCH and MCHR1 mRNA levels within the rat hypothalamus were up-regulated in (DIO) rats and MCH concentrations were significantly increased in the arcuate nuclei of DIO rats compared with their lean counterparts (Elliott, 2004). In MCHR-1 and leptin knockout knock out mice (*mchr-1 ob/ob* mice), an oral glucose load resulted in a lower blood glucose response and markedly lower insulin levels compared with the *ob/ob* mice despite no differences in body weight, food intake, or energy expenditure (Bjursell et al., 2006). Although central administration of ghrelin induced feeding (Horvath TL, 2002), this was independent from the presence of MCHR-1 (Bjursell et al., 2005). However,

the same study indicated, that growth hormone (GH) mRNA in the pituitary gland was markedly increased in response to ghrelin injection in the WT mice but was unaffected in the MCH receptor KO mice, which showed that MCH affects the growth hormone axis not through MCHR-1. Both the Arc and the LH have been discussed for their involvement in sensory, motivational, neuroendocrine, and autonomic pathways of feeding circuitries (Elmquist et al., 2001). Chronic food restriction causes c-Fos induction in the LH (Carr et al., 1998), which possibly accounts for the anticipation of food access (Angelles-Castellanos et al., 2004; Meynard et al., 2005;). I could demonstrate, that within scheduled fed rats, FD animals showed a clear neuronal activation within the Arc and the LH. Both regions have been shown to be directly related to feeding behaviour.In summary, further studies are needed to define if and how the MCH system moduates hormonal input information from the periphery. Furthermore, it remains to be determined, if the MCH system effect Arc neurons that express the neuropeptides NPY/AgRP or POMC/CART, possibly through its MCHR-1 receptor subtype, which further support the idea of a LHA-Arc feedback loop.

3.4.4.3. The MCH-system as a possible link between rewarding and motivational aspects of feeding

In search for addititional “hot spots” of specific neuronal activation in RFS rats, I observed c-Fos expression pattern strongly converge with the distribution of MCHR-1, in the nucleus accumbens shell, the BLA and the PVP most of the neurons that have shown MCHR-1 immunoreactivity exhibit nuclear c-Fos IR. Interestingly, all of these brain regions have been discussed for their modulatory actions within an appetite regulating network (Berthoud HR 2002). The common distribution of MCHR-1 and neuronal activation give rise to the assumptions that MCH acts as a feeding-related signal not only within its direct projections to LHA-connected hypothalamic regions, but additionally, by rewarding pathways that involve extrahypothalamic structures such as the nucleus accumbens, the amydala and thalamic regions. Concerning feeding behaviour, MCH has been shown to be involved in the appetitive activities, in addition to promoting food intake *per se* (Strubble and Van Dijk et al., 2002; Benoit et al., 2005). The nucleus accumbens is a ventral striatal nucleus with a critical role in both appetitive and consummatory phases of behaviour (Berridge and Robinson, 1998; Taha and Fields, 2005). In fact, it was shown that MCH signalling in the nucleus accumbens shell regulates feeding behaviour and modulates performance in the forced swim test, an in vivo testing to test depressive behaviour (Georgescu et al., 2005). Moreover, the nucleus accumbens shell is directly interconnected with the LHA by GABA-ergic projections and inhibition of the

Acb shell results in c-Fos expression within the LHA (Stratford et al., 1999).. Interestingly, a sub-nuclear gradient was detected with a mayor expression of c-Fos within the shell subdivision and to a lesser extend within the core division of the nucleus. This observation has been documented by others who have supposed a differential role for the nucleus accumbens sub-regions in anticipatory and feeding behaviour (Mendoza et al.,. 2005). We could detect neuronal activation and MCHR-1 within both the central and the basolateral amygdaloid nucleus, but c-Fos expression was comparably higher in FD than in FR rats. Connections between the basolateral and the lateral hypothalamus, is crucial for allowing learned cues to override satiety and promote eating in sated rats (Petrovich et al., 2002). Another key structure in this circuit is the paraventricular thalamic nucleus, which receives convergent input from orexin-coded hypothalamic energy-sensing and behavioural state-regulating neurons, as well as from circadian oscillators, and projects to cholinergic interneurons throughout the striatal complex (Kelley et al., 2005).

The brain reward mechanism is a key to trace the neuronal mechanism of appetite (Kalra et al., 2004; Kishi and Elmquist, 2005). Several lines of evidence have indicated, that the LHA, the nucleus accumbens shell, the amygdaloid nuclei and the paraventricular thalamic nucleus are part of a reward related circuitry within the brain (Saper et al., 2002). We could demonstrate that both c-Fos and MCHR-1 are co-expressed in FD animals Interestingly, the BLA sends substantial projections to the LHA (Petrovich et al., 2001; Kelley et al., 1999) and to the nucleus accumbens (Kirouac et al., 1995), which reflects an anatomical connection of the BLA with neural circuits that control feeding behaviour. Some authors argue, that the amygdala may be responsible for motivational aspects of feeding, the nucleus accumbens is involved in the translation of motivation into action and the LHA integrates information into motor outputs (Mogenson et al., 1980; Kelley et al., 2004; Wise et al., 2002). Besides acute effects on standard chow intake, intracerebroventricular (i.c.v.) administration of MCH also increased the intake of saccharin, which give further rise to the involvement of the MCH system on the palatability of food, independent from the opiod pathway (Furudono et al., 2006). Finally, it was demonstrated that MCH delivered into the nucleus accumbens shell (AcbSh) produced an intense hyperphagic response in satiated rats given the opportunity that hypothalamic regions is not the only MCH responsive brain region (Georgescu et al., 2005).

Rats restricted to a 2h feeding showed specific c-Fos expression in the thalamic paraventricular nucleus and anticipatory locomotor activity rhythm, but lesioning the thalamic PVN attenuated this rhythm, but not the light-dark entrained rhythm (Nakahara et al., 2004). We hypothesize that the RFS activated thalamic - amygdaloid- LHA- striatal circuitry evolved to coordinate

rewarding and motivational influences of feeding, especially in animals that are in a catabolic state such as the FD animals. In addition, activated regions of FR animals could serve to prolong the feeding central motivational state beyond the fulfillment of acute energy needs in order to promote "overeating" in order to store energy for the next period of food absence. Future research efforts may focus on a possible interaction of other neurotransmitter systems that have impact on the rewarding nature of food, including the central dopaminergic (Wang et al., 2002), the opiod (Levine, 2006) and the endocannabinoid system (Harrold, 2003).
In conclusion, I detected strong c-Fos induction after RFS in several regions that have been related to reward circuitries, including the LH, AcbSh, amygdaloid nuclei and thalamic structures. There was a high overlap of the distribution of c-Fos induction in FD animals with MCHR-1 IR within these regions. This supports that the central MCH system is involved in activation of reward and motivational pathways especially under food-restricted conditions.

3.4.5. Refeeding induces neurons of the neuroendocrine axis and of the autonomous system
I determined strongest expression of c-Fos in the SO and the PVN of the hypothalamus in FR animals. Initially, these regions have been studied for their influence in neuroendocrine effector pathways.
Satiety induces c-Fos in the nucleus tractus solitarius, an important relay station for autonomous afferent information. Finally, strongest and significant expression of c-Fos was detected in satied, food restricted animals within the nucleus of the solitary tract. This is supported by the observation, that within the Sol, gastric nutrient infusion causes c-Fos expression of neurons that are in contact with MCH IR (Zheng et al., 2005). In our study, Sol displays only weak MCHR-1 IR and of these, only single neurons display c-Fos IR. Other studies have suggested that MCH and MCHR-1 are present in the nodose ganglia, which suggest an additional site of action for the MCH system via vagal afferent neurons, that are interconnected with the Sol (Burdyga et al., 2006). Additionally, gastrointestinal peptides such as cholecystokinin (CCK) released from the gut or neural information from gastric tension, inhibit orexigenic neurons (Luckman, 2003), proposing an inhibitory pathway via vagal afferent neurons. This connectivity ultimately inhibit appetite, and perhaps inhibit orexigenic MCH neurons located within the LHA, as we could not detect any colocalisation for the c-Fos signal with MCH in the LH of both FD and FR rats.

In conclusion, I detected a strong degree of overlap of c-Fos expression with MCHR-1 IR under FD conditions not only in brain regions that have been shown to directly related to

hunger (hypothalamic regions), but related pathways that are interconnected with these structures such as the AcbSh, amydalaloid nuclei and the thalamic structures. Thus, these regions may be also relevant in the mediation of MCH orexigenic effects. Additionally, MCH seem to influence satiety related pathways, possibly through the hypothalamic neuroendocrine axis with the PVN and SO as neuroanatomical substrates.

3. Conclusions of the thesis

To summarize, my results further imply a role for MCHR-1 in the regulation of food intake and body weight, but MCHR-1 seems to be also involved in stress response, fluid hoemostasis, reproduction and feeding-related cues such as rewarding aspects. Thus, MCHR-1 seem to be an essential structure for the generation of MCH`s actions on these physiological and behavioural aspects.

Because of the high complexity of neuronal pathways leading ultimately to specific behaviour, the aim of this work was to further biochemically and functionally characterize the MCH system within the rat brain. Moreover, these efforts may identify brain structures that are targeted by MCH or MCHR-1 antagonist treatment, respectively.

One aspect was to further describe the central MCH system, its effects on energy hoemostasis and possible effects on related pathways within the hypothalamus. A great advantage of double-labelling fluorescence immunohistochemistry is the ability to identify the neurochemical properties of single neurons within brain tissue sections on a high resolution.

In the present study I used a selective MCHR-1 antibody as a tool to visualize the neurochemical properties of MCHR-1 immunoreactive neurons within the rat hypothalamus. Based on neuromorphological criteria, I demonstrated a possible interaction of the central MCH system with several other neuropeptidergic systems. As most of the known hypothalamic neuropeptides have characteristic physiological and behavioural properties mediated through CNS pathways, the present observation that MCHR-1 immunoreactivity co-localizes with orexigenic and anorexigenic neuropeptides within feeding-related hypothalamic nuclei underline the complex reciprocal communication between these neuropeptidergic pathways. It remains open, if MCH inhibit parallel, orexigenic pathways (such as the OxA or NPY pathways) or distinct anorexigenic pathways (such as the melanocortin pathway) by negative feedback mechanisms and with an involvement of MCHR-1. A possible detection of the interaction of orexigenic and anorexigenic pathways may be of relevance for the examination of synergistic anorexigenic effect of two anti-obesity compounds. This attempt may be

beneficial for long-term treatment modalities and possibly improve drug safety in a pharmaceutical intervention in massive obese patients.

Moreover, a here described colocalization of MCHR-1 with VP and CRH immunoreactive material in the PVN indicate MCHR-1 interaction with central regulatory pathways regulating fluid homeostasis and stress response. This observation is strengthened by here described *in vivo* data of acute MCH treatment or chronic MCHR-1 treatment on water intake, as determined after central MCH or compound B4 treatment. Given the observation that VP is a neuropeptide with antidiuretic and vasoconstrictive properties, both involved in the pathogenesis of hypertension, a special interest should be investigated on effects of MCHR-1 antagonists on the vasopressinergic tone. As one MCHR-1 antagonist was reported to compound-induce an adverse cardiovascular profile (Kym et al., 2005), it remains open if these effects are compound- or compound class- specific. Consequently, follow-up studies may address the influence of MCHR-1 antagonists on hypothalamic VP release with a special focus on bodies fluid- and electrolyte regulation. These observations may hint at putative, critical points for drug safety of MCHR-1 antagonists, and should be taken serious for ongoing *in vivo* studies using MCHR-1 antagonist.

A number of studies have implicated a role of the MCH system on stress response, possibly through the CRH system. Because food deprivation can be viewed as a stressor *per se*, it may be of relevance to minimize negative effects of the CRH system. Additionally, the CRH system has been reported as an important factor in mood and depression, and a number of behavioural studies have propose antidepressive and anxiolytic actions of MCHR-1 antagonist. Thus, MCHR-1 antagonists may evoke psychological well-beeing (antidepressive effects) during fasting episodes or binge eating episodes. It remains open, on which level of the stress axis MCH acts (hypothalamus, pituitary or both), thus additional experiments are needed.

Since the isolation of MCH and the initial observation, that MCH induces aggregation of melanin granules in the skin of teleost fish, the notion of MCH `s physiological functions has greatly expanded. The prominent discovery in the field of MCH research was the identification of MCH`s effects in the regulation of energy homeostasis. In the recent years, numerous studies underlined the orexigenic actions of MCH and its functional MCHR-1 receptor subtype and as a consequence, a number of MCHR-1 antagonist have been characterised as potent and selective anti-obesity agents. *In vitro* pharmacology has been defined the here introduced MCHR-1 antagonist B4 as a selective and potent agent to inhibit MCH signalling on its

MCHR-1 receptor. In vivo studies have shown that the compound B4 is orally available and has potent effects to decrease food intake and body weight in rodents.

Patents of both selective and potent *in vitro* MCHR-1 antagonists by several pharmaceutical companies confirms that MCHR-1 is suitable biological target structure. Moreover, these compound class have been determined as anorexigenic, centrally acting substances *in vivo*. Although the efficacy of these drugs, especially compound B4 seem unconceivable in several rodent models and have been successfully transferred to rhesus monkeys (shown by Dr. Leo Thomas et al., in a poster presentation at the NAASO meeting, 2005, P2-224) it remains open if this MCHR-1 antagonist also shows these strong anorexigenic properties in human. In addition, one important issue will contribute to the direct comparison of efficacy and the safety profile of MCHR-1 antagonists with available anti-obesity drugs that are already on the market, such as the SSRI sibutramine (reductil) or the CB-1R antagonist rimonabant (accomplia).

The here presented *in vivo* data of MCH and MCHR-1 antagonist treatment underlines as proof of principle experiments, that pharmacological intervention on the central MCH system promotes changes in feeding and body weight. The overal results of the here evaluated immunohistochemical analysis of c-Fos was shown to serve as a suitable tool to identify neuronal activation patterns. As a consequence, further studies are worth to profile the specific neuronal activation pattern of compound B4 either to substantiate the identified SO and PVN as hypothalamic "hot spots" after compound B4 treatment.

Expression of the c-Fos gene is feasible to show "activated" neurons, however MCHR-1 antagonist treatment pharmacologically inhibit MCH signalling, a mayor part deals with the question, which neurons are activated by endogeneous MCH. The here used experimental design of RFS was not introduced to model any of the eating disorders, nor obesity, but RFS promotes a strong physiological, time-dependent, orexigenic stimulus. The observed eating pattern of RFS treated animals was shown to induce a distinct neuronal activation pattern in several hypothalamic regions, forebrain structures and subcortical regions including several amygdaloid and thalamic nuclei. As the observed activation pattern may imply for a general orexigenic stimulus, the involvement the MCH system in this stimulatory process was verified by the observation, that numerous activated neurons by food-deprivation showed MCHR-1 immunoreactivity, thus defined as MCH responsive neurons. These neurons were situated in brain regions that have been shown to be involved in feeding anticipatory behaviour (DMH, PVP), rewarding aspects of food (nucleus accumbens shell division) and emotional aspects (amydgaloid nuclei). Interestingly, in food-restricted, satiated animals, hypothalamic regions of neuroendocrine and autonomous effector pathways, namely the PVN, SO, and the Sol

anatomically situated in the brainstem , were heavily activated. In addition, PVN and SO neurons exhibited strong MCHR-1 expression, which implies MCHR-1 as part of the neuroendocrine axis.

Interestingly, non invasive, functional neuroimaging techniques such as functional magnet resonance tomography (fMRT) or positron emission tomography (PET) have been used to trace neuronal activation patterns in humans after feeding related stimuli (Taratanni and DelParigi 2003; Hinton et al., 2004). However, great disadvantages of these *in vivo* imaging techniques are high costs. In advance of these technologies, a similar experimental background such as a RFS experiment opens the possibility to convey results from animal experiments to humans studies. Functional brain mapping studies will further detect local neuronal activity after discrete stimuli, and aspect of examination may involve detection of activated brain structures under specific pathophysiological states (such as in hunger states). In the future, administration of MCHR-1 antagonists in preclinical studies may help to profile central activatory patterns and give insights into the mechanism of actions of anti-obesity agents.

Epidemiological data of public health care systems indicate that the prevalence of eating disorders and obesity are rising. Especially the increasing number of obese patients in the western world with severe comorbidities demonstrate that there is an urgent need for successful anti-obesity therapies. Hopefully, future pharmacological therapies will support obese individuals on their hard way to loose weight and faciliate obese to change behaviour for a long-therm maintainance of bodies energy homeostasis.

Historically, the “ponderostat” was hypothesid to act as a ligament between external and internal factors. It is unconceivable, that complex feedback loops of a number of factors keep energy homeostasis stable within the body and as a consequence, capture body weight in an individual, physiological range. My work points out, that the central MCH system seem to be an essential part of energy homeostasis.

The central MCH system may reflect a ponderostat *per se* influencing feeding, fluid and other essential physiological responses. Therefore it should be kept in mind that this system may be especially sensitive in terms of energy depletion and may modulate other hormonal systems through its diverse connectivities in response to its pharmacological inhibition.

5. References

Abbott, C.R., Kennedy, A.R., Wren, A.M., Rossi, M., Murphy, K.G., Seal, L.J., Todd, J.F., Ghatei, M.A., Small, C.J., Bloom, S.R., 2003. Identification of hypothalamic nuclei involved in the orexigenic effect of melanin-concentrating hormone. Endocrinology 144, 3943-3949.

Adamantidis, A., Thomas, E., Foidart, A., Tyhon, A., Coumans, B., Minet, A., Tirelli, E., Seutin, V., Grisar, T., Lakaye, B., 2005. Disrupting the melanin-concentrating hormone receptor 1 in mice leads to cognitive deficits and alterations of NMDA receptor function. Eur.J.Neurosci. 21, 2837-2844.

Aguilera, G., Rabadan-Diehl, C., 2000. Vasopressinergic regulation of the hypothalamic-pituitary-adrenal axis: implications for stress adaptation. Regul.Pept. 96, 23-29.

Alberti, K.G., Zimmet, P., 2006. The metabolic syndrome: time to reflect. Curr.Diab.Rep. 6, 259-261.

Alon, T., Friedman, J.M., 2006. Late-onset leanness in mice with targeted ablation of melanin concentrating hormone neurons. J.N

Ahima RS, Flier JS,. 2000. Leptin Annu.Rev. Physiol. 62:413-437

Anand BK, Brobeck JR, 1951. Hypothalamic control of food intake in rats and cats. Yale J Biol Med 24:123-146.

Angeles-Castellanos, M., guilar-Roblero, R., Escobar, C., 2004. c-Fos expression in hypothalamic nuclei of food-entrained rats. Am.J.Physiol Regul.Integr.Comp Physiol 286, R158-R165.

Angeles-Castellanos, M., Mendoza, J., Escobar, C., 2006. Restricted feeding schedules phase shift daily rhythms of c-Fos and protein Per1 immunoreactivity in corticolimbic regions in rats. Neuroscience 26, 389-397.

Antin J, Gibbs J, Holt J, Young RC, Smith GP,. 1975. Cholecystokinin elecits the complete inhibition of satiety in rats. Physiol Psychol 69: 784-790.

Alvarenga, T.A., Andersen, M.L., Papale, L.A., Antunes, I.B., Tufik, S., 2005. Influence of long-term food restriction on sleep pattern in male rats. Brain Res. 1057, 49-56.

An, S., Cutler, G., Zhao, J.J., Huang, S.G., Tian, H., Li, W., Liang, L., Rich, M., Bakleh, A., Du, J., Chen, J.L., Dai, K., 2001. Identification and characterization of a melanin-concentrating hormone receptor. Proc.Natl.Acad.Sci.U.S.A 98, 7576-7581.

Arienzo, R., Clark, D.E., Cramp, S., Daly, S., Dyke, H.J., Lockey, P., Norman, D., Roach, A.G., Stuttle, K., Tomlinson, M., Wong, M., Wren, S.P., 2004. Structure-activity relationships of a novel series of melanin-concentrating hormone (MCH) receptor antagonists. Bioorg.Med.Chem.Lett. 14, 4099-4102.

Arienzo, R., Cramp, S., Dyke, H.J., Lockey, P.M., Norman, D., Roach, A.G., Smith, P., Wong, M., Wren, S.P., 2006. Quinazoline and benzimidazole MCH-1R antagonists. Bioorg.Med.Chem.Lett.

Arima, H., Aguilera, G., 2000. Vasopressin and oxytocin neurones of hypothalamic supraoptic and paraventricular nuclei co-express mRNA for Type-1 and Type-2 corticotropin-releasing hormone receptors. J.Neuroendocrinol. 12, 833-842.

Armellini, F., Zamboni, M., Robbi, R., Todesco, T., Rigo, L., Bergamo-Andreis, I.A., Bosello, O., 1993. Total and intra-abdominal fat measurements by ultrasound and computerized tomography. Int.J.Obes.Relat Metab Disord. 17, 209-214.

Astrand, A., Bohlooly, Y., Larsdotter, S., Mahlapuu, M., Andersen, H., Tornell, J., Ohlsson, C., Snaith, M., Morgan, D.G., 2004. Mice lacking melanin-concentrating hormone receptor 1 demonstrate increased heart rate associated with altered autonomic activity. Am.J.Physiol Regul.Integr.Comp Physiol 287, R749-R758.

Backberg, M., Hervieu, G., Wilson, S., Meister, B., 2002. Orexin receptor-1 (OX-R1) immunoreactivity in chemically identified neurons of the hypothalamus: focus on orexin targets involved in control of food and water intake. Eur.J.Neurosci. 15, 315-328.

Baker, B.I., Bird, D.J., 2002. Neuronal organization of the melanin-concentrating hormone system in primitive actinopterygians: evolutionary changes leading to teleosts. J.Comp Neurol. 442, 99-114.

Barber, L.D., Baker, B.I., Penny, J.C., Eberle, A.N., 1987. Melanin concentrating hormone inhibits the release of alpha MSH from teleost pituitary glands. Gen.Comp Endocrinol. 65, 79-86.

Barsh, Schwartz 2000

Bates, S.H., Myers, M.G., Jr., 2003. The role of leptin receptor signaling in feeding and neuroendocrine function. Trends Endocrinol.Metab 14, 447-452.

Bayer, L., Poncet, F., Fellmann, D., Griffond, B., 1999. Melanin-concentrating hormone expression in slice cultures of rat hypothalamus is not affected by 2-deoxyglucose. Neurosci.Lett. 267, 77-80.

Bednarek, M.A., MacNeil, T., Kalyani, R.N., Tang, R., Van der Ploeg, L.H., Weinberg, D.H., 2001. Selective, high affinity peptide antagonists of alpha-melanotropin action at human melanocortin receptor 4: their synthesis and biological evaluation in vitro. J.Med.Chem. 44, 3665-3672.

Bednarek, M.A., Feighner, S.D., Hreniuk, D.L., Palyha, O.C., Morin, N.R., Sadowski, S.J., MacNeil, D.J., Howard, A.D., Van der Ploeg, L.H., 2001. Short segment of human melanin-concentrating hormone that is sufficient for full activation of human melanin-concentrating hormone receptors 1 and 2. Biochemistry 40, 9379-9386.

Bednarek, M.A., Hreniuk, D.L., Tan, C., Palyha, O.C., MacNeil, D.J., Van der Ploeg, L.H., Howard, A.D., Feighner, S.D., 2002. Synthesis and biological evaluation in vitro of selective, high affinity peptide antagonists of human melanin-concentrating hormone action at human melanin-concentrating hormone receptor 1. Biochemistry 41, 6383-6390.
Bell, C.G., Meyre, D., Samson, C., Boyle, C., Lecoeur, C., Tauber, M., Jouret, B., Jaquet, D., Levy-Marchal, C., Charles, M.A., Weill, J., Gibson, F., Mein, C.A., Froguel, P., Walley, A.J., 2005. Association of melanin-concentrating hormone receptor 1 5' polymorphism with early-onset extreme obesity. Diabetes 54, 3049-3055.
Bell, C.G., Walley, A.J., Froguel, P., 2005. The genetics of human obesity. Nat.Rev.Genet. 6, 221-234.
Bernardis, L.L., Bellinger, L.L., 1993. The lateral hypothalamic area revisited: neuroanatomy, body weight regulation, neuroendocrinology and metabolism. Neurosci.Biobehav.Rev. 17, 141-193.
Berridge, K.C., Robinson, T.E., 1998. What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience? Brain Res.Brain Res.Rev. 28, 309-369.
Bell CG et al., 2005. The genetics of human obesity. Nat Rev. 6:221-234.
Benoit 2005
Bertile F, Oudart H, Maho YL, Raclot T., 2003. Recombinant leptin in the hypothalamic response to late fasing. Biochem Biophys Res Commun,310(3): 949-955.
Berthoud, H.R., 2002. Multiple neural systems controlling food intake and body weight. Neurosci.Biobehav.Rev. 26, 393-428.
Bittencourt, J.C., Presse, F., Arias, C., Peto, C., Vaughan, J., Nahon, J.L., Vale, W., Sawchenko, P.E., 1992. The melanin-concentrating hormone system of the rat brain: an immuno- and hybridization histochemical characterization. J.Comp Neurol. 319, 218-245.
Bittencourt, J.C., Sawchenko, P.E., 2000. Do centrally administered neuropeptides access cognate receptors?: an analysis in the central corticotropin-releasing factor system. J.Neurosci. 20, 1142-1156.
Björntorp 1984
Bjursell, M., Egecioglu, E., Gerdin, A.K., Svensson, L., Oscarsson, J., Morgan, D., Snaith, M., Tornell, J., Bohlooly, Y., 2005. Importance of melanin-concentrating hormone receptor for the acute effects of ghrelin. Biochem.Biophys.Res.Commun. 326, 759-765.
Bjursell, M., Gerdin, A.K., Ploj, K., Svensson, D., Svensson, L., Oscarsson, J., Snaith, M., Tornell, J., Bohlooly, Y., 2006. Melanin-concentrating hormone receptor 1 deficiency increases insulin sensitivity in obese leptin-deficient mice without affecting body weight. Diabetes 55, 725-733.
Bluet-Pajot, M.T., Presse, F., Voko, Z., Hoeger, C., Mounier, F., Epelbaum, J., Nahon, J.L., 1995. Neuropeptide-E-I antagonizes the action of melanin-concentrating hormone on stress-induced release of adrenocorticotropin in the rat. J.Neuroendocrinol. 7, 297-303.
Blundell, J.E., 1976. Letter: Strategies and tactics in the use of anti-obesity drugs. Lancet 1, 804.
Blundell, J.E., McArthur, R.A., 1979. Investigation of food consumption using a dietary self-selection procedure: effects of pharmacological manipulation and feeding schedules [proceedings]. Br.J.Pharmacol. 67, 436P-437P.
Bockaert, J., Pin, J.P., 1999. Molecular tinkering of G protein-coupled receptors: an evolutionary success. EMBO J. 18, 1723-1729.
Bohlooly, Y., Mahlapuu, M., Andersen, H., Astrand, A., Hjorth, S., Svensson, L., Tornell, J., Snaith, M.R., Morgan, D.G., Ohlsson, C., 2004. Osteoporosis in MCHR1-deficient mice. Biochem.Biophys.Res.Commun. 318, 964-969.
Borowsky, B., Durkin, M.M., Ogozalek, K., Marzabadi, M.R., DeLeon, J., Lagu, B., Heurich, R., Lichtblau, H., Shaposhnik, Z., Daniewska, I., Blackburn, T.P., Branchek, T.A., Gerald, C., Vaysse, P.J., Forray, C., 2002. Antidepressant, anxiolytic and anorectic effects of a melanin-concentrating hormone-1 receptor antagonist. Nat.Med. 8, 825-830.
Boutin, J.A., Suply, T., Audinot, V., Rodriguez, M., Beauverger, P., Nicolas, J.P., Galizzi, J.P., Fauchere, J.L., 2002. Melanin-concentrating hormone and its receptors: state of the art. Can.J.Physiol Pharmacol. 80, 388-395.
Bradley, P., 1978. The ponderostat and a physiological model of obesity. Am.J.Clin.Nutr. 31, 1975-1977.
Bradley, R.L., Kokkotou, E.G., Maratos-Flier, E., Cheatham, B., 2000. Melanin-concentrating hormone regulates leptin synthesis and secretion in rat adipocytes. Diabetes 49, 1073-1077.
Bray, G.A., 2006. Obesity: the disease. J.Med.Chem. 49, 4001-4007.
Breton 1993
Brobeck JR, 1946 Mechanism of the development of obesity in animals with hypothalamic lesions. Physiol Rev. 26:541-559.
Broberger, C., De, L.L., Sutcliffe, J.G., Hokfelt, T., 1998. Hypocretin/orexin- and melanin-concentrating hormone-expressing cells form distinct populations in the rodent lateral hypothalamus: relationship to the neuropeptide Y and agouti gene-related protein systems. J.Comp Neurol. 402, 460-474.

Broberger, C., 1999. Hypothalamic cocaine- and amphetamine-regulated transcript (CART) neurons: histochemical relationship to thyrotropin-releasing hormone, melanin-concentrating hormone, orexin/hypocretin and neuropeptide Y. Brain Res. 848, 101-113.

Broberger, C., 2005. Brain regulation of food intake and appetite: molecules and networks. J.Intern.Med. 258, 301-327.
Burdyga, G., Varro, A., Dimaline, R., Thompson, D.G., Dockray, G.J., 2006. Feeding-dependent depression of melanin-concentrating hormone and melanin-concentrating hormone receptor-1 expression in vagal afferent neurones. Neuroscience 137, 1405-1415.
Cabanac M., 2006. Adjustable set point: to honor Harold T. Hammel. J Appl. Physiol 100: 1338-1346.
Cabanac, M., Duclaux, R., Spector, N.H., 1971. Sensory feedback in regulation of body weight: is there a ponderostat? Nature 229, 125-127.
Cabanac, M., 2001. Regulation and the ponderostat. Int.J.Obes.Relat Metab Disord. 25 Suppl 5, S7-12.
Carai, M.A., Colombo, G., Maccioni, P., Gessa, G.L., 2006. Efficacy of rimonabant and other cannabinoid CB1 receptor antagonists in reducing food intake and body weight: preclinical and clinical data. CNS.Drug Rev. 12, 91-99.
Carpenter, A.J., Al-Barazanji, K.A., Barvian, K.K., Bishop, M.J., Britt, C.S., Cooper, J.P., Goetz, A.S., Grizzle, M.K., Hertzog, D.L., Ignar, D.M., Morgan, R.O., Peckham, G.E., Speake, J.D., Swain, W.R., 2006. Novel benzimidazole-based MCH R1 antagonists. Bioorg.Med.Chem.Lett. 16, 4994-5000.
Chaillou, E., Baumont, R., Tramu, G., Tillet, Y., 2000. Effect of feeding on Fos protein expression in sheep hypothalamus with special reference to the supraoptic and paraventricular nuclei: an immunohistochemical study. Eur.J.Neurosci. 12, 4515-4524.
Chaki, S., Yamaguchi, J., Yamada, H., Thomsen, W., Tran, T.A., Semple, G., Sekiguchi, Y., 2005. ATC0175: an orally active melanin-concentrating hormone receptor 1 antagonist for the potential treatment of depression and anxiety. CNS.Drug Rev. 11, 341-352.
Chaki, S., Funakoshi, T., Hirota-Okuno, S., Nishiguchi, M., Shimazaki, T., Iijima, M., Grottick, A.J., Kanuma, K., Omodera, K., Sekiguchi, Y., Okuyama, S., Tran, T.A., Semple, G., Thomsen, W., 2005. Anxiolytic- and antidepressant-like profile of ATC0065 and ATC0175: nonpeptidic and orally active melanin-concentrating hormone receptor 1 antagonists. J.Pharmacol.Exp.Ther. 313, 831-839.
Chambers, J., Ames, R.S., Bergsma, D., Muir, A., Fitzgerald, L.R., Hervieu, G., Dytko, G.M., Foley, J.J., Martin, J., Liu, W.S., Park, J., Ellis, C., Ganguly, S., Konchar, S., Cluderay, J., Leslie, R., Wilson, S., Sarau, H.M., 1999. Melanin-concentrating hormone is the cognate ligand for the orphan G-protein-coupled receptor SLC-1. N
Chaffer CL, Morris MJ,. 2000. The feeding response to Melanin-Concentrating Hormone is attenuated by Antagonism of the NPY Y1-Receptor in the rat. Endocrinology 143(1):191-197.
Chaudri O, Small C, Bloom S,. 2006. Gastrointestinal hormones regulating appetite.
Cheetham 2004
Chen H et al., (1996), Evidence that the diatetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. Cell 84:491-495ature 400, 261-265.
Chen, Y., Hu, C., Hsu, C.K., Zhang, Q., Bi, C., Asnicar, M., Hsiung, H.M., Fox, N., Slieker, L.J., Yang, D.D., Heiman, M.L., Shi, Y., 2002. Targeted disruption of the melanin-concentrating hormone receptor-1 results in hyperphagia and resistance to diet-induced obesity. Endocrinology 143, 2469-2477.
Chou, T.C., Scammell, T.E., Gooley, J.J., Gaus, S.E., Saper, C.B., Lu, J., 2003. Critical role of dorsomedial hypothalamic nucleus in a wide range of behavioral circadian rhythms. J.Neurosci. 23, 10691-10702.
Clark, D.E., Higgs, C., Wren, S.P., Dyke, H.J., Wong, M., Norman, D., Lockey, P.M., Roach, A.G., 2004. A virtual screening approach to finding novel and potent antagonists at the melanin-concentrating hormone 1 receptor. J.Med.Chem. 47, 3962-3971.
Clegg, D.J., Air, E.L., Benoit, S.C., Sakai, R.S., Seeley, R.J., Woods, S.C., 2003. Intraventricular melanin-concentrating hormone stimulates water intake independent of food intake. Am.J.Physiol Regul.Integr.Comp Physiol 284, R494-R499.
Cohen P et al., 2005. Selective deletion of leptin receptor in neurons leads to obesity. J Clin Invest 108:1113-1121.
Collins, C.A., Kym, P.R., 2003. Prospects for obesity treatment: MCH receptor antagonists. Curr.Opin.Investig.Drugs 4, 386-394.
Cone, R.D., 2005. Anatomy and regulation of the central melanocortin system. Nat.Neurosci. 8, 571-578.
Cullinan, W.E., Herman, J.P., Battaglia, D.F., Akil, H., Watson, S.J., 1995. Pattern and time course of immediate early gene expression in rat brain following acute stress. Neuroscience 64, 477-505.
Cummings, D.E., Schwartz, M.W., 2003. Genetics and pathophysiology of human obesity. Annu.Rev.Med. 54, 453-471.
Cvetkovic, V., Poncet, F., Fellmann, D., Griffond, B., Risold, P.Y., 2003. Diencephalic neurons producing melanin-concentrating hormone are influenced by local and multiple extra-hypothalamic tachykininergic projections through the neurokinin 3 receptor. Neuroscience 119, 1113-1145.
Cvetkovic, V., Brischoux, F., Griffond, B., Bernard, G., Jacquemard, C., Fellmann, D., Risold, P.Y., 2003.

Evidence of melanin-concentrating hormone-containing neurons supplying both cortical and neuroendocrine projections. Neuroscience 116, 31-35.
Dai, J., Van, D., V, Swaab, D.F., Buijs, R.M., 1998. Postmortem anterograde tracing of intrahypothalamic projections of the human dorsomedial nucleus of the hypothalamus. J.Comp Neurol. 401, 16-33.
Daniels SR, Arnett DK, Eckel RH, Gidding SS et al., 2005. Overweight in Children and Adolescents. Circulation 111:1999-2012.
De Kloet, E.R., 2003. Hormones, brain and stress. Endocr.Regul. 37, 51-68.
De, L.L., Kilduff, T.S., Peyron, C., Gao, X., Foye, P.E., Danielson, P.E., Fukuhara, C., Battenberg, E.L., Gautvik, V.T., Bartlett, F.S., Frankel, W.N., van den Pol, A.N., Bloom, F.E., Gautvik, K.M., Sutcliffe, J.G., 1998. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc.Natl.Acad.Sci.U.S.A 95, 322-327.
De Wardener HE., 2001. The hypothalamus and hypertension. Physiol Rev. 81(4):1599-658.
Della-Zuana, O., Presse, F., Ortola, C., Duhault, J., Nahon, J.L., Levens, N., 2002. Acute and chronic administration of melanin-concentrating hormone enhances food intake and body weight in Wistar and Sprague-Dawley rats. Int.J.Obes.Relat Metab Disord. 26, 1289-1295.
Deriaz, O., Fournier, G., Tremblay, A., Despres, J.P., Bouchard, C., 1992. Lean-body-mass composition and resting energy expenditure before and after long-term overfeeding. Am.J.Clin.Nutr. 56, 840-847.
Del Parigi A, Gaultier JF, Chen K et al., 2002. Neuroimaging and obesity: mapping the brain reponses to hunger and satiety in humans using positron emission tomography. Ann N Y Acad Sci 967:389-97.
Deurveilher, S., Semba, K., 2005. Indirect projections from the suprachiasmatic nucleus to major arousal-promoting cell groups in rat: implications for the circadian control of behavioural state. Neuroscience 130, 165-183.
Dhillo, W.S., Bloom, S.R., 2001. Hypothalamic peptides as drug targets for obesity. Curr.Opin.Pharmacol. 1, 651-655.
Diaz-Munoz, M., Vazquez-Martinez, O., guilar-Roblero, R., Escobar, C., 2000. Anticipatory changes in liver metabolism and entrainment of insulin, glucagon, and corticosterone in food-restricted rats. Am.J.Physiol Regul.Integr.Comp Physiol 279, R2048-R2056.
Dorton AM., 2005. The pituitary gland: embryology, physiology and pathophysiology.
Drazen, D.L., Woods, S.C., 2003. Peripheral signals in the control of satiety and hunger. Curr.Opin.Clin.Nutr.Metab Care 6, 621-629.
Drewnowski, A., 1999. Sweetness, appetite, and energy intake: physiological aspects. World Rev.Nutr.Diet. 85, 64-76.
Drozdz, R., Eberle, A.N., 1995. Binding sites for melanin-concentrating hormone (MCH) in brain synaptosomes and membranes from peripheral tissues identified with highly tritiated MCH. J.Recept.Signal.Transduct.Res. 15, 487-502.
Eberle, A.N., Mild, G., Schlumberger, S., Drozdz, R., Hintermann, E., Zumsteg, U., 2004. Expression and characterization of melanin-concentrating hormone receptors on mammalian cell lines. Peptides 25, 1585-1595.
Eckel 2003
Edwards CM, Abusnana S, Sunter D, Murphy KG, Ghatei MA, Bloom SR., 1999. The effect of the orexins on food intake: comparison with neuropeptide Y, melanin-concentrating hormone and Galanin. J Endocrinol. 160 (3): R7-12.
Elias, C.F., Saper, C.B., Maratos-Flier, E., Tritos, N.A., Lee, C., Kelly, J., Tatro, J.B., Hoffman, G.E., Ollmann, M.M., Barsh, G.S., Sakurai, T., Yanagisawa, M., Elmquist, J.K., 1998. Chemically defined projections linking the mediobasal hypothalamus and the lateral hypothalamic area. J.Comp Neurol. 402, 442-459.
Elias, C.F., Aschkenasi, C., Lee, C., Kelly, J., Ahima, R.S., Bjorbaek, C., Flier, J.S., Saper, C.B., Elmquist, J.K., 1999. Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. Neuron 23, 775-786.
Elias, C.F., Lee, C.E., Kelly, J.F., Ahima, R.S., Kuhar, M., Saper, C.B., Elmquist, J.K., 2001. Characterization of CART neurons in the rat and human hypothalamus. J.Comp Neurol. 432, 1-19.
Elliott, J.C., Harrold, J.A., Brodin, P., Enquist, K., Backman, A., Bystrom, M., Lindgren, K., King, P., Williams, G., 2004. Increases in melanin-concentrating hormone and MCH receptor levels in the hypothalamus of dietary-obese rats. Brain Res.Mol.Brain Res. 128, 150-159.
Elmquist, J.K., Maratos-Flier, E., Saper, C.B., Flier, J.S., 1998. Unraveling the central nervous system pathways underlying responses to leptin. Nat.Neurosci. 1, 445-450.
Elmquist, J.K., 2001. Hypothalamic pathways underlying the endocrine, autonomic, and behavioral effects of leptin. Int.J.Obes.Relat Metab Disord. 25 Suppl 5, S78-S82.
Elmquist, J.K., Coppari, R., Balthasar, N., Ichinose, M., Lowell, B.B., 2005. Identifying hypothalamic pathways controlling food intake, body weight, and glucose homeostasis. J.Comp Neurol. 493, 63-71.
Escobar, C., az-Munoz, M., Encinas, F., guilar-Roblero, R., 1998. Persistence of metabolic rhythmicity during fasting and its entrainment by restricted feeding schedules in rats. Am.J.Physiol 274, R1309-R1316.
Faintuch, J., Soeters, P.B., Osmo, H.G., 2006. Nutritional and metabolic abnormalities in pre-AIDS HIV infection. Nutrition 22, 683-690.
Felson, D.T., Anderson, J.J., Naimark, A., Walker, A.M., Meenan, R.F., 1988. Obesity and knee osteoarthritis.

The Framingham Study. Ann.Intern.Med. 109, 18-24.
Flegal, K.M., Carroll, M.D., Ogden, C.L., Johnson, C.L., 2002. Prevalence and trends in obesity among US adults, 1999-2000. JAMA 288, 1723-1727.
Ford, L.E., 1984. Some consequences of body size. Am.J.Physiol 247, H495-H507.
Forray, C., 2003. The MCH receptor family: feeding brain disorders? Curr.Opin.Pharmacol. 3, 85-89.
Frank GK, Bailer UF, Shannan H, Wagner, Wagner A, Kaye WH (2004) Neuroimaging studies in Eating Disorders. CNS Spectrums 9(7):539-548.
Frederich, R.C., Hamann, A., Anderson, S., Lollmann, B., Lowell, B.B., Flier, J.S., 1995. Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. Nat.Med. 1, 1311-1314.
Friedman, J.M., Halaas, J.L., 1998. Leptin and the regulation of body weight in mammals. Nature 395, 763-770.
Friedman, J.M., 2004. Modern science versus the stigma of obesity. Nat.Med. 10, 563-569.
Fujisawa 2003
Furudono Y, Ando C, Yamamoto C, Kobashi M, Yamamoto T., (2006).Involvement of specific orexigenic neuropeptides in sweetener-induced overconsumption in rats. Behav Brain Res 175(2): 241-248.
Gao, X.B., van den Pol, A.N., 2001. Melanin concentrating hormone depresses synaptic activity of glutamate and GABA neurons from rat lateral hypothalamus. J.Physiol 533, 237-252.
Gaultier JF et al., 2000. Differential brain responses to satiation in obese and lean men. Diabetes 49:838-846.
Gelfand, E.V., Cannon, C.P., 2006. Rimonabant: a selective blocker of the cannabinoid CB1 receptors for the management of obesity, smoking cessation and cardiometabolic risk factors. Expert.Opin.Investig.Drugs 15, 307-315.
Georgescu, D., Sears, R.M., Hommel, J.D., Barrot, M., Bolanos, C.A., Marsh, D.J., Bednarek, M.A., Bibb, J.A., Maratos-Flier, E., Nestler, E.J., DiLeone, R.J., 2005. The hypothalamic neuropeptide melanin-concentrating hormone acts in the nucleus accumbens to modulate feeding behavior and forced-swim performance. J.Neurosci. 25, 2933-2940.
Gibbs, J., Young, R.C., Smith, G.P., 1973. Cholecystokinin decreases food intake in rats. J.Comp Physiol Psychol. 84, 488-495.
Gibson, W.T., Pissios, P., Trombly, D.J., Luan, J., Keogh, J., Wareham, N.J., Maratos-Flier, E., O'Rahilly, S., Farooqi, I.S., 2004. Melanin-concentrating hormone receptor mutations and human obesity: functional analysis. Obes.Res. 12, 743-749.
Gil-Campos, M., Aguilera, C.M., Canete, R., Gil, A., 2006. Ghrelin: a hormone regulating food intake and energy homeostasis. Br.J.Nutr. 96, 201-226.
Gomori, A., Ishihara, A., Ito, M., Mashiko, S., Matsushita, H., Yumoto, M., Ito, M., Tanaka, T., Tokita, S., Moriya, M., Iwaasa, H., Kanatani, A., 2003. Chronic intracerebroventricular infusion of MCH causes obesity in mice. Melanin-concentrating hormone. Am.J.Physiol Endocrinol.Metab 284, E583-E588.
Gonzalez, M.I., Baker, B.I., Wilson, C.A., 1997. Stimulatory effect of melanin-concentrating hormone on luteinising hormone release. Neuroendocrinology 66, 254-262.
Goldsmith SR, 1987. Vasopression as vasopressor. Am J Med 1987;82(6):1213-9.
Gooley, J.J., Schomer, A., Saper, C.B., 2006. The dorsomedial hypothalamic nucleus is critical for the expression of food-entrainable circadian rhythms. Nat.Neurosci. 9, 398-407.
Gottschlich, **M.M**., Mayes, T., Khoury, J.C., Warden, G.D., 1993. Significance of obesity on nutritional, immunologic, hormonal, and clinical outcome parameters in burns. J.Am.Diet.Assoc. 93, 1261-1268.
Grassi-Zucconi, G., Menegazzi, M., De Prati, A.C., Bassetti, A., Montagnese, P., Mandile, P., Cosi, C., Bentivoglio, M., 1993. c-fos mRNA is spontaneously induced in the rat brain during the activity period of the circadian cycle. Eur.J.Neurosci. 5, 1071-1078.
Greenway, F.L., Caruso, M.K., 2005. Safety of obesity drugs. Expert.Opin.Drug Saf 4, 1083-1095.
Griffond, B., Baker, B.I., 2002. Cell and molecular cell biology of melanin-concentrating hormone. Int.Rev.Cytol. 213, 233-277.
Grill, H.J., Schwartz, M.W., Kaplan, J.M., Foxhall, J.S., Breininger, J., Baskin, D.G., 2002. Evidence that the caudal brainstem is a target for the inhibitory effect of leptin on food intake. Endocrinology 143, 239-246.
Grill HJ, Norgren R (1978). Chronically decerebrate rats demonstrate satiation but not bait shyness. Science 201:267-269.:611-614.
Grodstein, F., Goldman, M.B., Cramer, D.W., 1994. Body mass index and ovulatory infertility. Epidemiology 5, 247-250.
Grundy SM (2006). Drug Therapy of the Metabolic Syndrome: minimizing the emerging crisis in polypharmacy. Nat Rev Drug Discov. 5(4):295-309.
Guan, J.L., Uehara, K., Lu, S., Wang, Q.P., Funahashi, H., Sakurai, T., Yanagizawa, M., Shioda, S., 2002. Reciprocal synaptic relationships between orexin- and melanin-concentrating hormone-containing neurons in the rat lateral hypothalamus: a novel circuit implicated in feeding regulation. Int.J.Obes.Relat Metab Disord. 26, 1523-1532.
Gutkowska, J., ntunes-Rodrigues, J., McCann, S.M., 1997. Atrial natriuretic peptide in brain and pituitary gland. Physiol Rev. 77, 465-515.
Halford, J.C., 2006. Obesity drugs in clinical development. Curr.Opin.Investig.Drugs 7, 312-318.

Halford, J.C., 2006. Pharmacotherapy for obesity. Appetite 46, 6-10.
Handlon, A.L., Zhou, H., 2006. Melanin-concentrating hormone-1 receptor antagonists for the treatment of obesity. J.Med.Chem. 49, 4017-4022.
Hardy JD., 1969. Regulation of energy balance. Nature 223:629-631.
Harrold, J.A., Williams, G., 2003. The cannabinoid system: a role in both the homeostatic and hedonic control of eating? Br.J.Nutr. 90, 729-734.
Harthoorn, L.F., Sane, A., Nethe, M., Van Heerikhuize, J.J., 2005. Multi-transcriptional profiling of melanin-concentrating hormone and Orexin-Containing Neurons. Cell and Mol Neurobiology 25(8): 1209-1223.
Hawes BE, Kil E, Green B, O`Neill K, Fried S, Graziano MP,. 2000. The Melanin-Concentrating Hormone Receptor Couples to Multiple G Proteins to Activate Diverse Intracellular Signaling Pathways. Endocrinology 141(12): 4524-4532.
Heckl S, Pipkorn R, Nägele T, Vogel U, Küker W, Voigt K., 2004. Molecular imaging: Bridging the gap between neuroradiology and neurohistology. Histol. Histopathol 19:651-668. ncentrating hormone and orexin-containing neurons. Cell Mol.Neurobiol. 25, 1209-1223.
Heinrichs, S.C., De Souza, E.B., 1999. Corticotropin-releasing factor antagonists, binding-protein and receptors: implications for central nervous system disorders. Baillieres Best.Pract.Res.Clin.Endocrinol.Metab 13, 541-554.
Herdegen, T., Leah, J.D., 1998. Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. Brain Res.Brain Res.Rev. 28, 370-490.
Herman, J.P., Ostrander, M.M., Mueller, N.K., Figueiredo, H., 2005. Limbic system mechanisms of stress regulation: hypothalamo-pituitary-adrenocortical axis. Prog.Neuropsychopharmacol.Biol.Psychiatry 29, 1201-1213.
Herrera, D.G., Robertson, H.A., 1996. Activation of c-fos in the brain. Prog.Neurobiol. 50, 83-107.
Hertzog, D.L., Al-Barazanji, K.A., Bigham, E.C., Bishop, M.J., Britt, C.S., Carlton, D.L., Cooper, J.P., Daniels, A.J., Garrido, D.M., Goetz, A.S., Grizzle, M.K., Guo, Y.C., Handlon, A.L., Ignar, D.M., Morgan, R.O., Peat, A.J., Tavares, F.X., Zhou, H., 2006. The discovery and optimization of pyrimidinone-containing MCH R1 antagonists. Bioorg.Med.Chem.Lett. 16, 4723-4727.
Herve C, Fellmann D,. 1997. Changes in rat-Melanin Concentrating Hormone and Dynorphin messenger Ribonucleic acids induced by food deprivation. Neuropeptides 31(3): 237-42.
Hervieu GJ, Volant K, Grishina O, Descroix-Vagne M, Nahon JL., 1996. Similarities in cellular expression and functions of melanin-concentrating hormone and atrial natrizretic factor in the rat digestive tract. Endocrinology 137: 561-571.
Hervieu, G.J, 2003. Melanin-concentrating hormone functions in the nervous system: food intake and stress. Expert.Opin.Ther.Targets. 7, 495-511.
Hervieu, G.J., Cluderay, J.E., Harrison, D., Meakin, J., Maycox, P., Nasir, S., Leslie, R.A., 2000. The distribution of the mRNA and protein products of the melanin-concentrating hormone (MCH) receptor gene, slc-1, in the central nervous system of the rat. Eur.J.Neurosci. 12, 1194-1216.
Hervieu, G.J., Cluderay, J.E., Harrison, D.C., Roberts, J.C., Leslie, R.A., 2001. Gene expression and protein distribution of the orexin-1 receptor in the rat brain and spinal cord. Neuroscience 103, 777-797.
Hervieu, G.J., 2006. Further insights into the neurobiology of melanin-concentrating hormone in energy and mood balances. Expert.Opin.Ther.Targets. 10, 211-229.
Hewson, A.K., Dickson, S.L., 2000. Systemic administration of ghrelin induces Fos and Egr-1 proteins in the hypothalamic arcuate nucleus of fasted and fed rats. J.Neuroendocrinol. 12, 1047-1049.
Heymsfield 1999
Herzog ED, Muglia LJ,. 2006. You are when you eat. Nature Neurosci. 9(3): 300-302.
Hill, J., Duckworth, M., Murdock, P., Rennie, G., Sabido-David, C., Ames, R.S., Szekeres, P., Wilson, S., Bergsma, D.J., Gloger, I.S., Levy, D.S., Chambers, J.K., Muir, A.I., 2001. Molecular cloning and functional characterization of MCH2, a novel human MCH receptor. J.Biol.Chem. 276, 20125-20129.
Hillebrand, J.J., de, W.D., Adan, R.A., 2002. Neuropeptides, food intake and body weight regulation: a hypothalamic focus. Peptides 23, 2283-2306.
Hoffman, G.E., Lee, W.S., Smith, M.S., Abbud, R., Roberts, M.M., Robinson, A.G., Verbalis, J.G., 1993. c-Fos and Fos-related antigens as markers for neuronal activity: perspectives from neuroendocrine systems. NIDA Res.Monogr 125, 117-133.
Horvath, T.L., 2005. The hardship of obesity: a soft-wired hypothalamus. Nat.Neurosci. 8, 561-565.
Huang, C.Q., Baker, T., Schwarz, D., Fan, J., Heise, C.E., Zhang, M., Goodfellow, V.S., Markison, S., Gogas, K.R., Chen, T., Wang, X.C., Zhu, Y.F., 2005. 1-(4-Amino-phenyl)-pyrrolidin-3-yl-amine and 6-(3-amino-pyrrolidin-1-yl)-pyridin-3-yl-amine derivatives as melanin-concentrating hormone receptor-1 antagonists. Bioorg.Med.Chem.Lett. 15, 3701-3706.
Hübschle, T., Thom, E., Watson, A., Roth, J., Klaus, S., Meyerhof, W., 2001. Leptin-induced nuclear translocation of STAT3 immunoreactivity in hypothalamic nuclei involved in body weight regulation. J.Neurosci. 21, 2413-2424.
Huda, M.S., Wilding, J.P., Pinkney, J.H., 2006. Gut peptides and the regulation of appetite. Obes.Rev. 7, 163-

182.
Inoue, K., Zorrilla, E.P., Tabarin, A., Valdez, G.R., Iwasaki, S., Kiriike, N., Koob, G.F., 2004. Reduction of anxiety after restricted feeding in the rat: implication for eating disorders. Biol.Psychiatry 55, 1075-1081.
Inzunza, O., Seron-Ferre, M.J., Bravo, H., Torrealba, F., 2000. Tuberomammillary nucleus activation anticipates feeding under a restricted schedule in rats. Neurosci.Lett. 293, 139-142.
Ioannides-Demos, L.L., Proietto, J., Tonkin, A.M., McNeil, J.J., 2006. Safety of drug therapies used for weight loss and treatment of obesity. Drug Saf 29, 277-302.
Ito, M., Gomori, A., Ishihara, A., Oda, Z., Mashiko, S., Matsushita, H., Yumoto, M., Ito, M., Sano, H., Tokita, S., Moriya, M., Iwaasa, H., Kanatani, A., 2003. Characterization of MCH-mediated obesity in mice. Am.J.Physiol Endocrinol.Metab 284, E940-E945.
Janssen, I., Katzmarzyk, P.T., Boyce, W.F., Vereecken, C., Mulvihill, C., Roberts, C., Currie, C., Pickett, W., 2005. Comparison of overweight and obesity prevalence in school-aged youth from 34 countries and their relationships with physical activity and dietary patterns. Obes.Rev. 6, 123-132.
Jequier, E., 2002. Leptin signaling, adiposity, and energy balance. Ann.N.Y.Acad.Sci. 967, 379-388.
Jezova, D., Bartanusz, V., Westergren, I., Johansson, B.B., Rivier, J., Vale, W., Rivier, C., 1992. Rat melanin-concentrating hormone stimulates adrenocorticotropin secretion: evidence for a site of action in brain regions protected by the blood-brain barrier. Endocrinology 130, 1024-1029.
Johnstone, L.E., Fong, T.M., Leng, G., 2006. Neuronal activation in the hypothalamus and brainstem during feeding in rats. Cell Metab 4, 313-321.
Jordan, J., Scholze, J., Matiba, B., Wirth, A., Hauner, H., Sharma, A.M., 2005. Influence of Sibutramine on blood pressure: evidence from placebo-controlled trials. Int.J.Obes.(Lond) 29, 509-516.
Kalra, S.P., Dube, M.G., Pu, S., Xu, B., Horvath, T.L., Kalra, P.S., 1999. Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. Endocr.Rev. 20, 68-100.
Kalra, S.P., Kalra, P.S., 2004. Overlapping and interactive pathways regulating appetite and craving. J.Addict.Dis. 23, 5-21.
Kannel, W.B., 1985. Lipids, diabetes, and coronary heart disease: insights from the Framingham Study. Am.Heart J. 110, 1100-1107.
Kanuma, K., Omodera, K., Nishiguchi, M., Funakoshi, T., Chaki, S., Semple, G., Tran, T.A., Kramer, B., Hsu, D., Casper, M., Thomsen, B., Sekiguchi, Y., 2005. Lead optimization of 4-(dimethylamino)quinazolines, potent and selective antagonists for the melanin-concentrating hormone receptor 1. Bioorg.Med.Chem.Lett. 15, 3853-3856.
Kanuma, K., Omodera, K., Nishiguchi, M., Funakoshi, T., Chaki, S., Semple, G., Tran, T.A., Kramer, B., Hsu, D., Casper, M., Thomsen, B., Beeley, N., Sekiguchi, Y., 2005. Discovery of 4-(dimethylamino)quinazolines as potent and selective antagonists for the melanin-concentrating hormone receptor 1. Bioorg.Med.Chem.Lett. 15, 2565-2569.
Kas, M.J., van den, B.R., Baars, A.M., Lubbers, M., Lesscher, H.M., Hillebrand, J.J., Schuller, A.G., Pintar, J.E., Spruijt, B.M., 2004. Mu-opioid receptor knockout mice show diminished food-anticipatory activity. Eur.J.Neurosci. 20, 1624-1632.
Kela, J., Salmi, P., Rimondini-Giorgini, R., Heilig, M., Wahlestedt, C., 2003. Behavioural analysis of melanin-concentrating hormone in rats: evidence for orexigenic and anxiolytic properties. Regul.Pept. 114, 109-114.
Kelley, AE,. 1999 Functional Specificity of ventral striatal compartments in appetive behaviour.
Kelley, A.E., 2004. Ventral striatal control of appetitive motivation: role in ingestive behavior and reward-related learning. Neurosci.Biobehav.Rev. 27, 765-776.
Kelley A.E. , Baldo BA, Pratt WE, 2005. A proposed hypothalamic-thalamic-striatal axis for the integration of energy balance, arousal, and food reward. J Comp. Neurol.493(1): 72-85.
Kennedy AR, Todd JF, Stanley SA, Abbott CR, Small CJ, Ghatei MA, Bloom SR., 2000. Melanin-concentrating hormone (MCH) suppresses thyroid stimulating hormone (TSH) release, in vivo and in vitro, via the hypothalamus and the pituitary. Endocrinol. 142 (7): 3265-3268.
Kennedy, G.C., 1953. The role of depot fat in the hypothalamic control of food intake in the rat. Proc.R.Soc.Lond B Biol.Sci. 140, 578-596.
Kim, N., Meyers, K.M., Mendez-Andino, J.L., Warshakoon, N.C., Ji, W., Wos, J.A., Colson, A., Mitchell, M.C., Davis, J.R., Pinney, B.B., Reizes, O., Hu, X.E., 2006. Identification of substituted 4-aminopiperidines and 3-aminopyrrolidines as potent MCH-R1 antagonists for the treatment of obesity. Bioorg.Med.Chem.Lett. 16, 5445-5450.
King, P.J., 2005. The hypothalamus and obesity. Curr.Drug Targets. 6, 225-240.
Kirouac, G.J., Ganguly, P.K., 1995. Topographical organization in the nucleus accumbens of afferents from the basolateral amygdala and efferents to the lateral hypothalamus. Neuroscience 67, 625-630.
Kishi, T., Elmquist, J.K., 2005. Body weight is regulated by the brain: a link between feeding and emotion. Mol.Psychiatry 10, 132-146.
Kishi, T., Aschkenasi, C.J., Choi, B.J., Lopez, M.E., Lee, C.E., Liu, H., Hollenberg, A.N., Friedman, J.M., Elmquist, J.K., 2005. Neuropeptide Y Y1 receptor mRNA in rodent brain: distribution and colocalization with melanocortin-4 receptor. J.Comp Neurol. 482, 217-243.

Kiss, A., Mikkelsen, J.D., 2005. Oxytocin--anatomy and functional assignments: a minireview. Endocr.Regul. 39, 97-105.
Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., Kangawa, K., 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402, 656-660.
Kokkotou, E., Mastaitis, J.W., Qu, D., Hoersch, D., Slieker, L., Bonter, K., Tritos, N.A., Maratos-Flier, E., 2000. Characterization of [Phe(13), Tyr(19)]-MCH analog binding activity to the MCH receptor. Neuropeptides 34, 240-247.
Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K (1999). Ghrelin is a growth hormone releasing peptide from the stomach. Nature 402:656-660.
Koltermann OG, Insel J, Sackow M, Olefsky M,. 1980. Mechanisms of insulin resistence in human obesity. J Clin Invest 65:1272-1284.
Konkle ATM, Bielajew C (2004) Tracing the Neuroanatomical Profiles of Reward Pathways with marker for neuronal activation. Review in the Neurosciences, 15:383-414.
Kokkotou G, Tritos NA, Mastaitis JW, Slieker L, Maratos-Flier E., 2001. Melanin-concentrating hormone receptor is a target of leptin action in the mouse brain. Endocrinol. 142(2): 680-686.
Kokkotou, E., Jeon, J.Y., Wang, X., Marino, F.E., Carlson, M., Trombly, D.J., Maratos-Flier, E., 2005. Mice with MCH ablation resist diet-induced obesity through strain-specific mechanisms. Am.J.Physiol Regul.Integr.Comp Physiol 289, R117-R124.
Kolakowski, L.F., Jr., Jung, B.P., Nguyen, T., Johnson, M.P., Lynch, K.R., Cheng, R., Heng, H.H., George, S.R., O'Dowd, B.F., 1996. Characterization of a human gene related to genes encoding somatostatin receptors. FEBS Lett. 398, 253-258.
Koob, G.F., Heinrichs, S.C., 1999. A role for corticotropin releasing factor and urocortin in behavioral responses to stressors. Brain Res. 848, 141-152.
Kopelman, P.G., 2000. Obesity as a medical problem. Nature 404, 635-643.
Kotz, C.M., 2006. Integration of feeding and spontaneous physical activity: role for orexin. Physiol Behav. 88, 294-301.
Kotz, C.M., Wang, C., Teske, J.A., Thorpe, A.J., Novak, C.M., Kiwaki, K., Levine, J.A., 2006. Orexin A mediation of time spent moving in rats: neural mechanisms. Neuroscience 142, 29-36.
Kovacs, K.J., 1998. c-Fos as a transcription factor: a stressful (re)view from a functional map. Neurochem.Int. 33, 287-297.
Kowalski, T.J., McBriar, M.D., 2004. Therapeutic potential of melanin-concentrating hormone-1 receptor antagonists for the treatment of obesity. Expert.Opin.Investig.Drugs 13, 1113-1122.
Kowalski, T.J., Spar, B.D., Weig, B., Farley, C., Cook, J., Ghibaudi, L., Fried, S., O'neill, K., Del Vecchio, R.A., McBriar, M., Guzik, H., Clader, J., Hawes, B.E., Hwa, J., 2006. Effects of a selective melanin-concentrating hormone 1 receptor antagonist on food intake and energy homeostasis in diet-induced obese mice. Eur.J.Pharmacol. 535, 182-191.
Kringelbach, M.L., 2004. Food for thought: hedonic experience beyond homeostasis in the human brain. Neuroscience 126, 807-819.
Kym RP, Iyengar R, Souers AJ, Lynch JK et al. 2005 Discovery and characterisation of Aminooiperidinecoumarin MCHR-1 Antagonists. J Med Chem, 48:5888-5891.
Laferrere B, Abraham C, Russell CD, Bowers CY (2005). Growth hormone releasing peptide 2, like ghrelin, increases food intake in healthy men. J Clin Endocrinol Metab 90
Lamounier-Zepter, V., Ehrhart-Bornstein, M., Bornstein, S.R., 2006. Metabolic syndrome and the endocrine stress system. Horm.Metab Res. 38, 437-441.
Landry, G.J., Simon, M.M., Webb, I.C., Mistlberger, R.E., 2006. Persistence of a behavioral food-anticipatory circadian rhythm following dorsomedial hypothalamic ablation in rats. Am.J.Physiol Regul.Integr.Comp Physiol 290, R1527-R1534.
Larsson, B., 1991. Obesity, fat distribution and cardiovascular disease. Int.J.Obes. 15 Suppl 2, 53-57.
Lechan, R.M., Qi, Y., Berrodin, T.J., Davis, K.D., Schwartz, H.L., Strait, K.A., Oppenheimer, J.H., Lazar, M.A., 1993. Immunocytochemical delineation of thyroid hormone receptor beta 2-like immunoreactivity in the rat central nervous system. Endocrinology 132, 2461-2469.
Lechan, R.M., Fekete, C., 2004. Feedback regulation of thyrotropin-releasing hormone (TRH): mechanisms for the non-thyroidal illness syndrome. J.Endocrinol.Invest 27, 105-119.
Lechan, R.M., Fekete, C., 2006. The TRH neuron: a hypothalamic integrator of energy metabolism. Prog.Brain Res. 153, 209-235.
Leibowitz, S.F., Wortley, K.E., 2004. Hypothalamic control of energy balance: different peptides, different functions. Peptides 25, 473-504.
Lembo, P.M., Grazzini, E., Cao, J., Hubatsch, D.A., Pelletier, M., Hoffert, C., St-Onge, S., Pou, C., Labrecque, J., Groblewski, T., O'Donnell, D., Payza, K., Ahmad, S., Walker, P., 1999. The receptor for the orexigenic peptide melanin-concentrating hormone is a G-protein-coupled receptor. Nat.Cell Biol. 1, 267-271.
Levine, A.S., Kotz, C.M., Gosnell, B.A., 2003. Sugars: hedonic aspects, neuroregulation, and energy balance. Am.J.Clin.Nutr. 78, 834S-842S.

Levine, A.S., Kotz, C.M., Gosnell, B.A., 2003. Sugars and fats: the neurobiology of preference. J.Nutr. 133, 831S-834S.
Levine, A.S., 2006. The animal model in food intake regulation: examples from the opioid literature. Physiol Behav. 89, 92-96.
Levine, J.A., 2003. Non-exercise activity thermogenesis. Proc.Nutr.Soc. 62, 667-679.
Lin S, Huang XF (1999) Altered hypothalamic c-Fos-like immunoreactivity in diet-induced obese mice. Brain Res Bulletin 49(3):215-219.
Lowe, M.R., 2003. Self-regulation of energy intake in the prevention and treatment of obesity: is it feasible? Obes.Res. 11 Suppl, 44S-59S.
Luckman, S.M., Rosenzweig, I., Dickson, S.L., 1999. Activation of arcuate nucleus neurons by systemic administration of leptin and growth hormone-releasing peptide-6 in normal and fasted rats. Neuroendocrinology 70, 93-100.
Luckman, S.M., Lawrence, C.B., 2003. Anorectic brainstem peptides: more pieces to the puzzle. Trends Endocrinol.Metab 14, 60-65.
Ludwig, D.S., Mountjoy, K.G., Tatro, J.B., Gillette, J.A., Frederich, R.C., Flier, J.S., Maratos-Flier, E., 1998. Melanin-concentrating hormone: a functional melanocortin antagonist in the hypothalamus. Am.J.Physiol 274, E627-E633.
Ludwig, D.S., Tritos, N.A., Mastaitis, J.W., Kulkarni, R., Kokkotou, E., Elmquist, J., Lowell, B., Flier, J.S., Maratos-Flier, E., 2001. Melanin-concentrating hormone overexpression in transgenic mice leads to obesity and insulin resistance. J.Clin.Invest 107, 379-386.
Meynard MM, Valdes JL, Recabarren M, Seron-Ferre M, Torrealba F,. 2005 Specific activation of histaminergic neurons during daily feeding anticipatory behaviour in rats. Behav. Brain Res. 158(2):311-9.
Malecka-Tendera, E., Mazur, A., 2006. Childhood obesity: a pandemic of the twenty-first century. Int.J.Obes.(Lond) 30 Suppl 2, S1-S3.
Mantzoros, C.S., Flier, J.S., 2000. Editorial: leptin as a therapeutic agent--trials and tribulations. J.Clin.Endocrinol.Metab 85, 4000-4002.
Marsh, D.J., Weingarth, D.T., Novi, D.E., Chen, H.Y., Trumbauer, M.E., Chen, A.S., Guan, X.M., Jiang, M.M., Feng, Y., Camacho, R.E., Shen, Z., Frazier, E.G., Yu, H., Metzger, J.M., Kuca, S.J., Shearman, L.P., Gopal-Truter, S., MacNeil, D.J., Strack, A.M., MacIntyre, D.E., Van der Ploeg, L.H., Qian, S., 2002. Melanin-concentrating hormone 1 receptor-deficient mice are lean, hyperactive, and hyperphagic and have altered metabolism. Proc.Natl.Acad.Sci.U.S.A 99, 3240-3245.
Mashiko, S., Ishihara, A., Gomori, A., Moriya, R., Ito, M., Iwaasa, H., Matsuda, M., Feng, Y., Shen, Z., Marsh, D.J., Bednarek, M.A., MacNeil, D.J., Kanatani, A., 2005. Antiobesity effect of a melanin-concentrating hormone 1 receptor antagonist in diet-induced obese mice. Endocrinology 146, 3080-3086.
Maulon-Feraille, L., Della, Z.O., Suply, T., Rovere-Jovene, C., Audinot, V., Levens, N., Boutin, J.A., Duhault, J., Nahon, J.L., 2002. Appetite-boosting property of pro-melanin-concentrating hormone(131-165) (neuropeptide-glutamic acid-isoleucine) is associated with proteolytic resistance. J.Pharmacol.Exp.Ther. 302, 766-773.
McBriar, M.D., Guzik, H., Xu, R., Paruchova, J., Li, S., Palani, A., Clader, J.W., Greenlee, W.J., Hawes, B.E., Kowalski, T.J., O'neill, K., Spar, B., Weig, B., 2005. Discovery of bicycloalkyl urea melanin concentrating hormone receptor antagonists: orally efficacious antiobesity therapeutics. J.Med.Chem. 48, 2274-2277.
McBriar, M.D., 2006. Recent advances in the discovery of melanin-concentrating hormone receptor antagonists. Curr.Opin.Drug Discov.Devel. 9, 496-508.
McBriar, M.D., Guzik, H., Shapiro, S., Paruchova, J., Xu, R., Palani, A., Clader, J.W., Cox, K., Greenlee, W.J., Hawes, B.E., Kowalski, T.J., O'neill, K., Spar, B.D., Weig, B., Weston, D.J., Farley, C., Cook, J., 2006. Discovery of orally efficacious melanin-concentrating hormone receptor-1 antagonists as antiobesity agents. Synthesis, SAR, and biological evaluation of bicyclo[3.1.0]hexyl ureas. J.Med.Chem. 49, 2294-2310.
McMinn, J.E., Baskin, D.G., Schwartz, M.W., 2000. Neuroendocrine mechanisms regulating food intake and body weight. Obes.Rev. 1, 37-46.
Mendoza, J., Angeles-Castellanos, M., Escobar, C., 2005. Entrainment by a palatable meal induces food-anticipatory activity and c-Fos expression in reward-related areas of the brain. Neuroscience 133, 293-303.
Mendoza, J., Angeles-Castellanos, M., Escobar, C., 2005. Differential role of the accumbens Shell and Core subterritories in food-entrained rhythms of rats. Behav.Brain Res. 158, 133-142.
Meynard, M.M., Valdes, J.L., Recabarren, M., Seron-Ferre, M., Torrealba, F., 2005. Specific activation of histaminergic neurons during daily feeding anticipatory behavior in rats. Behav.Brain Res. 158, 311-319.
Mieda, M., Williams, S.C., Richardson, J.A., Tanaka, K., Yanagisawa, M., 2006. The dorsomedial hypothalamic nucleus as a putative food-entrainable circadian pacemaker. Proc.Natl.Acad.Sci.U.S.A 103, 12150-12155.
Mistlberger, R.E., 1994. Circadian food-anticipatory activity: formal models and physiological mechanisms. Neurosci.Biobehav.Rev. 18, 171-195.
Mitchell, J.E., Crow, S., 2006. Medical complications of anorexia nervosa and bulimia nervosa. Curr.Opin.Psychiatry 19, 438-443.
Mogenson, G.J., Jones, D.L., Yim, C.Y., 1980. From motivation to action: functional interface between the limbic system and the motor system. Prog.Neurobiol. 14, 69-97.

Mondal, M.S., Nakazato, M., Date, Y., Murakami, N., Yanagisawa, M., Matsukura, S., 1999. Widespread distribution of orexin in rat brain and its regulation upon fasting. Biochem.Biophys.Res.Commun. 256, 495-499.
Monzon, M.E., de Souza, M.M., Izquierdo, L.A., Izquierdo, I., Barros, D.M., de, B., Sr., 1999. Melanin-concentrating hormone (MCH) modifies memory retention in rats. Peptides 20, 1517-1519.
Monzon, M.E., de, B., Sr., 1999. Response to novelty after i.c.v. injection of melanin-concentrating hormone (MCH) in rats. Physiol Behav. 67, 813-817.
Monzon, M.E., Varas, M.M., de, B., Sr., 2001. Anxiogenesis induced by nitric oxide synthase inhibition and anxiolytic effect of melanin-concentrating hormone (MCH) in rat brain. Peptides 22, 1043-1047.
Morens, C., Norregaard, P., Receveur, J.M., van, D.G., Scheurink, A.J., 2005. Effects of MCH and a MCH1-receptor antagonist on (palatable) food and water intake. Brain Res. 1062, 32-38.
Morgan, J.I., Curran, T., 1991. Proto-oncogene transcription factors and epilepsy. Trends Pharmacol.Sci. 12, 343-349.
Mori, M., Harada, M., Terao, Y., Sugo, T., Watanabe, T., Shimomura, Y., Abe, M., Shintani, Y., Onda, H., Nishimura, O., Fujino, M., 2001. Cloning of a novel G protein-coupled receptor, SLT, a subtype of the melanin-concentrating hormone receptor. Biochem.Biophys.Res.Commun. 283, 1013-1018.
Morley, J.E., Thomas, D.R., Wilson, M.M., 2006. Cachexia: pathophysiology and clinical relevance. Am.J.Clin.Nutr. 83, 735-743.
Morton, G.J., Mystkowski, P., Matsumoto, A.M., Schwartz, M.W., 2004. Increased hypothalamic melanin concentrating hormone gene expression during energy restriction involves a melanocortin-independent, estrogen-sensitive mechanism. Peptides 25, 667-674.
Münzberg, H., Myers, M.G., Jr., 2005. Molecular and anatomical determinants of central leptin resistance. Nat.Neurosci. 8, 566-570.
Münzberg, H., Bjornholm, M., Bates, S.H., Myers, M.G., Jr., 2005. Leptin receptor action and mechanisms of leptin resistance. Cell Mol.Life Sci. 62, 642-652.
Murray, J.F., Adan, R.A., Walker, R., Baker, B.I., Thody, A.J., Nijenhuis, W.A., Yukitake, J., Wilson, C.A., 2000. Melanin-concentrating hormone, melanocortin receptors and regulation of luteinizing hormone release. J.Neuroendocrinol. 12, 217-223.
Murray JF, Hahn JD, Kennedy AR, Small CJ, Bloom SR, Haskell-Luevano C, Coen CW, Wilson CA,. 2006. Evidence for a stimulatory action of melanin-concentrating hormone on luteinising hormone release involving MCH1 and melanocortin 5 receptors. J Neuroendocrinol. 18(3): 157-167.
Nahon, J.L., Presse, F., Bittencourt, J.C., Sawchenko, P.E., Vale, W., 1989. The rat melanin-concentrating hormone messenger ribonucleic acid encodes multiple putative neuropeptides coexpressed in the dorsolateral hypothalamus. Endocrinology 125, 2056-2065.
Nahon, J.L., 1994. The melanin-concentrating hormone: from the peptide to the gene. Crit Rev.Neurobiol. 8, 221-262.
Nahon, J.L., 2006. The melanocortins and melanin-concentrating hormone in the central regulation of feeding behavior and energy homeostasis. C.R.Biol. 329, 623-638.
Nakahara K, Fukui K, Murakami N., 2006. Involvement of thalamic paraventricular nucleus in the anticipatory reaction under food restriction in the rat. J Vet Med Sci. 66(10): 1297-300.
Nestler, E.J., Carlezon, W.A., Jr., 2006. The mesolimbic dopamine reward circuit in depression. Biol.Psychiatry 59, 1151-1159.
Niimi, M., Sato, M., Tamaki, M., Wada, Y., Takahara, J., Kawanishi, K., 1995. Induction of Fos protein in the rat hypothalamus elicited by insulin-induced hypoglycemia. Neurosci.Res. 23, 361-364.
Niswender, K.D., Schwartz, M.W., 2003. Insulin and leptin revisited: adiposity signals with overlapping physiological and intracellular signaling capabilities. Front Neuroendocrinol. 24, 1-10.
Obici, S., Rossetti, L., 2003. Minireview: nutrient sensing and the regulation of insulin action and energy balance. Endocrinology 144, 5172-5178.
Ogden, C.L., Flegal, K.M., Carroll, M.D., Johnson, C.L., 2002. Prevalence and trends in overweight among US children and adolescents, 1999-2000. JAMA 288, 1728-1732.
Olszewski, P.K., Wirth, M.M., Shaw, T.J., Grace, M.K., Billington, C.J., Giraudo, S.Q., Levine, A.S., 2001. Role of alpha-MSH in the regulation of consummatory behavior: immunohistochemical evidence. Am.J.Physiol Regul.Integr.Comp Physiol 281, R673-R680.
Olszewski, P.K., Grace, M.K., Billington, C.J., Levine, A.S., 2003. Hypothalamic paraventricular injections of ghre
Olszewski PK, Wirth ME, Shaw TJ, Grace MK, Billington CJ, Giraudo SQ, Levine AS (2001). Role of a-MSH in the regulation of consummatory behaviour: immunohistochemical evidence. Am J Physiol Reg Integr Comp Physiol 281:R673-R680. lin: effect on feeding and c-Fos immunoreactivity. Peptides 24, 919-923.
O`Rahilly ,. 2003. Minireview: human obesity-lessons fro monogenic disorders. Endocrinology 144: 3757-3764.
Padwal, R., Li, S.K., Lau, D.C., 2003. Long-term pharmacotherapy for overweight and obesity: a systematic review and meta-analysis of randomized controlled trials. Int.J.Obes.Relat Metab Disord. 27, 1437-1446.
Pagotto, U., Marsicano, G., Cota, D., Lutz, B., Pasquali, R., 2006. The emerging role of the endocannabinoid system in endocrine regulation and energy balance. Endocr.Rev. 27, 73-100.

Palani, A., Shapiro, S., McBriar, M.D., Clader, J.W., Greenlee, W.J., O'neill, K., Hawes, B., 2005. Biaryl diamides as potent melanin concentrating hormone receptor 1 antagonists. Bioorg.Med.Chem.Lett. 15, 5234-5236.
Palani, A., Shapiro, S., McBriar, M.D., Clader, J.W., Greenlee, W.J., Spar, B., Kowalski, T.J., Farley, C., Cook, J., van, H.M., Weig, B., O'neill, K., Graziano, M., Hawes, B., 2005. Biaryl ureas as potent and orally efficacious melanin concentrating hormone receptor 1 antagonists for the treatment of obesity. J.Med.Chem. 48, 4746-4749.
Parkes, D.G., Vale, W.W., 1993. Contrasting actions of melanin-concentrating hormone and neuropeptide-E-I on posterior pituitary function. Ann.N.Y.Acad.Sci. 680, 588-590.
Parkes, D.G., 1996. Diuretic and natriuretic actions of melanin concentrating hormone in conscious sheep. J.Neuroendocrinol. 8, 57-63.
Pelletier, G., Guy, J., Desy, L., Li, S., Eberle, A.N., Vaudry, H., 1987. Melanin-concentrating hormone (MCH) is colocalized with alpha-melanocyte-stimulating hormone (alpha-MSH) in the rat but not in the human hypothalamus. Brain Res. 423, 247-253.
Pelleymounter MA,.1995. Effects of the obese gene product on body weight regulation in ob/ob mice. Science 269:540-543.
Pereira-da-Silva, M., De Souza, C.T., Gasparetti, A.L., Saad, M.J., Velloso, L.A., 2005. Melanin-concentrating hormone induces insulin resistance through a mechanism independent of body weight gain. J.Endocrinol. 186, 193-201.
Petrovich, G.D., Canteras, N.S., Swanson, L.W., 2001. Combinatorial amygdalar inputs to hippocampal domains and hypothalamic behavior systems. Brain Res.Brain Res.Rev. 38, 247-289.
Petrovich, G.D., Setlow, B., Holland, P.C., Gallagher, M., 2002. Amygdalo-hypothalamic circuit allows learned cues to override satiety and promote eating. J.Neurosci. 22, 8748-8753.
Peyron, C., Tighe, D.K., van den Pol, A.N., De, L.L., Heller, H.C., Sutcliffe, J.G., Kilduff, T.S., 1998. Neurons containing hypocretin (orexin) project to multiple neuronal systems. J.Neurosci. 18, 9996-10015.
Pissios, P., Maratos-Flier, E., 2003. Melanin-concentrating hormone: from fish skin to skinny mammals. Trends Endocrinol.Metab 14, 243-248.
Pissios P, Trombly DJ, Tzameli I, Maratos-Flier E,. 2001. Melanin-concentrating hormone Receptor-1 Activates Extracellular Signal-Regulated Kinase and Synergizes with Gs-Coupled Pathways. Endocrinology 144: 3514-3523.
Pittman QJ, Lawrence D. Central effects of arginine vasopressin on blood pressure in rats. Endocrinology 1982; 110(3):1058-60.
Plata-Salaman, C.R., 2000. Central nervous system mechanisms contributing to the cachexia-anorexia syndrome. Nutrition 16, 1009-1012.
Porter JP, Brody MJ,. 1986.Spinal vasopressin mechanisms of cardiovascular regulation. Am J Physiol., 1986. 251 (3pt2):R510-7.
Poulain DA, Wakerley JB. Electrophysiology of hypothalamic magnocellular neurons secreting oxytocin and vasopressin. Neurosci 1982; 7(4):773-808.
Prentice, A.M., Black, A.E., Coward, W.A., Cole, T.J., 1996. Energy expenditure in overweight and obese adults in affluent societies: an analysis of 319 doubly-labelled water measurements. Eur.J.Clin.Nutr. 50, 93-97.
Presse, F., Nahon, J.L., Fischer, W.H., Vale, W., 1990. Structure of the human melanin concentrating hormone mRNA. Mol.Endocrinol. 4, 632-637.
Qu, D., Ludwig, D.S., Gammeltoft, S., Piper, M., Pelleymounter, M.A., Cullen, M.J., Mathes, W.F., Przypek, R., Kanarek, R., Maratos-Flier, E., 1996. A role for melanin-concentrating hormone in the central regulation of feeding behaviour. Nature 380, 243-247.
Rashotte, M.E., Basco, P.S., Henderson, R.P., 1995. Daily cycles in body temperature, metabolic rate, and substrate utilization in pigeons: influence of amount and timing of food consumption. Physiol Behav. 57, 731-746.
Reed, G.W., Hill, J.O., 1996. Measuring the thermic effect of food. Am.J.Clin.Nutr. 63, 164-169.
Reinehr, T., de, S.G., Toschke, A.M., Andler, W., 2006. Long-term follow-up of cardiovascular disease risk factors in children after an obesity intervention. Am.J.Clin.Nutr. 84, 490-496.
Ring, R.H., 2005. The central vasopressinergic system: examining the opportunities for psychiatric drug development. Curr.Pharm.Des 11, 205-225.
Rodriguez, M., Beauverger, P., Naime, I., Rique, H., Ouvry, C., Souchaud, S., Dromaint, S., Nagel, N., Suply, T., Audinot, V., Boutin, J.A., Galizzi, J.P., 2001. Cloning and molecular characterization of the novel human melanin-concentrating hormone receptor MCH2. Mol.Pharmacol. 60, 632-639.
Rohner-Jeanrenaud, F., 2002. Aspects of the neuroendocrine regulation of body weight homeostasis. Ann.Endocrinol.(Paris) 63, 125-128.
Rokosz, L.L., Hobbs, D.W., 2006. Biological examination of melanin concentrating hormone receptor 1: multi-tasking from the hypothalamus. Drug News Perspect. 19, 273-286.
Rolls, B.J., Roe, L.S., Kral, T.V., Meengs, J.S., Wall, D.E., 2004. Increasing the portion size of a packaged snack increases energy intake in men and women. Appetite 42, 63-69.
Rossi, M., Choi, S.J., O'Shea, D., Miyoshi, T., Ghatei, M.A., Bloom, S.R., 1997. Melanin-concentrating hormone acutely stimulates feeding, but chronic administration has no effect on body weight. Endocrinology 138, 351-355.

Rowland NE, Roth JD, McMullen MR, Patel A, Cespedes AT., 2000. Dexfenfluramine and norfenfluramine: comparison of mechanism of action in feeding and brain Fos.ir studies. Am J Physiol Reg Integr Comp Physiol 278:R390-R399.
Roubenoff, R., 1996. Applications of bioelectrical impedance analysis for body composition to epidemiologic studies. Am.J.Clin.Nutr. 64, 459S-462S.
Sahu, A., 2002. Interactions of neuropeptide Y, hypocretin-I (orexin A) and melanin-concentrating hormone on feeding in rats. Brain Res. 944, 232-238.
Sahu, A., 2003. Leptin signaling in the hypothalamus: emphasis on energy homeostasis and leptin resistance. Front Neuroendocrinol. 24, 225-253.
Sailer, A.W., Sano, H., Zeng, Z., McDonald, T.P., Pan, J., Pong, S.S., Feighner, S.D., Tan, C.P., Fukami, T., Iwaasa, H., Hreniuk, D.L., Morin, N.R., Sadowski, S.J., Ito, M., Ito, M., Bansal, A., Ky, B., Figueroa, D.J., Jiang, Q., Austin, C.P., MacNeil, D.J., Ishihara, A., Ihara, M., Kanatani, A., Van der Ploeg, L.H., Howard, A.D., Liu, Q., 2001. Identification and characterization of a second melanin-concentrating hormone receptor, MCH-2R. Proc.Natl.Acad.Sci.U.S.A 98, 7564-7569.
Saito, Y., Nothacker, H.P., Wang, Z., Lin, S.H., Leslie, F., Civelli, O., 1999. Molecular characterization of the melanin-concentrating-hormone receptor. Nature 400, 265-269.
Saito, Y., Cheng, M., Leslie, F.M., Civelli, O., 2001. Expression of the melanin-concentrating hormone (MCH) receptor mRNA in the rat brain. J.Comp Neurol. 435, 26-40.
Saito, Y., Maruyama, K., 2006. Identification of melanin-concentrating hormone receptor and its impact on drug discovery. J.Exp.Zoolog.A Comp Exp.Biol. 305, 761-768.
Sakamaki, R., Uemoto, M., Inui, A., Asakawa, A., Ueno, N., Ishibashi, C., Hirono, S., Yukioka, H., Kato, A., Shinfuku, N., Kasuga, M., Katsuura, G., 2005. Melanin-concentrating hormone enhances sucrose intake. Int.J.Mol.Med. 15, 1033-1039.
Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R.M., Tanaka, H., Williams, S.C., Richarson, J.A., Kozlowski, G.P., Wilson, S., Arch, J.R., Buckingham, R.E., Haynes, A.C., Carr, S.A., Annan, R.S., McNulty, D.E., Liu, W.S., Terrett, J.A., Elshourbagy, N.A., Bergsma, D.J., Yanagisawa, M., 1998. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell 92, 1.
Saper, C.B., Chou, T.C., Elmquist, J.K., 2002. The need to feed: homeostatic and hedonic control of eating. Neuron 36, 199-211.
Sawchenko, P.E., 1998. Toward a new neurobiology of energy balance, appetite, and obesity: the anatomists weigh in. J.Comp Neurol. 402, 435-441.

Schlumberger, S.E., Talke-Messerer, C., Zumsteg, U., Eberle, A.N., 2002. Expression of receptors for melanin-concentrating hormone (MCH) in different tissues and cell lines. J.Recept.Signal.Transduct.Res. 22, 509-531.
Schiöth H,. 2006. G Protein-Coupled Receptors in Regulation of Body Weight. CNS & Neurological Disorders-Drug Targets, 5:241-249.
Schwartz MW, 2006. Central Nervous System Regulation of Food Intake. Obesity 14: 1S-8S.
Schwartz, M.W., Woods, S.C., Porte, D., Jr., Seeley, R.J., Baskin, D.G., 2000. Central nervous system control of food intake. Nature 404, 661-671.
Schwartz, M.W., Gelling, R.W., 2002. Rats lighten up with MCH antagonist. Nat.Med. 8, 779-781.
Segal 1987
Segal-Lieberman, G., Bradley, R.L., Kokkotou, E., Carlson, M., Trombly, D.J., Wang, X., Bates, S., Myers, M.G., Jr., Flier, J.S., Maratos-Flier, E., 2003. Melanin-concentrating hormone is a critical mediator of the leptin-deficient phenotype. Proc.Natl.Acad.Sci.U.S.A 100, 10085-10090.
Segal-Lieberman, G., Rubinfeld, H., Glick, M., Kronfeld-Schor, N., Shimon, I., 2006. Melanin-concentrating hormone stimulates human growth hormone secretion: a novel effect of MCH on the hypothalamic-pituitary axis. Am.J.Physiol Endocrinol.Metab 290, E982-E988. Segal, K.R., 1987. Comparison of indirect calorimetric measurements of resting energy expenditure with a ventilated hood, face mask, and mouthpiece. Am.J.Clin.Nutr. 45, 1420-1423.
Shearman, L.P., Camacho, R.E., Sloan, S.D., Zhou, D., Bednarek, M.A., Hreniuk, D.L., Feighner, S.D., Tan, C.P., Howard, A.D., Van der Ploeg, L.H., MacIntyre, D.E., Hickey, G.J., Strack, A.M., 2003. Chronic MCH-1 receptor modulation alters appetite, body weight and adiposity in rats. Eur.J.Pharmacol. 475, 37-47.
Sheng, M., Greenberg, M.E., 1990. The regulation and function of c-fos and other immediate early genes in the nervous system. Neuron 4, 477-485.
Shi, Y., 2004. Beyond skin color: emerging roles of melanin-concentrating hormone in energy homeostasis and other physiological functions. Peptides 25, 1605-1611.
Shimada, M., Tritos, N.A., Lowell, B.B., Flier, J.S., Maratos-Flier, E., 1998. Mice lacking melanin-concentrating hormone are hypophagic and lean. Nature 396, 670-674.
Shimazaki, T., Yoshimizu, T., Chaki, S., 2006. Melanin-concentrating hormone MCH1 receptor antagonists: a potential new approach to the treatment of depression and anxiety disorders. CNS.Drugs 20, 801-811.
Shindler, K.S., Roth, K.A., 1996. Double immunofluorescent staining using two unconjugated primary antisera

raised in the same species. J.Histochem.Cytochem. 44, 1331-1335.
Shor-Pozner G, Azar AP, Insinga S, Leibowitz SF,. 1985. Deficits in the control of food intake after hypothalamic paraventricular nucleus lesions. Physiol Behav. 35(6): 883-890.
Singh ME, Verty ANA, Price I, McGregor IS, Mallet PE., 2004. Modulation of morphine-induced Fos-immunoreactivity by the cannabinoid receptor antagonist SR 141716. Neuropharmacology 47:1157-1169.
Sjostrom, L., Rissanen, A., Andersen, T., Boldrin, M., Golay, A., Koppeschaar, H.P., Krempf, M., 1998. Randomised placebo-controlled trial of orlistat for weight loss and prevention of weight regain in obese patients. European Multicentre Orlistat Study Group. Lancet 352, 167-172.
Sjostrom, L., 2006. Analysis of the XENDOS study (Xenical in the Prevention of Diabetes in Obese Subjects). Endocr.Pract. 12 Suppl 1, 31-33.
Skofitsch, G., Jacobowitz, D.M., Zamir, N., 1985. Immunohistochemical localization of a melanin concentrating hormone-like peptide in the rat brain. Brain Res.Bull. 15, 635-649.
Smith, D.G., Davis, R.J., Rorick-Kehn, L., Morin, M., Witkin, J.M., McKinzie, D.L., Nomikos, G.G., Gehlert, D.R., 2006. Melanin-concentrating hormone-1 receptor modulates neuroendocrine, behavioral, and corticolimbic neurochemical stress responses in mice. Neuropsychopharmacology 31, 1135-1145.
Solomon, A., De Fanti, B.A., Martinez, J.A., 2005. Peripheral ghrelin participates in glucostatic feeding mechanisms and in the anorexigenic signalling mediated by CART and CRF neurons. Nutr.Neurosci. 8, 287-295.
Spiegel AM, Nagel EG,. 2006. NIH research on obesity and type 2 diabetes: providing the science evidence base for actions to improve health. Nat Med 12 (1): 67-69.
Spiegelman, B.M., Flier, J.S., 2001. Obesity and the regulation of energy balance. Cell 104, 531-543.

Starling EG, Verney, EB; The secretion of urine as studied on the isolated kidney. Proc R Soc London (Biol) 1924:321-363)
Steffen, K.J., Roerig, J.L., Mitchell, J.E., Uppala, S., 2006. Emerging drugs for eating disorder treatment. Expert.Opin.Emerg.Drugs 11, 315-336.
Stephan, F.K., 1986. Coupling between feeding- and light-entrainable circadian pacemakers in the rat. Physiol Behav. 38, 537-544.
Stephan, F.K., 2002. The "other" circadian system: food as a Zeitgeber. J.Biol.Rhythms 17, 284-292.
Strader, A.D., Woods, S.C., 2005. Gastrointestinal hormones and food intake. Gastroenterology 128, 175-191.
Stratford, T.R., Kelley, A.E., 1999. Evidence of a functional relationship between the nucleus accumbens shell and lateral hypothalamus subserving the control of feeding behavior. J.Neurosci. 19, 11040-11048.
Stratford, T.R., 2005. Activation of feeding-related neural circuitry after unilateral injections of muscimol into the nucleus accumbens shell. Brain Res. 1048, 241-250.
Strubble 2002
Sun G, Tian Z, Murata T, Narita K, Honda K, Higuchi T,. 2004 Central and peripheral immunoreactivity of melanin-concentrating hormone in hypothalamic obese and lactating rats. Neuroendocrinol. 16(1):79-83.
Suply, T., Della, Z.O., Audinot, V., Rodriguez, M., Beauverger, P., Duhault, J., Canet, E., Galizzi, J.P., Nahon, J.L., Levens, N., Boutin, J.A., 2001. SLC-1 receptor mediates effect of melanin-concentrating hormone on feeding behavior in rat: a structure-activity study. J.Pharmacol.Exp.Ther. 299, 137-146.
Suzuki, R., Shimojima, H., Funahashi, H., Nakajo, S., Yamada, S., Guan, J.L., Tsurugano, S., Uehara, K., Takeyama, Y., Kikuyama, S., Shioda, S., 2002. Orexin-1 receptor immunoreactivity in chemically identified target neurons in the rat hypothalamus. Neurosci.Lett. 324, 5-8.
Taha, S.A., Fields, H.L., 2005. Encoding of palatability and appetitive behaviors by distinct neuronal populations in the nucleus accumbens. J.Neurosci. 25, 1193-1202.
Takahashi, K., 2004. Translational medicine in fish-derived peptides: from fish endocrinology to human physiology and diseases. Endocr.J. 51, 1-17.
Takahashi K, Suzuki H, Totsune K, Murakami O, Satoh F, Sone M, Sasano H, Mouri T, Shibakara S,. 1995. Melanin-Concentrating hormone in Human and Rat. Neuroendocrinology 61:493-498.
Takekawa, S., Asami, A., Ishihara, Y., Terauchi, J., Kato, K., Shimomura, Y., Mori, M., Murakoshi, H., Kato, K., Suzuki, N., Nishimura, O., Fujino, M., 2002. T-226296: a novel, orally active and selective melanin-concentrating hormone receptor antagonist. Eur.J.Pharmacol. 438, 129-135.
Tan, C.P., Sano, H., Iwaasa, H., Pan, J., Sailer, A.W., Hreniuk, D.L., Feighner, S.D., Palyha, O.C., Pong, S.S., Figueroa, D.J., Austin, C.P., Jiang, M.M., Yu, H., Ito, J., Ito, M., Ito, M., Guan, X.M., MacNeil, D.J., Kanatani, A., Van der Ploeg, L.H., Howard, A.D., 2002. Melanin-concentrating hormone receptor subtypes 1 and 2: species-specific gene expression. Genomics 79, 785-792.
Tartaglia, L.A., 1997. The leptin receptor. J.Biol.Chem. 272, 6093-6096.
Tataranni, P.A., 2003. Treatment of obesity: should we target the individual or society? Curr.Pharm.Des 9, 1151-1163.
Tavares, F.X., Al-Barazanji, K.A., Bigham, E.C., Bishop, M.J., Britt, C.S., Carlton, D.L., Feldman, P.L., Goetz, A.S., Grizzle, M.K., Guo, Y.C., Handlon, A.L., Hertzog, D.L., Ignar, D.M., Lang, D.G., Ott, R.J., Peat, A.J.,.
Tavares, F.X., Al-Barazanji, K.A., Bigham, E.C., Bishop, M.J., Britt, C.S., Carlton, D.L., Feldman, P.L., Goetz, A.S., Grizzle, M.K., Ter Horst, G.J., Luiten, P.G., 1987. Phaseolus vulgaris leuco-agglutinin tracing of

intrahypothalamic connections of the lateral, ventromedial, dorsomedial and paraventricular hypothalamic nuclei in the rat. Brain Res.Bull. 18, 191-203.
Tavares, F.X., Al-Barazanji, K.A., Bishop, M.J., Britt, C.S., Carlton, D.L., Cooper, J.P., Feldman, P.L., Garrido, D.M., Goetz, A.S., Grizzle, M.K., Hertzog, D.L., Ignar, D.M., Lang, D.G., McIntyre, M.S., Ott, R.J., Peat, A.J., Tornaghi, G., Raiteri, R., Pozzato, C., Rispoli, A., Bramani, M., Cipolat, M., Craveri, A., 1994. Anthropometric or ultrasonic measurements in assessment of visceral fat? A comparative study. Int.J.Obes.Relat Metab Disord. 18, 771-775.
Ter Horst GJ, Luiten PG,. 1987. Phaseolus vulgaris leuco-agglutinin tracing of intrahypothalamic connections of the lateral, ventromedial, dorsomedial and paraventricular hypothalamic nuclei in the rat. Brain Res Bulletin 18(2): 191-203.
Timofeeva E, Richard D., Activation of the central nervous system in obese Zucker rats during food deprivation. J Comp Neurol 441(1):71-89.
Toschke AM, Beyerlein A, von Kries R., 2006. Children at high risk for overweight: a classification and regression trees analysis approach.
Toumaniantz, G., Bittencourt, J.C., Nahon, J.L., 1996. The rat melanin-concentrating hormone gene encodes an additional putative protein in a different reading frame. Endocrinology 137, 4518-4521.
Toumaniantz, G., Ferreira, P.C., Allaeys, I., Bittencourt, J.C., Nahon, J.L., 2000. Differential neuronal expression and projections of melanin-concentrating hormone (MCH) and MCH-gene-overprinted-polypeptide (MGOP) in the rat brain. Eur.J.Neurosci. 12, 4367-4380.
Tritos NA, Mastaitis JW, Kokkotou E, Maratos-Flier E., 2001. Characterisation of the melanin-concentrating hormone and preproorexin expression in the murine hypothalamus. Brain Res. 895 (1-2): 160-166.
Tschöp M, Weher C, Tataranni PA, Devanarayan V, Ravussin E, Heimann ML,. 2001. Circulating ghrelin levels are decreased in human obesity. Diabetes 50: 707-709.
Tyhon, A., Adamantidis, A., Foidart, A., Grisar, T., Lakaye, B., Tirelli, E., 2006. Mice lacking the melanin-concentrating hormone receptor-1 exhibit an atypical psychomotor susceptibility to cocaine and no conditioned cocaine response. Behav.Brain Res. 173, 94-103.
Unmehopa, U.A., Van Heerikhuize, J.J., Spijkstra, W., Woods, J.W., Howard, A.D., Zycband, E., Feighner, S.D., Hreniuk, D.L., Palyha, O.C., Guan, X.M., MacNeil, D.J., Van der Ploeg, L.H., Swaab, D.F., 2005. Increased melanin concentrating hormone receptor type I in the human hypothalamic infundibular nucleus in cachexia. J.Clin.Endocrinol.Metab 90, 2412-2419.
van den Pol, A.N., 2003. Weighing the role of hypothalamic feeding neurotransmitters. Neuron 40, 1059-1061.
Van Itallie, T.B., 2006. Sleep and energy balance: Interactive homeostatic systems. Metabolism 55, S30-S35.
Van Itallie TB, Smith NS, Quatermain D., 1977. Short-term and long-term components in the regulation of food intake: evidence for a modulatory role of carbohydrate status. Am.J.Clin.Nutr. 30: 742.
Varas M, Perez M, Monzon ME, Barioglio SR (2002). Melanin-concentrating hormone, hippocampal nitric oxide levels and memory retention. Peptides 23:2213-21.
Varas M, Perez M, Ramirez O, Barioglio SR (2002). Melanin-concentrating hormone increase hippocampal synaptic transmission in the rat. Peptides 23:151-5.
Verbalis JG, Blackburn RE, Hoffman GE, Stricker EM,. 1995. Establishing behavioral and physiological functions of central oxytocin: insightsfrom studies of oxytocin and ingestive behaviors. Adv Exp Med Biol 395: 209-225
Viale, A., Zhixing, Y., Breton, C., Pedeutour, F., Coquerel, A., Jordan, D., Nahon, J.L., 1997. The melanin-concentrating hormone gene in human: flanking region analysis, fine chromosome mapping, and tissue-specific expression. Brain Res.Mol.Brain Res. 46, 243-255.
Vitale, R.M., Zaccaro, L., Di, B.B., Fattorusso, R., Isernia, C., Amodeo, P., Pedone, C., Saviano, M., 2003. Conformational features of human melanin-concentrating hormone: an NMR and computational analysis. Chembiochem. 4, 73-81.
Volkow, N.D., Wise, R.A., 2005. How can drug addiction help us understand obesity? Nat.Neurosci. 8, 555-560.
Von Meyenfeld M,. 2005. Cancer-associated malnutrition: an introduction. Eur. J. Oncol Nurs 9 Suppl 2: S35-8.
Vrang, N., Larsen, P.J., Clausen, J.T., Kristensen, P., 1999. Neurochemical characterization of hypothalamic cocaine- amphetamine-regulated transcript neurons. J.Neurosci. 19, RC5.
Wang GJ et al., 2001. Brain dopamine and obesity. Lancet 357:354-357.
Wang, G.J., Volkow, N.D., Fowler, J.S., 2002. The role of dopamine in motivation for food in humans: implications for obesity. Expert.Opin.Ther.Targets. 6, 601-609.
Wang, S., Behan, J., O'neill, K., Weig, B., Fried, S., Laz, T., Bayne, M., Gustafson, E., Hawes, B.E., 2001. Identification and pharmacological characterization of a novel human melanin-concentrating hormone receptor, mch-r2. J.Biol.Chem. 276, 34664-34670.
Watts AG,. 2000. Understanding the neural control of ingestive behaviors: helping to separate cause from effect with dehydration-associated anorexia. Horm. Behav 37: 261-283.
Watts, A.G., Swanson, L.W., 1987. Efferent projections of the suprachiasmatic nucleus: II. Studies using retrograde transport of fluorescent dyes and simultaneous peptide immunohistochemistry in the rat. J.Comp Neurol. 258, 230-252.

Watts, A.G., Swanson, L.W., Sanchez-Watts, G., 1987. Efferent projections of the suprachiasmatic nucleus: I. Studies using anterograde transport of Phaseolus vulgaris leucoagglutinin in the rat. J.Comp Neurol. 258, 204-229.
Weigle, D.S., 2003. Pharmacological therapy of obesity: past, present, and future. J.Clin.Endocrinol.Metab 88, 2462-2469.
Wermter, A.K., Reichwald, K., Buch, T., Geller, F., Platzer, C., Huse, K., Hess, C., Remschmidt, H., Gudermann, T., Preibisch, G., Siegfried, W., Goldschmidt, H.P., Li, W.D., Price, R.A., Biebermann, H., Krude, H., Vollmert, C., Wichmann, H.E., Illig, T., Sorensen, T.I., Astrup, A., Larsen, L.H., Pedersen, O., Eberle, D., Clement, K., Blundell, J., Wabitsch, M., Schafer, H., Platzer, M., Hinney, A., Hebebrand, J., 2005. Mutation analysis of the MCHR1 gene in human obesity. Eur.J.Endocrinol. 152, 851-862.
Whitlock, B.K., Daniel, J.A., McMahon, C.D., Buonomo, F.C., Wagner, C.G., Steele, B., Sartin, J.L., 2005. Intracerebroventricular melanin-concentrating hormone stimulates food intake in sheep. Domest.Anim Endocrinol. 28, 224-232.
WHO (1998) Obesity-Preventing and Managing the global Epidemic. WHO: Geneva, 1998.
Williams, G., Bing, C., Cai, X.J., Harrold, J.A., King, P.J., Liu, X.H., 2001. The hypothalamus and the control of energy homeostasis: different circuits, different purposes. Physiol Behav. 74, 683-701.
Williams, G., Cai, X.J., Elliott, J.C., Harrold, J.A., 2004. Anabolic neuropeptides. Physiol Behav. 81, 211-222.
Williamson-Hughes, P.S., Grove, K.L., Smith, M.S., 2005. Melanin concentrating hormone (MCH): a novel neural pathway for regulation of GnRH neurons. Brain Res. 1041, 117-124.
Wise, R.A., 2002. Brain reward circuitry: insights from unsensed incentives. Neuron 36, 229-240.
Witty, D.R., Bateson, J., Hervieu, G.J., Al-Barazanji, K., Jeffrey, P., Hamprecht, D., Haynes, A., Johnson, C.N., Muir, A.I., O'Hanlon, P.J., Stemp, G., Stevens, A.J., Thewlis, K., Winborn, K.Y., 2006. Discovery of potent and stable conformationally constrained analogues of the MCH R1 antagonist SB-568849. Bioorg.Med.Chem.Lett. 16, 4872-4878.
Witty, D.R., Bateson, J.H., Hervieu, G.J., Jeffrey, P., Johnson, C.N., Muir, A.I., O'Hanlon, P.J., Stemp, G., Stevens, A.J., Thewlis, K.M., Wilson, S., Winborn, K.Y., 2006. SAR of biphenyl carboxamide ligands of the human melanin-concentrating hormone receptor 1 (MCH R1): discovery of antagonist SB-568849. Bioorg.Med.Chem.Lett. 16, 4865-4871.
Witty, D.R., Bateson, J., Hervieu, G.J., Al-Barazanji, K., Jeffrey, P., Hamprecht, D., Haynes, A., Johnson, C.N., Muir, A.I., O'Hanlon, P.J., Stemp, G., Stevens, A.J., Thewlis, K., Winborn, K.Y., 2006. Discovery of potent and stable conformationally constrained analogues of the MCH R1 antagonist SB-568849. Bioorg.Med.Chem.Lett. 16, 4872-4878.
Woods, S.C., Seeley, R.J., Rushing, P.A., D'Alessio, D., Tso, P., 2003. A controlled high-fat diet induces an obese syndrome in rats. J.Nutr. 133, 1081-1087.
Wu, W.L., Burnett, D.A., Caplen, M.A., Domalski, M.S., Bennett, C., Greenlee, W.J., Hawes, B.E., O'neill, K., Weig, B., Weston, D., Spar, B., Kowalski, T., 2006. Design and synthesis of orally efficacious benzimidazoles as melanin-concentrating hormone receptor 1 antagonists. Bioorg.Med.Chem.Lett. 16, 3674-3678.
Xu, R., Li, S., Paruchova, J., McBriar, M.D., Guzik, H., Palani, A., Clader, J.W., Cox, K., Greenlee, W.J., Hawes, B.E., Kowalski, T.J., O'neill, K., Spar, B.D., Weig, B., Weston, D.J., 2006. Bicyclic[4.1.0]heptanes as phenyl replacements for melanin concentrating hormone receptor antagonists. Bioorg.Med.Chem. 14, 3285-3299.
Yeomans, M.R., Blundell, J.E., Leshem, M., 2004. Palatability: response to nutritional need or need-free stimulation of appetite? Br.J.Nutr. 92 Suppl 1, S3-14.
Zamir, N., Skofitsch, G., Jacobowitz, D.M., 1986. Distribution of immunoreactive melanin-concentrating hormone in the central nervous system of the rat. Brain Res. 373, 240-245.
Zamir, N., Skofitsch, G., Bannon, M.J., Jacobowitz, D.M., 1986. Melanin-concentrating hormone: unique peptide neuronal system in the rat brain and pituitary gland. Proc.Natl.Acad.Sci.U.S.A 83, 1528-1531.
Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., Friedman, J.M., 1994. Positional cloning of the mouse obese gene and its human homologue. Nature 372, 425-432.
Zheng, H., Corkern, M., Stoyanova, I., Patterson, L.M., Tian, R., Berthoud, H.R., 2003. Peptides that regulate food intake: appetite-inducing accumbens manipulation activates hypothalamic orexin neurons and inhibits POMC neurons. Am.J.Physiol Regul.Integr.Comp Physiol 284, R1436-R1444.
Zheng, H., Patterson, L.M., Morrison, C., Banfield, B.W., Randall, J.A., Browning, K.N., Travagli, R.A., Berthoud, H.R., 2005. Melanin concentrating hormone innervation of caudal brainstem areas involved in gastrointestinal functions and energy balance. Neuroscience 135, 611-625.
Zhou, D., Shen, Z., Strack, A.M., Marsh, D.J., Shearman, L.P., 2005. Enhanced running wheel activity of both Mch1r- and Pmch-deficient mice. Regul.Pept. 124, 53-63.
Zhou, H.Q., 2006. Potent, Selective, and Orally Efficacious Antagonists of Melanin-Concentrating Hormone Receptor 1. J.Med.Chem. 49, 7095-7107ntrol. Endocrinology 144, 3749-3756.
Zhou, H.Q., 2006. 6-(4-chlorophenyl)-3-substituted-thieno[3,2-d]pyrimidin-4(3H)-one-based melanin-concentrating hormone receptor 1 antagonist. J.Med.Chem. 49, 7108-7118.

(a) **Sahu A**,. (2002). Interaction of neuropeptide Y, hypocretin-I (orexin A) and melanin-concentrating hormone on feeding in rats. Brain Res 944: 232-238.
(b) **Hagan MM**, Rushing PA, Pritchard LM, Schwartz MW et al,. 2000. Long-term orexigenic effects of AgRP-(83-132) involve mechanisms other than melanocortin receptor blockade. Am J Physiol Intr Comp Physiol 279(1): R47-52
(c) **Kyrkouli SE**, Stanley BG, Leibowitz SF,. 1986. Galanin: stimulation of feeding by medial hypothalamic injection of this novel peptide. Eur J Pharmacol 122(1):159-60.
(d) **Krasnow SM**, Fraley GS, Schuh SM, Baumgartner JW, Clifton DK, Steiner RA,. 2003. A role for galanin-like peptide in the integration of feeding, body weight regulation and reproduction in the mouse. Endocrinology 144(3):813-822.
(e) **Pierroz DD**, Ziotopoulou M, Ungsunan L, Moschos S, Flier JS, Mantsoros CS.2002. Effects of acute and chronic administration of the melanocortin agonist MTII in mice with diet-induced obesity. Diabetes 51(5):1337-45.
(f) **Lambert PD**, Couceyro PR, McGirr KM, Dall Vechia SE, Smith Y, Kuhar MJ., (1998). CART peptides in the central control of feeding and interactions with NPY. Synapse 29(4):293-8.
(g) **Arase K**, York DA, Shimizu H, Shargill N, Bray GA,. 1988. Effects of corticotropin-releasing factor on food intake and brown adipose tissue thermogenesis in rats. Am J Physiol 255 (3Pt 2):E255-9.

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7. Eidestattliche Erklärung

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Hiermit versichere ich, dass ich die Arbeit selbst verfasst und keine andere als die von mir angegebenen Quellen und Hilfsmittel benutzt habe. Die Arbeit wurde an keiner anderen Hochschule zur Begutachtung eingereicht.

Biberach den

Thomas Appl