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Experimental models for investigating IAPP regulation of food intake in rats and mice

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ABSTRACT

Background: Maintenance of energy balance is one of the fundamental processes of living organisms and involves complex regulatory pathways. Appetite regulation is an important component of this, because it modulates energy intake. Islet amyloid polypeptide (IAPP or Amylin) is a 37-amino-acid peptide that is produced primarily by the β-cells in the pancreas and is co-secreted with insulin in response to a meal. A reduction in food intake and body weight is seen following several routes and modes of IAPP administration in rodents, and the peptide has been suggested to be necessary for normal satiety to occur.
Objectives: The present studies investigated whether IAPP acts as a satiety hormone. In addition, the mechanisms through which IAPP exerts its effects were investigated.
Methods: The effect of chronic IAPP treatment on energy homeostasis were investigated in rats by subcutaneous (SC) infusion of IAPP (25 pmol/kg-min) for 2-7 days. Ad libitum fed and pair fed rats were used as controls for the IAPP groups. The effects of peripheral and central administration of IAPP were compared by analyzing food intake, meal pattern, and body weight in rats after 7 days of SC infusion of IAPP (0, 0.25, 2.5, or 25 pmol/kg-min) or intracerebroventricular (ICV) infusion (0, 0.025, 0.25 or 2.5 pmol/kg-min). The expression of neuropeptide Y (NPY) mRNA, agouti-related protein (AgRP) mRNA, and proopiomelanocortin (POMC) mRNA was measured in the arcuate nucleus of the hypothalamus of rats after ICV infusion (2.5 pmol IAPP/kg-min for 5 days). Pair-fed and ad libitum fed rats receiving vehicle only were used as controls. Food intake, meal pattern, and body weight were measured during a long-term, 27-week study in 8 adult male IAPP knockout mice (IAPP-/-, strain C57BL/6) and 8 adult male wild-type mice (IAPP +/+, strain C57BL/6). After the long-term experiment was completed, a short-term experiment was conducted in which food intake and body weight were analyzed in the mice after a 3-day infusion of IAPP (25 pmol/kg-min).
Results: Transient inhibition of food intake following IAPP infusion was seen in all the rat experiments. The minimal effective dose for SC infusion was 2.5 pmol/kg-min compared to 0.25 pml/kg-min for the ICV infusion. SC infusion of IAPP decreased the weight of epididymal fat pads and lowered circulating levels of triglycerides, free fatty acids, leptin, and insulin. Glucose metabolism and protein metabolism were largely unchanged. No changes in expression of AgRP or NPY mRNA were seen after ICV infusion of IAPP, but a decrease in POMC mRNA was seen in both IAPP and pair-fed animals. In the mouse experiments, no differences in food intake or body weight were seen between knockout and wild-type mice during the long-term study. In the short-term study, food intake was significantly lower in the knockout mice than in the wild-type group.
Conclusion: The data from these studies provide support for the hypothesis that IAPP is a satiety hormone that acts primarily in the brain to inhibit food intake. Inhibition of food intake does appear to be mediated by POMC at the time point studied. The lack of differences in food intake and body weight between IAPP knockout (KO) and wild-type mice indicate that food intake can be controlled in the absence of IAPP. The more marked anorectic effect seen in the KO mice during IAPP infusion suggests that IAPP receptors and/or IAPP post-receptor signalling pathways are up-regulated in mice lacking endogenous IAPP.

Keywords: adiposity, agouti-related protein, amylin, body weight, food intake, hypothalamus, IAPP, knockout mice, leptin, meal pattern, neuropeptide Y, proopiomelanocortin, satiety.

LIST OF PUBLICATIONS

This thesis is based on the papers listed below. Roman numerals will be used to refer to the papers in the text of the thesis.

I. B. Isaksson, F. Wang, J. Permert, M. Olsson, B. Fruin, M.K. Herrington, L. Enochsson, C. Erlanson-Albertsson and U. Arnelo.
Chronically administered islet amyloid polypeptide in rats serves as an adiposity inhibitor and regulates energy homeostasis.
Pancreatology 2005;5:29-36.

II. M. Olsson, M.K. Herrington, R.D. Reidelberger, J. Permert and U. Arnelo.
Comparison of the effects of chronic central administration and chronic peripheral administration of islet amyloid polypeptide on food intake and meal pattern in the rat.
Peptides 2007;28:1416-1423.

III. M. Olsson, M.K. Herrington, R.D. Reidelberger, J. Permert, S. Gebre-Medhin and U. Arnelo.
Food intake and meal pattern in IAPP knockout mice with and without infusion of exogenous IAPP.
Scandinavian Journal of Gastroenterology 2012;47:191-196.

IV. M. Olsson, M.K. Herrington, A. Jansson, J. Permert and U. Arnelo.
On the regulation of mRNA expression of proopiomelanocortin, neuropeptide Y, and agouti-related protein in the hypothalamus by chronic central infusion of islet amyloid polypeptide.
(In manuscript).

The following paper is not part of the actual thesis but was published during the study period. Reference is made to the paper in the text of the thesis.

S. Mirshamsi, M. Olsson, U. Arnelo, J.M. Kinsella, J. Permert, M.L. Ashford.
BVT.3531 reduces body weight and activates K(ATP) channels in isolated arcuate neurons in rats.
Regulatory Peptides 2007;141:19-24.

CONTENTS

LIST OF ABBREVIATIONS

aCSF	artificial cerebrospinal fluid
AgRP	agouti-related protein
AM	adrenomedullin
ANOVA	analysis of variance
AP	area postrema
ARC	arcuate nucleus
BSTL	bed nucleus of the stria terminalis
BW	body weight
CCK	cholecystokinin
Ce	central amygdaloid nucleus
CGRP	calcitonin gene-related peptide
CT	calcitonin
DEPC	diethylpyrocarbonate
DFI	dark food intake
EDTA	ethylenediaminetetraacetic acid
FI	food intake
IAPP	islet amyloid polypeptide, amylin
ICV	intracerebroventricular
IP	intraperitoneal
KO	knockout
LFI	light food intake
LPS	lateral parabrachial nucleus
MII	minimum intermeal interval
MMS	minimum meal size
MS	meal size
mRNA	messenger ribonucleic acid
NM	number of meals
NPY	neuropeptide Y
NTS	nucleus of the solitary tract
PBS	phosphate-buffered saline
PEEK	polyetheretherketone
POMC	proopiomelanocortin
RAMPs	receptor activity modifying proteins
SC	subcutaneous
sCT	salmon calcitonin
SEM	standard error of the mean
SSC	sodium chloride-sodium citrate buffer
TEA	triethanolamine
TFI	total food intake
TNM	total number of meals
TRIS	tris (hydroxymethyl) aminomethane
UPC2	uncoupling protein 2
WT	wild-type

1 INTRODUCTION

ISLET AMYLOID POLYPEPTIDE (IAPP, AMYLIN)

1.1 CHEMICAL CHARACTERISTICS AND SECRETION OF IAPP

IAPP is a 37-amino-acid peptide that is produced primarily by the β-cells in the pancreatic islets but also by other endocrine cells in the pancreas and the gut; it is co-released with insulin in response to a meal (Ahrén *et al*. 1992, Arnelo *et al*. 1998, Butler *et al*. 1990, Clark *et al*. 1989, Cooper GJ. 1994, Lukinius *et al*. 1989, Mulder *et al*. 1997, Ogawa *et al*. 1990, Westermark *et al*. 1987). An increase in secretion of IAPP relative to insulin has been discovered in several animal models of obesity and/or diabetes (O´Brien *et al*. 1991, Ogawa *et al*. 1990), but other studies have shown no difference or a decrease of IAPP secretion relative to insulin. The reason for this difference in secretion of IAPP and insulin is not clear, but the gene expression and/or translation rates of the two polypeptides are independently controlled (Gedulin *et al*. 1991, O´Brien *et al*. 1991).

IAPP, which has a molecular mass of 3850 Daltons, was first isolated from human and cat islet amyloid and from amyloid produced by a human insulinoma (Cooper *et al*. 1987, Westermark *et al.* 1986, Westermark *et al*. 1987).

The mouse IAPP gene spans about 5.8 kb on chromosome 6 and, like the rat and human IAPP genes (on chromosome 12), consists of three exons encoding prepro-amylin (Ekawa *et al*. 1997). Mouse and rat IAPP have identical sequences and differ from human IAPP by 6 amino acids (Butler *et al*. 2004, Leffert *et al*. 1989). Human IAPP forms amyloid because it contains an amyloidogenic sequence (residues 25-29) (Westermark *et al*. 1990). Because of the substitution of three proline residues in the amyloidogenic region, mouse IAPP and rat IAPP do not form fibrils or amyloid and are not toxic to neurons (Lim *et al*. 2008).

1.2 IAPP RECEPTORS

IAPP binding sites are located throughout the brain (including the nucleus accumbens, hypothalamus, area postrema, lamina terminalis, and the nucleus of the solitary tract (NTS)) and in the lung, stomach, spleen, and liver (Beaumont *et al*. 1993, Bhogal *et al*. 1992, Bhogal *et al*. 1993, Sexton *et al*. 1994). Because both the area postrema of the

hindbrain and third ventricle/median eminence area in the hypothalamus lack functional blood-brain barriers (Banks and Kastin 1998, Fry *et al*. 2007), plasma peptides can enter the brain and influence neuronal activity in these regions.

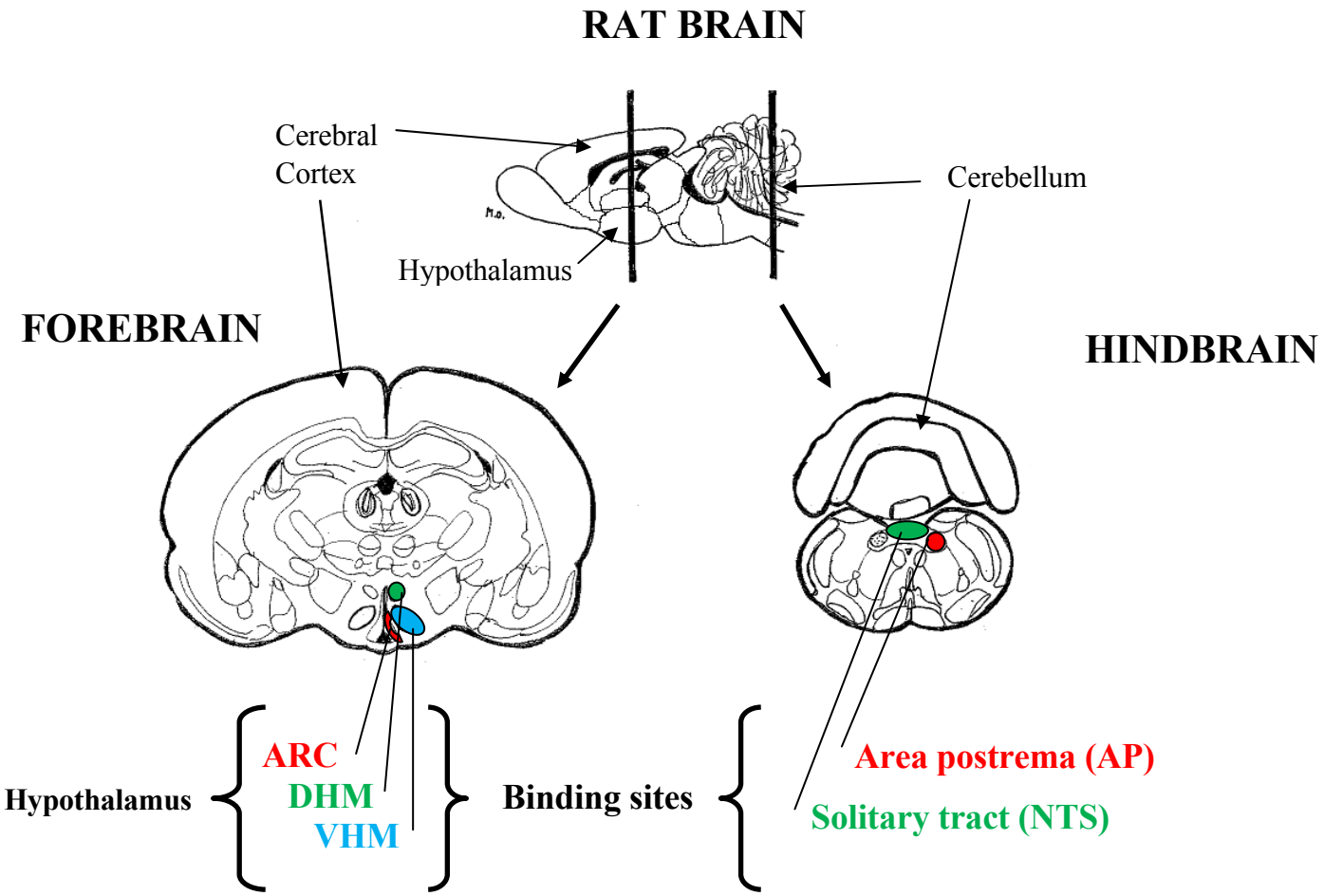


Fig 1. *ARC,DHM and VHM nuclei of the hypothalamus and AP and NTS in the hindbrain are the predominant locations of high IAPP affinity binding sites in the brain. Modified from Sexton et al. (Sexton et al. 1994). Top drawing show medial view of the rat brain halved in the median plane. Left drawing show frontal section through the plane of the hypothalamus. Right drawing show frontal section through the plane of medulla oblongata.*

So far, no specific receptors for IAPP have been discovered. Modification of the calcitonin receptor (a seven-transmembrane G-protein-coupled receptor) by receptor activity modifying proteins (RAMPs), using RAMP1 or RAMP3, results in a receptor with relatively high affinity for IAPP (Muff *et al*. 1999). Peripheral and central administration of the IAPP receptor antagonist acetyl-[Asn^{30},Tyr^{32}]sCT-(8-32) (AC187) stimulates food intake in rats (Mollet *et al*. 2004, Reidelberger *et al*. 2004, Rushing *et*

al. 2000a), but does not completely block, amylin-induced anorexia (Reidelberger *et al*. 2004). Another IAPP receptor antagonist is $hCGRP_{8-37}$ which has been shown to block IAPP induced vasodilatation in rats (Wang *et al*. 1991).

1.3 EFFECTS OF IAPP

Studies of islet amyloid polypeptide provide evidence that IAPP reduces food intake, inhibits gastric emptying, reduces adipose energy reserves, inhibits insulin secretion, causes peripheral insulin resistance *in vivo*, and increases body temperature (Arnelo *et a*l. 1996a, Balasubramaniam *et al.* 1991, Barth *et al*. 2003, Chance *et al.* 1991, Clementi *et al*. 1996, Fehmann *et al*. 1990, Johnson *et al*. 1990, Molina *et al*. 1990, Leighton and Cooper 1988, Lutz *et al.* 1994, Lutz TA 2006, Ohsawa *et al*. 1989, Wickbom *et al.* 2008, Wielinga *et al*. 2010, Young A. 1997). Because of its anorexic effects, IAPP has been suggested as a candidate for the treatment of obesity (Lutz TA. 2012). The stable IAPP analog pramlintide has been shown to reduce caloric intake and body weight in obese and normal weight people (Aronne *et al*. 2007, Chapman *et al*. 2007, Smith *et al*. 2007) and to aid in the management of food intake and body weight in patients with diabetes mellitus (Edelman SV. 2008).

Regulation of food intake is a complex process that involves a number of peripheral and central signals, including gut hormones, pancreatic hormones, and neurotransmitters (Blundell J. 1991, Blundell *et al*. 2012, Erlanson-Albertsson and York 1997, Hellström *et al*. 2004, Näslund and Hellström 2007, Moran and Dailey 2011). Some of these, such as ghrelin, agouti-related protein (AgRP) and neuropeptide Y (NPY), stimulate appetite and food intake; in contrast, food intake is inhibited by IAPP, cholecystokinin (CCK), glucagon-like peptide-1(GLP-1), enterostatin, leptin, insulin, and pro-opiomelanocortin (POMC) (Balasubramaniam *et al*. 1991, Biebermann *et al*. 2012, De Silva *et al*. 2011, Donahey *et al*. 1998, Erlanson-Albertsson C and York 1997, Hagan *et al*. 1999, Hagan *et al*. 2000, Hellström *et al*. 2004, Näslund *et al.* 2005, Shargill *et al*. 1991, Stanley *et al*. 1993). Synergistic interactions in the inhibition of food intake have been seen between IAPP and cholecystokinin (CCK) (Bhavsar *et al*. 1998) and between IAPP and leptin (Osto *et al*. 2007, Trevaskis *et al*. 2008). Interactions between IAPP and insulin have also been observed (Osto *et al*. 2007, Rushing *et al*. 2000b).

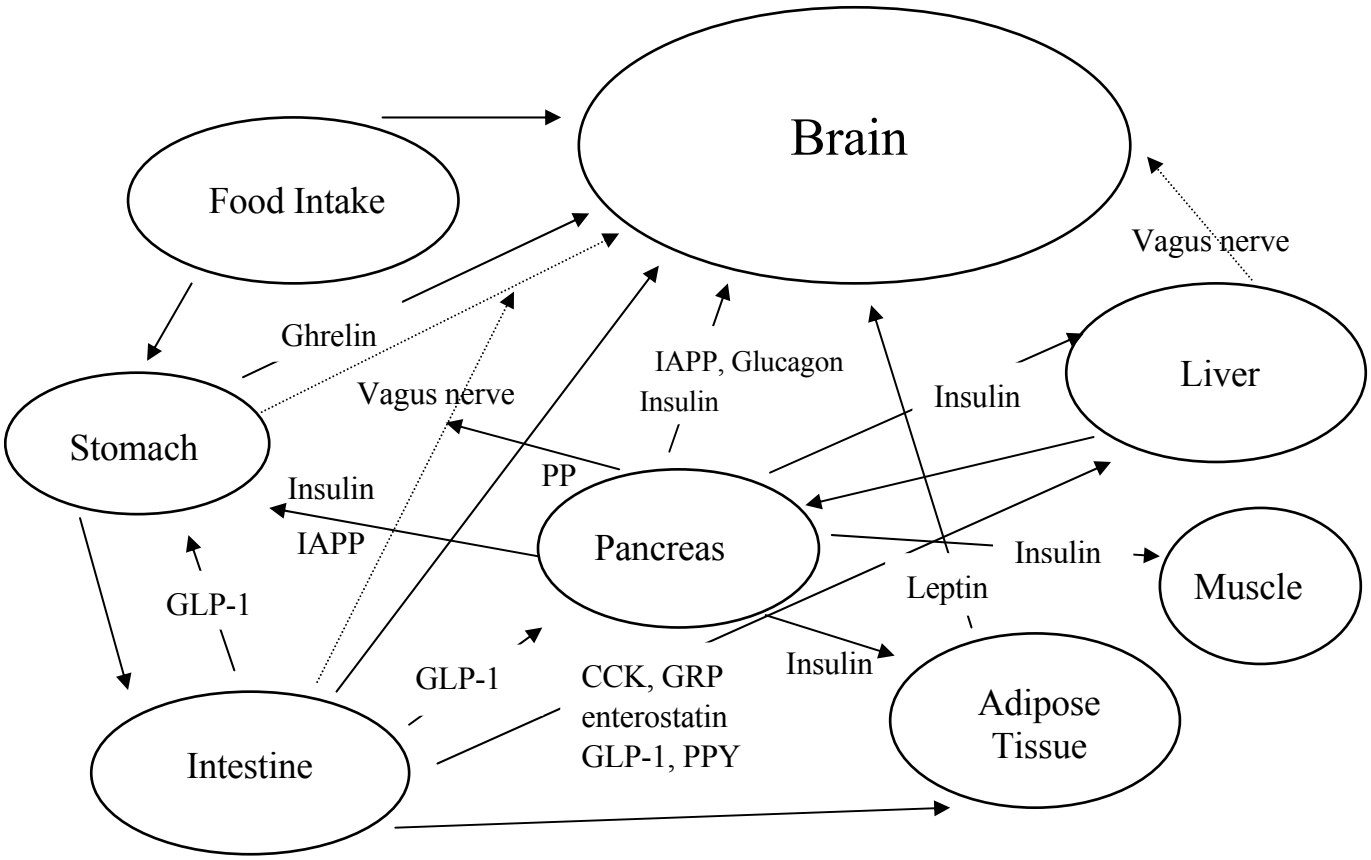


Fig 2. *Simple scheme of gastrointestinal peptides and the relationship with different target areas describing the complexity of food intake regulation.*

IAPP has been shown to bind to the area postrema (AP) in the hind brain and to activate multiple nervous system regions implicated in energy homeostasis. These include the lateral parabrachial nucleus (LPB), the central amygdala, the lateral hypothalamus, the nucleus of the solitary tract (NTS), the central amygdaloid nucleus (Ce), and the lateral subdivision of the bed nucleus of the stria terminalis (BSTL) (Reidiger *et al*. 2004, Rowland *et al*. 1997). These areas are interconnected and are linked to the hypothalamus, which is the main integrative centre for controlling energy balance (Saper CB. 2002). Both the AP in the hindbrain and the third ventricle/median eminence in the hypothalamus lack functional blood-brain barrier (Banks *et al*. 1998, Fry *et al*. 2007).

IAPP has been shown to increase c-fos (a marker of neuronal activation) in the NTS, LPB and Ce, probably as an effect secondary to its activation of AP neurons (Reidiger *et al*. 2001, Reidiger *et al*. 2004, Rowland and Richmond 1999). Pathways activated by IAPP, shown with c-fos, also project to the arcuate nucleus (ARC) and the ventromedial nucleus of the hypothalamus (Potes and Lutz 2010). The ARC contains populations of neurons that express neuropeptide Y (NPY), agouti-related protein

(AgRP), and proopiomelanocortin (POMC) (Baker *et al*. 1995, Qiu *et al*. 2001, Uchoa *et al*. 2012).

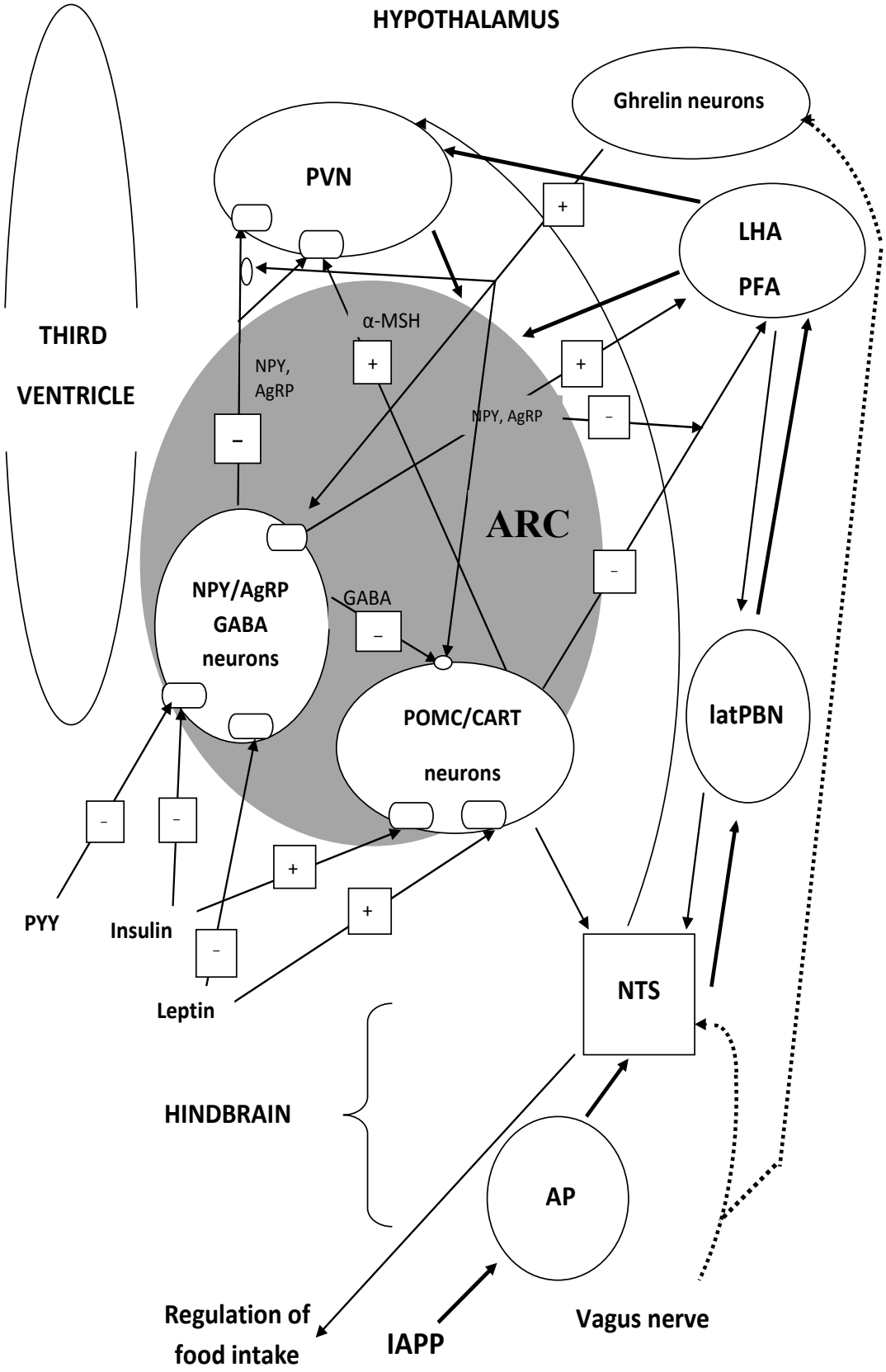


Fig 3. *The hypothalamus and hindbrain; central signals and interactions between different nucleii. Modified from Authesserre et al, Institut Européen de Chimie et Biologie, www.cellbiol.net.*

1.4 EXPERIMENTAL MODELS

The effects of IAPP on food intake and metabolism have been studied through bolus injection (Bhavsar *et al.* 1998**,** Lutz *et al*. 1994, Morley and Flood 1991, Rowland *et al*. 1999**)**, acute (hours) (Arnelo *et al.* 1996a, Reidelberger *et al.* 2001) and chronic (days) (Arnelo *et al.* 1996b, Lutz *et al.* 2001, Mack *et al*. 2007) infusion of exogenous IAPP, primarily in rats and mice. Osmotic mini-pumps can provide continuous subcutaneous infusions without disturbing the animals (Arnelo *et al*. 1996b), and in-dwelling intracerebroventricular (ICV) catheters deliver IAPP centrally without interfering with an animal´s activities and access to food (Wielinga *et al*. 2010). Similarly, IV and or intraperitoneal (IP) catheters tethered to infusion swivels can allow free movement of animals during continuous or intermittent peptide infusion (Arnelo *et al*. 1996a, Arnelo *et al*. 1998, Reidelberger *et al*. 2001, Chelikani PK. 2007).

Computerized collection of food intake data at very frequent intervals allows determination of both total food intake and meal pattern (meal size, meal number, intake during the light and dark periods) (Arnelo *et al*. 1996b, Arnelo *et al*. 1998, Reidelberger *et al*. 2001, Reidelberger *et al*. 2004, Woltman *et al*. 1995). The studies in Paper III required the development of a method for collecting detailed food intake and meal pattern data and for analyzing meal patterns in mice. This included changing the rat feeding system to a system suitable for mice, which are much smaller rodents. Previously, that kind of meal pattern analysis had been done only in rats.

Molecular biology technique was used to develop a knockout mouse that lacks a functional gene for IAPP (Gebre-Medhin *et al*. 1998). These mice were developed by replacing exon 3 in the murine IAPP locus with a neomycin-resistance gene (*neo*)-selection marker (Gebre-Medhin *et al*. 1998). Thus IAPP knockout mice provide an opportunity to investigate the physiological role of IAPP by observing the processes that occur in its absence and investigating the effects that administration of the missing gene produce has on the animal.

Transgenic mice expressing human IAPP have been developed using a human IAPP recombinant construct fused to either the rat insulin-2 promoter (Höppener *et al*. 1993, D'Alessio *et al*. 1994) or the rat insulin-1 promoter (Fox *et al.* 1993). Transgenic mice with the rat IAPP gene rather than the human IAPP gene have also been used to investigate the effects of chronic overproduction of IAPP (Höppener *et al*. 1993). In addition, transgenic rats have been developed using the human gene attached to the rat insulin-2 promoter (Butler *et al*. 2004).

2 AIMS

The studies in this thesis investigated the role of IAPP as a satiety hormone and the mechanisms through which it exerts its effects.

The specific aims were to:

1. Examine the effects of exogenous IAPP on food intake, adiposity, and energy metabolism in rats and mice. (Papers I, II, III, IV).

2. Determine whether the route of administration or the dose of exogenous IAPP affects its action in rats. (Paper II).

3. Investigate whether ICV infusion of IAPP causes changes in the expression of mRNA for neuropeptide Y, agouti-related protein, and proopiomelanocortin in the arcuate nucleus of rats. (Paper IV).

4. Determine whether endogenous IAPP is necessary for maintenance of normal body weight and a normal response to exogenous IAPP in mice. (Paper III).

3 MATERIALS AND METHODS

This section presents an overview of some of the methodologies used in the different papers. Additional details are included in the individual papers.

3.1 ANIMALS AND HOUSING CONDITIONS

Adult male Sprague-Dawley rats were used in the experiments in Papers I, II, and IV. In Paper III, adult male IAPP knockout mice (IAPP–/–, strain C57BL/6) and wild-type mice (IAPP+/+, strain C57BL/6) were used. The knockout mice were generated by homologous recombination (Gebre-Medhin *et al*. 1998). All animals were housed individually in temperature-controlled rooms with a 12:12h dark:light period. Plastic shoebox-type cages were used in Papers I and IV; metal metabolic cages were used in Papers II and III. In order to obtain accurate food intake information all the cages had wire floors that kept the animals from eating their feces. In both Papers II and III, the cage had an eating veranda attached to the cage where the rat or the mouse could reach its food. The feeding unit and the 16 units making the feeding system are shown in Fig. 4a and Fig. 4b.

a)  **b)** 

Fig 4. a) *The feeding unit consisting of a metabolic cage, an eating veranda, a food bowl, and a plate (to collect spillage) on a balance.* ***b)*** *16 feeding units, via a black box (switch), connected to an IBM-compatible computer making up the feeding system.*

In Paper II, the veranda was 9.5 cm deep x 8 cm wide x 10 cm high with a 3 cm diameter hole in the base. In Paper III, the cages had a metal cylindrical side compartment with a hole that was 1.5 cm in diameter through which the mice ate finely

powdered rodent chow. Numerous 4 mm diameter holes in the side compartment produced an open lattice that allowed any food that was dropped during feeding to fall to the bowl or balance.

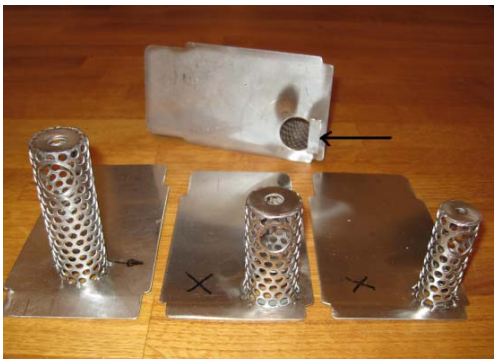
a)  **b)** 

Fig 5. a) *Example of different eating veranda prototypes. The arrow points to the little threshold described in Paper III.* ***b)*** *The mouse and its eating veranda during ongoing experiment.*

All the animals were fed standard rodent chow. In Papers I and IV, ground rat chow was mixed with sucrose and evaporated milk in a ratio of 500 g:400 g:354 ml to form a paste. This kind of food was chosen as an alternative to the finely-powdered chow used in meal pattern studies because it is known to minimize food spillage (Blevins *et al*. 2000). In Papers II and III, finely-powdered rodent chow R36 (Lactamin, Vadstena, Sweden) was used. In the pair-feeding studies (Papers I and IV), food-intake data were recorded once each day by manually weighing the food bowls. For the papers in which meal pattern was analyzed, food bowl weight was recorded at 16-second intervals (Paper II) or 2-minute intervals (Paper III) throughout each 24 hour period.

All animals had free access to food, except for individuals in the pair-fed groups in Papers I and IV. During the period of IAPP administration in Papers I and IV, pair-fed animals received the amount of food consumed the previous day by their counterparts in the IAPP groups. All animals had free access to water.

Animals were allowed to become accommodated to the housing conditions before the start of the studies. At the end of the experiments, the animals were anesthetized with sodium pentobarbital (60 mg/kg IP) and then killed by decapitation (Papers I, II, and IV) or by cervical dislocation prior to clipping the heart (Paper III).

3.2 IAPP

Rat IAPP, which is identical in sequence to mouse IAPP, was used for all the studies. For subcutaneous administration, IAPP was dissolved in 0.15 M sodium chloride and then infused at a dose of 25 pmol/kg-min in Papers I and III, and at doses of 0.25 pmol/kg-min, 2.5 pmol/kg-min, and 25 pmol/kg-min in Paper II using osmotic mini-pumps. Control animals in Papers I and II received vehicle only. For intracerebroventricular administration in Papers II and IV, IAPP was dissolved in artificial cerebrospinal fluid (aCSF) and administered through a catheter at doses of 0.025, 0.25, and 2.5 pmol/kg-min (Paper II) or 2.5 pmol/kg-min (Paper IV). Control rats in Paper II received vehicle only. The doses were chosen on the basis of data from previous experiments in rodents (Arnelo *et al*. 1996b, Arnelo *et al*. 2000, Fürnsinn *et al*. 1994, Rushing *et al*. 2000a).

3.3 SURGICAL PROCEDURES

3.3.1 Anesthesia

Before implantation of osmotic mini-pumps, the rats in Papers I, II, and IV were premedicated with midazolam at a dose of 1mg/kg IP (Paper I) or 0.7 mg/kg SC (Papers II and IV). The animals were then anesthetized with ketamine and xylazine (Paper I: 70 mg/kg and 10 mg/kg, respectively, IP; Papers II and IV 60 mg/kg and 9 mg/kg, respectively, IP). Brief isoflurane anesthesia was used with the mice in Paper III.

Before the insertion of intracerebroventricular catheters in Papers II and IV, the rats were premedicated with 0.5 mg midazolam SC and then anesthetized with Pentothal Natrium (40 mg/kg) and ketamine (40 mg/kg) IP.

3.3.2 Osmotic mini-pumps

Osmotic mini-pumps (Alzet, AlzaCorp., USA) were implanted subcutaneously on the back of the animals in the interscapular groove, Fig. 6a. The mini-pumps were used to administer IAPP or vehicle subcutaneously for 2, 3, 5, 6, or 7 days in Paper I, for 7 days in Paper II, and for 3 days in Paper III. Osmotic mini-pumps were attached to intracerebroventricular catheters for delivery of IAPP for 5 days (Paper IV) or 7 days (Paper II), Fig 6b.

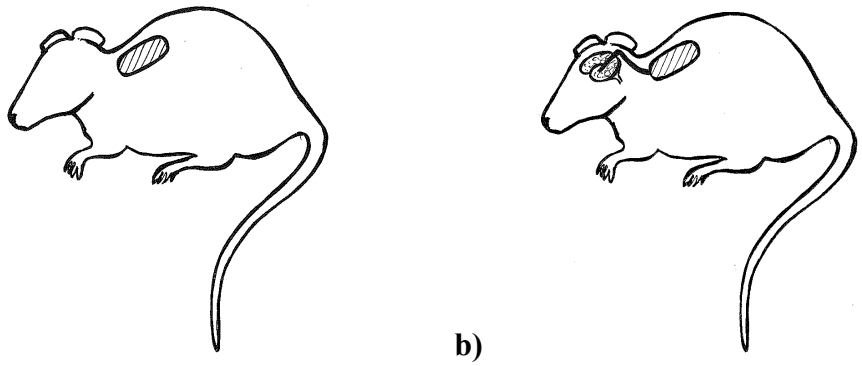


Fig 6. a) *Subcutaneous IAPP administration by osmotic mini-pump* **b)** *Intracerebroventricular IAPP administration, indwelling ICV catheters connected to the osmotic mini-pump.*

3.3.3 ICV catheters

For the experiments involving central administration of IAPP, the rats had indwelling ICV catheters inserted 1-2 weeks before the start of IAPP infusion. The catheters were constructed from PEEK (polyetheretherketone) tubing (i.d. 0.25 mm, o.d. 0.65 mm) and Micro-Renathane tubing (i.d. 0.36 mm, o.d. 0.80 mm). After the catheters were filled with aCSF, they were plugged with metal stylets and inserted into the third ventricle of the brain under aseptic conditions using a small-animal stereotaxic instrument. *The Rat Brain in Stereotaxic Coordinates* (Paxinos and Watson 1982) was used as the basis for selection of the coordinates (0.8 mm anterior to bregma, 5.9 mm below the surface of the brain, 1.0 mm lateral to the midline). A catheter angle of -10 degrees was used in order to avoid larger vessels and decrease the amount of blood loss. After the catheter was in place, it was attached to the bone with cement and tunneled down to the back of the rat. When the osmotic mini-pump was inserted at the start of the peptide infusion, the catheter was attached to the pump. At the end of the experiment in Paper II, methylene blue was injected into the catheters to allow later visual determination of their position. The cannulae ends were verified to be located in the third ventricle by microscope. In Paper IV, the rat brains were collected and frozen on dry ice.

3.4 MEASUREMENT OF FOOD INTAKE

3.4.1 Total food intake

Total food intake (TFI) or daily food intake is the amount of food an animal consumes during a 24-h period. In Papers I and IV, TFI was recorded each day by manually weighing the food bowls. In the studies of meal patterns (Papers II and III), animals were housed in custom-made metabolic cages, and their food bowls were attached to balances with a sensitivity of 0.01 g. Bowl weights were recorded every 16 seconds in Paper II and every 2 minutes in Paper III, using an IBM-compatible computer. The computerized data collection system permitted precise measurement of food intake and allowed calculation of meal pattern, something that is not possible if bowls are weighed by hand at various intervals. Corrections for spillage were made in all the papers.

3.4.2 Meal pattern analysis

Meal patterns were investigated in rats in Paper II by determining average meal size (MS) and average meal number (NM) for each day. Individual meals were defined using a minimum meal size (MMS) criterion of 50 mg and a minimum intermeal interval (MII) criterion of 5 minutes, criteria that have been used previously (Woltman *et al*. 1995). The 5-min MII criterion is considered to be the most effective in separating the log- or exponential-survivorship curve for interbout intervals into within- and between-meal intervals in rats with free access to food (Castonguay *et al*. 1986, Meguid *et al*. 1990, Woltman *et al*. 1995). Circadian food intake *i.e* food intake during the light and dark periods was also calculated for each day in paper II and III.

MMS criterion and MII criterion for mice have not been established. Therefore, following several pilot experiments in which different criteria were used, we found that a 20 mg MMS and a 10 min MII gave a reasonable number of meals with little loss of food intake data over 24 hours. Therefore, in Paper III, meal patterns were investigated in mice by using a MMS criterion of 20 mg and a MII criterion of 10 minutes.

Average 72-h meal numbers and meal sizes were determined in weeks 1, 7, 14, 21, and 27 in the long-term knockout mouse experiment. NM and MS were calculated daily in the short-term knockout mouse study.

3.5 PHYSIOLOGICAL MEASUREMENTS

3.5.1 Body weight

Body weight was recorded daily in Papers I and IV. In Paper II, body weight was determined before pump insertion and after the 7-day infusion period. In Paper III, the mice were weighed weekly in the long-term experiment and on days 0 and 6 in the short-term experiment.

3.5.2 Circulating nutrients and peptides

In Paper I, blood was obtained by heart puncture and collected in empty tubes or in tubes containing aprotinin and EDTA. Serum glucose and lactate concentrations were determined enzymatically (Wang *et al*. 1998). Free fatty acids were determined using an acyl-CoA oxidase-based kit; triglycerides were determined by bioluminescence and enzymatic colorimetry (Hellmér *et al*. 1989). Insulin and leptin were measured using radioimmunoassay kits.

3.5.3 Blood urea nitrogen

In Paper I, blood was collected after a 2- or 6-day IAPP infusion, and blood urea nitrogen (BUN) was determined using the diacetyl method (verbal communication with dr B. Fruin).

3.5.4 Body composition

In Paper I, the epididymal fat pads were resected and weighed as a measure of adiposity after 2- and 6-day infusions of IAPP. The liver and soleus muscle were also resected, snap frozen, and homogenized in ice-cold buffers for determination of protein, DNA, and glycogen content. Protein was determined using a BCA protein assay kit. DNA was determined using the assay described by Labarca *et al.* (Labarca and Paicen 1980); glycogen was measured using the protocol described by Lust *et al.* (Lust *et al*. 1975).

3.5.5 Glucose transport in skeletal muscle

In Paper I, soleus muscles were resected from rats after a 3-day infusion of IAPP and were incubated with 0, 0.7, 1.4, or 7 nM insulin. [^{3}H]-3-O-methylglucose and [^{14}C]

mannitol were added for the last 10 minutes of incubation to give final concentrations of 8 and 12 mM, respectively. After the muscle samples were dissolved in sodium hydroxide (Wallberg-Henriksson *et al*. 1987), the isotopes were counted simultaneously. The counts were used to calculate the extracellular space and the intracellular concentration of 3-O-methylglucose.

3.5.6 mRNA for uncoupling protein 2 (UCP2)

In Paper I, the expression of UCP2 mRNA after a 5-day infusion of IAPP was determined in soleus muscle and epididymal fat pads that were resected from rats and snap-frozen in liquid nitrogen. After total RNA was extracted from the samples (Chomczynski and Sacchi 1987), UCP2 mRNA was determined as described by Isaksson *et al*. (Isaksson *et al*. 2002).

3.5.7 Fluorescence in situ hybridization (FISH)

In Paper IV, the rat brains were collected and frozen on dry ice at the end of the 5-day infusion of IAPP, and 14 μm coronal brain sections were cut from the hypothalamus. As described previously in protocols for detecting mRNA with the FISH technique (Breininger *et al*. 2000) the sections were treated with paraformaldehyde, washed in phosphate buffer, dehydrated with alcohol, and delipidated with chloroform. The slides were air-dried after additional treatment with 100% alcohol. NPY-digoxin labeled probes were added and the slides were incubated, washed, dehydrated, and air dried. Two consecutive slides containing 4 hypothalamic sections, including ARC, from each rat were then used for fluorescence microscopic imaging and the distribution of NPY-immunofluorecence was evaluated using a red fluorescent dye (Cy 3).

3.5.8 mRNA in situ hybridization (ISH)

In Paper IV, the 14 μm coronal hypothalamic brain sections (see section 3.5.7) were treated with paraformaldehyde, acetylated in triethanolamine, dehydrated with alcohol, and delipidated with chloroform. The slides were air-dried after additional treatment with 100% alcohol. Riboprobes that had been transcribed from cRNA of rat NPY, rat AgRP, and rat POMC and labeled with [^{33}P]UTP were placed on the slides. The slides were then incubated, washed, dehydrated, and air dried. Slides containing 6 hypothala-

mic sections from each rat were put onto a phosphoimager screen for 4 days, and the radioactivity was recorded. The ARC areas of the screens were analyzed using Optiquant™ Image Analysis Software and a Cyclone phosphoimager. Levels of mRNA for neuropeptide Y (NPY), agouti-related protein (AgRP), and proopiomelanocortin (POMC) were calculated by subtracting the background values from the measured intensities. These methods were previously described by Schwartz et al. (Schwartz *et al.* 1997).

3.6 STATISTICAL ANALYSIS

In all the papers, values are presented as means ± SEM. P values less than 0.05 were considered statistically significant.

In Papers I, III and IV, data were analyzed using JMP 4 software (SAS Institute, Inc.). In Papers I, III and IV, analysis of variance and Tukey-Kramers test were used to test the statistical significance of multiple comparisons. Unpaired student´s t tests were used for comparisons of two means in all the papers.

In Paper II, body weight data was evaluated by one-way analysis of variance (ANOVA). Effects of IAPP on food intake and meal parameters were evaluated using a 2-factor repeated measures ANOVA, with IAPP dose as the between-group factor and the day of infusion as the within-group factor. Planned comparisons of treatment means were evaluated by direct contrasts of means (least significant difference method) using the statistical program SYSTAT.

In Paper III, repeated measures data were analyzed using the MIXED procedure in SAS®System 9.1 (SAS Institute Inc., Cary, NC, USA) (Kirk RE. 1995, Littell *et al*. 2006). The within-group factor was time. First- and second-order polynomial trends were also investigated to determine whether trends in the groups differed over time. Different covariance pattern mixed models were tested: compound symmetry, first order autoregressive, and heterogeneous autoregressive. The covariance structure with the smallest value of the Akaike's Information Criterion was considered most desirable.

3.7 ETHICAL APPROVAL

The animal studies in Papers I, II, III, and IV were approved by the Animal Research Ethical Committee for Southern Stockholm (Stockholms södra försöksdjuretiska nämnd).

4 REVIEW OF THE PAPERS

4.1 PAPER I

"Chronically administered islet amyloid polypeptide in rats serves as an adiposity inhibitor and regulates energy homeostasis".

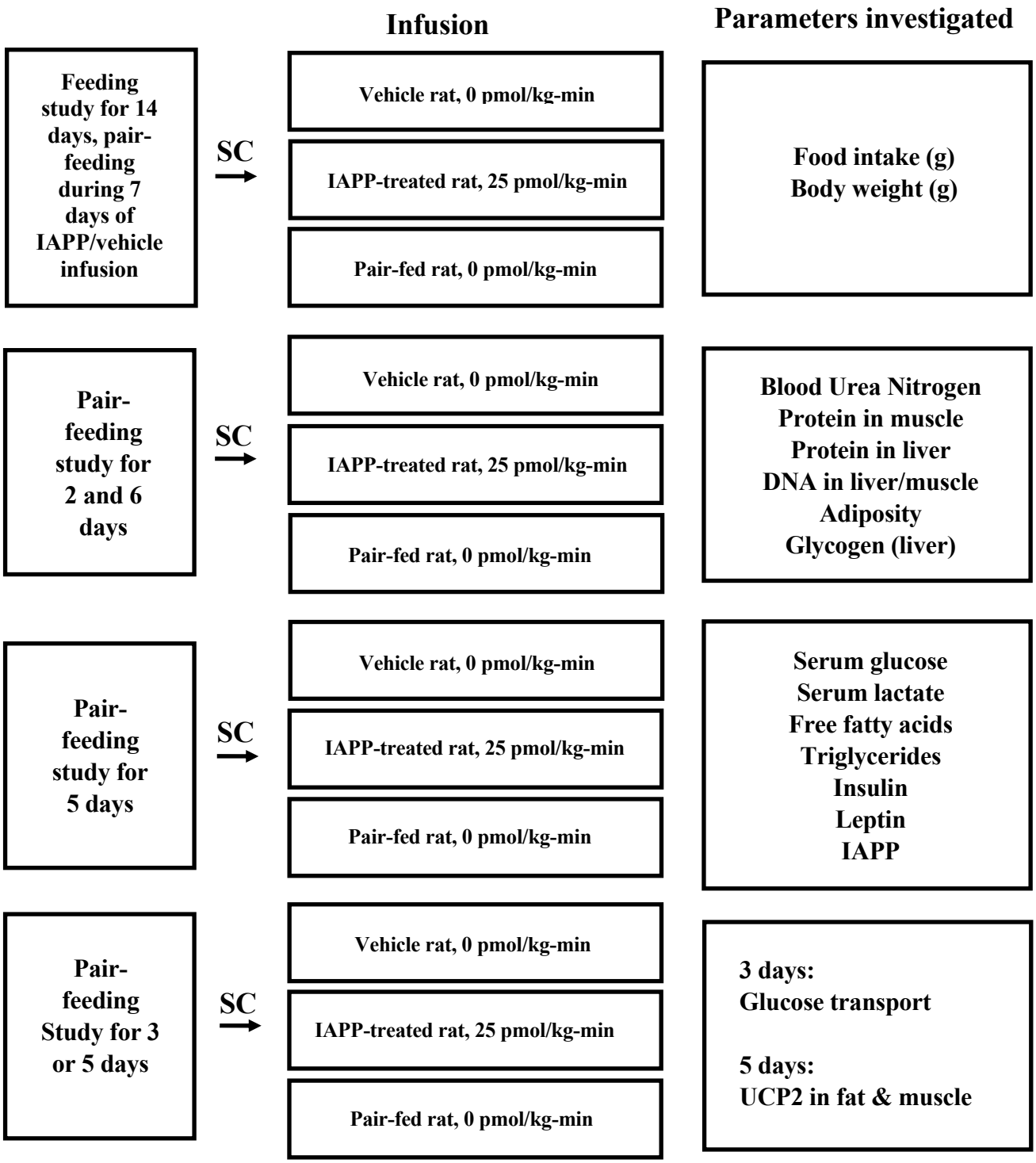


Fig 7. *Study design of feeding/metabolic experiments in Paper I.*

The effects of chronic IAPP treatment on different aspects of energy homeostasis were investigated in rats after subcutaneous infusion of IAPP (25 pmol/kg-min) for 2-7 days. The IAPP-treated rats were compared to saline-infused control groups in which the animals were either fed ad libitum or were pair-fed to the IAPP-treated rats. In the studies of food intake, body weight, and adiposity, the pair-fed animals were pair-fed for the 7 days when the IAPP rats received IAPP and were then fed ad libitum for the next week.

In the IAPP rats, food intake was significantly reduced in days 1-6 compared to the ad libitum fed animals ($p < 0.05$). After the IAPP infusion, food intake in the rats that had received IAPP was no different from the ad libitum group during days 7-10. It was higher than the ad libitum group on days 11-14 ($p < 0.05$). The most marked decrease in food intake in the IAPP-treated rats was seen on day 1. The IAPP group had significantly lower body weight gain during days 1-8 ($p < 0.05$) that the ad libitum fed rats. The epididymal fat pads of the IAPP-treated rats were smaller than those in the pair-fed group after the 6-day infusion period ($p < 0.05$).

After 5 days of IAPP infusion, circulating IAPP was significantly elevated in the IAPP-treated group compared with the ad libitum and pair-fed groups (both $p < 0.001$). The IAPP-treated group had lower insulin concentrations than the ad libitum fed group ($p < 0.05$) and lower leptin concentrations that either the ad libitum or pair-fed groups (both $p < 0.05$).

Serum triglycerides and free fatty acids were significantly reduced in the IAPP group compared with the pair-fed rats (both $p < 0.05$). Glucose and protein metabolism in the IAPP-treated rats was largely unchanged. No significant differences were seen in serum glucose and lactate concentrations. The only significant difference seen in glucose transport in skeletal muscle occurred at 1.4 nM insulin, with the rate of transport at that dose being significantly lower in the IAPP group than in the pair-fed group. No differences were seen in UCP2 mRNA levels in epididymal fat or soleus muscle or in the protein, glycogen, and DNA content of liver or soleus muscle tissue. Blood urea nitrogen levels in the pair-fed rats were higher on days 2 and 6 than those in the IAPP-treated animals ($p < 0.01$ and $p < 0.05$, respectively) and were higher on day 2 than those in the ad libitum fed group ($p < 0.05$).

4.2 PAPER II

"Comparison of the effects of chronic central administration and chronic peripheral administration of islet amyloid polypeptide on food intake and meal pattern in the rat".

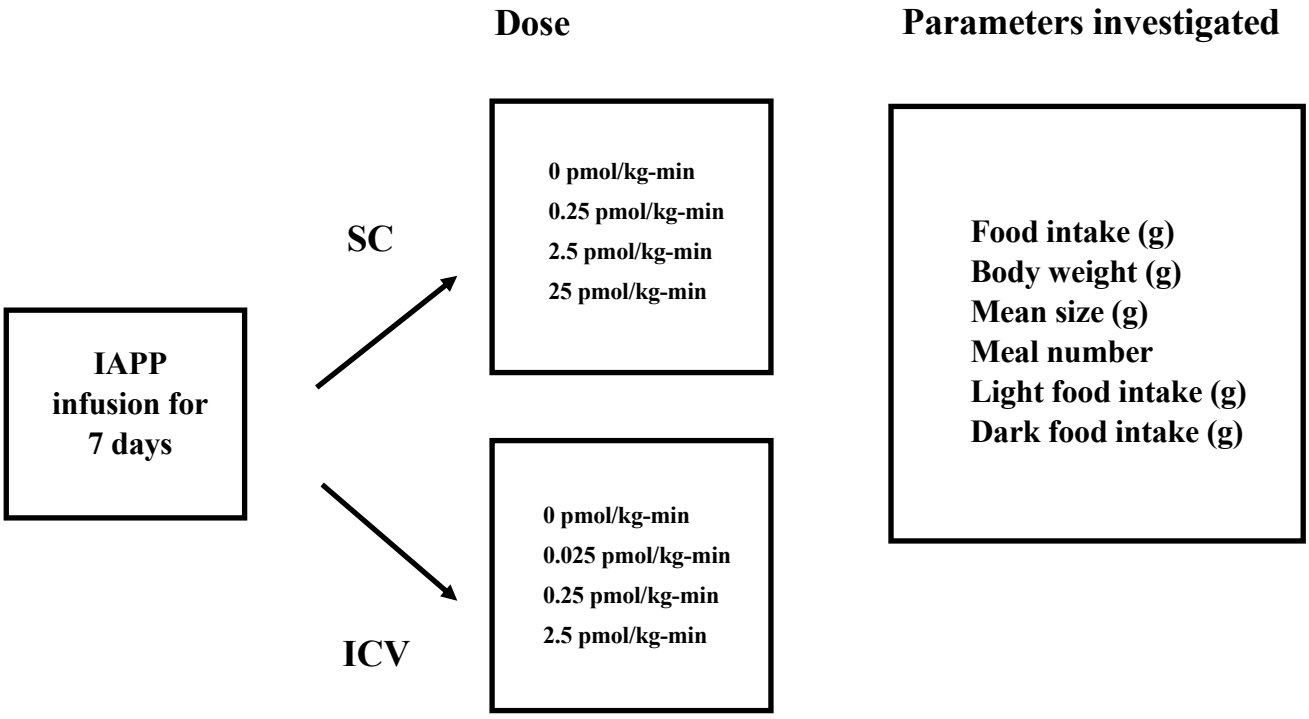


Fig 8. *Study design for SC and ICV chronic IAPP infusion experiment where the effects on food intake and meal pattern were studied.*

These experiments tested the following hypothesis: If IAPP decreases food intake by acting as a hormonal signal to the brain, then central administration should be more potent than peripheral administration and both methods should affect meal pattern similarly. The studies compared the dose-dependent effects of peripheral and central administration of IAPP on food intake, meal pattern, and body weight in rats.

Peripheral administration of IAPP was studied by subcutaneous infusion of IAPP in rats for 7 days at doses of 0, 0.25, 2.5 and 25 pmol/kg-min using osmotic mini-pumps. Central administration was studied by intracerebroventricular infusion of IAPP for 7 days at doses of 0, 0.025, 0.25 and 2.5 pmol/kg-min using osmotic mini-pump connected to previously-implanted ICV catheters.

A transient, dose-dependent independent inhibition of food intake was seen with both SC and ICV administration. The maximum effect was seen on day 2 for both routes of administration (both $p < 0.001$). The minimal effective doses were 2.5 pmol/kg-min for SC administration and 0.25 pmol/kg-min for ICV infusion. The

decrease in food intake produced by infusion of IAPP was due mainly to decreased MS, although a significant decrease in NM also occurred at the highest SC and ICV doses. SC administration produced a larger, more persistent decrease in food intake during the light period than in the dark period, while ICV infusion caused a larger, more persistent decrease during the dark period. IAPP significantly decreased body weight gain during the infusion period only at the highest SC dose (25 pmol/ kg-min) and the highest ICV dose (2.5 pmol /kg-min), both $p < 0.001$.

4.3 PAPER III

"Food intake and meal pattern in IAPP knockout mice with and without infusion of exogenous IAPP".

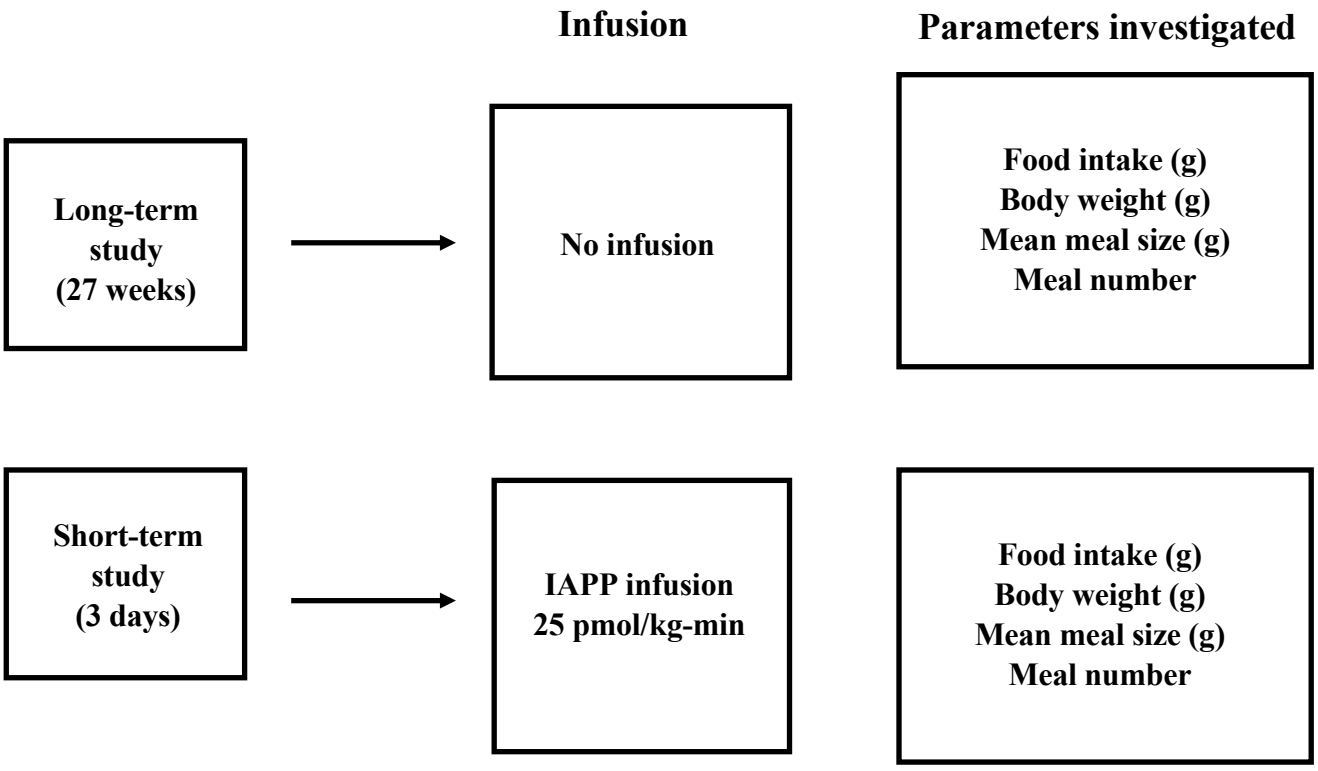


Fig 9. *Study design for investigating food intake and meal pattern in IAPP KO and WT mice with (short term study) and without administration of IAP (long term study).*

The long-term study in this paper tested the following hypothesis: If IAPP is necessary for normal satiety, mice lacking IAPP would be expected to have higher total food intake and, consequently, higher body weights than mice with normal IAPP production. The short-term study used infusion of IAPP to test the hypothesis that lack of endogenous IAPP would have an effect on the inhibition of food intake by exogenous IAPP.

Because only data collection systems for use with rats were available at the time of the studies, a method for obtaining detailed data on food intake in mice was developed before the start of the experiments. Pilot studies were conducted, and observations from these experiments were used as the basis for modifying the side cylinder (the eating veranda) of a metabolic cage for rats. The new side cylinder that was developed was shorter and narrower than the one used with rats. It had a smaller hole above the food

bowl and had a 3 mm-high metal plate at the entrance to the cylinder. Numerous holes allowed food that was dropped during feeding to fall from the side compartment to the bowl or balance.

In the long-term study, food intake and meal patterns were recorded during weeks 1, 7, 14, 21, and 27 in 8 adult male IAPP KO mice (IAPP-/-, strain C57BL/6) and 8 adult male WT mice (IAPP +/+, strain C57BL/6). The mice averaged about 4 months of age at the start of the experiment. There were no significant differences in body weight between the WT and KO mice at the beginning of the long-term experiment or during the course of the study. No statistically-significant differences were seen in total food intake between the KO and WT mice during any of the individual study weeks or when the weeks were combined. In weeks 7, 14, 21, and 27, meal number was significantly higher in both WT and KO mice compared with week 1 (all $p < 0.0001$). For both WT and KO mice, meal size was significantly lower during weeks 7, 14, 21, and 27 compared with week 1 (all $p < 0.05$). No significant differences in meal size or meal number were seen between WT and KO mice at the start of the long-term experiment or during any of the selected weeks in that experiment.

A short-term experiment was conducted in the same mice two weeks after the end of the long-term study. The effects of infusion of exogenous IAPP in knockout and wild mice was investigated using osmotic mini-pumps that were implanted subcutaneously in all the mice and delivered rat IAPP at a dose of 25 pmol/kg-min for three days. There were no significant differences in weight between the groups on day 0 or at the end of the experiment. Food intake in the KO mice was significantly lower than that in the WT mice on days 1 and 2 (both $p < 0.01$) (Fig. 10). There were no significant differences in meal number ($p = 0.17$) or meal size ($p = 0.07$) between WT and KO mice. Compared with the amount of food eaten on day 0, food intake was significantly reduced in both groups on days 1–3 (WT $p < 0.0001$, $p < 0.0001$, and $p < 0.01$, respectively; KO $p < 0.0001$, $p < 0.0001$, and $p < 0.001$, respectively). Compared with day 0, both WT and KO mice had a significant decrease in meal number on days 1–3 (all $p < 0.0001$). Compared with day 0, meal size decreased significantly on day 1 in both groups (both $p < 0.01$) but not on days 2 or 3.

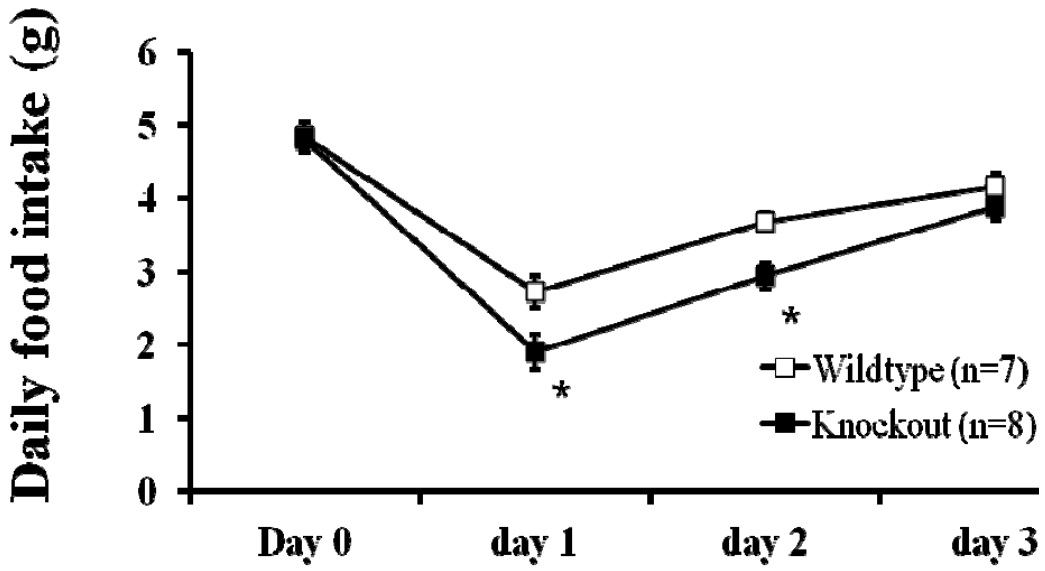


Fig 10. *Daily food intake during SC IAPP (25 pmol/kg-min) infusion in IAPP KO mice or WT mice. Day 0 denotes the day before insertion of the osmotic minipump. Values are means ± SEM. * P < 0.01.*

4.4 PAPER IV

"On the regulation of mRNA expression of proopiomelanocortin, neuropeptide Y, and agouti-related protein in the hypothalamus by chronic central infusion of islet amyloid polypeptide".

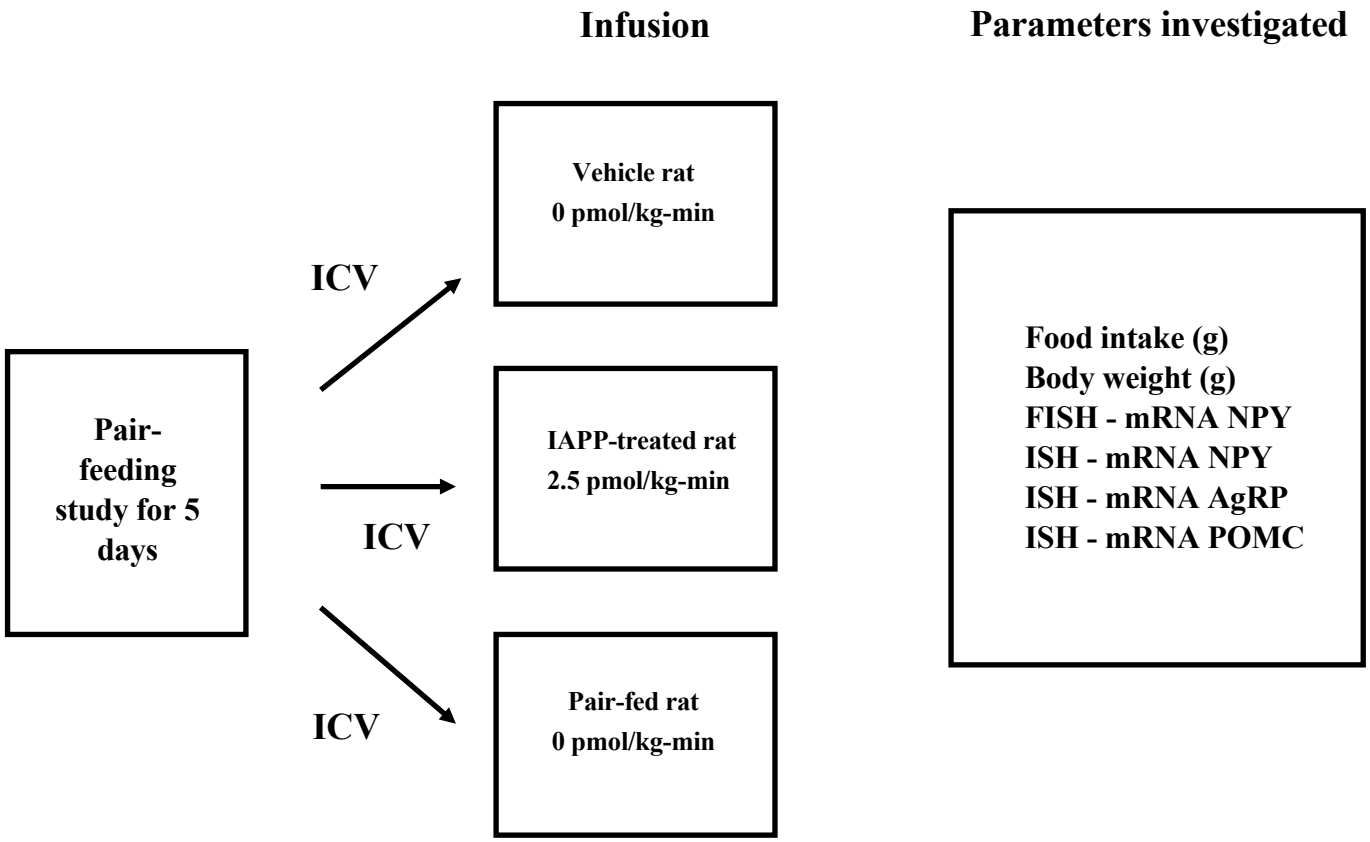


Fig 11. *Study design of pair-feeding experiment where the effects of chronically ICV IAPP administration on mRNA expression of NPY, AgRP and POMC were studied.*

This paper tested the hypothesis that intracerebroventicular infusion of IAPP would change mRNA expression of neuropeptide Y (NPY), agouti-related protein (AgRP) and proopiomelanocortin (POMC) in the arcuate nucleus (ARC) of rats. The study design, Fig.11, included three groups of rats: a group that received ICV infusion of IAPP in the third ventricle at a dose of 2.5 pmol IAPP/kg-min for 5 days, a group that received ICV infusion of vehicle only, and a group that was pair-fed to the IAPP rats and received ICV infusion of vehicle. The pair-fed animals, which were given the amount of food consumed by their counterparts the previous day, were included in the experiment to help determine whether any changes seen in the IAPP group were primary effects of IAPP or whether they were secondary effects caused by a decrease in food intake. Food intake and body weight were measured daily. In situ hybridization (ISH) was used to

measure mRNA in 14 μm coronal brain sections cut from the hypothalamus. Fluorescence in situ hybridization (FISH) was also used to evaluate mRNA NPY-immunostaining in 14 μm coronal hypothalamic brain sections (Fig.12).

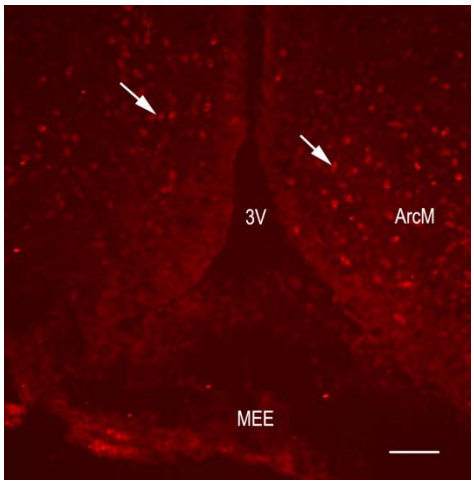


Fig 12. *NPY mRNA expression (shown with red fluorescence die) in ARC in the hypothalmus using the FISH technique. Arrows point at cells positive for NPY content. 3V=third ventricle, ArcM= arcuate nucleus (medial part), MME=medial eminence (external layer).*

Cumulative food intake for days 1-5 was significantly less in both the IAPP-animals and the pair-fed rats compared to vehicle-treated rats (both $p < 0.05$). Body weight increased during the experiment in the vehicle-treated rats, while the body weight of the IAPP-treated and pair-fed rats decreased significantly (both $p < 0.05$ compared to vehicle-treated). Food intake was significantly decreased in both IAPP and pair-fed animals during days 2-5 compared to vehicle treated control rats.

No significant differences in mRNA expression were seen among the groups for either NPY or AgRP (Figs. 13a-c and Figs. 14a-c). POMC mRNA was significantly higher in the vehicle group than in the pair-fed rats and IAPP rats (both $p < 0.05$). There was also a significant difference between the IAPP and pair-fed animals ($p < 0.05$), with the lowest levels of POMC mRNA in the pair-fed animals (Figs. 15a-c).

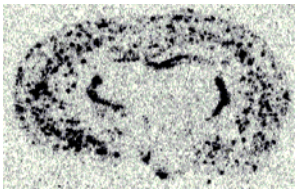
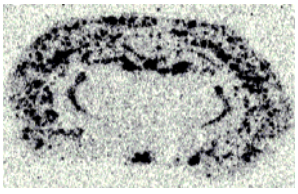
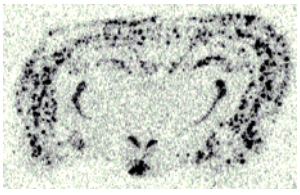
a)  **b)**  **c)** 

Fig 13. *NPY mRNA expression in ARC. The pictures show frontal section through the plane of the hypothalamus.* ***a)*** *in vehicle rat* ***b)*** *in pair-fed rat* ***c)*** *in IAPP-treated rat using the ISH technique.*

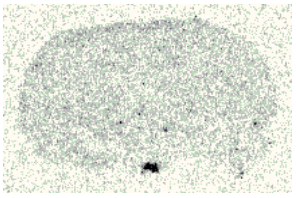
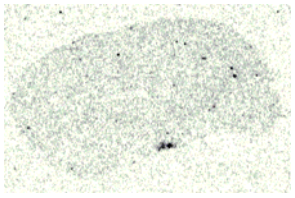
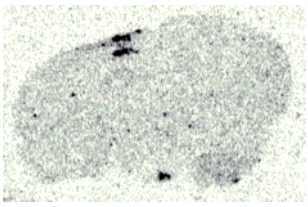
a)  **b)**  **c)** 

Fig 14. *AgRP mRNA expression in ARC. The pictures show frontal section through the plane of the hypothalamus.* ***a)*** *in vehicle rat* ***b)*** *in pair-fed rat* ***c)*** *in IAPP-treated rat using the ISH technique*.

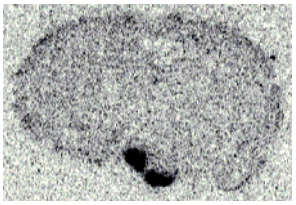
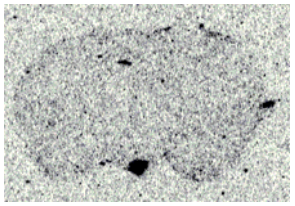
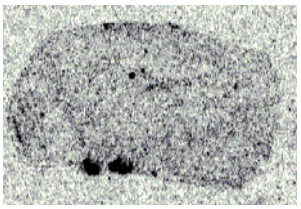
a)  **b)**  **c)** 

Fig 15. *POMC mRNA expression in ARC. The pictures show frontal section through the plane of the hypothalamus.* ***a)*** *in vehicle rat* ***b)*** *in pair-fed rat* ***c)*** *in IAPP-treated rat using the ISH technique.*

5 DISCUSSION

The experiments in this thesis have investigated the role of IAPP as a satiety hormone and have examined some of the mechanisms through which it exerts its effects.

5.1 IAPP AS A SATIETY HORMONE

The following criteria for evaluating a potential satiety hormone (Grossman MI. 1977, Grossman MI. 1979a, Grossman MI. 1979b, Reidelberger *et al*. 1994) have been used as a framework in the current studies to investigate IAPP as a satiety hormone and examine the mechanisms of its effects:

1. Intravenous administration of the peptide inhibits food intake.
2. Food intake increases plasma levels of the peptide.
3. Food intake is inhibited by intravenous administration of the peptide at doses that reproduce the postprandial rise of the endogenous peptide in plasma.
4. Food intake is stimulated by blocking the action of the endogenous peptide or knocking out the gene.

Subcutaneous administration of the peptide significantly inhibited food intake in all four of the papers, which supports criterion #1. These findings are consistent with data from previous studies using subcutaneous or intracerebroventricular infusions of IAPP in rodents (Arnelo *et al*. 1996b, Baldo and Kelly 2001, Chance *et al*. 1992, Chance *et al*. 1993, Clementi *et al*. 1996, Lutz *et al*. 1994, Lutz *et al*. 1998a, Rushing *et al*. 2000a). Previous studies have shown that the inhibition of food intake by IAPP is not due to malaise or nonspecific effects such as a reduction of water intake (Lutz *et al*. 1995b, Morley *et al*. 1997). A dose response study was conducted in rats in Paper II; in the other papers only a single dose of IAPP was used.

The postprandial increases in circulating IAPP that have been seen in humans (Butler *et al*. 1990) and rats (Arnelo *et al.* 1998, Pieber *et al.* 1994) support criterion #2. In a study in rats (Arnelo *et al*. 1998), food intake increased IAPP from a fasting level of 10.8 ± 0.5 pM to a peak of 19.0 ± 1.0 pM. Intravenous infusion of IAPP at a dose of 1 pmol/kg-min, which would increase plasma IAPP by an amount comparable to that produced by a meal, has been found to inhibit food intake (Reidelberger *et al*. 2001). This data provides evidence that IAPP meets Criterion #3. In Paper I, the circulating IAPP levels (318 ± 38 pM) seen in the rats receiving 25 pmol/kg-min

IAPP subcutaneously were comparable to those seen in genetically obese rats (Huang *et al*. 1992) and in transgenic rats with the human IAPP gene (Ahrén *et al.* 1998, D'Alessio *et al*. 1994).

Criterion #4 has been evaluated in previous studies by use of IAPP receptor blockers. Administration of AC187 increases food intake in rats when administered centrally (Rushing *et al*. 2001) or peripherally (Reidelberger *et al*. 2004). However, it does not completely block the anorexia produced by exogenous IAPP (Reidelberger *et al*. 2004), which indicates either that AC187 has insufficient access to the CT/RAMP receptors or that IAPP also acts through another receptor not blocked by AC187. Other IAPP receptor blockers such as $hCGRP_{8-37}$, AC413 and sCT_{8-32} have also been investigated (Bailey *et al*. 2012); however, these were found to be less specific than AC187.

If IAPP is required for normal satiety to occur, animals lacking IAPP would be expected to have higher food intakes than those with normal IAPP production. However, food intake did not differ significantly between wild-type and IAPP KO mice in the 27-week long-term experiment in Paper III. This finding, which is consistent with previous studies of food intake in IAPP KO mice using other experimental designs (Dacquin *et al.* 2004, Gebre-Medhin *et al*. 1998, Turek *et al.* 2010), indicates that food intake can be regulated effectively in the absence of IAPP and suggests redundancy in the control of food intake. Knockout mice lacking the gene for cholecystokinin (a peptide hormone that inhibits food intake) have also been found to have normal food intake compared to wild-type littermates (Lo *et al*. 2008). On days 1 and 2 during the short-term IAPP infusion in KO and wild-type mice, food intake was significantly lower in the KO mice. The more marked anorectic effect of IAPP in the KO mice suggests that IAPP receptors and/or IAPP post-receptor signaling pathways were up-regulated in the mice that lacked endogenous IAPP.

5.2 EFFECTS OF IAPP

5.2.1 Body weight and body composition

Both rats and mice in Papers I, II, and IV had a decrease in body weight or in body weight gain as a result of IAPP infusion. This is consistent with findings in previous studies using subcutaneous infusion (Arnelo *et al*. 1996b, Arnelo *et al.* 2000). Compared to saline-infused rats, animals receiving a 2-week ICV infusion of IAPP had lower body weights regardless whether their body weight had previously been

decreased by fasting, had been increased by voluntary overfeeding or had been unmanipulated (Wielinga *et al*. 2010).

No differences in body weight between the wild-type and IAPP KO mice were seen in Paper III during either the long-term or short-term experiments, which indicates that food intake can be regulated effectively in the absence of IAPP and suggests redundancy in the control of food intake. Cholecystokinin knockout mice have also been found to have normal body weights compared to wild-type littermates (Lo *et al.* 2008), which provides further support for redundancy in the control mechanism. No differences in body weight were seen between IAPP KO mice and wild-type mice in a study of bone resorption (Dacquin *et al.* 2004). In another investigation, male IAPP KO mice were reported to have significantly higher body mass after 18 weeks, but the large inter-individual variation that was observed led the authors to argue that the altered body weight might reflect the hybrid genetic background of the animals in that study (Gebre-Medhin *et al*. 1998).

In Paper I, the epididymal fat pad was decreased in the rats following a 7-day SC IAPP infusion. This result is consistent with a previous study in which retroperitoneal fat decreased in rats after a 10-day IAPP infusion into the third ventricle of the brain (Rushing *et al.* 2000a). The normal BUN levels and skeletal muscle composition in the IAPP group in Paper I suggest that the reduction in body weight seen in the IAPP rats was not caused by protein depletion in skeletal muscles.

When rats in the pair-fed group in Paper I were given free access to food, they immediately overate and quickly normalized their body weight. In contrast, rats in the IAPP group took a longer time to normalize their food intake and body weight. One possible explanation is that the osmotic pumps might have continued releasing IAPP after the anticipated end of the IAPP infusion. Alternatively, IAPP may have exerted residual biological effects that persisted after the end of the IAPP infusion. The pair-fed animals were semi-starved and were craving food; they were far more keen on overeating than the satisfied IAPP-treated animals.

5.2.2 Insulin

The effect of IAPP on insulin secretion has been controversial. Most studies have shown that IAPP inhibits insulin secretion (Ahrén *et al.* 1998, Bennet *et al*. 1994, Dégano *et al.* 1993, Fürnsinn *et al*. 1994, Mather *et al*. 2002, Wang *et al.* 1999). However, some other studies have reported no effect on insulin secretion (Broderick *et*

al. 1991, Tedstone *et al.* 1990). The hypoinsulinemia seen in the IAPP group in Paper I supports the hypothesis that IAPP inhibits insulin secretion. Because no significant hypoinsulinemia was seen in the pair-fed group in Paper I, the hypoinsulinemia observed in the IAPP group appears to be independent of the decrease in food intake. The hypoinsulinemia in the IAPP group may partly be a result of the decreased adiposity in these rats because basal plasma insulin levels are influenced by the degree of adiposity (Polonsky *et al*. 1988).

5.2.3 Glucose, lipid, and protein metabolism

Circulating glucose concentrations and tissue glycogen content were unchanged in the rats receiving IAPP infusion in Paper I. Glucose transport in isolated skeletal muscles was largely unchanged. These results indicate either that glucose homeostasis was not disturbed during chronic IAPP exposure or that glucose homeostasis had already been restored when the rats were examined. These findings are in concert with previous results from Arnelo *et al.* who found unaltered glucose metabolism *i.e.* glucose metabolic rate during hyperinsulinemic euglycemic clamp in rats following 2 or 5 days of SC infusion of IAPP (Arnelo *et al.* 1997).

The decrease in serum free fatty acids and triglycerides in the IAPP group in Paper I is consistent with the reduction in adiposity in that group. Because a previous study using a 4-hour IAPP treatment found enhanced lipolysis and increased circulating lipids in rats (Ye *et al*. 2001), it is possible that lipolysis was temporarily reduced in the Paper I study and caused the reduction in adiposity. Because body fat mass is the primary determinant of circulating leptin levels (Cnop *et al*. 2002), the decreased adiposity in the IAPP group may be responsible for the hypoleptinemia seen in that group. Chronic ICV infusion of IAPP in rats has been shown to reduce the quotient of carbon dioxide production and oxygen consumption, implying a preferential oxidation of fat (Wielinga *et al*. 2010).

Blood urea nitrogen and tissue protein content were unchanged in the IAPP group in Paper I, indicating that protein metabolism was not disturbed by the IAPP treatment. However, the pair-fed rats in the study had an elevated BUN level as well as decreased body weight, which suggests that the pair-fed rats experienced metabolic changes associated with caloric restriction (Giesecke *et al*. 1989). Uncoupling protein 2 (UCP2) expression in intra-abdominal fat and skeletal muscles was not changed during IAPP

exposure in Paper I, indicating that UCP2 is not involved in the metabolic changes induced by IAPP in those tissues.

5.3 MECHANISMS OF IAPP ACTION

5.3.1 Food intake patterns

There are a number of different ways in which eating behavior can be altered. An increase or decrease in total food intake during a 24-hour period can reflect changes in the number of meals and/or the size of individual meals. In addition, the proportion of the total food intake that occurs at different times of days can be changed.

Analysis of meal patterns requires collection of data at very frequent intervals throughout the study period. The computerized systems used in Papers II and III allowed detailed information to be recorded automatically without disturbing the animals.

5.3.1.1 Development of a data collection system for mice

The experiments in Paper III required the development of a method for computerized data collection in mice, because no suitable system was available. Pilot studies showed that, because of their small size, mice housed in the type of metabolic cage used for rats in Paper II and could turn around in the side compartment. This led to their defecating or urinating into the food bowl or onto the balance, which reduced the accuracy of food intake measurement. In addition, the mice could easily carry food back into the cage, and the food hole in the compartment was large enough that some mice were able to escape. Observations from pilot studies were used to design a modified side cylinder for the cage that was shorter and narrower and had a smaller hole above the food bowl. A threshold (a 3 mm-high metal plate) at the base of the cylinder entrance discouraged the mice from turning around. Numerous 4 mm diameter holes in the side compartment created a lattice that allowed food that was dropped during feeding to fall to the bowl or balance (Figs. 5a and 5b).

5.3.1.2 Food intake during light and dark periods

In the subcutaneous infusion experiment in Paper II, a transient reduction in food intake was seen in the IAPP rats during the dark period, but a more persistent reduction was seen during the light period. This is consistent with previous data from SC infusion

(Arnelo *et al*. 1996b). In the ICV experiment in Paper II, however, the reduction in food intake during the dark period was more persistent and more marked than the reduction seen during the light period, which suggests that IAPP did not act on the same target or targets when administered centrally as it did when administered peripherally.

A rat usually eats 80% of its daily food intake during the dark phase (Le Magnen J. 1992). In mice, 70% or more of the total food intake typically occurs during the dark period (Petersen *et al*. 1981, Strohmayer and Smith 1987). However, in Paper III, only about half of the food intake occurred during the dark period. Interestingly, knockout mice for the satiety hormone cholecystokin have been shown to eat more food during the light period ($p < 0.04$) and less food during the dark period than their wild-type littermates, although total food intake was not different between the CCK KO mice and the wild-type mice (Lo *et al.* 2008).

5.3.1.3 Meal size and meal number

In Paper II, the decrease in food intake in the IAPP-treated rats was mainly due to decreased meal size, although a significant decrease in meal number was also seen at the highest SC and ICV doses. This is consistent with studies showing a decrease in meal size after IP injection (Lutz *et al*. 1995b) or IV infusion of IAPP (Reidelberger *et al*. 2001). However, a decrease in meal number has also been seen with IAPP infusion (Arnelo *et al.* 1996b).

In general satiety factors are known to control amount of food consumed by affecting meal size, meal number, or both (Brobeck JR. 1955, Smith JC. 2000). A meal is traditionally defined as a cluster of smaller feeding bouts that are separated from other feeding clusters by an inter-meal interval where feeding is absent. IAPP is suggested foremost to reduce eating via meal size caused by meal termination due to satiety (Lutz TA. 2012). Boyle *et al.* showed that the parameter that affected the amount of food ingested was meal size rather than meal number (Boyle *et al*. 2012). We have previously shown that ICV infusion of BVT.3531, which is an analogue of 3-guandino-propionic acid, also reduced food intake by decreasing meal size (Mirshamsi *et al*. 2007).

In Paper III, the average meal number was slightly higher in the KO mice than in the wild-type mice in the long-term experiment, but the difference did not reach statistical significance. In the short-term IAPP-infusion experiment in the KO mice,

food intake was significantly lower in the KO mice than in the wild-type animals, but neither meal number ($p = 0.17$) nor meal size ($p = 0.07$) was significantly different between the groups. It is possible that the difference in meal size might have reached significance if the group size had been larger.

5.3.1.4 Transient inhibition of food intake

The inhibition of feeding by IAPP in Papers I, II, III, and IV was most marked at the beginning of the infusion and was attenuated later in the experiments. This is in line with previous IAPP studies with chronic administration of the peptide (Arnelo *et al*. 1996b, Arnelo *et al*. 2000, Lutz *et al*. 2001, Roth *et al.* 2006, Weilinga *et al.* 2010). Similarly, transient inhibition of food intake has been seen for continuous infusion of other anorexigenic substances, including PYY(3-36) (Chelikani *et al*. 2006), glucagonlike peptide-1 (Donahey *et al*. 1998), cholecystokinin (Lukaszewski and Praissman 1988) and leptin (Pal and Sahu 2003, Sahu A. 2002). Early substance-induced reductions in daily food intake and adiposity may elicit a delayed compensatory response in order to restore energy balance, perhaps mediated by a reduction in leptin signaling to the brain (Friedman JM. 2002). Another possibility is that continuous or frequent administration of the substances causes desensitization and down-regulation of G-protein coupled receptors. In contrast to the effect of continuous administration of satiety hormones, a sustained reduction of daily food intake in rats was seen was seen for 10 days with intermittent administration of PYY(3-36) (Chelikani *et al*. 2006). In experiments using intermittent intraperitoneal infusion of salmon calcitonin (a homolog of IAPP), none of the ten dosing regimens produced a sustained 25-35% reduction in daily food intake for more than 5 days, although there were moderate decreases in body weight and adiposity across the 7-week study period (Chelikani *et al*. 2007). This lack of sustained reduction in food intake may indicate that the IAPP and similar satiety factors causes desensitization and down-regulation of G-protein coupled receptors.

5.3.2 Pathways in the periphery and brain

The minimal effective dose during SC infusion (2.5 pmol/kg-min) in Paper II was 10-fold higher than that for ICV administration (0.25 pmol/kg-min). These doses are similar to doses used in earlier separate SC studies (Arnelo *et al*. 1996b, Arnelo *et al.* 1998, Reidelberger *et al*. 2001) and ICV experiments (Rushing PA. 2000a). The

differences in potency seen with different routes of administration indicate that the inhibition of food intake by IAPP is due primarily by effecting the brain. That hypothesis is supported by experiments showing that neither subdiaphragmatic vagotomy and nor capsaicin denervation of peripheral sensory nerves attenuates anorexic responses to IAPP (Lutz *et al*. 1994, Lutz *et al*. 1995a, Lutz *et al*. 1998b). The difference between the effects of IAPP on meal pattern with the two methods of administration suggests that IAPP does not act on the same target or targets when administered centrally as it does when it is administered peripherally. This is consistent with data from studies showing that experimental lesions in the area postrema/nucleus of the solitary tract of the hindbrain attenuated the anorexic response to IP, but not ICV, injection of IAPP (Lutz *et al*. 1995a, Lutz *et al*. 1998a, Lutz *et al*. 2001).

IAPP may be acting, at least in part, through modification of neuropeptides in the brain. A significant increase in POMC mRNA in the ARC of diet-induced obesity prone rats has been reported after 22 days of subcutaneous infusion of IAPP (Roth *et al.* 2006). However, in Paper IV, POMC mRNA in the ARC was significantly decreased in both the IAPP and pair-fed group compared to the controls after 5 days of ICV IAPP infusion. In contrast, a study by Barth *et al.* showing no change in POMC mRNA in the ARC 60 or 120 minutes after intraperitoneal administration of IAPP (50 µg/kg) (Barth *et al*. 2003).

In Paper IV, no significant differences in AgRP mRNA were seen in either the IAPP rats or their pair-fed controls. This is consistent with the study by Barth *et al.* in which no changes in AgRP mRNA were seen after IP injection of IAPP (Barth *et al*. 2003). However, Sucajtys-Szulc *et al*. saw an increase in AgRP mRNA after 30 days of food restriction in rats (Sucajtys-Szulc *et al.* 2010).

No significant differences in NPY mRNA were seen in the IAPP group or the pair-fed rats in Paper IV, a finding that is consistent with studies in rats showing no change in NPY mRNA in the ARC after IP injection of IAPP (Barth *et al*. 2003) and no change in NPY content in the ARC after central infusion of IAPP into the lateral ventricle (0.5 nmol/day for 6 days) (Morris and Nguyen 2001). However, Roth *et al*. reported that SC infusion of IAPP increased NPY mRNA in the ARC of diet-induced obesity prone rats and their pair-fed controls (Roth *et al*. 2006). An increase in NPY content has been seen in the hypothalamus after 5 days of subcutaneous infusion of IAPP (25 pmol) (Arnelo *et al*. 2000).

Differences in experimental design *(e.g.* dose of IAPP, method of administration, species and strain of animals used, diets, and feeding schedules) may be the reason for

the inconsistent data about the effect of IAPP on POMC, AgRP, and NPY mRNA expression in the ARC. If that is the case, the variability in results would reflect the complexity of the system regulating food intake and body weight.

6 CONCLUSIONS

The experiments in Papers I-IV provide the following information about the effects of IAPP and the mechanisms mediating its actions:

1. IAPP consistently reduced food intake in a dose-dependent manner. Subcutaneously-infused IAPP and intracerebroventricularly-infused IAPP differed in their effects on meal pattern. ICV IAPP had a 10-fold lower minimal effective dose than SC IAPP. This suggests that SC- and ICV-administered IAPP act through different targets and that IAPP inhibits food intake by acting primarily in the brain.

2. Chronic infusion of IAPP led to changed lipid metabolism that was characterized by hypolipidemia, hypoleptinemia, and decreased adiposity. Glucose and protein homeostasis were largely unchanged.

3. No changes in mRNA for neuropeptide Y or agouti-related protein were seen in the arcuate nucleus of the hypothalamus. There was a change in mRNA propiomelanocortin (POMC). This suggests that the anorexic effects of IAPP may be mediated through POMC at the time point studied.

4. Food intake and meal pattern were not changed in IAPP knockout mice compared to wild-type controls. This does not rule out that IAPP´s necessary for normal satiety to occur but rather suggests redundancy in the control of food intake.

5. Inhibition of food intake in response to IAPP infusion was more marked in IAPP knockout mice than in wild-type mice. This suggests that IAPP receptors and/or IAPP post-receptor signaling pathways are up-regulated in mice lacking endogenous IAPP.

7 FUTURE DIRECTIONS

Further studies of the mechanisms through which IAPP exerts its anorexic effects may provide information that could be used to counteract the world-wide increase in obesity and its associated health problems. In Papers I-IV, subcutaneous and ICV infusions of IAPP produced transient decreases of food intake. Investigations of the mechanisms responsible for the attenuation in anorexic effects would be valuable in the search for medication that has a long-term effect on food intake.

- In Paper II, SC and ICV infusion of IAPP had different effects on food intake during the light and dark periods. Studies of circadian rhythms and IAPP could help clarify the role of IAPP in the control of food intake.

- Studies to determine whether intermittent infusion of IAPP in combination with other anorexigenic substances could produce a sustained reduction in food intake and body weight would provide data that could be useful in evaluating the potential of IAPP as an anti-obesity medication.

- Because the mechanisms of interaction between IAPP, insulin, and leptin are not well understood, studies of the effects of insulin and leptin in IAPP knock-out mice could provide insights into the pathways involved. Development of knockout mice that lack functional genes for both IAPP and leptin would provide a valuable model for investigating the interdependence of the two peptides.

- Experiments looking at the effects IAPP on UPC2 expression in the pancreas would also be a promising line of investigation. Studies in ob/ob mice have shown that UPC2 decreases insulin secretion (Zhang *et al*. 2001), so it is possible that UPC2 may mediate IAPP's inhibition of insulin secretion. Investigation of the effects of IAPP on the expression of uncoupling proteins other than UPC2 could also provide valuable data.

- It would be useful to investigate how reduction in food intake by IAPP and by semi-starvation (pair-feeding regimen) would affect c-fos expression in the brain. In addition it would be valuable to investigate the effect of different doses and at different time points. Studies linking c-fos expression with measurement of mRNA levels in the brain would help to better understand the role of IAPPs control of food intake.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING

För att människor och djur skall överleva är det viktigt att det finns en balans mellan hur mycket energi kroppen förbrukar och hur mycket den tar upp. Styrningen av denna process är mycket komplicerad och inbegriper flera olika organ i kroppen där hjärnan har en överordnad roll. Aptit, hunger och mättnad, vad som styr hur vi äter har i detta sammanhang stor betydelse. IAPP (eller Amylin) är ett litet protein som bland annat tillverkas i bukspottskörtelns s.k. β-celler, där även insulin produceras.

IAPP släpps ut till blodbanan tillsammans med insulin som ett svar på en måltid. Tidigare djurförsök på råtta och mus har visat att djuret äter mindre och går ner i vikt om det behandlas med IAPP. Forskning har även visat att IAPP inte ger illamående eller obehag vilket ha givit upphov till teorier om att IAPP är ett s k mättnadshormon. Om så är fallet och var och hur IAPP orsakar sin mättande effekt är frågor som detta avhandlingsarbete sökt svar på.

I alla våra arbeten har vi sett att tillförsel av IAPP minskar födointaget. I ett av arbetena på råtta undersöktes vi effekten av en veckas IAPP-tillförsel på kroppens energibalans. Råttorna hade under hela tiden fri tillgång till mat och jämfördes med två likvärdiga grupper av djur som inte fick IAPP. Den ena gruppen hade fri tillgång till mat och den andra gruppen tilldelades lika mycket mat som IAPP-djuren ätit under dygnet innan, med andra ord kan man säga att de djur som fått IAPP-infusion bestämde hur mycket mat de parfödda djuren skulle få i sina matskålar. IAPP tillförsel medförde att en viss typ av fettväv i bukhålan minskade och att halterna av blodfetterna triglycerider och fria fettsyror sjönk. Dessutom sjönk halterna av hormonerna leptin och insulin i blodbanan men ämnesomsättningen med avseende på kolhydrater och äggviteämnen var i stort opåverkade.

I ett annat djurförsök, också på råtta, jämfördes vi om olika sätt att tillföra IAPP gjorde skillnad. Vi undersökte också hur dos och sätt att tillföra IAPP påverkade intaget av föda. En grupp råttor fick IAPP i en liten pump som opererats in i underhuden och den andra gruppen fick IAPP via en tunn slang direkt ner i hjärnans hålrum, ventrikel. Födointag, måltidsmönster och kroppsvikt studerades och jämfördes. Vi fann att det var 10 gånger mer effektivt att ge IAPP direkt i hjärnans hålrum, jämfört med att ge det i

underhudsfettet. Måltidsmönstret d v s hur djuren åt varierade mellan de två grupperna. Dessa fynd talar för att IAPP huvudsakligen påverkar hjärnan för att utöva sin effekt. Det varierande måltidsmönstret i vår försöksmodell talar för att IAPP utövat sin effekt på olika ställen i hjärnan.

I ett tredje arbete, studerades tre grupper av råttor. Råttor med fri tillgång till mat, med IAPP-behandling till hjärnan (grupp ett) respektive utan IAPP-behandling (grupp två) samt en grupp med parutfodrade djur, (grupp tre). I den tredje gruppen där djuren inte fick IAPP-behandling tilldelades djuren lika mycket mat som de IAPP-behandlade djuren åt dygnet innan, på samma sätt som beskrivits tidigare i texten. Vi undersökte sedan hur gener som förmedlar signaler i nervsystemet påverkas. Vi fann att generna för ett litet protein som förkortas POMC (proopiomelanocortin) ”tystas ner”, vilket brukar leda till att en mindre mängd POMC tillverkas, hos de råttor som fått IAPP men även de råttor som fått lika mycket mat som IAPP råttorna. Detta talar för att POMC kan vara inblandat i hur födointag regleras.

Födointag, måltidsmönster och kroppsviktsutveckling undersöktes i ett 27 veckor långt försök med vuxna möss. Så kallade IAPP knockout-möss, där genen som styr tillverkningen av IAPP saknas och dessa möss därmed har total avsaknad av kroppseget IAPP. IAPP knockout mössen studerades och jämfördes med vanliga möss (med kroppsegen IAPP-tillverkning). Efter den långa delen av försöket, utfördes ett kortare försök under 3 dagar, där födointag, måltidsmönster och kroppsvikt studerades under samtidig IAPP-tillförsel. I det längre musförsöket kunde ingen skillnad på födointag och kroppsvikt noteras vid jämförelse mellan IAPP knockout-möss och kontrollmöss. IAPP-tillförsel till mössen minskade matintaget både hos vanliga och IAPP knockout-möss. Ett ytterligare minskat intag sågs hos IAPP knockout-möss jämfört med de vanliga mössen. Resultaten kan tolkas som om att IAPP knockout-möss är mer känsliga för IAPPs anorektiska effekt då de inte har någon egen produktion av denna peptid och reagerar mer på den än de möss som har eget IAPP i kroppen.

Sammantaget styrker data från dessa studier hypotesen att IAPP är ett mättnadshormon. Den tiofaldiga skillnaden i lägsta effektiva dos mellan perifert och till hjärnan tillfört IAPP talar för att IAPP framförallt utövar sin effekt i hjärnan. Våra försök visar på att IAPP bidrar till minskad mängd POMC i hjärnan och kan därmed vara en del i hur IAPP verkar. Avsaknad av påverkan på födointag och kroppsvikt hos IAPP knockout-

möss innebär inte att IAPP inte är nödvändigt för att känna normal mättnad utan tyder snarast på att födointaget kan kontrolleras på annat sätt med hjälp av andra mättnads-faktorer. Detta har setts vid studier av andra viktiga mättnadsfaktorer och belyser hur vist naturen har anordnat viktiga parallella system för att bevara arternas överlevnad. Man kan likna hela systemet med mättnadsfaktorer med morgonrusningen i trafiken. Blockerar man en korsning så att bilarna inte kan ta sig igenom så hittar trafiken andra vägar att ta sig fram på.

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