Doctoral Thesis

Effect of visceral and metabolic signals on orexigenic neuropeptides in the brain

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EFFECT OF VISCERAL AND METABOLIC SIGNALS ON OREXIGENIC NEUROPEPTIDES IN THE BRAIN

A dissertation submitted to the

SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

for the degree of
Doctor of Sciences

presented by

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I SUMMARY

Food intake is controlled by mechanisms of hunger and satiety. Peripheral signals reflect the energy status of the body and are integrated and interpreted by specialized brain regions. Sensing of an energy deficit activates feelings of hunger and initiates motor programs leading to food-seeking behaviour and food intake. The process of ingestion and digestion releases a chain of peripheral signals, which finally lead to meal termination and satiety. The aim of this thesis was to analyze the role of central orexigenic (food-intake stimulating) neuropeptides in mechanisms of increased food intake and their modulation by visceral and metabolic signals.

In a first set of experiments, we analyzed food intake and expression of hypothalamic orexigenic neuropeptides [e.g. orexin-A, melanin-concentrating hormone (MCH) and neuropeptide Y (NPY)] in response to a hypoglycaemic dose of insulin (50 IU/kg) in male Sprague-Dawley rats. Insulin was injected intraperitoneally (IP) to induce hyperphagia. The satiety peptide cholecystokinin (CCK) infused IP during meals or continuously for 60 minutes antagonized hyperphagia. Hypothalamic prepro-orexin and MCH mRNA were significantly increased in insulin-injected hypoglycaemic rats, as compared with fed controls. Continuous 60-minute IP infusion of CCK normalized hypothalamic prepro-orexin and MCH mRNA. NPY mRNA was not changed by either treatment. This suggests that pathways activated by insulin-induced hypoglycaemia and peripheral CCK-derived signals converge on hypothalamic orexin and MCH neurones. The changes in prepro-orexin and MCH mRNA expression suggest a role for orexin and MCH in insulin hyperphagia and its counteraction by CCK.

The second set of experiments used 48 hours of fasting to induce hyperphagia and to examine the roles of orexigenic peptides and the effect of IP CCK. Orexin-A peptide levels were measured in the lateral hypothalamic area (LHA) and in several target areas of orexin neurones. As prepro-orexin mRNA increases in the LHA of fasted animals, we expected a fasting-induced change of orexin-A peptide in the target areas of orexin neurones. We found a fasting-induced decrease in LHA orexin-A, an effect inverse to
the results on the mRNA level. An increased release and turnover at the synapse may explain this reduction of orexin-A peptide in the face of increased mRNA. Thus, the reduced peptide content might in fact reflect increased orexin signalling in fasting. Orexin-A in the medial hypothalamus was unchanged by fasting and by CCK. In the posterior brainstem, however, CCK increased the orexin-A content in fasted animals. This suggests a role for orexin-A in the posterior brainstem in the sensing of a CCK-derived signal and, possibly, in the attenuation of hyperphagia. In the septum, the combination of fasting and CCK reduced orexin-A compared with the fed controls. The septum is thought to have inhibiting effects on food intake induced by LHA activation. Thus, depending on the brain area examined, orexin-A seems to be regulated differently. This may reflect different sub-functions of orexin-A in energy balance control in these brain areas.

A third set of experiments was conducted to analyze the role of peroxisome-proliferator-activated receptor β (PPARβ) in insulin hypoglycaemia and hyperphagia and its influence on the hypothalamic orexigenic neuropeptides orexin-A, MCH and NPY. To address these questions, PPARβ-knockout (KO) mice with genetic background C57BL/6J and the corresponding wild-type (WT) mice were used. The KO mice showed a slightly more pronounced hyperphagia and hypoglycaemia after IP injection of insulin (2 U/kg) than the WT mice. Insulin tended to reduce liver glycogen in WT mice but not in KO mice. This may explain the greater hypoglycaemia and hyperphagia after insulin in KO mice. Insulin increased hypothalamic MCH and NPY peptide content in KO and in WT mice. Insulin reduced hypothalamic orexin-A content in WT mice, but had no effect on orexin-A in KO mice. Thus, orexin-A does not appear to be necessary for insulin-induced hyperphagia in KO mice. In general, the results suggest a role for PPARβ in control of glucose metabolism and a connection to some orexigenic neuropeptides.

The results of this thesis provide further evidence for a role of the hypothalamic orexigenic neuropeptides orexin-A, MCH and NPY, in the control of food intake. A better understanding of the mechanisms of hunger and satiety may help to develop
pharmacological interventions against disorders of food intake and diseases related to disturbed energy balance regulation.
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In der zweiten Serie von Experimenten untersuchten wir die Mechanismen der erhöhten Nahrungsaufnahme am Modell eines Futterentzugs für 48 Stunden und die Antagonisierung der Hyperphagie durch IP Injektion von CCK. Wir massen den Gehalt


1 GENERAL INTRODUCTION

Control of food intake and energy balance in mammals involves many organs and physiological systems. The central nervous system senses the nutritional and energy state of the body by integrating peripheral signals, and responds with an orchestrated behavioural, autonomic and endocrine output to regulate short-term and long-term energy homeostasis and ultimately to preserve the structure and functions of the organism.

The psychophysiological concepts of “hunger” and “satiety” describe the brain/body representation of increased or decreased homeostatic need for energy. The physiological mechanisms behind these concepts are very complex. From the behavioural point of view, the transition from a registered energy deficit to “hunger” and finally – after eating - to “satiety” includes an initiation phase, which is generated by the internal state and the incentive value of the food. This initiation leads to a change in drive state and selection of the appropriate motor program for the next phase, the procurement phase. Procurement is non-stereotypic and volitional and involves foraging behaviour. It is followed by consummation, consisting of stereotypic and rhythmic movements. The process of satiation or a competing behaviour leads to meal termination. Satiety arises and a different internal state is reached (23; 176).

Food ingestion and digestion as well as nutrient absorption in the gut trigger a variety of signals which are sent to the brain. The entry of food into the gut causes the release of various gastrointestinal peptides, which contribute to the development of satiety. One important pre-absorptive gastrointestinal satiety signal is cholecystokinin (CCK), a peptide secreted by duodenal and jejunal mucosa cells after contact with protein or fats contained in the chyme. CCK binds to two CCK receptor subtypes: (1) the CCK-A receptor, in rats found in the pancreas, the gall bladder, the stomach, on vagal afferent and enteric neurones, and at a number of brain sites; and (2) the CCK-B receptor, which is widely distributed in the brain, but is also present in the afferent vagus and is found in the stomach and in some immune cells (25; 26; 44; 89; 99; 111; 132; 146). CCK
stimulates pancreatic secretion, gallbladder contraction (hence its name: chole = gall, cyste = bladder, kinein = to move), and intestinal motility, and inhibits gastric emptying (116). CCK is produced in the brain as well and is involved in diverse physiological functions such as feeding, pain, anxiety, sexual behaviour, sleep and memory (47). Besides CCK, other visceral molecules are known to affect regulation of energy homeostasis, as peptide YY, glucagon-like-peptide-1, which both inhibit food intake, and ghrelin, which stimulates food intake (119).

Exogenous CCK was shown to inhibit food intake in 1973 (71). It was proposed to mimic the physiological actions of endogenous CCK because it reduced meal size and duration without any signs of toxicity (71). Further studies confirmed the satiating effect of the hormone in different species such as rat, pig, rhesus monkey and humans (5; 70; 71; 88). Also, CCK injected intraperitoneally (IP) into sham-fed rats elicited the complete behavioural satiety sequence (6). This behavioural satiety sequence describes the transition from eating to grooming followed by resting; it can be used to discriminate between interfering and physiological effects of satiation and satiety (79). CCK has been shown not to disrupt the behavioural satiety sequence, but to shorten the latency until resting starts, i.e. to promote satiety. Convincing evidence for a physiological role of CCK in satiety is provided by (1) the finding that CCK receptor antagonists stimulate food intake in various species including man, and that (2) rats with a non-functional CCK-A receptor are hyperphagic and obese (114; 135; 136).

The feeding inhibitory effect of CCK is mediated by CCK-A receptors and the vagus nerve (11; 101; 136). The action of CCK on vagal afferents reduces food intake directly, whereas the effect of CCK on the pyloric sphincter affects food intake indirectly, by slowing gastric emptying (115; 116). Exogenous CCK increases the expression of c-fos, a marker for enhanced neuronal activity, in several brain regions including the locus coeruleus/subcoeruleus nucleus (LC/SC), the nucleus of the solitary tract (NST), the area postrema (AP), and the paraventricular nucleus (PVN) (108; 109; 123). This c-fos induction depends also on CCK-A receptors and on the integrity of vagal afferents (40; 49; 65; 108). The NST and the parabrachial nucleus (PBN) in the pons are important
relay stations of visceral and taste information carried by the trigeminal (V), facial (VII),
glossopharyngeal (IX) and the vagus nerves (X). From there the nutrient-related signals
are conveyed to the hypothalamus, the central nucleus of the amygdala, the bed
nucleus of the stria terminalis, the thalamus and the cortex, to mention only some of the
central areas [reviewed in: (160)].

The hypothalamus controls body homeostasis, i.e., the “milieu intérieur”, by acting on
the endocrine system and the autonomic nervous system, and by interacting with brain
areas that regulate motivation to activate the appropriate behavioural programme. In
the 1940s and 1950s, a “dual centre” concept of food intake control was developed, in
which the lateral hypothalamic area (LHA) was considered to be a “feeding centre” and
the ventromedial hypothalamus (VMH) a “satiety centre” (2; 3; 51; 164). Current
knowledge about functional neuroanatomy and neurochemistry is much more detailed.
Discrete neurones producing neurotransmitters and neuropeptides with specific energy
homeostasis-related functions were detected and are the object of scientific scrutiny.
Neurones in the arcuate nucleus (ARC), located around the base of the third ventricle
and above the median eminence, produce the orexigenic peptides neuropeptide Y
(NPY) and agouti gene-related protein (AGRP) as well as the anorexigens pro¬
opiromelanocortin (POMC) and cocaine-and amphetamine-regulated transcript (CART)
(32; 57). POMC is the precursor of α-melanocyte-stimulating hormone (α-MSH) that
inhibits food intake. The NPY/AGRP- and POMC/CART-systems are thought to work
like an “accelerator” and “brake” for food intake control. NPY is one of the food-intake
stimulants we were interested in. If injected icv or into the PVN, it increases food intake
(43; 98; 163) and if NPY is administered chronically into the PVN, body weight and body
fat mass increase (162). NPY concentrations in the PVN and the ARC increase in
fasted rats, as does overall hypothalamic NPY mRNA (19; 29; 140). In addition to the
control of energy homeostasis, NPY is involved in several physiological functions,
including anxiety, blood pressure regulation and circadian rhythms (170).

Recently identified orexigenic substances produced in the LHA “feeding centre” include
the orexins, orexin-A and orexin-B (syn. hypocretin-1 and hypocretin-2), two peptides
produced from one precursor molecule called prepro-orexin (50; 145). The orexins were originally identified as ligands for two orphan G-protein-coupled receptors (145). The orexin-1 receptor (OR-1) has a greater affinity for orexin-A and is coupled to Gq G-proteins. The orexin-2 receptor (OR-2) binds orexin-A and orexin-B with equal affinity and is coupled to Gq and G\(\alpha_i\) (144; 189) G-proteins. Orexinergic neurones project widely to the entire neuroaxis: cortical, diencephalic, mesencephalic brain areas, and areas in the brainstem are innervated (129). Orexin-A seems to regulate short-term energy homeostasis, as shown by chronic icv infusions, which increased daytime food intake and decreased nighttime food intake, but not total daily food intake or body weight (186). The role of orexin in the control of eating behaviour is emphasized by the finding, that orexin-deficient mice (pre-pro-orexin knockout mice and orexin/ataxin-3 mice) are hypophagic (80; 181). Besides their functions in the control of food intake and metabolism, the orexins were also found to control arousal state and autonomic processes [functions of orexins are reviewed in: (64; 142-144; 168)]. LHA neurones also produce melanin-concentrating hormone (MCH), which – like orexin – stimulates food intake when administered intracerebroventricularly (icv) (133; 138; 145). MCH binds to a G-protein-coupled receptor, SLC-1, which also originally was an orphan receptor (90) [for reviews on MCH see: (73; 86)]. Neurones producing MCH send projections throughout the brain, like the orexin neurones (27; 158). Genetically obese mice were demonstrated to overexpress MCH mRNA and chronic icv MCH seems to cause obesity in mice (72; 133). Therefore, MCH appears to be involved in both short-term and long-term energy balance control. Further, MCH is implicated in other physiological functions like stress, anxiety and depression (83) [for review on the role of the diverse hypothalamic nuclei and neuropeptides in energy homeostasis, see: (179)]. Figure 1 shows the anatomy of hypothalamic neuropeptide systems.
The simplistic “dual centre” idea has long been abandoned because it became clear that brain areas other than the LHA and VMH contribute to the control of food intake and energy balance. For instance, Grill and Kaplan (75) propose a “distributionist” model of food intake control comprising the hypothalamic nuclei and the brainstem as main integrative systems, capable of interoception and driving local effector systems to produce output. The brainstem contains several areas known to be involved in energy homeostasis control, such as the NST, the AP, the dorsal motor nucleus of the vagus (DMNX), the PBN and the dorsal raphé nucleus (DR). Reciprocal connections link brainstem and forebrain networks for food intake control (159).
In some of our experiments, we administered hypoglycaemic doses of insulin to increase food intake. In contrast to this food-intake-stimulating effect of insulin-induced hypoglycaemia, central insulin also can have anorexigenic effects, and if delivered continuously, leads to body-weight loss (106; 149). Circulating levels of insulin are proportional to body fat mass. Insulin plays a role in short-term and longer-term energy homeostasis, as e.g. mice with a neuron-specific insulin-receptor knockout show increased food intake and body weight (34). Streptozotocin-induced diabetic rats become hyperphagic; this hyperphagia can be blunted by icv infusions of insulin during six days, suggesting a role for decreased brain insulin in diabetic hyperphagia (157). Additionally, ARC NPY mRNA levels are increased in streptozotocin-diabetic rats, an effect also antagonized by six-day icv insulin (157). As shown in Fig.1, circulating insulin gains access to the brain and inhibits hypothalamic NPY/AGRP neurones, while it stimulates POMC/CART neurones, to reduce food intake.

Peroxisome-proliferator-activated receptors (PPARs) were recently found to be involved in regulatory processes relevant for disturbances of lipid and glucose homeostasis including type 2 diabetes (172). The name PPAR is derived from the observation that some drugs can increase the number and size of peroxisomes in the liver and a few other organs of rodents, an effect which is paralleled by alterations in lipid metabolism (95; 112; 113). Such drugs are called peroxisome proliferators. Peroxisomes are organelles that partly β-oxidize long-chain fatty acids, which then are fully oxidized in mitochondria. Peroxisomes also detoxify hydrogen peroxide. PPARβ (the Xenopus laevis form) or δ (the mammalian orthologue) belongs to the class of PPARs which are members of the superfamily of ligand-activated nuclear transcription factors (87). PPARβ is expressed ubiquitously, in contrast to PPARα, which is highly expressed in liver, heart, kidney, skeletal muscle and brown adipose tissue, and PPARγ, which is mainly found in white and brown adipose tissues, but also in large intestine and spleen (30). Fatty acids, eicosanoids and prostaglandins are the natural ligands for PPARs. PPARs play a role in fatty acid oxidation and storage: PPARα enhances fatty acid oxidation in the liver, while PPARγ promotes lipid storage and is important for adipocyte differentiation (20). The fibrates, hypolipidaemic drugs, are synthetic ligands for PPARα,
whereas the thiazolidinediones act via PPARγ to increase insulin sensitivity (33; 97). Much less is known about the functions of PPARβ than the other two PPAR isoforms, but the results of recent studies suggest a complex role for PPARβ in lipid homeostasis, also involving other organs than adipose tissue (16; 174). For example, those PPARβ-deficient mice that survive despite placental defects, show decreased adiposity (16; 128). If PPARβ is artificially expressed in adipose tissue, mice are resistant to high-fat diet- and genetically-induced obesity (174). By contrast, PPARβ-deficient mice were shown to be more susceptible to obesity (174).

This thesis addressed the roles of the hypothalamic orexigenic neuropeptides orexin, MCH and NPY and of the transcription factor PPARβ in experimentally-induced states of increased hunger, and tested whether the peripheral satiety signal CCK would influence the expression and/or the levels of the above neuropeptides in specific brain areas.
2 AIM OF OUR EXPERIMENTS

In a first set of experiments, we treated male Sprague-Dawley rats with high, hypoglycaemic doses of insulin and tried to antagonize the resulting hyperphagia by intrameal or continuous IP CCK infusions. The central neural substrates of both CCK’s satiating action and of insulin hyperphagia are not yet fully identified. We hypothesized that hypothalamic orexigens might be involved in insulin hypoglycaemia-induced hyperphagia and that CCK might modulate both hyperphagia and the expression of orexigenic neuropeptides in the hypothalamus. We therefore measured mRNA levels of prepro-orexin, MCH and NPY in the hypothalamus of insulin hypoglycaemic animals infused IP with CCK or phosphate-buffered saline (PBS), as compared with fed euglycaemic control animals.

In a second set of experiments, we used 48 h of fasting to increase hunger in rats. Again, we were interested in hyperphagia as well as its counteraction by CCK and possible neural substrates. Orexin-A peptide content of several brain areas involved in control of food intake and supplied by orexinergic neurones was determined in CCK- or PBS-injected animals that were either fed or fasted.

The final series of experiments, conducted with PPARβ-knockout and wild-type control mice, investigated whether a lack of PPARβ affects the hyperphagia induced by hypoglycaemic doses of insulin. The feeding response of the mice to insulin and associated levels of hypothalamic orexin-A, MCH and NPY were analyzed, as were blood glucose and liver glycogen content.

Overall, this thesis addressed the question whether changes in food intake induced by hypoglycaemia or fasting are accompanied by changes in central orexigenic peptide mRNA or peptide levels, and whether such changes might be instrumental for the observed changes in feeding. To understand the mechanisms that control food intake and to know the peripheral and central signalling pathways involved, may help to develop pharmacological interventions against disordered eating and its consequences.
3 EFFECT OF CCK-8 ON INSULIN-INDUCED HYPERPHAGIA AND HYPOTHALAMIC OREXIGENIC NEUROPEPTIDE EXPRESSION IN THE RAT

3.1 INTRODUCTION

The lateral hypothalamic area (LHA) has been implicated in the control of food intake since the early 1950s, when lesion and electrical stimulation studies led to the concept of a LHA “feeding centre” (2; 3; 51). High doses of insulin, which produce hypoglycaemia and hyperphagia in normal rats, fail to stimulate eating in LHA-lesioned rats (61), suggesting that the LHA is intimately involved in insulin-induced hyperphagia. The LHA contains glucose-sensitive neurones, which react to decreases in blood glucose (125) and might therefore be involved in insulin-induced hyperphagia. However, the neurochemical mediators of insulin hyperphagia are still largely unknown.

Orexin-A and orexin-B (hypocretin-1 and hypocretin-2) are two related LHA neuropeptides discovered in 1998 by two independent groups (50; 145). Orexin-A is thought to be involved in control of energy homeostasis as well as other functions, such as arousal, sleep/wake, stress and autonomic functions (143; 154; 168). Insulin-induced hypoglycaemia increases orexin mRNA (37; 74) and induces c-fos expression in LHA neurones (120), notably in orexin neurones of the LHA (117). Melanin-concentrating hormone (MCH) is another neuropeptide found in the LHA (158), which increases food intake when injected centrally (133). Insulin-induced hypoglycaemia stimulates MCH neuronal activity and increases MCH mRNA levels (14). Also, neuropeptide Y (NPY) is a hypothalamic orexigen, mainly produced in neurones of the arcuate nucleus (43; 162; 163). NPY plays a role in some, but not all, conditions in which food intake increases; intriguingly, NPY-deficient mice show normal food intake and body weight (163).

Cholecystokinin (CCK) is a putative gastrointestinal satiety signal, released in the small intestine in response to food digestion. CCK has been shown to inhibit food intake in different species, including rats and humans (70; 71). The actions of peripheral CCK are mainly mediated through CCK-A receptors and depend on intact vagal afferents.
Peripherally administered CCK activates the medial portion of the nucleus of the solitary tract, the lateral parabrachial nucleus, and other nuclei such as the area postrema, the paraventricular nucleus of the hypothalamus, the supraoptic nuclei, and the central amygdaloid nucleus. Activation of brain nuclei by peripheral CCK depends on CCK-A receptors and is mediated by vagal afferents. Whether CCK is able to inhibit insulin-induced hyperphagia is unknown.

This study addressed the role of hypothalamic orexin, MCH and NPY in insulin-induced hyperphagia in the rat. We examined the effects of peripheral CCK-8 on insulin-induced hyperphagia and on changes in the hypothalamic mRNA levels of prepro-orexin, MCH, and NPY induced by insulin hypoglycaemia when no food was available. We found that peripheral CCK is able to counteract insulin-induced hyperphagia and that this effect is accompanied by a normalization of the hypothalamic expression of prepro-orexin and MCH.

3.2 MATERIALS AND METHODS

3.2.1 Animals and housing conditions. Male Sprague-Dawley rats were kept in a temperature-controlled room (22 ± 0.5 °C) on a 12h:12h light-dark cycle. Rats had ad libitum access to water and powdered rodent chow (Nafag, Gossau, Switzerland) and were single-housed in open-topped Plexiglas cages (37x21x41 cm) designed for remotely controlled infusions and concomitant food intake pattern recordings. Before the experiments, rats were handled daily and adapted to the housing conditions. The experimental procedures were approved by the Kanton of Zurich’s Animal Use and Care Committee.

3.2.2 Surgery. Food was removed 6-12 hours before surgery. Rats were anaesthetized by intraperitoneal (IP) injection (110 μl/100 g BW) of a 1:4 (v/v) mixture of xylazine (20 mg/mL, NARCOXYL-2⁰; Veterinaria, Zurich, Switzerland) and ketamine (100 mg/mL, NARKETAN-10⁰; Chassot, Berne, Switzerland). The IP catheters were assembled as
previously described for hepatic-portal vein catheters (166) and sterilized with ethylene-
oxide at 37 °C before implantation. The protruding dorsal end of a silicone catheter
(Ulrich AG, St. Gallen, Switzerland, ID 0.63 mm, OD 1.19 mm, length 15.5 cm for a
350-g rat) was slipped on to a modified cannula with a screw top, reinforced with a
2.5cm-piece of a larger silicon catheter (ID 1.02 mm, OD 2.16 mm) and led through a
folded monofilament knitted polypropylene surgical mesh (BARD MESH®; Davol Inc., RI,
USA) to improve adhesion to skin and fascia. The IP catheters were exteriorized and
fixed in the interscapular area as described previously for hepatic-portal vein catheters
(155; 166). The dorsal end was led subcutaneously from the neck to a 4-cm incision in
the abdomen, pulled through a small hole in the abdominal wall and fixed inside with
non-absorbable silk thread. At the intraperitoneal end of the catheter, a very small piece
of larger tubing (ID 1.02 mm, OD 2.16 mm) was attached to prevent the catheter from
being pulled out through the hole. Immediately after surgery, an intramuscular injection
of the analgesic metamizolum was given (100 µl, 500 mg/mL; VETALGIN®; Veterinaria,
Zurich, Switzerland). On the day of surgery and on the two following days animals
received antibiotic treatment (Chloramphenicol, 30 mg/kg injected subcutaneously and
twice daily; CHLOROSOL-25®; for Switzerland: Dr. E. Gräub AG, Berne). Catheters
were flushed daily with 0.5 mL 0.9% sterile saline during the first post-operative week
and every second day thereafter.

3.2.3 Adaptation and experimental procedure. After 7 days of recovery from surgery,
rats were adapted to the experimental procedures for 5 days. For experiments with
insulin injections, two hours after lights on, the animals were injected IP with 0.9%
saline. Also, animals received several intrameal saline infusions on the days prior to the
experiment. For adaptation to 60-min continuous infusions, 0.9% saline infusions of
increasing duration were given until 60 min were reached. The infusion pumps (model
A99, Razel, Stamford, CT) were remotely controlled, and the rats’ feeding activity was
observed by infrared cameras. Food intake patterns were recorded and analyzed as
previously described (96). Before and after infusions, the tubing connecting the pumps
and the animals was flushed with 2-3 mL of 1% benzyl alcohol and saline.
3.2.4 Compounds. Sulphated CCK-8 (cholecystokinin octapeptide, ammonium salt; Bachem, Switzerland) was dissolved in sterile phosphate buffered saline (PBS), frozen in aliquots, and administered at the doses specified for each experiment. For all insulin-hyperphagia studies, intermediate-duration porcine insulin (INSULATARD MC SUIS\textsuperscript{®}; Novo Nordisk, Bagsvaerd, Denmark) was injected IP at a dose of 50 IU/kg body weight.

3.2.5 Experimental protocols

3.2.5.1 Experiment 1: intrameal CCK-8 infusions. The effect of three repetitive intrameal IP CCK-8 infusions on feeding behaviour during the dark phase was examined in 12 rats weighing 470 ± 9.3 g (mean ± S.E.M.), which had free access to food (n = 6 per group). After baseline measurements, CCK-8 or control infusions were tested in a cross-over design. During each of the first three spontaneous dark-phase meals CCK-8 (4 μg/kg) or PBS was infused IP. The infusions were initiated by remote control 30 seconds after meal onset and lasted for 3 minutes. The criteria for meal onset were a decrease of food cup weight by at least 0.3 g and concomitant feeding activity visually verified on the monitor. The infusion pumps were adjusted to yield an infusion rate of 280 μL/min.

3.2.5.2 Experiment 2: intrameal CCK-8 infusions during insulin-induced hypoglycaemia. We studied the effects of CCK-8 on insulin-induced hyperphagia and on hypothalamic neuropeptide expression during the light phase because we aimed for an experimental setup which finally – in a terminal experiment – could show changes in neuropeptide expression, and also because several findings indicate that such changes might be more robust during the light than during the dark phase. Firstly, the increase in food intake induced by intracerebroventricular orexin-A appears to be more pronounced during daytime (186) and particularly the early light phase (82; 145). Further, previous studies which found an increase in prepro-orexin mRNA in response to insulin-induced hypoglycaemia were done in the light phase (37). Food cups were closed only between the insulin injections and the start of the infusions (45 min). The effect of three repeated intrameal CCK-8 infusions was tested in 16 rats assigned to two groups (each n = 8) (body weight 409 ± 7 g). Both groups received saline injections combined with PBS infusions for baseline, and then insulin was injected at 2 h after lights on in combination
with infusions of either CCK-8 (4 μg/kg CCK-8) or PBS. Infusions were given for 3 minutes during the first three meals after insulin injection. The infusion rate to deliver the CCK-8 dose in three minutes was 224 μL/min.

3.2.5.3. **Experiment 3: continuous CCK-8 infusions during insulin-induced hypoglycaemia.** This experiment tested the effects of continuous 60-minute CCK infusions on insulin-induced hyperphagia in the light phase. Fifteen rats weighing 373 ± 6 g were assigned to two groups (n = 8 and 7, respectively). Both groups received saline injections combined with PBS infusions for baseline, and then insulin (50 IU/kg) was injected 2 h after lights on, together with 60-minute infusions of either CCK (32 μg/kg CCK-8) or PBS in a cross-over design. The CCK and PBS infusions started one hour after insulin injections. The infusion pumps were adjusted to deliver 5 mL over 60 min (83.3 μL/min). Food cups were closed between 15 min prior to injections and the infusion start (75 min).

3.2.5.4 **Experiment 4: neuropeptide expression and blood glucose.** In the terminal experiment, 20 rats weighing 460 ± 9 g were assigned to three groups. Two groups comprising 8 rats each had no access to food (food cups removed 15 min before injections), received insulin injections (50 IU/kg) and 60 minutes later a 30-minute infusion of either CCK-8 (32 μg/kg) or PBS. Food was removed in insulin-treated animals because Cai et al. found an increase in prepro-orexin mRNA in insulin-hypoglycaemic animals only when animals had no access to food (37). A third group (n=4) had *ad libitum* food access and received saline injections only. Individual animals' treatments were staggered by 8 minutes. Pilot experiments had shown that hypoglycaemia is well established 60 min after insulin injections (plasma glucose levels in hypoglycaemic rats (n = 5) were 3.2 ± 0.3 mmol/L, compared with 7.0 ± 0.1 mmol/L basal values (n = 10)) [P<0.0001]). Moreover, hyperphagia induced by hypoglycaemia was clearly reduced after 30 min of CCK infusion (0.4 ± 0.2 vs. 3.1 ± 0.7 g, n = 15 in each group [P<0.05]). We thus sacrificed the rats in the final experiment 90 minutes after insulin injection, i.e. 30 minutes after starting the CCK infusion.
The whole hypothalamus was dissected following "Maps and Guide to Microdissection of the Rat Brain" (Miklos Palkovits and Michael T. Brownstein, Elsevier) using a rodent brain slicer (ASI instruments, Warren, MI, USA). Samples were snap-frozen in liquid nitrogen and stored at -80 °C.

Total RNA was isolated by single-step method after Chomczynski and Sacchi (41). The RNA solution was pretreated with DNase1 (DNA-FREE®, Ambion; Austin, Texas, USA), and reverse transcription of total RNA (REVERSE TRANSCRIPTION SYSTEM®; Promega Corporation, Madison, WI, USA) was performed at 42 °C for 90 minutes.

Semi-quantitative analysis of mRNA expression of neuropeptides and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was carried out by RT-PCR with the number of cycles at which the band intensity increased linearly with the amount of RNA used. cDNA was amplified by PCR (PCR SUPERMIX®, Invitrogen; Carlsbad, CA, USA). For GAPDH, PCR was run for 30 cycles (1 min at 94 °C denaturation, 1 min at 55 °C annealing, 1 min at 72 °C elongation) with a final hold step at 4 °C. For the NPY, MCH and prepro-orexin, PCR was run for 40 cycles (one cycle 15 min at 50 °C, 10 min at 95 °C, then 40 cycles 15 sec 95 °C and 1 min 60 °C). Forward primers were as follows: AAC GAC CCC TTC ATT GAC CTC (GAPDH), TGG GTA TTT GGA CCA CTG CA (prepro-orexin), AAA ATG ATG AGAGCG GCTTCA (MCH), GGG GCT GTG TGG ACT GAC CCT GG (NPY) and reverse primers: TAC CGT ACC TGA CACCAGTAC (GAPDH), TGGTGTCTG GAG CTCAGG G (prepro-orexin), CGAGAT TCTGCTTGGAGC CT (MCH), GATGTA GTGTCG CAGAGCGGAG (NPY). cDNA was quantified by computerized densitometry (Scion image, Scion Corporation, Frederick, Maryland, USA), and the signals of NPY, MCH and prepro-orexin normalized by intensity of the house-keeping gene GAPDH.

3.2.5.4.1 Analysis of blood metabolites: Trunk blood was collected immediately after decapitation. 3 mg NaF/mL blood or 1.8 mg EDTA/mL blood was added as anticoagulant for analysis of glucose and insulin, respectively. Glucose concentration was determined by a standard enzymatic method with a Cobas Mira autoanalyzer.
(Glucose HK, Cobas Mira, Hoffmann LaRoche, Switzerland). Plasma insulin was determined by radioimmunoassay (Insulin RIA 100, Pharmacia&Upjohn Diagnostics AB, Uppsala, Sweden; 100% cross-reactivity with porcine insulin; using human standard).

3.2.6 Statistical analyses: Behavioural data were analyzed by one-way repeated measures ANOVA or by one-way factorial ANOVA followed by Fisher's PLSD post-hoc test. Neuropeptide mRNA and glucose data were analyzed using one-way ANOVA (factorial) followed by Fisher’s PLSD post-hoc. All analyses were done with StatView (SAS Corporation).

3.3 RESULTS

3.3.1 Experiment 1: intrameal CCK-8 infusions. Eleven rats completed the experiment; one was excluded because the catheter became clogged. Infusions of 4 μg/kg body weight CCK-8 reduced the size of the first three spontaneous nocturnal meals by about 50% (P<0.0001) (Fig. 1). Cumulative food intake was still significantly lower in CCK-infused animals at the end of infusions, when CCK-treated animals and control animals had consumed 5.8 ± 0.7 g and 7.6 ± 0.5 g, respectively (P<0.05) in 3.7 ± 0.3 (CCK) and 3.5 ± 0.4 meals (controls, P=0.48).
Fig. 1: Three repetitive intrameal infusions of CCK-8 (4 μg/kg) reduced meal size in rats. CCK-8 or an equivalent volume of vehicle (PBS) was infused (280 μl/min over 3 min) during the first 3 spontaneous meals in the dark phase. Data (mean ± S.E.M) represent sizes of three meals each of 11 rats (33 meals in total) for baseline, PBS, and CCK, respectively. (P<0.0001 in Fisher’s PLSD test after significant repeated measures ANOVA). Meals are defined in the text. CCK, sulphated cholecystokinin octapeptide; PBS, phosphate-buffered saline.

3.3.2 Experiment 2: intrameal CCK-8 infusions during insulin-induced hypoglycaemia. Fifteen rats completed the experiment; one had to be excluded because it detached its neckpiece. Insulin administration causing hypoglycaemia (3.2 ± 0.3 mmol/L, compared with 7.0 ± 0.1 mmol/L basal values [P<0.0001] in pilot experiment) increased cumulative food intake and meal size as compared with baseline conditions (P<0.05). CCK-8 treatment reduced meal size during hypoglycaemia (P<0.05) (Fig. 2), but a slight reduction in cumulative food intake (4.1 ± 1 g vs. 4.9 ± 0.8 g) did not reach statistical significance. During the infusion period, CCK and PBS-treated animals ate 2.6 ± 0.6 and 2.6 ± 0.4 meals, respectively (P=0.94).
Fig. 2: Three repetitive intrameal infusions of CCK-8 (4 μg/kg) blunted the increase in meal-size induced by IP insulin (50 IU/kg). IP CCK-8 or an equivalent volume of vehicle (PBS) was infused (224 μl/min over 3 min) during the first 3 meals after insulin injection in the light phase. Data (mean ± S.E.M) represent sizes of two meals measured in all 15 rats (30 meals) for baseline, hypoglycaemia, and hypoglycaemia/CCK, respectively (P<0.05 in Fisher’s PLSD test after significant factorial ANOVA). CCK, sulphated cholecystokinin octapeptide; PBS, phosphate-buffered saline; hypo, insulin-hypoglycaemic rats.

3.3.3 Experiment 3: continuous CCK-8 infusions during insulin-induced hypoglycaemia. Fourteen rats completed the experiment, one being excluded because of technical problems. Fifty IU/kg insulin causing hypoglycaemia (see above) significantly increased meal size (P<0.0001) and 60-min cumulative food intake (P<0.05). Sixty minutes continuous infusion of 32 μg/kg CCK-8 antagonized (P<0.0001) the enhancement of meal size by hypoglycaemia and completely abolished its hyperphagic effect (P<0.0001) (Fig. 3A, 3B and 4). In addition, CCK infusion reduced the number of meals during the infusion period (CCK 0.3 ± 0.2 meals vs. controls 0.8 ± 0.1 meals, P<0.05). Ninety min after insulin injections, marked hyperphagia was evident (baseline, 1.0 ± 0.3 g vs. insulin/PBS, 3.1 ± 0.7 g; P<0.05). Moreover, hyperphagia was clearly reduced after 30 min CCK infusion to 0.4 ± 0.2 g in CCK treated, hypoglycaemic rats (P<0.05). The
timing of the terminal experiment was based on these results and on the plasma glucose data from the pilot experiment (see Methods).

Fig. 3: Sixty min continuous infusion of CCK-8 (32 μg/kg) prevented the increase in meal size (3A) and cumulative food intake (3B) induced by IP insulin (50 IU/kg). CCK-8 or an equivalent volume of vehicle (PBS) was infused (83 μl/min) for 60 min, starting one hour after insulin injections. Data (mean ± S.E.M.) represent size of the single meal eaten and cumulative food intake during the 60-min infusion period of 14 rats for baseline, hypoglycaemia, and hypoglycaemia/CCK, respectively (P<0.0001 and P<0.05 in Fisher's PLSD after significant repeated measures ANOVA). CCK, sulphated cholecystokinin octapeptide; PBS, phosphate-buffered saline; hypo, insulin-hypoglycaemic rats.
Fig. 4: Injection of insulin (50 IU/kg) increased cumulative food intake in rats. 60 min continuous infusion of CCK-8 (32 μg/kg) antagonized this effect. Infusions of CCK-8 or an equivalent volume of vehicle buffer (PBS) were given over 60 min, starting one hour after insulin injections. Data (mean ± S.E.M.) represent food intakes during infusion and at time after insulin injection each of 14 animals for baseline, hypoglycaemia, and hypoglycaemia/CCK, respectively (P<0.05 in Fisher’s PLSD after significant repeated measures ANOVA). CCK, sulphated cholecystokinin octapeptide; PBS, phosphate-buffered saline; hypo, insulin-hypoglycaemic rats.

3.3.4 Experiment 4: neuropeptide expression and blood glucose.
IP insulin (50 IU/kg) led to a massive increase in plasma insulin concentration (saline-injected rats: 21.3 ± 5.45 μU/mL insulin; all insulin-injected rats (whether CCK- or PBS-infused): >240 ± 0 μU/mL insulin [all above the assay range]; insulin was only determined to control for the accuracy of IP insulin injections). This was accompanied by a significant decrease in plasma glucose from 5.3 ± 0.4 mmol/L (saline/ad lib) to 2.0 ± 0.2 mmol/L (insulin/PBS) (P<0.0001), or to 2.8 ± 0.2 mmol/L (insulin/CCK) (P<0.0001). Thus, CCK infusion significantly attenuated hypoglycaemia to 2.8 ± 0.2 mmol/L (insulin/CCK) (P<0.05). Compared with values in ad-libitum fed and saline-injected animals, IP insulin (50 IU/kg) increased the hypothalamic level of prepro-orexin mRNA by 30% (P<0.05) and that of MCH mRNA by 52% (P<0.05) in rats without access to
food. Continuous, 60-min IP CCK infusion (32 μg/kg) blunted the insulin-induced increase in expression of prepro-orexin and MCH (P<0.05). NPY mRNA expression was not significantly changed by either treatment (Fig. 5A, 5B, 5C).

![Graphs showing mRNA expression](image)

Fig. 5: Insulin (50 IU/kg, IP) increased expression of prepro-orexin mRNA (5A) and of MCH mRNA (5B), and IP CCK-8 infusion (32 μg/kg) abolished this increase. NPY mRNA was not changed significantly (5C). Data (mean ± S.E.M.) represent ratios of band intensities of prepro-orexin/GAPDH, of MCH/GAPDH and of NPY/GAPDH for n=4 animals with food ad lib, n=8 animals with insulin injection and CCK infusion and n=8 animals with insulin injection and PBS infusion (for prepro-orexin and for MCH mRNA P<0.05 in Fisher's PLSD after significant factorial ANOVA). CCK, sulphated cholecystokinin octapeptide; PBS, phosphate-buffered saline; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hypo, insulin-hypoglycaemic rats.
3.4 DISCUSSION

Intrameal IP CCK infusion reduced spontaneous dark-phase meal size in rats, confirming the well-known satiating and meal-terminating effect of the hormone (70; 71). Extending these data, we report here for the first time that repetitive intrameal or continuous IP CCK administration also reduced the increased meal size and hyperphagia caused by hypoglycaemic doses of insulin. These results suggest that CCK may interact with glucose signalling in the control of eating. This assumption is supported by the observed normalization of the hypoglycaemia-induced increase in hypothalamic prepro-orexin and MCH expression by peripheral CCK. The behavioural interaction between insulin and CCK, and the parallel changes in orexigenic neuropeptide expression, is particularly interesting because the central neural mechanisms of both CCK and glucose signalling are still poorly understood. Our results implicate hypothalamic orexin and MCH as brain targets of the CCK-activated ascending satiety pathway.

In 1984, West et al. reported a persistent reduction of meal size by CCK infusions given during every meal for 6 days. Total food intake was not affected, however, because the animals compensated for the decrease in meal size by increasing meal frequency (177). Here, we observed no increase in the number of meals by CCK during the infusion period. Thus, cumulative food intake was still lower in CCK-treated rats at the end of continuous infusions and after three repeated nocturnal intrameal infusions. Cumulative food intake was also somewhat lower after light phase intrameal CCK infusions, but this difference was not significant. Together, these findings suggest that the animals do not immediately compensate for a transient CCK-induced reduction of food intake.

We used a high dose of insulin to induce hypoglycaemia that produced pronounced hyperphagia. This is an extreme state that cannot be compared to the physiological dips in blood glucose thought to initiate normal meals. In the terminal experiment, animals had no access to food, but were sacrificed 90 min after insulin injections, a time at which the rats were observed to be not yet very drowsy because of hypoglycaemia.
In our behavioural studies, the rats seemed to be aroused by the hypoglycaemia, since they only showed minor resting behaviour after eating and CCK infusions even during light phase. Insulin accessing the brain can inhibit feeding (106; 149). The high hypoglycaemic dose we used produced pronounced hyperphagia that evidently over rode any food-intake reducing effect of insulin itself.

In demonstrating that insulin-induced hypoglycaemia and hyperphagia are accompanied by increased hypothalamic levels of prepro-orexin and MCH mRNA, our results confirm previous reports of increased expression of hypothalamic orexin (37; 74) and of increased MCH mRNA (14) by hypoglycaemic doses of insulin. Our novel finding is, however, that CCK, in addition to markedly reducing hypoglycaemia-induced hyperphagia, almost normalized the hypoglycaemia-induced increases in hypothalamic prepro-orexin and MCH expression. Increased activities of these hypothalamic orexigenic peptidergic pathways seem to be important for the response to insulin hypoglycaemia and may be instrumental in driving hypoglycaemia-induced hyperphagia. By contrast, we did not find convincing changes in hypothalamic NPY levels.

A recent study showed that intracerebroventricular (icv) orexin antagonized the hypophagic effect of CCK (9). This, together with our results, suggests that the orexin system and the peripheral CCK satiety system are functionally connected. More specifically, our results show that CCK and the orexin system interact in regulating a crucial physiological response to hypoglycaemia, namely increased hunger and food intake. Orexin is assumed to provide a link between metabolic state and motivated behaviour. The animal must be able to adjust arousal state and eating behaviour to its energetic needs. Profound insulin hypoglycaemia might be a signal which primes the orexin system for food-seeking behaviour. The administration of exogenous CCK mimics the consequences of food ingestion, and this might block the orexin system and thus inhibit food seeking and ingestion. Some authors have implicated an inhibiting influence of several feeding-related signals like gastric distension, glucose or other nutrients, on orexin neurones, since in their experiments, hypoglycaemic animals allowed to feed did not show any increase in hypothalamic prepro-orexin mRNA (36;
Therefore, the effect of CCK on prepro orexin levels might partly be due to inhibition of gastric emptying and prolonged activation of mechanoreceptors. By contrast, as already mentioned, some authors reported an increase in orexin expression induced by hypoglycaemia in animals with access to food (74).

CCK also reduced the hypoglycaemia-induced increase in MCH mRNA expression. MCH is thought to play a role in energy homeostasis because centrally administered MCH stimulates food intake (133) and because MCH-deficient mice are hypophagic and lean (151). Recently, a novel reciprocal hypothalamic circuit including orexin and MCH has been proposed (77). Our results suggest that MCH-dependent mechanisms are also involved in hypoglycaemia-induced hyperphagia and that this mechanism, too, is modulated by peripheral CCK.

Hypothalamic NPY mRNA and peptide content are increased in several conditions of hyperphagia, e.g. after fasting, food restriction or in insulin-dependent diabetes (150; 178). Our present finding that hypothalamic NPY expression was not significantly altered during insulin-induced hypoglycaemia is consistent with two other studies that reported no increase in hypothalamic NPY level or expression in response to acute or chronic insulin-induced hypoglycaemia and hyperphagia (45; 54). Interestingly, insulin itself acts to decrease food intake; it crosses the blood-brain barrier and changes hypothalamic neuropeptide status, including decreased activity of NPY neurones. The hyperinsulinaemia produced by high doses of exogenous insulin might thus counteract any stimulation of NPY neurones by neuroglycopenia (45).

We measured neuropeptide expression in blocks of whole hypothalamus rather than in specific hypothalamic regions. In our view, this does not vitiate the determination of prepro orexin and MCH mRNA levels since these neuropeptides are expressed in two separate cell populations of the lateral hypothalamus only. Given accurate dissection and consistent landmarks, the dilution factor of using the whole hypothalamus should be essentially constant. NPY mRNA is produced mainly in the arcuate nucleus, but also in the periventricular area, the supraoptic nucleus and the suprachiasmatic nucleus (179).
Thus, differential changes of NPY expression in these subnuclei cannot be discriminated by whole hypothalamus analysis.

Because we tried to replicate the findings of Cai et al. (37), our insulin-treated rats had no access to food. Yet, it appears unlikely that the increases found in prepro-orexin and MCH mRNA were due to the lack of food because food was removed for only 105 minutes, and prepro-orexin mRNA as well as MCH mRNA were shown to be increased by insulin hypoglycaemia even in animals with access to food (14; 74).

It has to be mentioned, that our ad libitum-fed control group used in the terminal experiment comprised only four animals. This is a rather small number, and given the quite high variance of neuropeptide concentrations, we have to admit that Type 2 statistical errors may have occurred under these conditions.

Peripheral CCK acting on CCK-A receptors triggers vagal afferent signals which enter the brain at the level of the nucleus of the solitary tract. Interestingly, the LHA is not mentioned in studies in which brain activation by peripheral CCK was analyzed by c-fos induction, but neurones in the paraventricular nucleus are activated (102; 108). This does not completely exclude activation of the LHA by peripheral CCK, since c-fos-independent mechanisms of neuronal activation exist. The paraventricular nucleus receives input from arcuate NPY/agouti-related protein and pro-opiomelanocortin/cocaine- and amphetamine-regulated transcript neurones and from LHA orexin neurones (59; 60). IP CCK-8 antagonized eating induced by electrical stimulation of the lateral hypothalamus (147). These and our results indicate that IP CCK-8 exerts its satiating effect by targeting different hypothalamic systems, and this is associated with reductions in prepro-orexin and MCH expression.

The significantly higher plasma glucose levels in insulin-CCK rats than in insulin-PBS rats may also contribute to the observed effects on hypothalamic orexin expression. In acute insulin-induced hypoglycaemia, LHA prepro-orexin mRNA was increased (37; 74), and 33% of LHA orexin neurones were shown to be activated (117). Griffond et al.
reversed the insulin/hypoglycaemia-induced increase in prepro-orexin mRNA by injecting glucose to re-establish euglycaemia (74). In contrast to hypoglycaemia, 2-DG, which also induces neuroglycopenia and triggers eating, was not found to increase prepro-orexin mRNA (37). 2-DG is a glucose antimetabolite and after 2-DG administration, animals become hyperglycaemic because of physiological counter-regulation. This hyperglycaemia may theoretically prevent any changes in prepro-orexin mRNA. The LHA also includes glucose-sensitive neurones, which are activated by falling blood glucose levels and inhibited by an increase; these have not yet been fully characterized neurochemically (125). Orexin cells could be part of the glucose-sensing system of the LHA (117), reacting mainly to marked decreases in plasma glucose. One recent study reported that glucose-sensing neurones were not found to contain orexin-A, but juxtaposition of hypothalamic orexin and glucose-sensitive neurones suggests synaptic contacts between these two cell groups, and they may work together in regulating glucoprivic eating (100). The MCH cells are also putative candidates for being the glucose-sensitive neurones of the LHA. MCH mRNA increases in the LHA after IP 2-DG, while 2-DG-induced glucoprivation in LHA cell cultures did not change MCH expression (18). Thus, the role of the MCH cells in food-intake regulation, especially by glucose, remains to be elucidated.

Our results strongly suggest that increased activities of the orexin and MCH systems may mediate, at least in part, the hyperphagic effects of insulin-induced hypoglycaemia. This may include effects leading to increased food intake that are counteracted by IP CCK. CCK may change neuropeptide expression by activating mechanisms dependent on CCK-A receptors and the vagus and/or by interacting with a glucose-dependent pathway. The changes in blood glucose levels and CCK, both, may directly influence the prepro-orexin and MCH mRNA expression or indirectly through glucose-sensing neurones which interact with MCH and orexin cells.

In conclusion, CCK-8 antagonizes hypoglycaemia-induced hyperphagia and normalizes the hypoglycaemia-induced increase in the hypothalamic expression of the orexigenic neuropeptides, orexin and MCH. Thus, hypoglycaemic insulin doses appear to activate
a circuit containing orexin and MCH neurones, and these neurones appear to be among the targets of the CCK-activated ascending satiety pathways. Studies analyzing the role of the diverse receptor subtypes of these neuropeptides in different brain areas should help to clarify the interaction between hypoglycaemia and peripheral CCK in the control of food intake behaviour. In turn, this should bring us closer to rational and effective pharmacological manipulation of the central mechanisms regulating hunger and satiety.
4 EFFECT OF INTRAPERITONEAL CCK-8 ON FOOD INTAKE AND BRAIN OREXIN-A AFTER 48 HOURS OF FASTING IN THE RAT

4.1 INTRODUCTION

The lateral hypothalamic area (LHA) has long been implicated in food intake control as a "feeding centre" (3). In 1998, a novel pair of LHA neuropeptides was discovered, orexin-A and orexin-B (also called hypocretin-1 and hypocretin-2) (50; 145). Orexin-A and orexin-B are produced in the lateral and posterior hypothalamic areas, but also in the perifornical nucleus and in subthalamic areas such as the zona incerta, subincertal and subthalamic nuclei (50; 145). Centrally administered orexin-A and orexin-B stimulate food intake, and the LHA level of prepro-orexin mRNA is elevated in insulin-induced hypoglycaemia and in fasting (37; 74; 145). Insulin-induced hypoglycaemia also activates orexin neurones (117). The orexins are implicated in the control of energy homeostasis, arousal and autonomic processes (94; 142; 153; 168). More specifically, they may integrate information about the arousal and energy status and orchestrate the body's responses to such homeostatic challenges by their widespread projections (144; 184). Fasting causes a negative energy balance and is accompanied by an increase in arousal. Fasting increases LHA prepro-orexin mRNA expression and might also change the orexin-A peptide content in the LHA and in the target areas of orexinergic neurones (37; 145). Yet, other attempts to show such effects of fasting yielded inconsistent results (110; 167). To resolve this uncertainty, we used an enzyme immunoassay to analyze the orexin-A contents after 48 hours of fasting in the lateral and the medial hypothalamus and in brain areas receiving orexinergic fibres. We determined the orexin-A contents in the septum and in the posterior brainstem, where orexin-immunoreactive fibres are found in the nucleus of the solitary tract (NST), the area postrema (AP) and other areas (130). The septum is part of the limbic system and implicated in food intake control in addition to its presumed functions in learning, memory and reward (91; 122). The brainstem integrates peripheral signals related to control of food intake and energy homeostasis (159).
We have previously shown that intraperitoneal (IP) administration of the gastrointestinal satiety peptide cholecystokinin (CCK) attenuated the insulin hypoglycaemia-induced increases in food intake and in hypothalamic prepro-orexin mRNA (68), suggesting that an insulin hypoglycaemia-induced hunger signal and an opposing CCK-derived satiety signal converge on hypothalamic orexin neurones. The recently reported reversal of the feeding inhibitory effect of CCK by intracerebroventricular administration of orexin-A in mice (9) also suggests an interaction between CCK and the orexin system in the control of food intake. Peripheral CCK activates neurones in many of the regions that receive projections from LHA orexin-A neurones (102; 108). To address the potential functional interactions between afferent CCK signalling and the orexin system we assessed the effect of an IP injected anorectic CCK dose on the fasting-induced change in brain orexin-A content. Because blood glucose regulates orexin expression and the activity of orexin neurones (36; 37; 74; 117; 145), blood glucose values during and after fasting, and after CCK injection were determined.

4.2 MATERIALS AND METHODS

4.2.1 Animals and housing conditions. Adult male Sprague-Dawley rats (Charles River, Germany) were housed single-caged in a temperature-controlled room (22 ± 0.5° C) on a 12 h:12 h light-dark cycle. Except during experiments, rats had ad libitum access to water and rodent chow (Kliba-Nafag, Kaiseraugst, Switzerland). The rats were handled and weighed every day. All experimental procedures were approved by the Kanton of Zurich's Animal Use and Care Committee.

4.2.2 Substances. Sulphated CCK-8 (cholecystokinin octapeptide, ammonium salt, Bachem, Switzerland) was dissolved in phosphate-buffered saline (PBS) and frozen in aliquots.
4.2.3 Experimental Protocols

4.2.3.1 Experiment 1: Time-course of blood glucose during fasting. Twelve rats weighing 210 ± 10 g (mean ± S.E.M.) were used for this experiment. Food was removed from the cages 10 h after lights on. To track circadian changes in circulating glucose during the second day of fasting, blood glucose in fasting rats (n=6) was measured during the light phase and at the onset of the dark phase (i.e., after 41, 43, 45, 47, 49 and 51 h of fasting) and compared with the blood glucose concentration of ad libitum fed control rats (n=6). Blood samples (6.5 μL) were taken with a pipette directly from a tail incision and blood glucose concentration was measured by Glucotrend2® (Roche Diagnostics, Rotkreuz, Switzerland).

4.2.3.2 Experiment 2: CCK effect on feeding after 48 h fasting. Thirty-two rats weighing 263 ± 15 g were used for this experiment. After 48 h of fasting (started 10 h after lights on) rats were injected IP with 500 μL of PBS (n=10), 5 μg/kg CCK (n=11), or 10 μg/kg CCK (n=11). Five minutes after injections, pre-weighed food pellets were given to the animals and food intake was measured 15, 30, 45, 60, 90 and 120 min later.

4.2.3.3 Experiment 3: Blood glucose after fasting and CCK. Thirty-two rats weighing 323 ± 24 g were used. Based on the previously observed effect of continuous IP CCK infusions on plasma glucose in insulin hypoglycaemic rats (68), we examined whether a single IP CCK injection affected blood glucose in fasted rats. Rats were assigned to the following four groups: PBS/fasted (n=8), PBS/fed (n=8), 5 μg/kg CCK/fasted (n=8), 5 μg/kg CCK/fed (n=8). Every 30 min between 8 and 12 h after lights on, a rat of the fasted groups was submitted to 48 h fasting. CCK or PBS was injected (500 μL each) IP after 48 h of fasting at the end of light phase (between 8 and 12 h after lights on). Fifteen min after the injections, tail incisions for blood glucose measurements (Glucotrend2®, Roche Diagnostics, Rotkreuz, Switzerland) were performed as described above. The whole procedure was staggered to allow for 30 minutes/animal, and the whole experiment lasted for four days, with 8 animals per day (2 animals per group).
4.2.3.4 Experiment 4: Effect of 48 h fasting and CCK on orexin-A in the brain: Twenty-four rats weighing 329 ± 13 g were used. Animals were assigned to the following four groups: PBS/fasted (n=6), PBS/fed (n=6), 5 μg/kg CCK/fasted (n=6), 5 μg/kg CCK/fed (n=6). Every 40 min between 8 and 12 h after lights on, two rats (one of the CCK/fasted and one of the PBS/fasted group) were submitted to 48 h fasting. Thus, all animals were sacrificed during the last four hours of the light phase (between 8 and 12 h after lights on). We used the lower CCK dose (5 μg/kg) because it yielded approximately the same reduction of 30-minute food intake as the higher CCK dose (10 μg/kg). Injections and decapitation of the animals were staggered by 10 min with the injections given 32 min prior to sacrifice. Brains were removed immediately after decapitation, flushed with distilled water, frozen in 2-methylbutane (Fluka Chemie, Buchs, Switzerland), and stored at -80 °C.

4.2.3.4.1 Enzyme Immunoassay. Brain tissue was dissected according to Paxinos and Watson (127), using a rodent brain slicer (ASI Instruments, Warren, MI, USA). Tissue was immediately homogenized and homogenates were left on ice for 1 h. Brain tissue proteins were extracted using 1% (w/v) 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulphate (CHAPS®, Pierce Biotechnology, Rockford IL, USA). 600 μl CHAPS per 100 mg wet tissue were added for homogenization. Extracts were stored at -80 °C until peptide assays. Orexin-A was quantified by enzyme immunoassay (PhoenixPeptide, Belmont, California USA) in lateral hypothalamus, medial hypothalamus, posterior brainstem and the septum. All these brain areas were analyzed as a whole piece and not further dissected for orexin-A determinations.

Anterior-posterior landmarks for dissection were as follows. For the hypothalamus: between 0 and -4 mm relative to bregma (see Paxinos and Watson) = between anterior part of the optic chiasm and the pituitary; for septum: between 1 and 0 mm relative to bregma = 1 mm more anterior than hypothalamic slice; and for posterior brainstem: between -12 and -16 mm relative to bregma = anterior cut in the middle of the cerebellum. The slice containing the septum was further dissected and dorsally cut at the level of the corpus callosum and ventrally at level of the anterior commissure;
laterally, the septum was dissected at the level of the lateral ventricle. The slice containing the hypothalamus was cut dorsally to the third ventricle and laterally at the border to the temporal cortex (slice seen from posterior).

4.2.4 Statistical analyses: Experiment 1 was analyzed by one-way repeated measures ANOVA followed by Fisher’s PLSD post-hoc test. Experiments 2, 3 and 4 were analyzed by one-way ANOVA (factorial) followed by Fisher’s PLSD post hoc. All analyses were done with StatView (SAS Corporation).
4.3 RESULTS

4.3.1 Experiment 1: Time-course of blood glucose during fasting. Fasted rats lost about 10% body weight (from 208.5 ± 3.1 g to 187.4 ± 2.6 g) while ad libitum fed rats gained about 8% body weight (from 211.7 ± 5.4 g to 228.4 ± 5.1 g) relative to their weights at the beginning of the experiment. The blood glucose concentration in 41-51 h fasted rats was reduced by 33% (4.4 ± 0.1 mmol/L vs. 6.6 ± 0.1 mmol/L) (P<0.001) (Fig. 1). Prepro-orexin mRNA is increased and orexin neurones are activated by low blood glucose (37; 74; 117). We determined blood glucose values to detect any possible time window with particularly low blood glucose concentration during fasting. As the stability of the blood glucose concentration between 41 and 51 h of fasting did not suggest a particular time point, we decided to sacrifice the animals in the subsequent experiments at the end of light phase, i.e. at a circadian time at which basal values of orexin have been found to be lowest (66; 187). Also Fujiki et al. found increased hypocretin-1 (orexin-A) levels in the cerebrospinal fluid after 72 hours of fasting only if the animals were sacrificed towards the end of the light phase (66).

Fig. 1: Blood glucose levels during the light phase of the second day of fasting were constantly lower in fasted animals as compared with ad libitum-fed rats (4.4 ± 0.1 mmol/L vs. 6.6 ± 0.1 mmol/L) (P<0.001 in repeated measures ANOVA).
4.3.2 Experiment 2: CCK effect on feeding after 48 h fasting. Fasting caused an average weight loss of 13% relative to baseline (from 262.6 ± 2.6 to 229.4 ± 2.6 g). Previous experiments with on-line food intake recording revealed that undisturbed animals ate 0.9 ± 0.1 g/hour during the last four hours of the light phase (n = 38; data of baseline measurements in three independent experiments with different animals). Thus, with PBS-injected controls eating 5.4 ± 0.4 g during the first 30 min of food access, hyperphagia was clearly induced by 48 h of fasting (P<0.0001). Both CCK doses reduced food intake when injected at the end of the light phase after 48 h of fasting; 15-minute intake values were: PBS, 3.1 ± 0.2 g, CCK (5 μg/kg) 2.2 ± 0.2 g, and CCK (10 μg/kg) 2.0 ± 0.2 g (P<0.05 for both CCK doses). Thirty-minute cumulative food intake was still reduced in both CCK groups: PBS, 5.4 ± 0.4 g, CCK (5 μg/kg): 3.6 ± 0.2 g and CCK (10 μg/kg): 3.5 ± 0.4 g (P<0.05 for both CCK doses). These differences started to disappear at 50 minutes after injection (45 minutes after food access) (Fig. 2).

Fig.2: IP administration (5 min before food access; food access at time 0 min) of 10 μg/kg CCK and of 5 μg/kg CCK reduced 15- and 30-minute food intake (P<0.05 in Fisher’s PLSD after significant factorial ANOVA). These differences disappeared 50 minutes after injection (45 minutes after food access). Data are mean ± S.E.M. of cumulative food intakes at the times indicated of n=10 PBS-treated rats and n=11 5μg/kg CCK-treated rats and n=11 10 μg/kg CCK-treated rats. C5, 5 μg/kg CCK; C10, 10 μg/kg CCK; CCK, sulphated cholecystokinin octapeptide; PBS, phosphate-buffered saline.
4.3.3 Experiment 3: Blood glucose after fasting and CCK. Fasting reduced blood glucose by 34% (fed vs. fasted: 6.4 ± 0.1 mmol/L vs. 4.2 ± 0.1 mmol/L; P<0.0001) and caused an average weight loss of 8%, relative to baseline body weight (from 322.9 ± 8 g to 296.1 ± 7.7 g). Rats with ad libitum access to food gained 6% weight during the same time (from 326 ± 12.5 g to 344 ± 11.5 g). CCK did not affect blood glucose, neither after 48 h fasting (CCK/fasted: 4.3 ± 0.2 mmol/L vs. PBS/fasted: 4.2 ± 0.1 mmol/L; P=0.7) nor in ad libitum-fed rats (CCK/fed: 6.4 ± 0.1 mmol/L vs. PBS/fed: 6.4 ± 0.1 mmol/L; P=0.9).

4.3.4 Experiment 4: Effect of 48 h fasting and CCK on orexin-A peptide in the brain: Forty-eight hours of fasting caused an average weight loss of 7% (from 322 ± 3.2 g to 301 ± 3.7 g), whereas animals with continuous ad libitum access to food gained 4.5% weight during the experiment (from 330 ± 4.9 g to 345 ± 5.2 g). Fasting (PBS/fasted) appeared to reduce posterior brainstem orexin-A compared with PBS/fed animals, but the difference was not significant (P=0.08). Compared with fed animals treated with CCK (CCK/fed), the reduction by fasting reached statistical significance (P<0.05). CCK increased the posterior brainstem orexin-A content in fasted animals by 35% (CCK/fasted vs. PBS/fasted, P<0.05) (Fig. 3). In the lateral hypothalamus (LHA, Fig. 4), fasting (PBS/fasted) decreased orexin-A content compared to PBS/fed animals > 50% (P<0.05). In PBS/fasted animals, the LHA orexin-A level was also lower (-80%) than in CCK/fed animals (P<0.05). In CCK/fasted animals, LHA orexin-A was still lower than in PBS/fed animals (P=0.04). In the medial hypothalamus, no significant treatment differences were found. In the septum (Fig. 5), the combination of fasting and CCK (CCK/fasted) reduced the orexin-A level compared with the CCK/fed (by 17%) and PBS/fed (by 13%) groups (P<0.05).
Fig. 3: CCK increased the orexin-A level in posterior brainstem of fasted animals by 35% (P<0.05 in Fisher’s PLSD after significant factorial ANOVA). The orexin-A level appeared to be lowered by fasting (P=0.08 in Fisher’s PLSD after significant factorial ANOVA), but this was not significant. The combination of feeding and CCK (CCK/fed) increased the brainstem orexin-A level by 48% (P<0.05 in Fisher’s PLSD after significant factorial ANOVA). Data are mean ± S.E.M. of amount of neuropeptide in ng/mL homogenate of n=6 animals in each group. CCK, sulphated cholecystokinin octapeptide; PBS, phosphate buffered saline.
Fig. 4: Fasting decreased the LHA orexin-A content by 50% (P<0.05 in Fisher's PLSD after significant factorial ANOVA). CCK alone did not have significant effects, but the combination of CCK and feeding (CCK/fed) increased the orexin-A level compared with fasted animals >80% (P<0.05 in Fisher's PLSD after significant factorial ANOVA). Data are mean ± S.E.M. of amount of neuropeptide in ng/mL homogenate of n=6 animals in each group. CCK, sulphated cholecystokinin octapeptide; PBS, phosphate buffered saline.

Fig. 5: The combination of fasting and IP CCK injection reduced the septum orexin-A level compared with the CCK/fed and PBS/fed treatments by 17 and 13%, respectively (P<0.05 in Fisher's PLSD after significant factorial ANOVA). Data are mean ± S.E.M. of amount of neuropeptide in ng/mL homogenate of n=6 animals in each group. CCK, sulphated cholecystokinin octapeptide; PBS, phosphate buffered saline.
4.4 DISCUSSION

We investigated the effect of IP CCK on the orexin system in rats fasted for 48 h to increase hunger and to challenge the orexin neurones. IP CCK antagonized the fasting-induced hyperphagia. Previous studies of ours suggested a relationship between peripheral CCK and the hypothalamic orexin neurones. Our present data complement these findings by showing that pathways activated by peripheral CCK affect orexin-A containing neurones in some of the examined brain areas, such as the posterior brainstem and the septum. On the other hand, LHA orexin-A was not affected by CCK, but decreased by fasting, and medial hypothalamic orexin-A did not change at all.

We sacrificed the rats during the last four hours of the light phase, i.e., at a circadian time when orexin-A levels in rat cerebrospinal fluid and LHA as well as medial thalamic extracellular liquid were reported to be at minimal levels (66; 187). We chose this time point because cerebrospinal fluid orexin-A was only found to be increased by a 72 h fast, if rats were killed towards the end of light phase, but not at the begin of light phase, when basal orexin-A levels are high (66). Two earlier studies examined orexin levels in several brain regions after 48 hours of fasting in rats and reported discrepant results. In one study, orexin-A and orexin-B contents did not change significantly, but tended to increase in the LHA, and decreased strongly in orexin target brain areas (110). The other study reported a significant increase of orexin-A in the septal nuclei, whereas no other brain area showed any fasting-induced changes (167). As circadian time influences basal brain orexin-A levels and the response of orexin-A to fasting, different circadian time points of sacrifice might contribute to the inconsistent results of fasting experiments. Our findings show that orexin-A content of different brain areas is susceptible to manipulations such as fasting and/or IP CCK at the end of light phase.

For experiments 1 to 3 of this fasting study, we used the same group of animals. For experiment 4, a new group of animals was used. The animals of the second group were chosen to have a similar body weight during experiment 4 as the first group during experiment 3. The body weight of the first group changed during the experiments, but
we do not expect this body weight difference to be important for the outcome of our studies.

The reduction of orexin-A levels in the LHA of fasted control animals is opposite to what one might expect on the basis of the upregulation of hypothalamic prepro-orexin mRNA previously observed upon fasting (37; 145). The fasting-induced increase in the mRNA level suggests an increased "need" for orexin signalling induced by fasting that activates transcriptional processes. Increased neuronal activity induced by 24h of fasting, reflected by enhanced c-fos expression in LHA/perifornical orexin neurones, has been shown in the non-human primate (53). Neuropeptide transmitters are rapidly degraded after release into the synaptic cleft, and there is no reuptake into the presynaptic cell (165). The reduced measurable amount of orexin-A peptide in brain homogenates upon fasting may therefore be due to increased release and degradation, i.e. increased turnover, of orexin-A peptide at the synapses in target regions. In the posterior brainstem, CCK may somehow slow down this process and inhibit orexinergic signals, leading to an increase in measurable orexin-A levels.

Orexin-A administered directly into the LHA increases food intake and c-fos expression in several brain areas involved in food intake control, including the LHA, the NST and the lateral septal area (118). Orexin-A immunoreactive fibres are widely distributed and also found in nuclei of the medial hypothalamus, septal nuclei and several brainstem areas including the NST, AP and PBN (48; 130). Orexin neurones were proposed to sense the nutritional status of the individual (145) and to orchestrate the adaptive response to negative energy balance (144), which we induced here by fasting. The satiety hormone CCK may signal the development of a positive energy balance after food intake. The significant increase in posterior brainstem orexin-A levels in fasted animals induced by IP CCK suggests that posterior brainstem orexin-A is somehow influenced by the incoming CCK-derived signal and, perhaps, involved in the feeding suppressive effect of CCK in this situation. IP CCK induces c-fos also in the AP and NST (108), where CCK-activated vagal afferents terminate (134). Also, the NST expresses higher levels of orexin-receptor-1 than of receptor-2, suggesting that this
nucleus is a target for orexin-A (104). The NST may therefore be a candidate nucleus of the posterior brainstem, on which the CCK and the orexin-A signalling might converge. In addition to interacting in food intake control, orexin-A and CCK may also interact in the control of arousal in relation to feeding. IP CCK elicits and prolongs resting behaviour after food ingestion (103), and gastrointestinal factors are being implicated in the reduced sleep onset latency after ingestion of a meal (81). The importance of orexins in arousal processes is well established (see (143) for review). Kotz et al. showed that LHA orexin-A-induced feeding is not just a consequence of enhanced arousal, but that LHA-administered orexin-A is important for both activation of arousal and of feeding pathways (92). The coupling of the control of vigilance and metabolic state may help to adequately organize sleep/wake and food intake behaviour (28).

Changes in blood or brain glucose concentrations may have contributed to the fasting-induced reduction of LHA orexin-A. The observed decrease in orexin-A may in fact reflect an increase in orexin-A activity because of the above-mentioned putative changes in orexin-A turnover at the synapse. The LHA contains a subpopulation of glucose-sensitive neurones, a cell group activated by hypoglycaemia - mainly indirectly through vagal pathways mediated by the brainstem, including the NST (126; 169). A reduction in blood glucose increases the activity and the prepro-orexin mRNA expression of orexin neurones (37; 74; 117). Orexin cells have close contacts to glucose-sensitive neurones, and orexin-A excites LHA glucose-sensitive neurones in vitro (100). The role of blood glucose changes in the observed fasting-induced decrease in LHA orexin-A could be tested by injecting glucose to fasted animals to reinstall euglycaemia and measuring orexin-A levels in the pertinent brain areas. The antagonism of insulin hypoglycaemia-induced increases in LHA prepro-orexin mRNA by injecting euglycaemia-inducing doses of glucose has been demonstrated (74). An alternative explanation for the fasting-induced orexin-A changes might be an activation of LHA and orexin-A neurones that is related to their function in arousal processes (92; 143).
Orexin-A in the medial hypothalamus neither responded to fasting nor to CCK administration. Nuclei belonging to the medial hypothalamus are innervated by orexin neurones and express orexin receptors (104; 130). Our results do not suggest a role of medial hypothalamic orexin-A in the mechanisms activated by fasting and/or IP CCK.

The combination of fasting and CCK lowered the concentration of orexin-A in the septum as compared to the two groups of fed animals. Besides other functions, the septum appears to play a role in the control of feeding because septal lesions increase food intake (91). The lateral septum and the LHA are anatomically (137) and functionally connected (122; 171). The septum is thought to have inhibitory effects on the LHA with regards to food intake (122). A recent study showed that lateral septal injection of urocortin inhibited feeding induced by orexin-A administered to the LHA (173). Marcus et al. found the lateral and medial septal nucleus only to express orexin-receptor-2, while the dorsal septal nucleus only produced orexin-receptor-1 mRNA (104). The prominent roles of both orexin-A and peripheral CCK in the regulation of food intake suggest that the orexin-A changes that we found in the septum are related to mechanisms of energy homeostasis. However, these changes might also be related to other functions of the septum and the LHA, such as arousal or reward mechanisms, which are important contributors to the proper control of feeding behaviour as well (38; 92; 105; 183).

Our previous insulin-hypoglycaemia study revealed that continuously IP infused CCK lowered hypothalamic prepro-orexin mRNA, but a single IP CCK injection did not influence LHA orexin-A levels of fasted rats in the present study. Several reasons might account for this difference: First, the less pronounced alerting character of the fasting-induced mechanisms compared with insulin-induced hypoglycaemia may predispose the system to react differently to IP CCK in fasting than to IP CCK in insulin hypoglycaemia. Second, the different circadian timing of the two experiments might contribute, as animals in the insulin hypoglycaemia study were sacrificed at the beginning of the light phase and not at the end, as in the present study. We chose the end of light phase time point in the present study deliberately because a 72-h fast was found to increase CSF...
orexin levels only at the end of the light phase (66). Third, a continuous 60 min IP CCK infusion and a single IP CCK injection may activate different physiological mechanisms.

In summary, our data show that fasting and IP CCK affect the orexin-A content of the posterior brainstem, lateral hypothalamus, medial hypothalamus and septum differently. In the posterior brainstem, CCK increased orexin-A levels in fasted animals, and this change might be related to the CCK-induced inhibition of food intake after 48 h food deprivation. Blood glucose level-dependent mechanisms might contribute to the orexin changes after fasting in the LHA, but the exact role of blood glucose in this context still needs to be clarified. Future studies should examine why the changes in LHA orexin-A peptide induced by fasting are inverse to the observed changes in the mRNA level. Also, it is of interest to analyze which parts of the posterior brainstem areas that are innervated by orexin neurones are affected by IP CCK, and to test their role in the feeding response to fasting and CCK.
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PEROXISOME-PROLIFERATOR-ACTIVATED RECEPTOR BETA AFFECTS HYPOTHALAMIC OREXIGENIC NEUROPEPTIDE LEVELS IN INSULIN-INDUCED HYPOGLYCAEMIC MICE

5.1 INTRODUCTION

Peroxisome-proliferator-activated receptors (PPARs) belong to the superfamily of ligand-activated nuclear receptors and function as transcription factors. Three different types of PPARs are currently known. PPARα is mainly expressed in liver, heart, kidney, skeletal muscle, and brown adipose tissue (BAT), and PPARγ in BAT, white adipose tissue (WAT), large intestine and spleen; PPARβ (also called PPARδ) is ubiquitous (30). The PPARs serve as sensors for fat and regulate lipid and glucose homeostasis through their activities in liver, muscle and adipose tissue (62). Natural ligands for PPARs are fatty acids, eicosanoids and prostaglandins. PPARα agonists such as the fibrates lower plasma triglycerides, reduce adiposity, and, consequently, improve insulin sensitivity (78). The thiazolidinediones (TZD) or glitazones act on PPARγ and are insulin-sensitizers used for the treatment of type 2 diabetes (97).

PPARβ may play a role when fatty acid oxidation and energy expenditure are increased, since mice with a targeted activation of PPARβ in adipose tissue show reduced adiposity and even resistance to high-fat diet-induced obesity and to obesity due to mutation of the leptin receptor (174). If PPARβ-KO mice are exposed to a high-fat diet they gain more weight than corresponding WT mice, and this seems to be related to an impaired thermogenesis caused by decreased uncoupling-protein 1 (UCP1) expression (174).

Studies in our lab have shown that PPARβ-KO mice do not develop the typical hyperphagic response after a phase of anorexia induced by cerebral ischaemic injury (7) (see table 1 for physiological peculiarities of PPARβ-KO mice). The ischaemic injury in this model was located to the cerebral cortex, and the animals behaved normally. This study suggests that PPARβ can affect control of food intake and that this seems to be...
related in particular to an effect of PPARβ on NPY (7). Whether PPARβ is involved in the changes of orexigenic neuropeptides in other models of hyperphagia remains to be determined. To address this question, we used PPARβ-KO and WT mice with a C57BL/6J genetic background and examined their food intake and hypothalamic orexigenic neuropeptide responses to insulin-induced hyperphagia. We determined orexin-A, melanin-concentrating hormone (MCH) and NPY protein levels in the hypothalamus by enzyme immunoassay. These neuropeptides increase food intake when administered into the brain and their expression can be increased by some food-intake enhancing manipulations such as hypoglycaemic doses of insulin and fasting (42; 98; 133; 145). Whereas prepro-orexin mRNA was increased by fasting (37), orexin-A peptide content was found to be reduced in several brain areas in fasting rats (Gallmann et al., in preparation, (110)). Insulin-induced hypoglycaemia has been shown in the rat to increase hypothalamic mRNA of prepro-orexin and MCH and to activate the neurones producing these peptides (14; 37; 74; 117). In rats, insulin hypoglycaemia seems not to increase hypothalamic NPY mRNA (45; 54), while a recent study in NPY-knockout mice suggests that NPY is required for insulin hyperphagia (156). As changes in peripheral glucose levels may alter these neuropeptides, we also measured glucose and liver glycogen responses in PPARβ-KO and WT mice.

5.2 MATERIALS AND METHODS

5.2.1 Animals and housing conditions. Male PPARβ-KO and WT mice with C57BL/6J genetic background were kindly provided by the group of Prof. W. Wahli (Institut de Biologie Animale, Université de Lausanne, Switzerland). The animals were housed single-caged in a temperature-controlled room (22 ± 0.5°C) on a 12h:12h light-dark cycle with lights on at 07:00h. Except during experiments, animals had ad libitum access to standard pelleted rodent food (Provimi Kliba, Kaiseraugst, Switzerland) and water. The experimental procedures were approved by the Canton of Zurich’s Animal Use and Care Committee. Basal 24 h food intake was not different between WT and PPARβ KO (4.1 ±
0.3 g and 3.9 ± 0.2 g respectively). Mice were of 2-4 months old and WT and PPARβ KO mice had similar body weights (see below).

| No hyperphagia and reduced hypothalamic NPY mRNA after cortical lesion |
| Decreased basal levels of circulating GH |
| Altered tissue-dependent production of NGF (no brain NGF) |
| Resistance to LPS-induced anorexia and very low levels of circulating TNFα |
| Tissue-specific alterations in UCP2 mRNA regulation (brain) |
| Resistance to Toxoplasma gondii (reduced circulating parasite number) |
| Infection with Spirometra does not result in enhanced body weight gain |

Table 1: Previous studies in our lab had revealed several physiological peculiarities of PPARβ-KO mice compared with WT mice. GH = growth hormone; NGF = nerve growth factor; LPS = lipopolysaccharide; TNFα = tumor-necrosis-factor alpha; UCP2 = uncoupling protein 2;

5.2.2 Experimental procedure.

5.2.2.1 Experiment 1: Effect of insulin on food intake. Five wild-type mice and six PPARβ-KO mice weighing about 28 g were used. Two hours and 30 min after lights on, insulin (HUMINSULIN® Lilly; Eli Lilly and Company, Indianapolis, USA) was injected intraperitoneally (IP) at doses of 0.3, 0.5, 1 and 2 units/kg body weight to PPARβ-KO and WT controls to search for an insulin dose that produces differences in the feeding response of KO and WT mice. The 2 units/kg dose was repeated using 11 WT and 11 PPARβ-KO mice. Food intake was measured 30, 60, 120, and 180 min after injection.

5.2.2.2 Experiment 2: Effect of insulin on circulating glucose, liver glycogen, and hypothalamic neuropeptides. Thirteen WT (weighing 26.6 ± 0.6 g) and 13 PPARβ-KO (weighing 28.2 ± 1.3 g) mice were injected with 2 U/kg insulin based on the result of the dose-response experiment. Injections were given IP, 30 minutes after food removal (food was removed 1 hour after lights on). Ninety minutes after insulin injection, retro-orbital blood was sampled and tested for glucose by GLUCOTREND2® (Roche Diagnostics, Rotkreuz, Switzerland). Animals were immediately sacrificed by cervical
dislocation. Hypothalamus and liver were dissected, snap frozen in liquid nitrogen and stored at -80 °C. From control animals, five WT (weighing 29.4 ± 1 g) and 5 PPARβ-KO (weighing 27.3 ± 2.3 g) mice, food was also removed 1 h after lights on. They were sacrificed at the same circadian time as insulin-injected mice.

Brain tissue was homogenized and proteins were extracted using 1% (w/v) 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulphate (CHAPS®, Pierce Biotechnology Inc., Rockford IL, USA) (8). 600 µL of CHAPS solution were added per 100 mg of tissue. Extracts were stored at -80 °C until peptides were assayed. Orexin-A, MCH and NPY were quantified by enzyme immunoassay (PhoenixPeptide, Belmont, California USA).

Liver glycogen was extracted by thawing the liver samples in 2 ml 30% KOH for 30 min in a boiling water bath, adding 3.5 ml ethanol, and centrifuging 20 min at 3000 rpm. The precipitate was dried, redissolved in 2 ml of distilled water, and hydrolyzed with 2 ml 4 M HCl for 1.5 h in boiling water. The hydrolysate was neutralized with 2 M Na2CO3, filtered and assayed for glucose using an enzymatic kit (GLUCOSE HK®, nr. 1447513, Roche) and COBAS MIRA® analyzer for glucose measurements (Roche Diagnostics, Rotkreuz, Switzerland).

5.2.3 Statistics. Food intake and blood glucose data were analyzed by Mann-Whitney Test. Liver glycogen and neuropeptide data were analyzed by one-way factorial ANOVA, followed by Tukey-Kramer Multiple Comparisons Test post-hoc. All analyses were done with Instat (GraphPad Software Inc.). P values < 0.05 were considered significant.
5.3 RESULTS

5.3.1 Experiment 1. Effect of insulin on food intake.
Following IP insulin injection of 0.3, 0.5 and 1.0 U (data not shown) we observed a tendency for PPARß KO to eat more, but only 2 U insulin/kg body weight stimulated food intake in KO mice more than in WT mice at 60 minutes after injection (P = 0.02, Fig. 1). Sixty-minute food intake in insulin-injected animals was 0.25 ± 0.02 g and 0.14 ± 0.04 g for KO and WT mice, respectively. Normally, mice eat almost nothing (about 0.01 g in average) during 4 hours in the light phase, and therefore animals were clearly hyperphagic after 2 U/kg insulin. Data are shown as cumulative food intake.

Fig.1: Insulin (2 U/kg body weight, IP) induces a more pronounced hyperphagia in PPARß-KO (KO) mice than in corresponding wild type (WT) mice (60 minutes: P=0.02 in Mann-Whitney test). Data are mean ± S.E.M. cumulative food intakes of n=11 KO and n=11 WT mice at the times indicated.
5.3.2 Experiment 2. Effect of insulin on circulating glucose, liver glycogen, and hypothalamic neuropeptides.

Insulin (2 U/kg body weight) lowered blood glucose levels in both WT (7.2 ± 0.6 mmol/L vs. 2.9 ± 0.2 mmol/L) and in KO mice (6.8 ± 0.5 mmol/L vs. 2.2 ± 0.3 mmol/L) (Fig. 2). Basal glucose was not different between the two groups. Hypoglycaemia in KO was significantly lower than WT mice and (P = 0.026). Liver glycogen content was lower in KO than in WT normoglycaemic mice (P<0.001); insulin (2 U/kg) appeared to reduce liver glycogen in WT but not in KO mice, but this difference did not reach statistical significance (Fig. 3).

Fig.2: Insulin reduces blood glucose levels in both PPARß-KO and WT mice. Hypoglycaemia was more profound in KO mice than in WT mice (P=0.026 in Mann-Whitney test). Data are mean ± S.E.M. blood glucose in mmol/L of n=5 WT/sal, n=5 PBKO/sal, n=13 WT/ins and n=13 PBKO/ins mice. PBKO, PPARß-knockout mice; WT, wild-type mice; sal, saline-injected animals; ins, insulin-injected animals.
Fig. 3: Basal liver glycogen content was lower in PPARß-KO mice than in WT mice (P<0.001 in Tukey-Kramer Multiple Comparisons test after significant one-way ANOVA). WT mice tended to reduce their liver glycogen after insulin, whereas the KO mice do not change it. Data are mean ± S.E.M. liver glycogen in mg/g of tissue of n=5 animals in each group. PBKO, PPARß-knockout mice; WT, wild-type mice; sal, saline-injected animals; ins, insulin-injected animals.
Fig. 4: Insulin increased hypothalamic NPY content in both PPARβ-KO and WT mice (P<0.001 in Tukey-Kramer Multiple Comparisons test after significant one-way ANOVA). Data are mean ± S.E.M. peptide contents in ng/mL homogenate of n=5 animals in each group. NPY, neuropeptide Y; PBKO, PPARβ-knockout mice; WT, wild-type mice; sal, saline-injected animals; ins, insulin-injected animals.

Fig. 5: Insulin increased hypothalamic MCH content in both PPARβ-KO and WT mice (P<0.001 in Tukey-Kramer Multiple Comparisons test after significant one-way ANOVA). Further, as well basal levels as levels after insulin were higher in the KO mice (P<0.001 in Tukey-Kramer Multiple Comparisons test after significant one-way ANOVA). Data are
mean ± S.E.M. peptide contents in ng/mL homogenate of n=5 animals in each group. MCH, melanin-concentrating hormone; PBKO, PPARβ-knockout mice; WT, wild-type mice; sal, saline-injected animals; ins, insulin-injected animals.

![Graph showing orexin-A content in WT and PBKO mice with and without insulin]

Fig. 6: Insulin decreased hypothalamic orexin-A content only in WT mice (P<0.05 in Tukey-Kramer Multiple Comparisons test after significant one-way ANOVA). Basal and insulin-induced Orexin-A is lower in the PPARβ-KO mice than basal values of WT mice (P<0.01 in Tukey-Kramer Multiple Comparisons test after significant one-way ANOVA). Data are mean ± S.E.M. peptide contents in ng/mL homogenate of n=5 animals in each group. PBKO, PPARβ-knockout mice; WT, wild-type mice; sal, saline-injected animals; ins, insulin-injected animals.

5.4 DISCUSSION

Our findings show that the lack of PPARβ can lead to changes in basal hypothalamic levels of orexigenic peptides and to changes in liver glycogen content. Also, PPARβ modulates the effect of a hypoglycaemic insulin dose on these peptides. We further observed a slight difference in the food-intake response of PPARβ-KO mice to hypoglycaemic doses of insulin.

PPARβ-KO mice had significantly lower basal liver glycogen levels than WT mice that were unaffected by insulin. Yet, insulin appeared to reduce liver glycogen in WT mice, although this difference was not significant. Presumably, hypoglycaemic doses of
insulin mobilise glucose from liver glycogen stores to counteract hypoglycaemia. These results – together with the lower blood glucose levels after insulin in the KO mice – suggest a role for PPARβ in glucose metabolism. PPARβ KO mice may have a disordered glucose handling in the liver. The lower baseline liver glycogen may contribute to the more profound hypoglycaemia found in KO mice following insulin administration, and this may in turn be related to the slightly more pronounced hyperphagia of KO mice in response to insulin.

Our neuropeptide measurements suggest a role for PPARβ in the regulation of hypothalamic orexin-A and MCH, since the basal levels of these orexigens differed between euglycaemic KO and WT control mice. Basal orexin-A levels were lower in KO than in WT mice and were not changed by insulin administration. In contrast, basal MCH levels were higher in KO than in WT mice, and insulin increased hypothalamic MCH in both genotypes. Basal NPY was not different between KO and WT mice, and NPY was also increased in both genotypes after insulin administration. Insulin and saline-injected control animals did not have access to food for 90 min. This is presumably too short a period of “food deprivation” during the light phase to cause changes in hypothalamic neuropeptide levels usually observed in response to fasting. Therefore, the basal differences in hypothalamic neuropeptide contents between WT and KO mice are presumably due to the genetic differences, i.e. to the presence or absence of PPARβ-dependent signalling.

The hypothalamic orexin-A content was not changed by insulin in PPARβ KO mice, but insulin-induced hypoglycaemia and several other manipulations which induce hyperphagia have been shown to increase hypothalamic pre-pro-orexin mRNA (37; 74; 145). The lack of an effect of insulin on orexin-A in hypoglycaemic KO mice appears to suggest that orexin-A is not necessary for insulin-induced hyperphagia in this mouse model. Hypothalamic orexin-A content was decreased in WT mice after administration of insulin. This is in contrast to the increase of prepro-orexin mRNA found in insulin-induced hypoglycaemia in rats (37; 68; 74) but fits our and other authors’ results obtained in fasted rats which also show a decreased orexin-A peptide level in the LHA.
or other brain regions in response to insulin, respectively (Gallmann et al., in preparation, (110)). The seemingly paradoxical decrease at the peptide level and the concomitant increase at the mRNA level might be explained by an increased release and turnover at the orexinergic synapses, leading to decreased peptide and increased mRNA levels at the same time, as neuropeptide transmitters are rapidly degraded at the synapse (165). Based on this assumption, basal hypothalamic orexin-A levels in PPARβ-KO mice in fact may be high (instead of low), and these levels seem not to change after insulin administration.

Both the WT and KO mice responded to insulin administration with an increase in the hypothalamic MCH content. A role for MCH in glucose-related regulatory processes is suggested but not clear, since in vivo studies showed inconsistent effects of IP 2-DG on MCH neurones (18; 131). It has been shown, however, that insulin hypoglycaemia activates MCH cells (14). Since orexin and MCH neurones form a hypothalamic circuit, the basal differences in orexin-A and MCH found in euglycaemic KO mice may be a consequence of a cross-talk between these two neuronal populations (77). The finding of increased MCH and NPY levels in both hypoglycaemic KO and WT mice strongly implicates these orexigens in the regulatory processes activated by administration of hypoglycaemic doses of insulin, and perhaps in mediation of the hyperphagia in response to insulin.

Glucose utilization appears to modulate the activity of neurones thought to be involved in the control of food intake (4; 124). Such glucose-sensing neurones are found in the hypothalamic VMH, the LHA, the ARC and the PVN (4; 67; 93). Hypothalamic neuropeptides participate in the control of glucose homeostasis and glucose-dependent food intake control, and they respond differentially to various manipulations of glucose metabolism. Presumably, manipulations that change glucose handling in the liver may be associated with changes in neuropeptides. So far, only a few studies have attempted to analyze a putative relationship between orexigenic neuropeptides and liver glycogen regulation. One study showed that NPY Y1 receptor-deficient mice show increased glycogen synthesis if fasted for 6 hours (35). Thus, high NPY levels in hypoglycaemic
mice might be related to an increased liver glycogenolysis. We only observed a tendency for reduced liver glycogen by insulin hypoglycaemia in WT mice, although this may be an expected consequence of insulin hypoglycaemia. The observed differences in neuropeptide regulation between KO and WT mice may somehow be related to the differences in liver glycogen regulation, but this relationship has to be analyzed in future studies.

Since we have found baseline differences in hypothalamic orexin-A and MCH levels, but not in basal food intake of PPARβ-KO and WT mice, these neuropeptide changes may be a compensation for some other physiological changes that we did not determine, and they may be related to other physiological functions than control of food intake. Our data demonstrate a role for orexin-A, MCH, NPY and PPARβ in physiological mechanisms activated by hypoglycaemic doses of insulin in mice. But with our set of data, an interpretation of neuropeptide changes with respect to food-intake regulation is difficult, as the behavioural data were not very clear.

In conclusion, our findings reveal several central differences in the response of PPARβ-KO mice to induction of glucoprivation by hypoglycaemic doses of insulin. The lack of PPARβ seems to lead to a chronic decrease in orexin-A and an increase in MCH levels in the hypothalamus. Further, the regulation of these two neuropeptides in response to insulin is changed, i.e. the upregulation of MCH is increased and orexin-A does not respond. These results provide further evidence for a functional connectivity of peripheral glucose homeostasis, central orexigenic neuropeptide systems, and may be food intake control, and they suggest that PPARβ is an important component in these interactions.
This thesis tested whether the peripheral satiety peptide CCK, or the presence or absence of PPARβ, modulated the changes in hypothalamic orexigenic neuropeptides associated with experimental hyperphagia induced by insulin hypoglycaemia or fasting.

In the first set of experiments (described in Chapter 3), we induced hyperphagia in male rats by a hypoglycaemic dose of 50 IU/kg insulin (Insulatard MC suis, Novo Nordisk), based on Cai et al., who were able to activate orexin neurones by the same dose (36). Also according to Cai et al., and because light phase food intake stimulation by icv orexins is stronger than dark phase stimulation, experiments were conducted during the early light phase (36; 37; 56; 82; 145; 186). Insulin hypoglycaemia-induced hyperphagia was characterised by an increase in meal size and was blunted by IP intrameal and continuous infusions of CCK (4 µg/kg or 32 µg/kg, respectively). Thus, signalling activated by insulin hypoglycaemia and by peripheral CCK appears to act on a common, presumably neural, substrate to control food intake in these particular conditions. The meal-size reducing effect of CCK in this paradigm of hyperphagia supplements data showing that CCK terminates meals under normal conditions (5; 6; 63; 70; 71).

Insulin-induced hypoglycaemia increased hypothalamic prepro-orexin and MCH mRNA, while 60 min continuous IP infusion of CCK attenuated this increase. NPY did not change significantly in response to insulin hypoglycaemia and/or CCK, confirming previous results of others (45; 54). As we measured the NPY mRNA content of the whole hypothalamus, the possibility of NPY changes in discrete hypothalamic subregions cannot be discounted. This caveat should not apply to prepro-orexin and MCH mRNA because these two neuropeptides are only expressed in the LHA (27; 50; 145). The observed changes in prepro-orexin and MCH mRNA suggest a role for hypothalamic orexin and MCH neurones in CCK-dependent satiety mechanisms, and maybe in hyperphagia induced by hypoglycaemia. The changes in orexigenic neuropeptide expression in response to CCK under baseline conditions are of further interest, since the identity of central neuronal systems that integrate CCK-derived...
signalling in the normal physiological control of food intake is largely unknown. Additionally, the effect of other satiety signals on brain orexigenic neuropeptides could be assessed. Since various satiety signals presumably may affect the activity of hypothalamic circuits producing food-intake stimulating neuropeptides differently, testing other satiating signals may elucidate specific or more generalized aspects of food-intake regulation.

In Chapter 4, we describe experiments using 48 h of fasting to induce hyperphagia and to challenge orexigenic neuropeptides. To reduce food intake, IP CCK was injected at the end of the fast. Animals were sacrificed at the end of the light phase because fasting was previously found to increase hypocretin-1 (orexin-A) levels in cerebrospinal fluid only during this circadian time window (66). The orexin-A content of lateral and medial hypothalamus, posterior brainstem and septum was determined because these brain areas (1) participate in food intake regulation, (2) express orexin receptor 1 and/or receptor 2 mRNA in subregions, and (3) are innervated by orexinergic neurones (104; 130). The clear fasting-induced decrease in LHA orexin-A content appears paradoxical given the previously observed fasting-induced increase in LHA prepro-orexin mRNA (37; 145). Other authors who also reported a decrease in orexin-A content after a 48 h fast in several brain areas, explain this reduction by increased turnover of the peptide at the synapse (110). Therefore, fasting may in fact have increased orexin-A turnover in the LHA. The fasting-induced change in LHA orexin-A may be related to both control of energy homeostasis and arousal regulation (92; 143). Our data do not suggest medial hypothalamic orexin-A to be involved in mechanisms activated by fasting or IP CCK. Orexin-A in the septum and posterior brainstem responds to the state induced by the combination of fasting and CCK. The posterior brainstem increase in orexin-A induced by CCK in fasted animals might be related to the observed food intake reduction by CCK. Again, we would expect CCK to induce decreases in orexin-A, and in fact, CCK may decrease the increased turnover at orexinergic synapses, which could increase measurable orexin-A content in areas of secretion.
The study described in Chapter 5 of this thesis was conducted to assess the role of peroxisome-proliferator-activated receptor β (PPARβ) in insulin hypoglycaemia-induced hyperphagia. PPARβ-knockout (KO) mice showed lower blood glucose values in response to insulin administration (2 U/kg) than wild-type (WT) mice. Further, a stronger hyperphagia in KO mice was observed, but this food-intake difference was only weak. KO mice had lower basal liver glycogen content than WT animals. Liver glycogen was reduced in WT mice after insulin, but no insulin-induced changes were observed in KO mice. Therefore, the lower baseline glycogen levels may explain the stronger insulin-induced hypoglycaemia and perhaps hyperphagia in KO mice. A hypoglycaemic dose of insulin increased hypothalamic MCH and NPY in both WT and KO mice. Insulin reduced hypothalamic orexin-A in WT but not in KO mice. Thus, orexin-A does not appear to be necessary for insulin-induced hyperphagia in PPARβ-KO mice. Together, these findings suggest a role for PPARβ in control of glucose metabolism and perhaps food intake. Also, PPARβ seems to interact with hypothalamic orexigenic neuropeptide systems. Absence of PPARβ may somehow have changed glucose and/or lipid metabolism. Indirect sensing of these changes may have led to a different regulation of basal MCH and orexin-A in the KO mice, but also direct effects of PPARβ-dependent signalling on these neuropeptides may have caused the central changes.

What kind of physiological mechanisms might be responsible for the changes in hypothalamic orexigenic neuropeptides and their mRNA in insulin-induced hypoglycaemic or fasted animals and in CCK-treated rats?

A factor common to all experiments is low blood glucose. Our data show a different response to insulin-induced hypoglycaemia, depending on whether the mRNA (in rats) or the neuropeptides themselves (in mice) were measured. In our rat and mouse studies, some neuropeptides or their mRNA were changed by insulin-induced hypoglycaemia or by fasting; this provides further evidence for the hypothesis that reduced blood glucose levels are sensed and integrated by hypothalamic neuropeptide circuits. Rats injected with insulin responded with an increased expression of hypothalamic prepro-orexin mRNA and MCH mRNA, whereas NPY mRNA was not
changed. Mice showed increased MCH and NPY peptide levels; specifically, the WT mice reduced their hypothalamic orexin-A content after insulin administration, while KO mice showed consistently low orexin-A levels. A species difference, or a difference between peptide and mRNA regulation, may have caused both the divergent response of rats and mice to insulin-induced hypoglycaemia. The difference between the insulin doses used in these two studies may also play a role. The 48-h fasting study revealed a sensitivity of lateral hypothalamic orexin-A to food deprivation, since orexin-A levels in the LHA were reduced in fasted animals. Also, this effect might be related to the state of low blood glucose induced by fasting. Together with the clearly-demonstrated reactivity of orexin neurones to changes in circulating blood glucose (37; 74), the changes in prepro-orexin mRNA and orexin-A which we observed, suggest that orexin neurones are activated by hypoglycaemia. The orexin neurones even might be a substrate for central mechanisms activated by hypoglycaemia that are located upstream of the other two neuropeptides we were interested in. By their projection to MCH neurones, orexin neurones may influence the regulation of MCH in response to insulin-induced hypoglycaemia, but a direct interaction of MCH neurones with glucose-sensitive neurones of the LHA is also possible. With respect to NPY regulation, our mice and rat studies produced different outcomes. Since high insulin levels inhibit NPY neurones, the use of a very high insulin dose in our rat experiments may have counteracted any hypoglycaemia-induced increase in NPY mRNA (141), whereas the moderate dose used in our mice studies may allow the observed increase in hypothalamic NPY peptide content.

The importance of the LHA in glucoprivic eating is well known (15; 61; 175; 182). We confirm other authors' findings of increased LHA prepro-orexin mRNA in acute hypoglycaemia in rats (37). In our fasting experiment, LHA orexin-A levels changed in parallel with glucose levels, but, in contrast to the mRNA levels (37; 145), they decreased (a possible explanation for this discrepancy is given above). Visceral glucose sensors activate vagal afferents that project to the NST (39). The NST comprises a population of glucose-sensitive cells that are linked to hepatoporal glucose-sensitive afferent units (1; 107). Rising glucose levels in the hindbrain and the
viscera inhibit LHA glucose-sensitive neurones (21; 152). Seemingly, LHA glucose-sensitive neurones are activated mainly indirectly by changes in glucose in the hepatoportal system, mediated via the NTS and PBN (39; 126). Orexin-A excites glucose-sensitive neurones in the LHA in vitro, and orexin neurones presumably make synaptic contacts on them (100). In rats or mice with insulin-induced hypoglycaemia and in fasted rats, activation of glucose-sensitive cells may influence orexin and/or MCH expression in the LHA. In hypoglycaemic rats, the increased mRNA levels of prepro-orexin and MCH presumably drive the insulin-induced hyperphagia, but a role for NPY cannot be excluded, since NPY availability could have increased in discrete hypothalamic subregions. In hypoglycaemic WT mice, all three neuropeptides were changed and therefore may be implicated in the response that finally leads to hyperphagia. Conversely, the KO mice did not show changes in orexin-A, and this argues against participation of orexin-A in producing hyperphagia in KO mice.

Another factor implicated in the control of food intake was inevitably manipulated in our experiments: blood insulin levels were increased by IP injections. Insulin accesses the brain in the median eminence, a brain region where the blood-brain barrier shows selective permeability. ARC NPY neurones may respond to inhibition by insulin, since they are one of the targets of the feeding-suppressing effect exerted by centrally-acting insulin. States of low plasma insulin are usually accompanied by increased NPY and NPY mRNA content in the hypothalamus, and this is normalized by insulin administration (141). High levels of circulating insulin should normally decrease feeding; as large doses of insulin induce hyperphagia in both rats and mice, the rise in circulating insulin is presumably a weaker signal on food-intake regulation than the hypoglycaemia which it caused.

We tested the effect of IP CCK administration on brain orexigenic neuropeptides in two different paradigms of increased food intake in rats. These studies yielded the novel finding that some of these hypothalamic neuropeptides are targets (direct or indirect) of peripheral CCK. In both models of hyperphagia, CCK blunted the increased feeding. Peripheral CCK exerts its satiating effect mainly through activation of CCK-A receptors
On vagal afferents (69; 101; 148; 161). CCK-mediated inhibition of gastric emptying may also contribute to CCK satiety (44). Peripheral CCK induces c-fos in many brain areas, including the hypothalamic PVN (108), that receives NPYergic and orexinergic projections (180). We demonstrated that the reduction of insulin-induced hyperphagia by IP CCK infusions in rats is associated with reductions in hypothalamic prepro-orexin and MCH mRNA levels. Also, a single IP CCK injection increased the posterior brainstem orexin-A content of fasted rats. Primary vagal and secondary glucose-related effects of CCK may contribute to the prepro-orexin and MCH mRNA changes observed in the insulin-hypoglycaemia study. In fact, blood glucose may also act on hepatic vagal afferents (39; 52; 139). The role of vagus-dependent CCK signalling for the observed neuropeptide changes is yet to be established – which could be verified by study of vagally deafferented animals. Small amounts of glucose could be co-infused IP in PBS-treated hypoglycaemic rats to produce systemic blood glucose values similar to the values in hypoglycaemic CCK-infused rats, and the glucose effect on neuropeptide expression evaluated in this paradigm. The 48-h fasting study adds important information about effects of CCK on the orexinergic system: orexin-A in the posterior brainstem seems to be a target of IP CCK. Given the importance of posterior brainstem nuclei for energy homeostasis regulation, this effect may be associated with the CCK-induced reduction of hyperphagia.

Another novel result of the 48-h fasting study was the differential regulation of orexin-A in diverse brain areas, i.e. lateral hypothalamus, medial hypothalamus, septum and posterior brainstem. This further emphasizes the importance of co-operation between a variety of brain regions in the overall control of energy homeostasis and food intake. Therefore, our results confirm the integrative role of hypothalamic orexigenic neuropeptide circuits, but they additionally suggest that the orexinergic system orchestrates the activity of a series of other brain regions innervated by orexin neurones.

The examination of insulin-induced hypoglycaemia in PPARβ-KO and WT mice revealed clear effects of insulin and of the genetic lack of PPARβ on neuropeptide regulation. Our findings show that PPARβ participates in the regulation of glucose metabolism, not
only in fat metabolism as it has already been shown. Importantly, PPARβ-dependent regulatory processes seem to affect hypothalamic orexigenic neuropeptide circuits. Thus, PPARβ should be added to the list of physiological factors that interact with central integrating neuronal systems to control energy homeostasis. Only little is known about putative physiological roles of PPARβ in general. How lack of PPARβ-dependent signalling may have affected central neuropeptide circuits, remains to be answered by future studies.

Functionally, the neuropeptide and mRNA results have to be interpreted carefully, but a relationship to the control of energy homeostasis is likely, as we manipulated food intake and measured substances that have been demonstrated to be involved in the regulation of food intake and energy homeostasis.

Regarding food intake control in general, anatomical and pharmacological findings suggest that the orexin-, MCH- and NPY-producing neurones of the hypothalamus interact with each other. Two recent studies point to the existence of contacts between orexin and MCH neurones (17; 77) which form separate populations in the LHA (31; 58). LHA orexin neurones project to the arcuate nucleus – the main region producing NPY – and other hypothalamic nuclei involved in the control of feeding behaviour (84; 130). Pharmacological studies reveal interactions of orexin and NPY in food-intake regulation (55; 85; 121; 185). ARC NPY neurones also innervate MCH-containing cells in the LHA (58). Hypothalamic orexigenic and anorexigenic neuropeptides are thought to integrate peripheral energy balance signalling. Our data on orexigenic neuropeptide regulation support this hypothesis, since we showed that hypothalamic neuropeptides are affected by a hypoglycaemic dose of insulin and by fasting, by its functional counteraction through peripherally administered CCK, and by the genetic lack of PPARβ. Hypoglycaemia as induced by insulin or fasting deprives the brain of its principal fuel, the glucose. Functional interactions of orexigenic neuropeptide circuits with glucose-sensing neurones may be important for homeostatic regulatory processes activated by hypoglycaemia and finally leading to changed food intake.
Energy homeostasis is maintained by diverse brain circuits. Studies in the chronic decerebrate rat underscore the importance of the brainstem in feeding control (76). Orosensory and viscerosensory input to the brainstem is provided by cranial nerves. Viscerosensory input is carried primarily by the vagus nerve, which terminates in the AP and the NST (24). Orexin neurones send projections to the entire neuroaxis (130), and the same holds true for MCH neurones (27). Thus, orexin and perhaps also MCH neurones appear to be well placed to orchestrate the response of neural systems in different brain areas to negative energy balance, as induced by fasting or insulin hypoglycaemia. Sakurai et al. hypothesized that orexin neurones sense the nutritional state of the animal (145). To restore energy balance, the sensed information of a physiological need has to be translated into an “action”, i.e. changes in feeding behaviour and energy expenditure. This involves brain systems controlling motivation and reward mechanisms to activate the appetitive behaviour of food seeking in states of hunger. It has been shown that orexin neurones are activated by appetite-inducing manipulations of the nucleus accumbens (188), one of the corticolimbic structures that process the “liking” and “wanting” of food (22). Orexin cells are thought to participate in the control of both arousal state and energy balance, and together with a putative role in feeding motivation, they may help the animal to seek food in states of hunger.

In summary, the experiments conducted as part of this thesis suggest a role for orexin and for MCH in the mediation of insulin hypoglycaemia-induced hyperphagia in the rat, and a role for orexin-A in the orchestrated response activated by fasting in the rat. The influence of CCK (a visceral signal) on orexigenic neuropeptide networks was shown in two paradigms of increased food intake. Blood glucose (a metabolic signal) may partly modulate these effects. The results of our studies in PPARβ-KO and corresponding WT mice suggest that PPARβ is also involved in the interplay between glucose metabolism, orexigenic neuropeptides, and food intake.

The increasing prevalence of obesity and obesity-related disorders such as non-insulin dependent diabetes and cardiovascular diseases in industrialized countries demands strategies against this epidemic that include pharmacological intervention. Because of
the complexity of systems regulating energy balance, detailed analysis and knowledge of these mechanisms is a prerequisite for development of drugs to effectively treat disordered eating and obesity. Given the gravity and magnitude of the obesity pandemic, one can only hope that new knowledge about the central and peripheral systems controlling food intake will ultimately be translated into therapeutic reality.
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7 REFERENCES


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Curriculum vitae

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