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Oxytocin as an appetite suppressant that reduces feeding reward

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Abstract

In the environment in which palatable and highly caloric foods are readily available, eating behavior is oftentimes not dictated by the necessity to replenish lacking energy, but rather by the pleasure of consumption. Centrally acting oxytocin (OT) is known to promote termination of feeding to protect internal milieu by preventing excessive stomach distension, hyperosmolality and ingestion of toxins. Initial evidence suggests that another possible role for OT in mechanisms governing food intake is to reduce consumption of select palatable tastants. This thesis explores the question whether OT is as an appetite suppressant that reduces feeding reward.

The first set of experiments addresses whether OT affects intake of (a) all carbohydrates, (b) only sweet carbohydrates or (c) sweet non-carbohydrate saccharin in mice. In those studies, generalized injection of a blood brain barrier penetrant OT receptor antagonist, L-368,899, significantly increased the intake of sweet (sucrose, glucose, fructose, polycose) and non-sweet (cornstarch) carbohydrates and promoted a trend approaching significance in saccharin consumption. Consumption of carbohydrate-enriched foods led to an increase in OT mRNA levels in the hypothalamus.

The second set of studies identifies the nucleus accumbens core (AcbC), a key component of the reward system, as a site that mediates anorexigenic effects of OT. Rats injected with OT directly in the AcbC showed a decreased intake of

sucrose and saccharin solutions as well as of standard chow. This treatment did not cause taste aversion, hence the outcome was not due to sickness/malaise. The effects of AcbC OT on feeding could be observed only in animals offered a meal in a non-social environment. Once a social setting (devoid of direct antagonistic interactions between individuals) of a meal was introduced, AcbC OT failed to reduce feeding. AcbC levels of OT receptor transcript were affected by exposure to palatable food as well as by food deprivation.

The third and final set of studies shows that aberrant integrity of neuronal circuitry within the neuroendocrine and reward systems due to genetic deletion of connexin 36 (Cx36) gap junctions leads to dysregulation of the OT system's functioning in the Cx36 KO mouse. This dysregulation is associated with hypersensitivity to aversive properties of foods, reduced interest in feeding for reward (palatable carbohydrates and saccharin) and abnormal ingestion of energy.

Overall, the findings suggest that OT diminishes feeding for reward, particularly the intake of palatable carbohydrates and saccharin, by acting – at least in part – via the reward system. OT appears to be part of central mechanisms that cross-link homeostasis-driven and palatability-related (i.e., flavor- and macronutrient-specific) termination of consumption.

A Jean-jacques

“On n’existe pas sans faire” De Beauvoir

List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Herisson FM**, Brooks LL, Waas JR, Levine AS, Olszewski PK. **Functional relationship between oxytocin and appetite for carbohydrates versus saccharin**. *Neuroreport. 2014 Aug 20;25(12):909-14. (published)*

II. **Herisson FM,** Waas JR , Fredriksson R, Schiöth HB, Levine AS, Olszewski PK**. Oxytocin acting in the nucleus accumbens core decreases food intake**. *Journal of Neuroendocrinology, 2016, 28, 10.1111/jne.12381* (*published*)

III. **Herisson FM**, Bird S, Sleigh JW, Levine AS, Olszewski PK. **Connexin 36 deletion diminishes feeding for reward.** (submitted)

List of papers not included in this thesis

- Eriksson A, Williams MJ, Voisin S, Hansson I, Krishnan A, Philippot G, Yamskova O, **Herisson FM**, Dnyansagar R, Moschonis G, Manios Y, Chrousos GP, Olszewski PK, Frediksson R, Schiöth HB. **Implication of coronin 7 in body weight regulation in humans, mice and flies.** *BMC Neurosci. 2015 Mar 14;16:13*

- Olszewski PK, Waas JR, Brooks LL, **Herisson FM**, Levine AS. **Oxytocin receptor blockade reduces acquisition but not retrieval of taste aversion and blunts responsiveness of amygdala neurons to an aversive stimulus.** *Peptides. 2013 Dec;50:36-41.*

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Abbreviations

Acb	Nucleus accumbens
AcbC	Nucleus accumbens core
AcbSh	Nucleus accumbens shell
AgRP	Agouti-related protein
AMY	Amygdala
AP	Area postrema
ARC	Arcuate nucleus
AV3V	Anteroventral third ventricle
BBB	Blood-brain barrier
BNST	Bed nucleus of the stria terminalis
b. wt	Body weight
CeA	Central nucleus of the amygdala
CCK	Cholecystokinin
CNS	Central nervous system
CTA	Conditioned taste aversion
Cx	Connexin
DAG	1,2-diacylglycerol
DMH	Dorsomedial hypothalamic nucleus
DMNV	Dorsal motor nucleus of the vagus
DVC	Dorsal vagal complex
Gαβ	G protein, α and β subunits
GABA	γ-aminobutyric acid
GDP	Guanosine diphosphate
GI	Gastrointestinal
GLP-1	Glucagon-like peptide-1
GPCR	G protein-coupled receptors
GTP	Guanosine triphosphate
ICV	Injection into the cerebral ventricle
IP	Intraperitoneal
IP3	Inositol triphosphate
KO	Knockout
LHA	Lateral hypothalamus
LiCl	Lithium chloride
MPOA	Medial preoptic area
mRNA	messenger RNA (ribonucleic acid)
NaCl	Sodium chloride/saline
NTS	Nucleus tractus solitarii/nucleus of the solitary tract
OB	Olfactory bulb
ORA	Oxytocin receptor antagonist
OT	Oxytocin
OTr	Oxytocin receptor
PBN	Parabrachial nucleus
PFA	Paraformaldehyde

PFC	Prefrontal cortex
PIP2	phosphatidyl-inositol-4-5-diphosphate
PKC	Protein kinase C
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus of the hypothalamus
SC	Subcutaneous
SON	Supraoptic nucleus
TBS	Tris-buffered saline
VMH	Ventromedial hypothalamic nucleus
VP	Vasopressin
VTA	Ventral tegmental area
WT	Wild type

Chapter 1

Introduction and Aims

Obesity is a public health issue worldwide and overeating greatly contributes to its prevalence. It is therefore essential to understand the mechanisms that regulate eating behavior and to identify new therapeutic targets. While the disruption of the "hunger-satiety" signaling oftentimes leads to excessive body weight [1], it is apparent that laboratory animals and humans oftentimes eat for reasons other than hunger. In the obesogenic environment, the usual reason to initiate and maintain food intake is a pleasant taste of food, i.e., feeding reward. When feeding reward comes into play, satiety is delayed. Therefore, the current research effort to limit excessive food intake underlying obesity is directed towards identifying mechanisms and molecules that promote early satiety in the context of eating for pleasure. Initial evidence suggests that oxytocin (OT) may act as a feeding inhibitor that cross-links satiety and reward. The exact nature of the functional relationship between OT and the regulation of palatability-driven appetite remains unclear. Thus, in this thesis I explore whether OT is as an appetite suppressant that reduces feeding reward.

1.1. Oxytocin and receptors

1.1.1 Oxytocin

The neuropeptide hormone oxytocin is a nonapeptide (Cys–Tyr–Ile–Gln–Asn–Cys–Pro–Leu–$GlyNH_2$) (Figure. 1.1) with a sulfur bridge between the two cysteines. OT was extracted from the mammalian pituitary in 1950, and it became the first sequenced and structurally-defined neuropeptide [2]. This neuropeptide is evolutionarily conserved across phyla and has been detected in most vertebrate and several invertebrate species [3-6]. Some variability of the OT amino acid sequences has been shown in vertebrates, for example, in cartilaginous fishes (isotocin [S4,I8]-oxytocin) and in birds [7], reptiles and amphibians (mesotocin [I8]-oxytocin) [8-10]. There is also some variability in the OT-like molecular structure in invertebrates. For example, inotocin has been identified in ants [11], whereas some insects, such as Drosophila melanogaster, lack the OT molecule whatsoever [5]. Acher et al. date the precursor of OT gene at 500 million years [12]. The fact that OT is highly conserved among the plethora of phyla and species strongly indicates that it plays a crucial role relevant to the basic functioning of the organism. The proposed function of OT in the control of energy intake and nutrient preference/selectivity further explored in this thesis, fits in the notion of the critical place of OT in regulatory mechanisms that are most essential in the survival of the organism: energy and nutrient homeostasis ensure the proper biochemical environment for cellular and system physiological responses to occur. Though it is not the topic of this thesis, it should be mentioned that OT's role in such elementary functions is not limited to food

intake, but it also includes e.g. reproduction and associative learning (from Caenorhabditis elegans to Eisenia foetida to humans [12-15]).

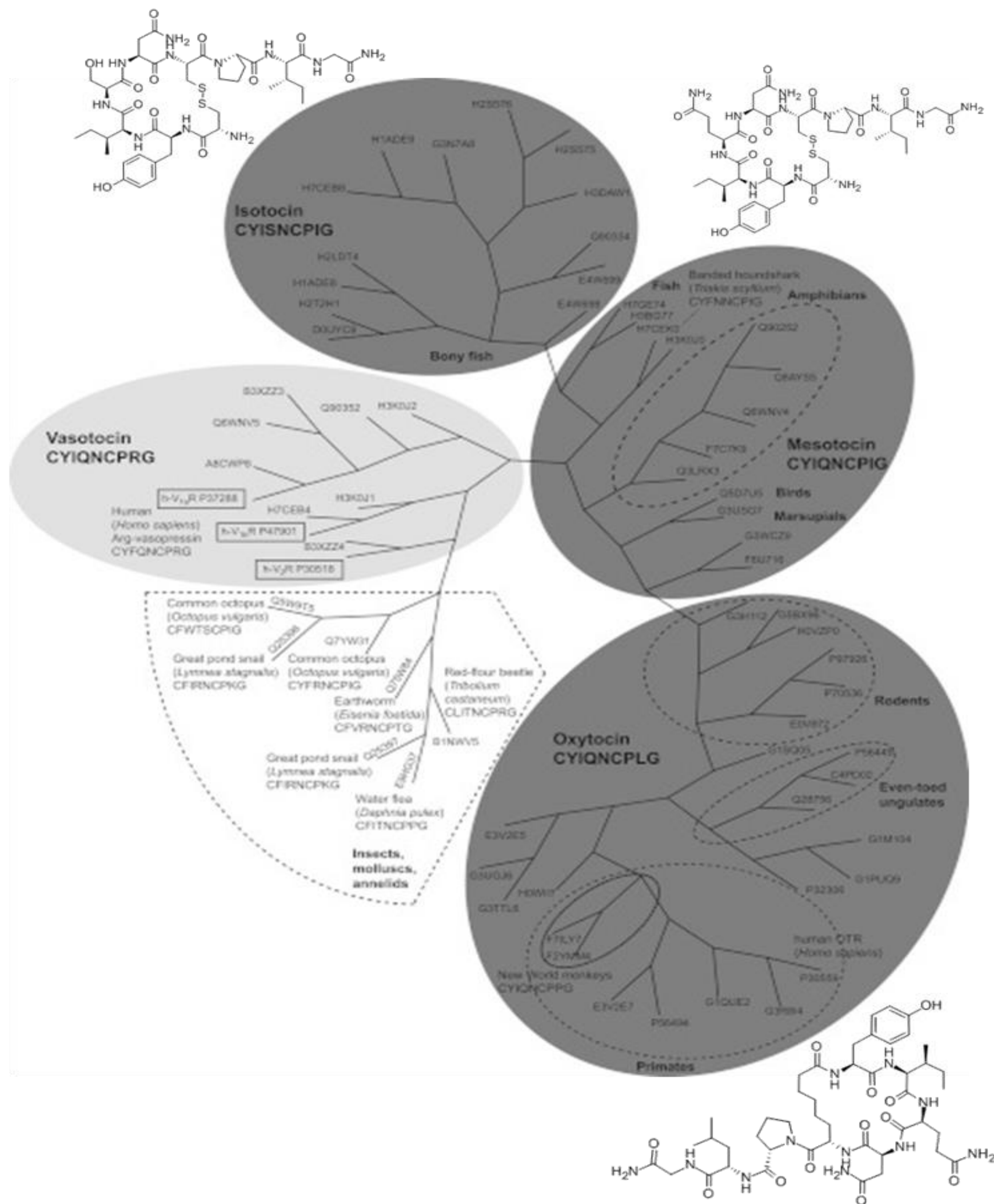

Figure 1.1: A phylogenetic tree including the structure of OT and OT-related peptides and their receptors. Figure adapted from Koehbach J et al 2013 [16].

The OT gene is localized in humans in chromosomal loci 20p13 [17]. After being transcribed, translated and spliced, an inactive gene product precursor protein that contains the signal peptide (at the N-terminus), OT, and neurophysin (at the

C-terminus), is formed. The full structure of the OT precursor protein is shown in Figure 1.2. The OT prepropeptide is package into the granules of the Golgi apparatus. The prepropeptide undergoes cleavage and modifications during the post-Golgi transport phase to axon terminals [18-20].

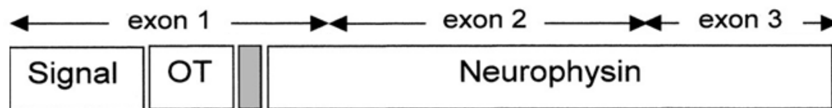

Figure 1.2: Domain organization of preprooxytocin. Figure adapted from from Gimpl G et al. 2001 [20]

In the central nervous system (CNS), OT is synthesized mainly in the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus as well as in hypothalamic magnocellular accessory neurons located between the PVN and SON [21]. Both the PVN and SON contain OT magnocellular populations, whereas the PVN hosts also parvocellular OT neurons. Magnocellular neurons release OT primarily to the general circulation via the posterior pituitary. On the other hand, parvocellular OT neurons send their projections not only to the neurohypophysis, but also to a variety of central sites. Importantly, many of the brain areas targeted by the OT system are involved in the regulation of food intake. Those sites include, for example, the components of the reward system (the nucleus accumbens and ventral tegmental area) as well as the energy balance-related CNS regions (for example, the nucleus of the solitary tract and the dorsal motor nucleus of the vagus) (Figure.1.3) [22, 23]. Therefore, CNS-derived OT controls feeding as well as other physiological and behavioral outcomes via its receptors scattered throughout the brain as well as in peripheral organs [19, 24-27].

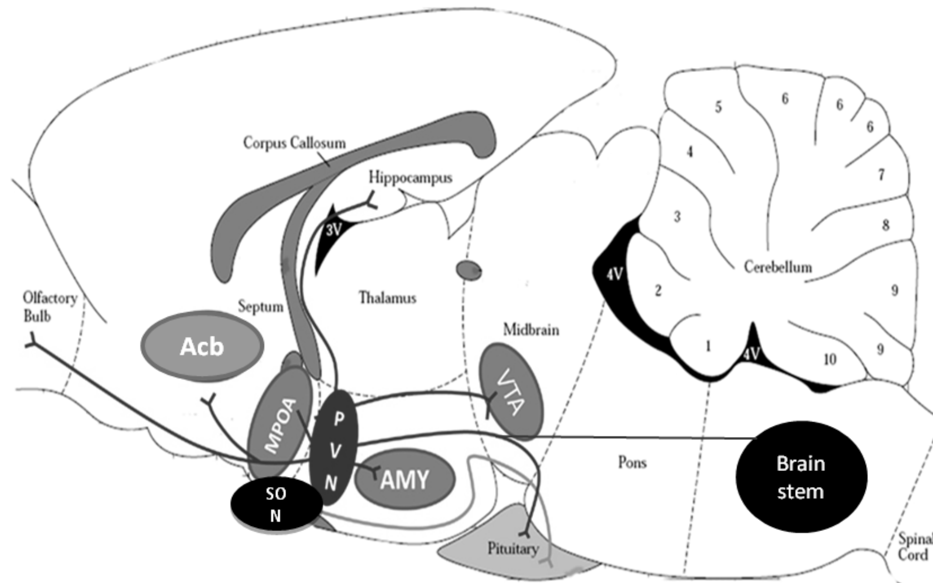

Figure 1.3: Parvocellular neurons of the PVN project to a variety of brain areas where OTr is expressed. Magnocellular neurons of the PVN (red) and SON (black) project to the posterior pituitary to release OT to the general circulation. It should be noted that the distribution of the OTr is very vast and it incorporates sites that govern a wide number of processes, such as reward (VTA and Acb), stress (AMY), learning and memory (hippocampus), maternal behavior (MPOA/BNST and OB) and periphery-CNS relay mechanisms (brain stem). Figure adapted from Rutherford HJ et al. 2011 [28]. (PVN: Paraventricular nucleus of the hypothalamus, SON: Supraoptic nucleus, VTA: Ventral tegmental area, Acb : Nucleus accumbens, AMY: Amygdala, MPOA: Medial Preoptic Area, BNST: Bed nucleus of the stria terminalis OB: Olfactory bulb).

1.1.2 Oxytocin receptor

The oxytocin receptor (OTr) gene, containing three introns and four exons, is localized in humans on chromosomal loci 3p25 and 3p26.2 [29, 30], and its transcription is controlled by several transcription factors, including inflammatory and immune mediators, cAMP, estrogen and steroids [31-33].

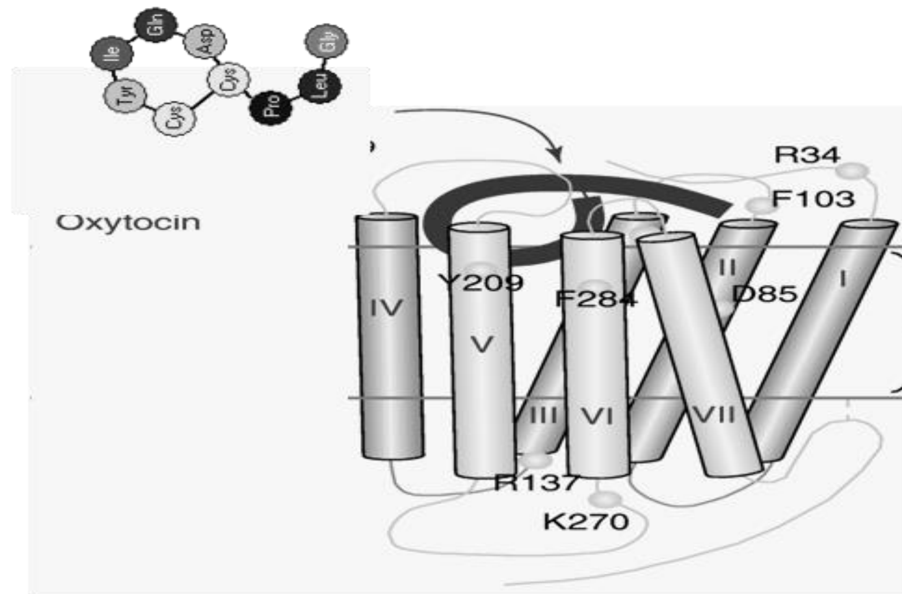

Figure 1.4: Schematic representation of the OTr structure with its seven transmembrane domains and the interaction site with the OT molecule. Figure adapted from Zingg HH et al. 2003 [34].

The OTr belongs to the superfamily of the rhodopsin-type (class I) G protein-coupled receptors (GPCR) having seven transmembrane domains and the α helix and it is highly conserved in mammalian genomes Figure 1.4. In Class I GPCR family, the amino acid aspartic acid in domain 2 is important in the process of receptor activation. [35]. The native OT molecule shows the superior affinity for the OTr. It should be mentioned, however, that also the endogenous vasopressin (VP) can bind the OTr, but the affinity for of the OTr for OT is 10 times higher than for VP, and the concentration of VP necessary to bind the OTr - 100 times higher than OT [36, 37].

The OTr binding site for its ligand is formed by extracellular loops 2 and 4 and part of the transmembrane domains 2 and 4 (Figure 1.4). In fact, the binding of an agonist peptide to transmembrane domains of the OTr on the cell surface changes receptor’s conformation and activates the hydrolysis of GDP to GTP in

the G protein, separating it into the α and β subunits. This separation followed by hydrolysis by phosphorylase C-β of phosphatidyl-inositol-4-5-diphosphate (PIP2) into inositol triphosphate (IP3) and 1,2-diacylglycerol (DAG). DAG allows activation of protein kinase C and IP3, release of Ca^{2+} and initiation of cellular responses [38-41], such as cell excitability processes, transmitter release and/or synthesis of proteins [42] (Figure 1.5).

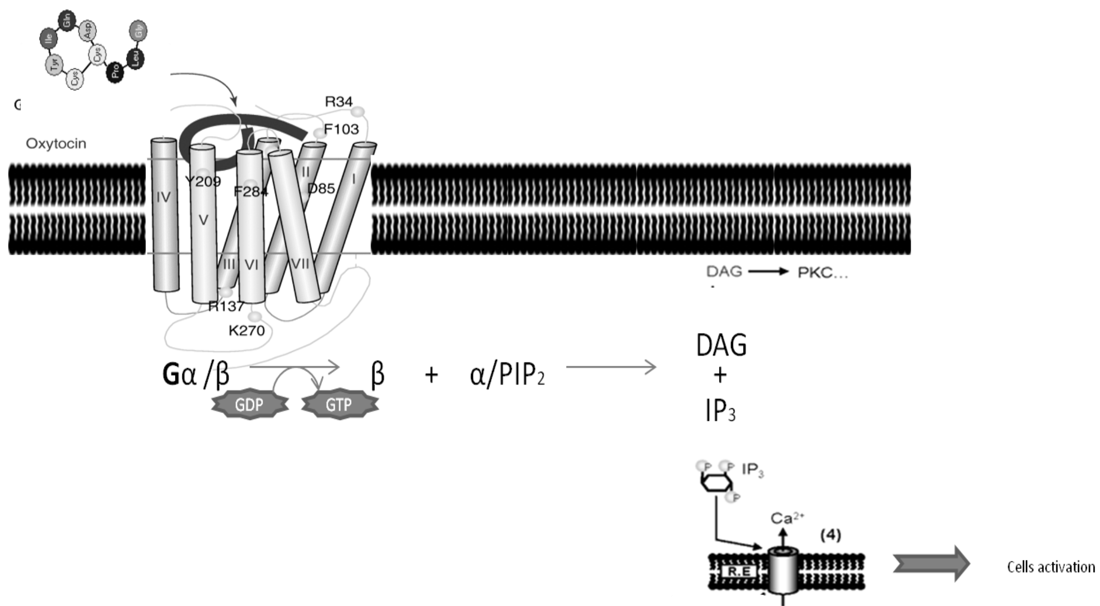

Figure 1.5: Activation of OTr signaling pathway by OT. G protein, α and β subunits (Gαβ) C-β of phosphatidyl-inositol-4-5-diphosphate (PIP2), inositol triphosphate (IP3) and 1,2-diacylglycerol (DAG), Guanosine-5'-triphosphate (GTP), guanosine diphosphate (GDP), Protein kinase C (PKC).

The endogenous OT molecule has a relatively short half-life and a very limited ability to cross the blood-brain barrier (BBB) [43]. These two issues are considered to be significant obstacles in using OT in various pharmacological application scenarios in which the native ligand is to act the CNS receptors. Therefore, substantial research effort has been directed towards developing synthetic ligands of the OTr. The past two decades of studies have produced the

development of OTr agonists and antagonists characterized by improved BBB permeability parameters and/or longer half-life (an overview of currently available ligands is presented in Table 1.1).

Table 1.1: Oxytocin receptor ligands

Agonist		Antagonist	
Peptide	Non-peptide	Peptide	Non-peptide
Carbetocin	WAY-267,464	Atosiban	L-368,899
Demoxytocin		Barusiban	L-371,257
Lipo-oxytocin-1			L-372,662
Merotocin			SSR-126,768
Oxytocin			Epelsiban
			Retosiban
			WAY-162,720

L-368,899 is one of the most frequently used OTr antagonists in research [44,45] (Figure 1.6). L-368,899 is a unique ligand that rapidly penetrates into the brain due to its capacity to cross the BBB [45,46] with accumulation in areas of the limbic system [46]. It has been employed in studies on, among others, central regulation of food intake or sexual activity [47,48].

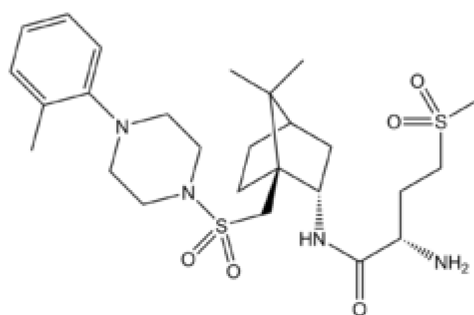

Figure 1 6: Chemical structure of [^{14}C]L-368,899

Approximately 80% of OT receptors are present in the CNS [49,50]. The distribution of this receptor mirrors the involvement of OT in numerous

physiological and behavioral processes, from food intake to stress to maternal behavior (see Section 1.2 for details on key functions of OT). OTr has been found in specific sites involved in the regulation of food intake, such as the nucleus accumbens (Acb), the ventral tegmental area (VTA), the hypothalamus and the brainstem [51-54]. The OTr mRNA has been also detected in the anterior olfactory nucleus, ventral pallidum, limbic system (bed nucleus of the stria terminalis, central amygdaloid nucleus, ventral subiculum), and hypothalamus [50, 55][56], with no major differences in distribution between males and females [49] (Figure 1.3; Table 1.2).

Table 1.2: The distribution of the OTr in the rats CNS. **A**dapted from Gimpl G et al. [20].

Limbic system	Hypothalamus	Brainstem
Lateral septal nucleus	Ventromedial hypothalamic nucleusb	Substantia nigra pars compacta
Bed nucleus of stria terminalis	Anterior medial preoptic area	Ventral and dorsal tegmental area
Amygdaloid-hippocampal area	Supraoptic nucleus	Central gray
Central amygdaloid nucleus	Paraventricular nucleus	Dorsal raphe nucleus
Medial amygdaloid nucleus	Posterior hypothalamic area	Reticular nuclei
Basolateral amygdaloid nucleus	Supramammillary nucleus	Medial vestibular nucleus
Dorsal subiculum	Lateral mammillary nucleus	Hypoglossus nucleus
Ventral subiculum	Medial mammillary nucleus	Dorsal motor nucleus of the vagus nerve
Nucleus accumbens		Inferior olive nucleus
		Substantia gelatinosa of trigeminal nucleus

1.2. Main behavioral and physiological effects of oxytocin

The vast peripheral and central distribution of the OTr underlies multiple functions played by OT, including the regulation of maternal, sexual, aggressive, affiliative, grooming and feeding behavior and physiology [57].

OT's roles in parturition and in the milk ejection reflex have been most avidly studied. OT induces contractions of the smooth muscle of the uterus leading to parturition. Expression of OTr mRNA and density of this receptor on the surface of the myometrium increase in the presence of estradiol [58][59,60], which precedes the active phase of the birth process. Following parturition, OT levels remain high and OT starts playing a crucial function in facilitating milk ejection [61]. Stimulation of tactile receptors by suckling, generates impulses transmitted from the nipple to the spinal cord and then to OT neurons in the hypothalamus [57,62].

Not only does OT ensure the proper physiological responses during and after parturition, but it also facilitates the development of the appropriate repertoire of a mother's social responses to the young [38-40,63,64]. It is particularly important in initiating maternal behavior [41]. Noteworthy, human studies show also the link between OT and both maternal and paternal responses to a newborn [65]. Furthermore, epigenetics (genomic imprinting) may control this hormonal and behavioral responsiveness to the offspring via the paternal and maternal genome [66,67].

The social bonding effects of OT extend well beyond parent-child relations. In humans, OT mediates the benefits of positive social interaction and emotions

stemming from non-family bonds. OT is released in the brain and into the periphery by stimuli such as touch, warmth, etc. Consequently, OT may facilitate physiological and behavioral effects induced by social interaction [68]. Administration of OT has been reported to increase feelings of affiliation to one's parents, decrease stress and anxiety in both men and women and promote effects such as the reduction in blood pressure and cortisol levels [69-71]. Intranasal administration of OT causes increased subjective feelings of attachment, generosity, sensitivity to emotion and maintenance of trust [69,72-74]. Indeed, in game-scenario experiments, intranasal OT prevented the loss of trust in an opponent even after the trust has been breached by this opponent before [75]. OT also improves the ability to infer the mental state of others from physical cues (facial expressions) [76,77]. Recently, the beneficial clinical effects of OT treatment on positive social interactions in autism (disorder characterized by impaired social interactions and communication) have been reported [78]. In laboratory animals, central administration of OT agonists enhances social recognition, memory for peers, the development of partner preference, and partner bonding [79]. Importantly, centrally administered OT promotes termination of food intake (Sections 1.3 and 1.4 describe the findings in detail) [80]. Experiments in rodents have shown that OT inhibits food intake through receptors localized throughout the brain. Indeed, central OT administration reduces food intake and prolongs latency to eat [81]. In addition, anorexia-inducing agents such as lithium chloride (LiCl) stimulate OT secretion into the periphery and in the CNS leading to the termination of food intake [82,83]. OT knockout (KO) mice show increased preference for palatable (sweet) carbohydrates [47].

1.3 Mechanisms controlling food intake: focus on the role of oxytocin

Proper energy balance is critical for survival of the organism. Maintenance of energy homeostasis depends on a variety of mechanisms, including those that regulate food consumption. Importantly, food intake should not be viewed in a simplistic manner as a mere means of acquiring calories, but rather a set of complex physiological and (consequently) behavioral processes. They allow the animal to obtain energy and diverse nutrients, without jeopardizing internal milieu (e.g., by inadvertently introducing toxins or an excessive osmotic load). Therefore, the complexity of appetite regulating mechanisms stems from the ever-present need to have consummatory behavior adapt to constant changes in the internal (such as neurohormonal, osmotic, chemoreceptor-, mechanoreceptor- and nutrient-derived signals) and external environment (light-dark cycle; social context, etc.). While this aspect of food intake control – for the purpose of this thesis dubbed "homeostatic" – is a key component affecting consumption, one should not forget that appetite is shaped by another critical factor, i.e., feeding reward. [84]. In fact, mechanisms promoting ingestive behavior driven by pleasant taste have been suggested to play such a significant role in modifying one's consumption profile that, under many circumstances, they may outweigh homeostatic controls of feeding [85].

Noteworthy, OT has been speculated to affect both homeostatic and reward-related ingestive behavior. The vast majority of evidence points to the brainstem-hypothalamus OT reciprocal pathways as mediators of homeostatic

aspects of food intake regulation, including responsiveness to changes in plasma osmolality, excessive distension of the stomach and avoidance of foods whose ingestion might cause sickness/malaise. Much less is known about the involvement of OT in feeding for pleasure, although evidence gathered to date suggests that OT diminishes appetite for sweet and/or carbohydrate tastants – at least partially – via the ventral tegmental area (VTA). Sections 1.3.1 and 1.3.2 provide more theoretical background on the involvement of OT in specific mechanisms affecting ingestive behavior and physiological processes related to feeding.

1.3.1. Oxytocin and homeostatic control of food intake

1.3.1.1. Oxytocin and satiety-driven termination of food intake

Early on it became apparent that the hypothalamus is a key central region responsible for the regulation of appetite. One of the pioneering studies showed that deletion of the PVN in rodents produces excessive food consumption and result in the obese phenotype [86]. Subsequent experiments indicated that the PVN is not the sole player in feeding regulatory mechanisms and a host of other neuroanatomical substrates of the hypothalamus were added to the list of – using the nomenclature of those early reports – “satiety/hunger centers”. Those sites include the SON, ventromedial hypothalamus (VMH), dorsomedial hypothalamic nucleus (DMH), lateral hypothalamus (LHA) and arcuate nucleus (ARC)[87,88]. Among those, the ARC has been shown to be directly affected by the presence of adipose-derived hormone, leptin, in the general circulation [89] [90]. The question arose as to how else (other than through the direct input into the ARC) the hypothalamic areas receive relevant signals that modify

neuroendocrine responses accompanying changes in ingestive behavior associated with hunger and satiety. It led to identification of the dorsal vagal complex (DVC), the nucleus of the solitary tract (NTS), dorsal motor nucleus of the vagus (DMNV) and area postrema (AP) as a “relay” region that integrates neural (mainly, vagally mediated), hormonal (e.g. cholecystokinin) and biochemical/chemical (plasma ion/toxin profile) input related to consummatory activity. Data strongly suggest neuroanatomical and functional reciprocity in the appetite-related functioning of the brainstem-hypothalamic circuit (and that includes the OT neuronal populations which both receive brainstem-derived innervation and project toward the DVC [91]. Figure 1.9 shows a schematic representation of an example of feeding-related neuroendocrine circuit that incorporates peripheral signaling and the brainstem-hypothalamic pathways.

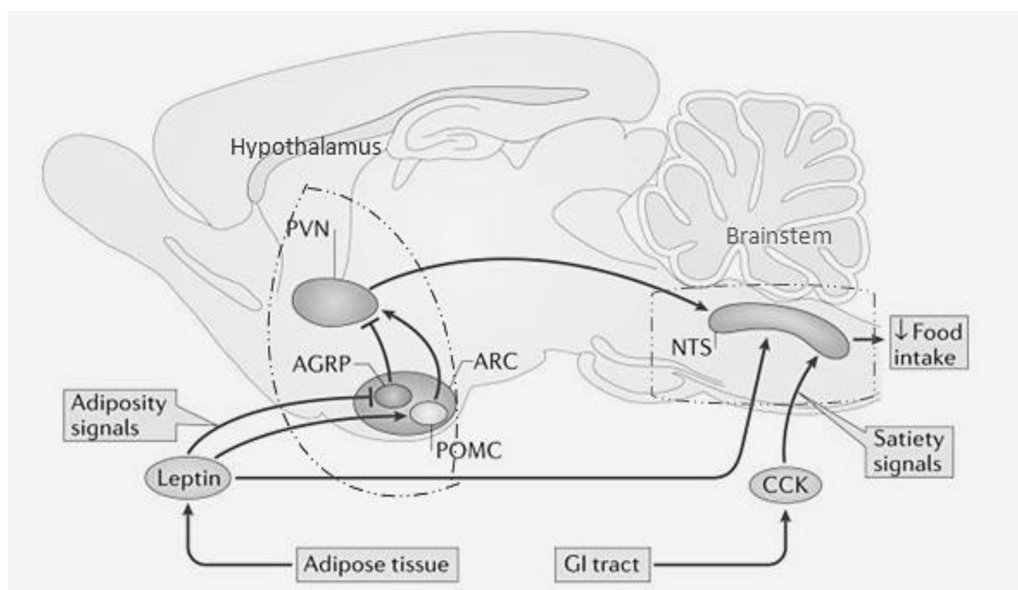

Figure 1.7: Homeostatic control of food intake by the CNS. The hypothalamus and the brainstem integrate signals from the periphery: Cholecystokinin (CCK) released from the GI (gastrointestinal) tract affects the NTS (nucleus of the solitary tract) and leptin from the adipose tissue affects AGRP (Agouti-related protein) and POMC (proopiomelanocortin) neurons in the hypothalamic arcuate nucleus (ARC). Figure adapted from Morton GJ et al. 2014 [92].

The fact that OT receptors are present throughout the brain areas controlling consumption for energy serves as the neuroanatomical basis suggesting the

involvement of OT in satiety processes. The OTr has been found in the DVC and the hypothalamus (including the PVN, SON, VMH and LHA). A decrease in energy-driven food intake has been found in laboratory animals injected with OT and synthetic OTr agonists into the cerebral ventricle (ICV) as well into specific sites, such as the VMH and NTS [93-95]. The initial article showing anorexigenic effect of ICV OT in food-deprived rats was published in the 1990's by Arletti et al. [81]. These authors showed that ICV OT inhibited chow intake in food-deprived and food-unrestricted rats. Moreover, ICV OT increased the latency to begin the meal and reduced time spent eating. The anorexigenic effects were abolished by a pre-treatment with an OTr antagonist. Since that initial report, a number of authors have generated data that support the notion of the meal-end, satiety-related activation of the OT system and the link between the energy status and the OT system's activity. For example, the number of Fos-positive OT neurons in the PVN and SON is significantly higher at the end than at the beginning of a scheduled meal in rodents [96]. Release of OT into the peripheral circulation occurs upon discontinuation of consummatory activity [97]. Real-time PCR analyses have revealed that OT system gene expression profile differs in animals subjected to various calorie availability regimens [47]. Finally, peripheral, ICV and direct site-specific intra-PVN injections of peptides that decrease appetite, such as alpha-melanocyte stimulating hormone (alpha-MSH) and glucagon-like peptide-1 (GLP-1), stimulate OT signaling [98,99]. Though the main consensus on the involvement of OT in feeding control stipulates that OT reduces appetite primarily via the central "pool" of its receptor, also peripheral (intraperitoneal; IP) injections of OT decrease ingestive behavior [81]. Baskin et al [100] found that peripheral injections of OT decrease food intake, but the blockade of the OTr in

the hindbrain does not completely abolish peripheral OT-induced anorexia, but only somewhat alleviates the hypophagic effects of OT. To some extent, these appetite changes might also be secondary to energy metabolism- and adiposity-related effects induced by the peptide. For example, Maejima and colleagues found that peripheral OT in mice reduces not only feeding, but also body weight and visceral fat mass, and it ameliorates glucose intolerance [101]. In line with those findings, OT has been shown to increase the expression of stearoyl-coenzyme A desaturase 1, as well as the content of N-oleoyl-phosphatidylethanolamine, the biosynthetic precursor of the oleic acid-derived PPAR-alpha activator, oleoylethanolamide, in the adipose tissue [102].

1.3.1.2 Stomach distension and osmolality

The risks posed by stomach distension that exceeds this organ's capacity as well as by ion imbalance (due to salt loading) are critical ingestive behavior-derived challenges to homeostasis. In order to prevent these two parameters from reaching values that endanger the animal's well-being, there needs to be a feeding regulatory system which will promote an immediate termination of any consummatory activity. OT has been suggested to be a component of this mechanism.

In rats, elevated sodium levels or dehydration (resulting in an increase in sodium concentration) increase neurohypophyseal OT secretion and promote concurrent inhibition of food intake [103]. Indeed, obliteration of the periventricular area of the rostral AV3V region in rats decreases their drinking response after an injection of hypertonic saline and decreases the posterior pituitary OT release [103]. ICV administration of OT decreases salt consumption and this effect is

reversible by an OTr antagonist [104]. Genetic deletion of the OTr promotes a decrease in salt consumption [105, 106]. Elevated OT plasma levels in rats lead to an increase the in urinary sodium concentration, the effect that can be abolished by an OTr blockade [107]. Furthermore, mice overexpressing OT mRNA consume two time less sodium compared to their wild-type (WT) counterparts [108]. One of the mechanisms underlying this effect might be the relationship between OT and angiotensin in facilitating renal sodium excretion and maintaining osmotic balance. For example, it has been shown that inhibition of OT release stimulates angiotensin secretion [104].

OT neuronal activity is elevated also upon increase in stomach distension (when large amounts of food are introduced into the stomach) and coincides with meal termination [109]. Information on the GI (gastrointestinal) motility is relayed into the CNS by the vagal afferents, eventually reaching OT neuronal populations in the hypothalamus [110]. OT affects gastric motility via NTS and DMN pathways [111,112]. ICV OT injections or electric stimulation of the PVN have been shown to dose-dependently decrease gastric motility in rats, the effect reversed by an OTr antagonist [113].

1.3.1.3. Oxytocin and aversion / toxicity

Aside from nutritive and non-nutritive (and, in both cases: palatability affecting) components, food may contain toxic chemicals. Ingestion of such substances may lead to malaise and sickness and, under some circumstances (depending on the amount ingested as well as physiological and pathophysiological status of the organism, to name a few), it may lead to serious health consequences. Furthermore, not only toxins per se, but also some nutritive and non-nutritive

food components – especially at high volumes and/or concentrations (and when ingested rapidly) - can produce toxic-like effects. The most desirable behavioral response upon inadvertent consumption of sickness producing tastants is an abrupt discontinuation of consummatory activity and – if a given tastant has easily distinguishable characteristics – learning to avoid this tastant in the future.

In the laboratory setting, the process of malaise/sickness-driven anorexia and subsequent avoidance of foods associated with the development of unpleasant GI sensations, is typically studied by employing a conditioned taste aversion (CTA) paradigm. A CTA is a classical Pavlovian phenomenon in which avoidance of a tastant upon subsequent presentations is generated by pairing the initial exposure to this tastant with an injection of a GI discomfort inducing substance [114], such as cholecystokinin (CCK), copper sulphate [115, 116] or – most frequently used - lithium chloride (LiCl) [117-119].

Administration of CTA inducing agents activates a vast array of neural and neuroendocrine pathways, and these circuits include OT. It has been shown that systemic infusion of LiCl induces c-Fos immunoreactivity in several brain regions, including the AP, NTS, lateral parabrachial nucleus, SON, PVN, and central nucleus of the amygdala (CeA). Vagal afferents reaching the NTS and DMNV mediate the neural input relevant to, among others, changes in gastric motility caused by toxins. One of neuronal populations affected by the vagal input contains GLP-1 synthesizing cells in the NTS that project directly to the hypothalamus and terminate in the vicinity of OT perikarya [120]; GLP-1 receptors are present on OT neurons and their ligands stimulate OT cells [121]. GLP-1 promotes CTAs, whereas antagonism of GLP-1 receptors alleviates aversive consequences [122]. Notably, GLP-1 NTS neurons are activated by LiCl.

Concurrently, in the SON and PVN of animals subjected to an aversive treatment, OT cells show a dramatic increase in colocalization with c-Fos, an immediate-early gene product [123]. This elevated OT neuronal activity is accompanied by a surge in circulating OT [124,125].

Due to the unique neuroanatomical distribution of OT neurons, for many years OT was viewed as the "final component" of central pathways that mediate CTA and some authors even doubted whether OT is a player in CTA processing at all or just a mere indicator of activity within aversion-related neural networks [126, 127] [128, 129]. For example, AP-facilitated chemoreception allows the toxin to be recognized directly by the brainstem [130]. Ablation of this particular hindbrain site critically impairs the animal's ability to develop a proper CTA response, though hypophagia in LiCl-treated AP-lesioned rats remains (and so does the increased OT tone upon toxin administration) [131]. Altogether, toxin-derived activation of the DVC engages downstream pathways that include hypothalamic OT cell populations [123,132]. As to whether OT is a key player in CTA was addressed in the study in which the effect of OTr blockade on CTA acquisition was established. It revealed that a BBB-penetrant OT antagonist, L-36,899, prevents the development of CTA, thus pointed to fact that an available OTr underlies the ability of the organism to acquire aversion.

1.3.2 Oxytocin and the reward system: emphasis on feeding for pleasure

Energy needs are not the sole motivation underlying ingestive behavior. One of the main reasons supporting the drive to consume food is pleasant taste. This pleasure of eating comes from activating appropriate taste receptors, from

certain postabsorptive processes [133] and – most importantly – from increasing activity of a specific set of brain sites that promote eating for pleasure, collectively referred to as the reward system [134, 135]. Two key components of the reward system are the Acb and the VTA. They contain cells expressing reward mediators, such as opioids, dopamine, serotonin and endocannabinoids [136-142]. Ingestion of palatable food increases the activity of the reward areas and affects the expression of genes associated with feeding reward in this circuit [143].

There are neuroanatomical and functional foundations underlying the relationship between reward sites and the OT system. The Acb and VTA express a high density of OTr, and PVN OT neurons have been shown to innervate Acb and VTA [20, 144, 145]. OT terminals have been found in the vicinity of perikarya and axons of mesolimbic neurons [144-146]. Notably, this cross-link with the mesolimbic reward system and the OT system is reciprocal. For example, various subtypes of opioid receptors have been detected in the SON and PVN [147, 148], hypothalamic OT neurons express opioid receptors [149, 150] and the release of OT and activity of OT cells is affected by direct administration of OTr ligands [47].

The cross-link between OT and reward appears to be broad and include non-feeding rewards: from naturally pleasant contexts of social and reproductive behaviors to intake of alcohol and administration of drugs of abuse). Cocaine treatment has been found to change OTr binding density in the bed nucleus of the stria terminalis [151]. Direct AcbC infusions of OT decrease methamphetamine-seeking in rats [152]. It also attenuates methamphetamine-induced conditioned place preference [153]. Qi et al. investigated the effects of

OT on methamphetamine-induced extracellular levels of glutamate and γ-aminobutyric acid (GABA) in the medial prefrontal cortex. They found that OT decreases glutamate release and increases γ- GABA in this site, and a selective inhibitor of OTr, antagonizes these effects of the native OT molecule [154]. Acb OT administration hampers the development, maintenance, and primed reinstatement of alcohol-induced conditioned place preference [155]. ICV OT in mice promotes the development of a conditioned social preference [156].

While the role of OT in the regulation of energy homeostasis is well established, the concept of a possible function in feeding reward has emerged relatively recently. Knockout (KO) mouse studies have shown that deletion of the OT gene leads to overconsumption of palatable sucrose solutions regardless of whether sugary tastants are offered during the light or dark phase of the LD cycle or during enhanced anxiogenic conditions [157, 158]. OT KOs given a choice between two tastants (water was used as a control ingestant in those two-bottle tests), display a greater preference for sucrose and for other palatable carbohydrate solutions even if these solutions are not sweet (e.g., cornstarch emulsion). The OT-null murine strain also drinks more saccharin (a non-caloric and non-carbohydrate sweetener) [157]. Higher preferences for palatable foods/solutions in OT KOs do not expand onto fat: a similar intake of a palatable lipid emulsion, Intralipid, was observed in KOs and the wild-type (WT) controls [159].

The OT KO mouse data are largely consistent with the outcomes of studies utilizing non-KO animal models. For example, OT mRNA is upregulated in the hypothalamus of rats eating scheduled, volume-unrestricted, sugary diet

compared to standard food [160]. Immunohistochemical comparison of hypothalamic OT neuronal activity levels (through detecting c-Fos-OT co-localization) shows a significantly higher proportion of activated hypothalamic OT neurons in sucrose than fat consuming animals. It should be noted that regardless of a composition of a meal, activation of OT neurons is much higher at the time of termination rather than initiation of a meal, which underscores the role of central OT as a general satiety mediator [47]. Moreover, injections of a non-peptide OTr antagonist, L-368,899, in choice and no-choice feeding scenarios [161], increase carbohydrate intake, without affecting fat consumption [47]. Finally, a recent report by Mullis et al. showed that direct VTA administration of OT decreases palatability-driven sucrose consumption and the effect is reversible by a pre-treatment with L-368,899. The same group of investigators found that blockade of the OTr in the VTA leads to overconsumption of sugar [162]. Despite the fact that another key reward site, the Acb, expresses the OTr in both the shell (AcbSh) and core (AcbC) subdivisions [163,164], there has been no attempt to define whether OT acting through this region affects food intake.

Finally, one should take into consideration that any behavior driven by reward, including food intake, is heavily influenced by social environment in which an individual animal is immersed. This social context component – depending on its quality (e.g. the place in hierarchy or the presence/absence of antagonistic relations) has a capacity to shift responsiveness to rewarding stimuli. For example, in a conditioned place preference paradigm, preference for cocaine can be changed by social interaction [165]; lesions of the AcbC induce preference for

the social compartment, whereas lesions of the AcbSh - for the cocaine compartment [166]. Rats maintained in a social setting consume more alcohol than when they are single-housed [167]. Also, subordinate rodents or rodents subjected to frequent social defeat scenarios, display enhanced appetite and obesity [168-170]. Particular caution should be applied when analyzing feeding reward-related effects of OT as this neurohormone has been implicated in a vast array of social behaviors, such as pair bonding, mother-infant bonding, and social approach and recognition [63, 171-173] [174-176]. Initial evidence already points to scenarios in which the magnitude of anorexigenic effects of an OTr antagonist, L-368,899, depends on social hierarchy in mice [177]. It is unclear though whether the reward system itself participates in this socially mediated shift in effectiveness of the drug.

1.4 Oxytocin as an appetite suppressant acting in the wide network of central sites: functional relationship between oxytocin and gap junction protein, connexin 36?

Importantly, OT does not act as a sole player in the regulation of homeostatic or rewarding aspects of appetite. OT is part of a large network of pathways and sites, comprising multiple genes, neurotransmitters, and neuropeptides. The ability of OT to effectively modify consumption depends on structural and functional integrity of the relevant brain circuits. This structural and functional integrity is assured by multiple molecules that include, to name a few, cytoskeletal proteins, synaptic molecules, and transcription factors [178-180]. As shown in Chapter 4 section 4.1 of this thesis, a subset of gap junctions, i.e. those

whose structure relies on the presence of connexin 36 proteins, are critical elements whose genetic elimination leads to a broad spectrum of perturbed appetite: feeding for energy, feeding for reward and avoidance of tainted foods. As OT is involved in the regulation of the very same aspects of food intake, it has led me to explore a question presented in detail in Chapter 4 section 4.2 of whether there is a functional relationship between OT and Cx36.

1.4.1. Cx36 gap junction

A gap junction channel is formed by transmembrane fixation of hexameric structures termed connexons. Connexons consist of six membrane proteins belonging to the connexin family (Cx; Figure 1.12). Gap junctions allow intercellular communication between adjacent cells by permitting the bidirectional passage of ions and small molecules [181-186]. Twenty genes code for Cxs in mice and 21 in humans [181]. Six Cxs (Cx26, Cx29, Cx30, Cx32, Cx36 and Cx43) have been identified in the mammalian CNS, but in the reward system, connexin 36 (Cx36) is predominant [187]. Cx36 channels can be targeted pharmacologically with quinine as their blocker [188,189].

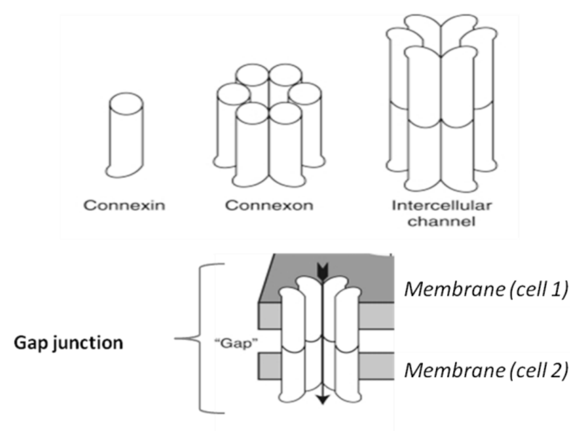

Figure 1.8: The structure of a gap junction. Figure adapted from Goodenough DA 2009 [190].

Studies have shown that, aside from reward areas, most endocrine neurons contain Cx36 [187], and that Cx36 gap junctions are implicated in maintaining the structural and functional integrity of circuits by facilitating the proper control of neuropeptidergic and neurohormonal tone [191, 192]. In mesolimbic areas, Cx36 is expressed in the VTA [193,194] and the Acb [195]. Allison and al. suggested that an intercommunication between GABA neurons in the VTA is mediated by Cx36 gap junctions [193,196]. Signaling within the networks that encompass Cx36 affects ethanol consumption: a recent study in demonstrated that Cx36 KO mice consume significantly less ethanol than WTs and this phenomenon is associated with an upregulation of the D2 receptor. [196]. Moreover, the rhythm oscillation of dopamine neurons regulated by VTA GABA affects the Acb and other components of the limbic system [197]. In rats, a decrease of Cx36 mRNA expression in the Acb occurs after amphetamine and cocaine treatments [198,199]. What is unknown at present is whether Cx36 gap junctions are an essential component of circuits through which OT reduces appetite.

Aims

The **overarching aim** of this doctoral research was **to determine whether OT suppresses appetite for palatable foods**. This project, utilizing mice and rats as model organisms, encompassed three specific aims:

Specific Aim 1: Defining a relationship between peripherally administered BBB-penetrant OTr antagonist and palatable diet intake. Defining whether OTr blockade increases the intake of: (a) all or only sweet carbohydrates or (b) any sweet food regardless its macronutrient content (Chapter 2).

Specific Aim 2: Defining whether the nucleus accumbens directly mediates the effects of OT on appetite and whether intra-accumbal OT modifies feeding for energy and/or reward in non-social and social contexts (Chapter 3).

Specific Aim 3: Understanding whether OT affects feeding for reward and/or homeostasis when brain circuits through which it acts are devoid of Cx36, a key protein that forms gap junctions in neuroendocrine and reward regions (Chapter 4).

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Chapter 2

Oxytocin receptor blockade enhances appetite for carbohydrates, particularly sucrose

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Abstract

Centrally acting oxytocin (OT) inhibits feeding. Recent evidence suggests a link between OT and control of carbohydrate and saccharin intake, but it is unclear whether OT affects appetite for only carbohydrates, especially sweet ones, or sweet tastants regardless of their carbohydrate content. Therefore, a blood-brain barrier penetrant OTr antagonist, L-368,899, was administered in mice and intake of liquid diets containing carbohydrates sucrose, glucose, fructose, polycose, or cornstarch or the non-carbohydrate, non-caloric sweetener saccharin was studied in episodic intake paradigms: one in which only one tastant was available; and the other, in which a choice between a carbohydrate (sucrose, glucose, or fructose) and saccharin was given. We also used RT-PCR to examine hypothalamic OT mRNA levels in mice given short-term access to sucrose, cornstarch, or saccharin. In the no-choice paradigm, L-368,899 increased the intake of all carbohydrates, whereas its effect on saccharin consumption showed only a trend. A 10-times lower dose (0.3 mg/kg) stimulated

intake of sucrose than other carbohydrates. In the choice test, a very low 0.1 mg/kg dose of L-368,899 doubled the proportion of sucrose consumption relative to saccharin, but did not affect fructose or glucose preference. OT gene expression increased after sucrose and CS but not saccharin exposure compared to controls, however a higher level of significance was detected in the sucrose group. We conclude that OT inhibits appetite for carbohydrates. Sucrose consumption greatly enhances OT gene expression and it is particularly sensitive to OTr blockade, suggesting a special functional relationship between OT and sugar intake.

2.1. Introduction

A neurohormone oxytocin (OT) produced mainly in the hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei inhibits food consumption. Central infusions of OT reduce chow intake and OT neurons are activated at meal termination [1,2]. Large food loads [3-5] , an increase in plasma osmolality [6], treatments with emetic agents [7] , and injections with satiety regulating neuroactive endogenous substances [3,8,9] increase OT neuronal activity. Genetic deletion of the OTr gene underlies obesity in the murine KO strain [10] . While the role of OT in consumption-related homeostasis is well established, most recent evidence suggests OT is involved in feeding reward. OT appears to serve as a cross-link bridging homeostatic and reward-related satiety. OT neuronal activity is particularly robust in response to manipulations that reduce intake of rewarding foods, e.g. to naloxone injections [11], and it is greatly diminished by orexigenic doses of opioid receptor agonists [12]. Long-term

habitual consumption of sugar is associated with diminished OT neuronal activity in response to a food load, suggesting that OT dysregulation leads to reward-driven overeating [13].

Importantly, initial studies suggest that OT seems to affect preferentially intake of those rewarding tastants that are sweet and/or high in carbohydrates. OT KO mice display enhanced preference for sugar and other carbohydrates and OT gene deletion is also associated with increased appetite for saccharin [13,14]. OTr blockade enhances appetite for sucrose [5,14,15]. Central injections of OT reduce the intake of the monosaccharide glucose, but only in food-deprived rats [4]. To add to the confusion, OTr KOs have unchanged preference for sucrose [16]. Furthermore, OT KOs drink elevated amounts of saccharin, but OTr blockade in thirsty animals does not increase preference for saccharin in the saccharin-vs-water two-bottle test [17].

Overall, it is still unclear whether OT affects appetite for only carbohydrates, especially sweet ones, or sweet tastants regardless of their carbohydrate content. Therefore, we administered a blood-brain barrier (BBB) penetrant, potent and selective antagonist of the mouse OTr, L-368,899 [18] to examine its effect on the intake of solutions containing sucrose, glucose, fructose, polycose, cornstarch (CS) or saccharin. Two paradigms were used: (1) only one tastant was available and the dose-response curves were established, and (2) a choice between two sweet ingestants, a carbohydrate (sucrose, glucose or fructose) vs. non-carbohydrate saccharin, was studied in mice injected with a very low dose of the antagonist. We also used real-time PCR (RT-PCR) to examine changes in

hypothalamic OT mRNA levels in mice given short-term access to sucrose, CS, or saccharin solutions.

2.2. Methods

2.2.1. Animals

Male C57BL/6J mice (26±3 g; AgResearch, NZ), housed individually (LD 12:12; lights on at 0700) at 21–22°C, had unlimited access to chow (Teklad) and water unless noted otherwise. The procedures were approved by the University of Waikato animal ethics committee.

2.2.2. Effect of OTr blockade on consumption in the no-choice single-bottle paradigm

Animals (n=8/group) were accustomed to having access to palatable 0.1% saccharin, 10% sucrose, 30% sucrose, 10% fructose, 10% polycose or 10% glucose, given alone, for 2 h (1000–1200 h) per day for 3 days. In the CS study, to stimulate intake of the carbohydrate, mice were food-deprived overnight and then they received access for 2 h (1000–1200) to a single bottle containing a 10% CS suspension (as CS is insoluble in water, 0.3% xanthan gum was added to the liquids in this experiment as described previously in [19]). Chow and water were removed for the 2-h period. Animals started drinking right after the solution was presented and finished within 50 min. On day 4, 5 min prior to solution exposure, mice were injected IP with saline or 0.1, 0.3, 1 and 3 mg/kg b.wt. L-368,899 (Tocris), at a dose range known to affect consumption [5]. Bottles were weighed; the amount of the solution consumed was corrected for spillage and reported in g/kg of b.wt. One-way ANOVA followed by Fisher's post-hoc test was used to

establish the effect of L-368,899. P values ≤ 0.05 were considered significantly different.

2.2.3. Effect of OTr blockade on sugars vs saccharin preference

Mice had been pre-exposed to 0.1% saccharin, 10% sucrose, 10% fructose or 10% glucose for 24h several days before the beginning of the trials in order to avoid neophobia. Animals (n=6/group) were accustomed to having access to two bottles for 3 days: one containing saccharin and the other containing (a) sucrose or (b) fructose or (c) glucose, for 2 h (1000–1200). Food was removed from the hoppers for the 2-h period of tastant presentation. Five min prior to the presentation of the bottles, mice were injected IP with saline or 0.1 mg/kg L-368,899, and the dose of the antagonist was selected as a low, sub-threshold dose insufficient to increase the intake of any carbohydrate tested in a single-bottle paradigm in the no-choice paradigm described above and in previous studies (e.g. [5]). The amount of the solution consumed corrected for spillage was calculated in g/kg of b. wt. and the data were expressed as % of sweet carbohydrate solution (e.g. sucrose) intake in total fluid (e.g. sucrose + saccharin) intake. A t-test was used to establish whether L-368,899 affected preference for carbohydrate vs non-carbohydrate. P values ≤ 0.05 were considered significantly different.

2.2.4. OT mRNA levels in mice consuming sucrose, CS or saccharin

On two subsequent days, water bottles were removed 2h before the beginning of the dark phase. At lights off, the animals (n=7-8/group) were given access to 3 ml of water (control) or the 10% CS, 10% sucrose or 0.1% saccharin solutions/suspensions (prepared as in 2.2) which represented ca 40% of their

night-time water intake. The fluid was consumed by all but 2 mice (1 from the water and 1 from the CS group – excluded from the study) between 3 and 4 h. At 5 h, water bottles were placed back in the cages. We did not observe differences in night-time chow (4.3-4.8 g range) and water (4.0-4.7 ml range) intakes between the groups. On the second day at lights on, chow and water were removed. The animals were decapitated (7:00-8:00), hypothalamic dissected and placed in RNAlater (Ambion) overnight (4°C). A standard protocol of sample preparation and RT-PCR was followed (details in [5]) and for brevity reasons, only the main elements are described herein. Samples were homogenized in TRIzol (Ambion); RNA was extracted with chloroform and precipitated in isopropanol. After centrifuging, the pellet was washed, air dried, and dissolved in the DNase buffer (NEB). The samples were treated with RNase-free DNase I (37°C, 1h; Merck) and the absence of genomic DNA was established by PCR of 5% template in the PCR mix [$MgCl_2$-free buffer, 50mM $MgCl_2$, Tween, 20mM dNTP, forward and reverse primers, Taq polymerase, and MilliQ H_2O; 10µl total volume]. 0.5µl 100ng/µl genomic DNA was added as a positive control, and 0.5µl MilliQ H_2O as a negative one. The product was analysed with electrophoresis. To synthesize cDNA, 5µg RNA samples (concentration determined spectrophotometrically) were diluted with MilliQ H_2O to 12µl. RNA was reverse transcribed in the Master Mix (Promega; 20µl]. Samples were incubated for 1h (37°C), followed by PCR to confirm cDNA synthesis. RT-PCR reactions were performed in duplicates; negative controls were included. 25ng of sample cDNA template was used per primer (OT primer sequences: cggtggatctcggactgaac (fwd) and tagcaggcggaggtcagag (rev)). Each RT-PCR (20µl total volume) contained 2µl $MgCl_2$-free buffer 10×, 0.2µl 20mM dNTP, 1.6µl 50mM $MgCl_2$, 0.05µl forward and

reverse primer (100pmol/µl), 1 µl DMSO, 0.5µl Sybr Green (1:50,000), 0.08µl Taq polymerase, and 9.52µl MilliQ H_2O. The amplification step included denaturation (95°C, 3min), and 40 cycles of denaturing (95°C, 20s), annealing (30s), and elongation (72°C, 30s). Expression of four housekeeping genes, β-actin, β-tubulin, glyceraldehyde-3-phosphate-dehydrogenase, and ribosomal protein, was used to calculate normalization factors (GeNorm). Primer efficiencies were calculated with LinRegPCR, and C_t values were corrected for differences in primer efficiencies. Differences between groups were analysed with ANOVA followed by Fisher's test with significance set at $P \leq 0.05$.

2.3. Results

In the no-choice paradigm, L-368,899 increased the intake of all carbohydrates (Figure 2.1), including the CS, whereas its effect on saccharin consumption only approached significance. A dose as low as 0.3 mg/kg elevated 10% and 30% sucrose consumption (10%: 0.3 mg/kg b. wt. p=0.025; 1 mg/kg b. wt. p=0.008; 30%: 0.3 mg/kg b. wt. p=0.012; 1 mg/kg b. wt. p=0.027). Fructose, glucose, polycose and CS intakes were also increased, however the lowest effective dose of L-368,899 (3 mg/kg) was ten times higher (fructose: p=0.016 ; glucose: p=0.020; polycose: p=0.031; CS: p=0.039). L-368,899 did not affect appetite for saccharin; although there was a trend at 3mg/kg (p=0.088).

In a two-bottle test, mice given access to a sugar (sucrose, glucose or fructose) and saccharin were pre-treated with a 0.1 mg/kg dose of L-368,899, sub-threshold in the no-choice experiment. L-368,899 doubled the proportion of

sucrose consumption relative to saccharin (p= 0.006), but had no effect on the preference for fructose or glucose versus saccharin (Figure 2.2).

Hypothalamic OT gene expression (Figure 2.3) was higher in animals exposed to sucrose and CS compared to controls, however a higher level of significance was detected in the group fed sucrose (p=0.008) than CS (p=0.036). Saccharin exposure did not affect OT mRNA levels.

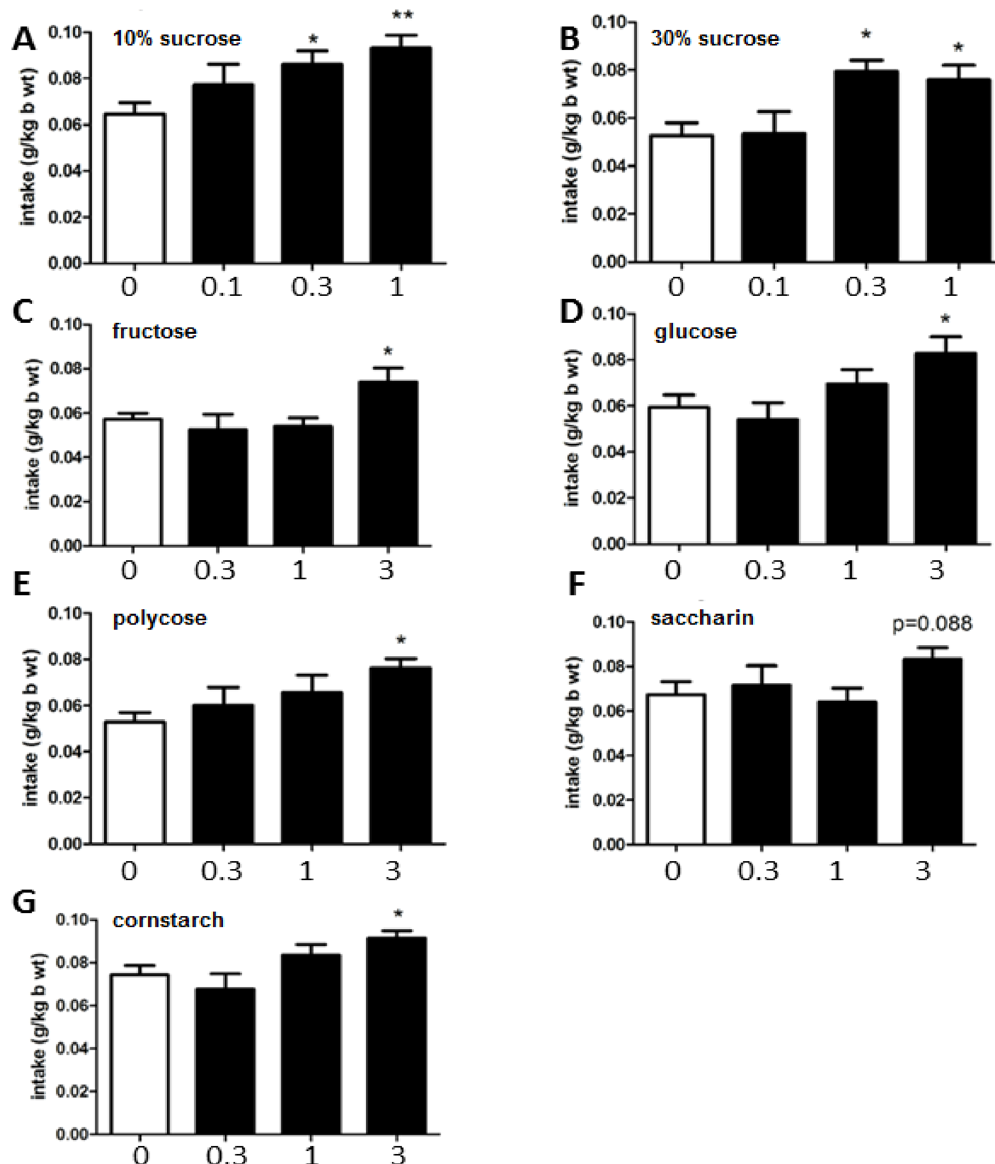

Figure 2.1: The effect of the OTr antagonist, L-368,899, at 0 (vehicle), 0.1, 0.3, 1 and 3 mg kg/b. wt on the intake of solutions containing (a) 10% sucrose, (b) 30% sucrose, (c) 10% fructose, (d) 10% glucose, (e) 10% polycose, (f) 0.1% saccharin and (g) 10% CS. * - p<0.05; ** - p<0.01

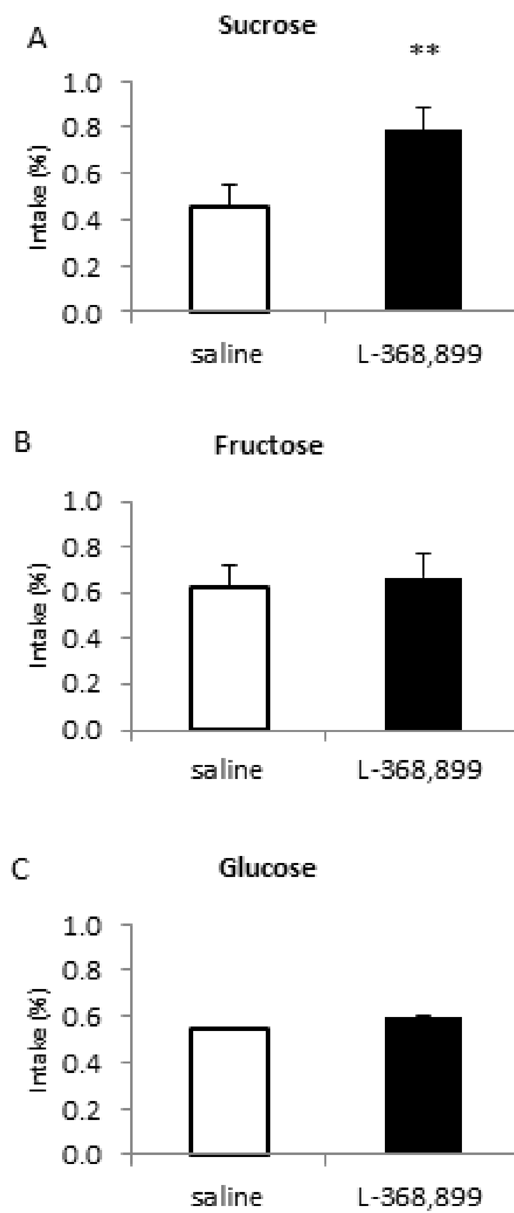

Figure 2.2: The effect of the OTr antagonist, L-368,899, at 0 (saline vehicle), 0.1mg kg/b. wt on the intake of solutions containing (a) 10% sucrose , (b) 10% fructose, (c) 10% glucose (carbohydrate) vs 0.1% saccharin (non-carbohydrate) expressed as the % of carbohydrate solution consumed in the total volume of ingested tastants. ** -p<0.01

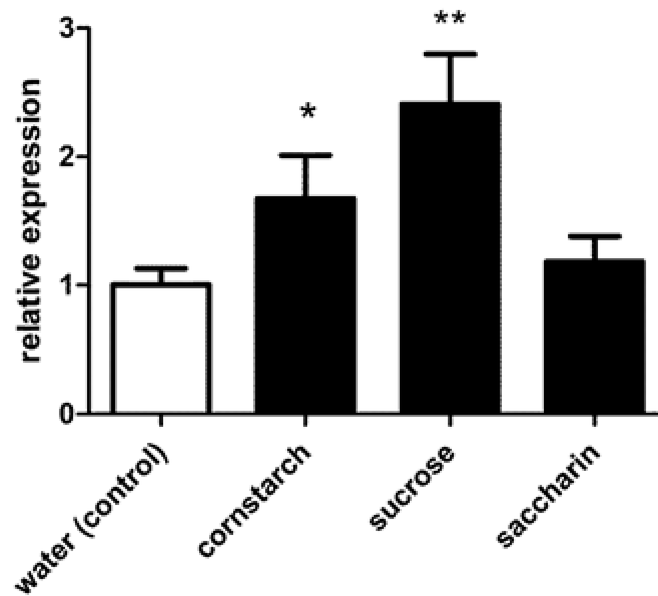

Figure 2.3: Hypothalamic OT gene expression established with RT-PCR. On two consecutive nights, mice were given access to 3 ml of 10% sucrose, 10% cornstarch or 0.1% saccharin solutions; controls were given water. * - $p<0.05$; ** - $p<0.01$

2.4. Discussion

The current set of experiments confirms that OT affects appetite for carbohydrates and it points to a special functional relationship between OT and sucrose intake. Antagonism of the OTr in the no-choice paradigm led to an increase in the intake of all carbohydrates, i.e., sucrose, glucose and fructose, polycose and CS, but it should be emphasized that the consumption of sucrose was induced by the antagonist dose that was 10 times lower than that needed to affect intake of the remaining carbohydrates. Importantly, blockade of the OTr stimulated appetite for carbohydrates independent of their sweetness and rewarding value: bland CS and palatable sweet carbohydrates were ingested more avidly after the treatment. This is in concert with the KO animal data in which the genetic deletion of the OT gene caused overconsumption of a variety

of carbohydrates, including starch [14]. Therefore, a sweet flavor does not seem to be a prerequisite underlying the effectiveness of OT in regulating carbohydrate-specific satiety and neither is the complexity of their structure (i.e., mono-, di- and polysaccharide). Furthermore, the effect of L-368,899 on sucrose intake remained even after the three-fold increase in the concentration of sucrose in the solution; hence, the link between carbohydrate consumption and OT does not appear to be modified by the caloric density of the tastant.

Aside from the episodic intake of carbohydrates being decreased by OTr antagonism, we found that short-term enrichment of the diet with carbohydrates (either sucrose or CS) caused up-regulation of the OT transcript in the hypothalamus compared to controls. This is in line with the OT mRNA findings in sucrose- versus regular diet-exposed rats [20]. Noteworthy, a greater increase in our RT-PCR study was found in sucrose- than in CS-fed mice despite the same amount of the carbohydrate containing fluid consumed by the respective groups.

Unlike appetite for carbohydrates, saccharin intake was not elevated by OTr blockade, though the highest dose of the compound induced the consummatory response that showed a trend approaching significance. Also, our RT-PCR experiment showed that hypothalamic OT gene expression was not affected by short-term saccharin exposure. Though these data are in contrast to OT KO murine studies in which saccharin overconsumption was noted [14], they are in line with antagonist injection experiments that showed the lack of the effect of L-368,899 on preference for saccharin in water-deprived mice given a choice between the saccharin solution and water [17]. Overall, this suggests that the

involvement of the OTr in saccharin intake regulation is not as critical as in appetite for carbohydrates, but obviously considering the trend in saccharin consumption reported herein and the positive KO mouse data [14], it cannot be negated. Importantly, it appears that this is not the sweet flavor (note saccharin vs. CS intakes) but rather the carbohydrate content of an ingestant that defines the link between OT and feeding control.

The two-bottle preference tests confirmed the proposed special relationship between OT and the control of sucrose intake. The very low dose of L-368,899 that was found to be insufficient to affect the intake of any of the carbohydrates in the no-choice paradigm, increased the preference for sucrose vs saccharin in the two-bottle test. It did not affect the relative preference for the other two carbohydrates, fructose and glucose, which strengthens the claim that the OTr plays a role in sucrose-specific satiety. The fact that there was no change of preference for fructose and glucose vs. saccharin implies that calories, at least in the relatively energy-dilute liquid diets, are not the causative factor behind the shift in preference in the sucrose vs. saccharin scenario.

In summary, the present study shows that antagonism of the OT receptor enhances appetite for carbohydrates and this role is independent from sweet flavor. Sucrose consumption greatly enhances OT gene expression and appears to be particularly sensitive to the OTr blockade, which strengthens the notion that there is a special functional relationship between OT and sucrose intake, and suggests that OT may serve as a key neuroregulator of sucrose-specific satiety.

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Chapter 3

Oxytocin acting via the nucleus accumbens core decreases food intake driven by hunger and by reward in rats offered a meal in a non-social setting

Abstract

Central oxytocin (OT) promotes feeding termination in response to homeostatic challenges, such as excessive stomach distension, salt loading, and toxicity. OT has also been proposed to affect feeding reward by decreasing consumption of palatable carbohydrates and sweet tastants. As the OT receptor (OTr) is expressed in the nucleus accumbens core (AcbC) and shell (AcbSh), a site regulating diverse aspects of eating behavior, we investigated whether OT acting therein affects appetite in rats. First, we examined whether direct AcbC and AcbSh OT injections affect hunger- and palatability-driven consumption. We found that only AcbC OT infusions decrease deprivation-induced chow intake and reduce consumption of palatable sucrose and saccharin solutions in non-deprived animals. These effects were abolished by a pre-treatment with an OTr

antagonist, L-368,899, injected in the same site. AcbC OT at an anorexigenic dose did not induce a conditioned taste aversion, which indicates that AcbC OT-driven anorexia is not caused by sickness/malaise. The appetite-specific effect of AcbC OT is supported by the real-time PCR analysis of OTr mRNA in the AcbC which revealed that food deprivation elevates OTr mRNA expression, whereas saccharin solution intake decreases OTr transcript levels. We also used c-Fos immunohistochemistry as a marker of neuronal activity. We found that AcbC OT injection increases activation of the AcbC, hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei, and – within the PVN and SON – it increases the percentage of activated OT cells. Finally, considering the fact that OT plays a significant role in social behaviour, we examined whether offering animals a meal in a social setting would modify their hypophagic response to AcbC OT injections. We found that a social context abolishes anorexigenic effects of AcbC OT. We conclude that OT acting via the AcbC decreases food intake driven by hunger and by reward in rats offered a meal in a non-social setting.

3.1. Introduction

Oxytocin (OT) promotes feeding termination mainly through central mechanisms [1, 2]. An increased level of OT neuronal activity and OT release occurs at the end of a meal and in response to excessive plasma osmolality and stomach distension [3,4]. In 1990, Arletti et al showed that intracerebroventricular (ICV) OT injections cause a marked reduction in food intake [5]. Since that initial study, many authors have confirmed the anorexigenic effect of OT and have begun identifying brain sites mediating OT-driven food termination [6]. The majority of

work has focused on the brainstem component of the circuitry: OT infusions in the nucleus of the solitary tract (NTS) [2] inhibit feeding, and the activity within the OT NTS-PVN pathway has been functionally linked with neuroendocrine regulators of energy homeostasis (especially those involved in gastrointestinal and osmotic control), including cholecystokinin (CCK), glucagon-like-peptide-1 (GLP-1), and peptide YY (PYY) [7]. Furthermore, OT administration in the ventromedial hypothalamic nucleus (VMH) acutely decreases chow intake and increases energy expenditure in rats [7, 8].

OT acting outside the brainstem-hypothalamic pathways also promotes hypophagia. Mullis et al found that OT administered in the ventral tegmental area (VTA) in rats causes a dose-dependent decrease in deprivation-induced chow intake and affects sucrose consumption [9]. Their findings were in agreement with other reports suggesting that OT appears to serve as a cross-link between mechanisms that bridge termination of consumption due to “homeostatic” (i.e., related mainly to stomach distension and osmolality) and palatability-specific (i.e., related to sweet flavor and carbohydrate) satiety. For example, OT knockout mice consume sweet carbohydrates and saccharin less avidly than wild-type controls. Pharmacological blockade of the OTr elevates intake of sweet tastants in sated mice [10], whereas OT administration reduces intake of glucose in food-deprived rats [11]. Opioid receptor antagonist injections that decrease palatable sucrose intake also activate OT neurons [12], and OT gene expression is increased after a high sugar diet intake in rats [13].

Despite the presence of the OTr in the core (C) and shell (Sh) subdivisions [2, 14, 15] of the nucleus accumbens (Acb), another key extrahypothalamic appetite

regulating area, thus far there have been no reports delineating potential involvement of this region in mediating OT's effects on food intake. Extensive studies on the relationship between AcbSh and feeding control have shown that opioid and dopamine signaling in the AcbSh affects particularly hedonics of consumption [15, 16], whereas glutamate and GABA in this subdivision regulate also energy intake [17] [18]. Less is known about AcbC, though several authors have reported that GLP-1 in the AcbC affects meal size and rewarding aspects of food consumption [19-21].

In the current set of studies, we investigated whether OT acting in the Acb affects appetite in rats. First, we examined whether AcbC and/or AcbSh injections of OT decrease hunger- and palatability-driven consumption. Once we established that only the AcbC injections produce hypophagia, we focused on providing a more detailed characterization of the role of the AcbC OT in feeding regulation. A conditioned taste aversion (CTA) paradigm was used to examine whether AcbC OT-driven anorexia is related to malaise. Considering the fact that OT plays a significant role in social behavior [22-24], we also examined how OT injected in the AcbC affects feeding in a social setting. Finally, we determined changes in c-Fos immunoreactivity in feeding-related brain sites after AcbC infusion of an anorexigenic dose of OT, and we analyzed changes in OTr mRNA expression in rats exposed to food deprivation and to a palatable diet.

3.2. Materials and Methods

3.2.1. Animals. Adult male Sprague-Dawley rats weighing ca 335 g at the beginning of the experiment were housed individually in plastic cages with LD

12:12 (lights on at 07:00; temp.: 20–22 °C). Tap water and standard laboratory chow (Teklad) were available *ad libitum* unless noted otherwise. All procedures received prior approval from the University of Waikato ethics committee.

3.2.2. Surgeries. Rats used for intracranial infusion studies were anesthetized with IP ketamine (100 mg/kg) / xylazine (20 mg/kg) and equipped unilaterally with a 7.5-mm stainless steel cannula (23-gauge, Plastics One, Australia) aimed at the AcbC or AcbSh. Stereotaxic coordinates for the AcbC were: 1.5 mm anteroposterior; -1.4 mm mediolateral to bregma; -5.5 mm dorsoventral to skull surface, and for the AcbSh: 1.5 mm, -0.8 mm; -7 mm, respectively. The injector protruded 0.5 mm below the tip of the cannula. Dental cement was used to secure the cannula to three screws inserted in the skull. Rats received Caprofen (5mg/ml, SC) immediately following the surgery. They were allowed to recover for at least 7 days. After the completion of all behavioral experiments, rats were sacrificed and cannula placement was assessed in immunohistochemically processed brains (see Section 3.2.11).

3.2.3. Injections. OT (Phoenix) and OTr antagonist (L-368,899; Tocris) were injected with Hamilton syringes in a volume of 0.5µl over 1 min. Drugs were dissolved in isotonic saline.

3.2.4. Effect of OT in the AcbC versus AcbSh on deprivation-induced chow intake. Rats were deprived of chow overnight. Five min before food presentation (10:00), they were injected in the AcbC or AcbSh with 0 (saline), 0.3, 1 and 3µg OT (n=8-9/group). Chow was weighed before and 2, 4 and 24 h after the treatment.

3.2.5. Effect of OT in the AcbC versus AcbSh on the intake of sweet palatable solutions. We followed a similar protocol as in (PMID: 24893201). In brief, animals (n=8-12/group) were accustomed to having access to a bottle of a 10% sucrose solution for 2 h/day (10:00–12:00) for 3 days (chow and water were removed for the 2 h). On day 4, just before sucrose presentation, rats were injected in the AcbC or AcbSh with 0 (saline), 0.1, 0.3, 1 and 3μg OT (n=8/group) and the amount of consumed solution was established 2 h post-injection. The same approach was employed to study the effect of OT on 0.1% saccharin intake, however, 0, 0.01, 0.03, 0.1, 0.3 and 3μg doses of OT were used.

3.2.6. Effect of AcbC OTr antagonist pre-treatment on the ability of AcbC OT to decrease deprivation-induced feeding. Rats were deprived of chow overnight. Just prior to regaining access to chow (10:00), they received two AcbC injections spaced 10 min apart: (a) saline followed by saline; (b) saline followed 1μg OT (lowest effective OT dose based on Exp 1); (c) 0.3μg L-368,899 followed by 1μg OT; (d) 1μl L-368,899 followed by 1μg OT; and (e) 3μg L-368,899 followed by 1μg OT (n=7/group). Food intake was measured 2 and 4 h post-injection. L-368,899 doses were chosen based on previous reports [10].

3.2.7. Effect of AcbC OTr antagonist pre-treatment on the ability of AcbC OT to decrease consumption of sucrose and saccharin solutions. As described for Exp 2.5, animals were accustomed to having access to a bottle of a 10% sucrose (or 0.1% saccharin) solution for 2 h/day (10:00–12:00) for 3 days. On day 4, just before sucrose presentation, rats were double-injected (10 min apart) in the AcbC with (a) saline followed by saline, (b) saline

followed 0.3μg OT, (c) 1μl L-368,899 followed by 0.3μg OT. Before the 0.1% saccharin presentation, rats were treated in the same manner except for the doses of OT and L-368,899, which were 0.03μg and 3μl, respectively (n=7-8/group). Consumption was measured 2 h post-injection.

3.2.8. Effect of AcbC OT on deprivation-induced and palatability-induced consumption in the social context. In order to assess the effect of AcbC OT on food intake in the social context, single-housed rats were episodically placed in a predictable social setting in which food intake occurred. We designed an apparatus which was a subdivided standard Macrolon cage with a metal grid lid (with an overhead food hopper and bottle holder on one side of the cage). It was subdivided into two identical chambers (L48cm x W19cm) by a transparent, Plexiglas partition wall, containing multiple ø1.5cm round openings, which allowed the rats placed simultaneously in each chamber for incomplete socialization (visual, auditory, olfactory and partial tactile) devoid of major direct interactions (especially antagonistic ones) that could impair animals' ability to access food. All animals used in these studies were accustomed to being in the apparatus and having food presented therein in at least 8 separate 1-h training sessions (starting between 10:00 and 12:00). The chambers were wiped with ethanol after each use. During the pharmacological studies, only one of the two animals simultaneously present in the apparatus received an injection of saline or OT.

To examine the effect of OT on deprivation-induced feeding in the social setting, animals had chow removed overnight. Afterwards, two rats were placed simultaneously in the social context apparatus (one rat per chamber) wherein

pre-weighed chow was put in the hopper. Just prior to the session, one of the two rats received an injection of 0 (saline), 1 or 3µg OT (n=8-10/group) in the AcbC, and food intake was measured 1 h later.

To assess the effect of OT on intake of sweet palatable tastants in the social setting, rats were accustomed to getting episodic access to a single bottle of 10% sucrose or 0.1% saccharin solution similarly to the non-social scenario described in Section 2.5. On the experimental day, two rats were placed simultaneously in the social context apparatus (one rat per chamber) wherein they gained access to individual pre-weighed bottles containing a sweet solution. Just prior to the session, one of the two rats received an injection of saline) or OT. 0.03, 0.1, 0.3 and 1µg doses of OT were injected in the sucrose-exposed animals, whereas 0.03, 0.1 and 0.3µg OT, in rats given the saccharin solution (n=6-8/group). Bottles were collected and weight before and after 1h post-injection.

3.2.9. AcbC OT and CTA development. Rats were accustomed to having access to water for 2 h (11:00–13:00) per day for 3 days. Food was removed from hoppers for the 2-h period of scheduled fluid presentation. On day 4, rats were given a novel 0.1% saccharin solution instead of water for 60 min. Afterwards they received an AcbC injection of saline or 1µg OT (an effective anorexigenic dose based on feeding experiments) (n=5/group). On day 5, a two-bottle preference test (saccharin versus water) was used to assess acquisition of a CTA to the saccharin solution [56-58]. Bottles were weighed and percentages of the saccharin solution intake (out of cumulative, i.e. saccharin plus water, intake) were calculated.

3.2.10. Consummatory behavior data analysis. All food intake experiments utilizing single OT injections were analyzed with one-way ANOVA followed by Fischer's post-hoc test. In the double injection experiments, first the effect of OT against saline control was confirmed with a t-test and then the effect of the antagonist pretreatment on OT-induced anorexia was determined with one-way ANOVA followed by Fischer's post-hoc test. The CTA OT versus saline two group comparison was done with a t-test. Values were considered significant when $p<0.05$.

3.2.11. c-Fos immunoreactivity in feeding-related brain sites in response to AcbC OT injection. Rats received a single injection of saline or 3µg OT in the AcbC (n=5-6/group). Injections were performed between 10:00 and 12:00. Food and water were removed immediately after the drug treatment. 60 min later, animals were anesthetized and perfused with 50 ml of saline followed by 500 ml of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Brains were excised and postfixed overnight in PFA (4°C). Coronal 60-µm Vibratome (Leica) sections were processed for c-Fos or c-Fos+OT immunostaining. The tissue was treated for 10 min in 3% H_2O_2 in 10% methanol (in TBS, pH 7.4) and incubated overnight at 4°C in the goat anti-c-Fos antibody (1:2000; Santa Cruz). Subsequently sections were incubated for 1h in the rabbit-anti-goat antibody (Vector) and then in the avidin-biotin complex (1h; Vector). Peroxidase was visualized with 0.05% diaminobenzidine, 0.01 H_2O_2, and 0.2% nickel sulfate (all Sigma). All incubations were done in a mixture of 0.25% gelatin and 0.5% Triton X-100 (Sigma) in TBS. Intermediate rinsing was done with TBS. Following the completion of c-Fos staining, sections were further processed for

visualization of OT. The procedure was similar to that used to identify c-Fos. However, rabbit anti-oxytocin was used as the primary antibody (1:15000; Millipore, Temecula, CA), and nickel sulfate was omitted from the DAB solution so as to obtain a brown color. Sections were mounted on gelatinized slides, dried, dehydrated in ethanol, soaked in xylene, and embedded in Entellan (Merck). The number of c-Fos positive nuclei was counted bilaterally in all regions of interest (4-5 sections containing a given site per animal) except for the AcbC where c-Fos IR was assessed ipsilaterally to the cannula, using Scion Image software. Densities of Fos positive nuclei (per mm^2) were averaged per rat and then per group. In the double staining analysis, the following estimates were assessed per section and then per region: the total number of OT neurons and the number of OT neurons positive for c-Fos. Cells were counted bilaterally, and the percentage of OT neurons containing c-Fos-positive nuclei was tabulated. A t-test was used to determine differences between the two groups (significance set at $p<0.05$).

3.2.12. Effect of regular diet versus sweet diet and regular diet versus food deprivation on OTr gene expression levels in the AcbC

In order to assess the effect of sweet palatable solution exposure on AcbC OTr mRNA levels, rats were given access to either chow and water (control) or to chow and 0.1% saccharin solution (instead of water) for 48h (*n* = 8/group). In a separate experiment assessing the effect of food deprivation on OTr mRNA levels, rats were either maintained on ad libitum access to chow and water (control) or chow was removed for 24 hours prior to decapitation (*n* = 8/group).

The animals were decapitated (10:00-12:00) and the AcbC was collected excised, immersed in RNAlater (Ambion), kept at room temperature for 2 h and, thereafter, stored at −80 °C (as described in our earlier publication [13]).

3.2.12.1. RNA isolation, cDNA synthesis and real-time PCR. Samples were sonicated in TRIzol (Invitrogen), chloroform was added to the homogenate, which was then centrifuged at 10,000 × *g* at 4°C for 15 min. The water phase was transferred to a new tube, and RNA was precipitated with isopropanol. The pellets were washed with 75% ethanol, air dried, and dissolved in RNAse-free water. DNA was removed with DNAse I treatment (Roche; 4 h, 37 °C), and the enzyme was inactivated by heating the samples at 75°C for 15 min. The absence of genomic DNA was determined by the PCR analysis with primers for the RNA extractions with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on the DNAse-treated RNA. RNA concentration was measured with a NanoDrop® ND-1000. cDNA was synthesized with MMLV reverse transcriptase (GE Healthcare), using random hexamers as primers according to the manufacturer's specifications. cDNA was analyzed with a MyIQ thermal cycler (Bio-Rad). Each rtPCR reaction with a total volume of 20 µl contained cDNA synthesized from 25 ng total RNA, 0.25 M each primer, 20 mM Tris/HCl (pH 8.4), 50 nM KCl, 4 mM $MgCl2$, 0.2 mM dNTP, SYBR Green (1:50000). Reactions were performed with 0.02 U/l Taq DNA polymerase (Invitrogen) under the following conditions: initial denaturation for 4 min at 95 °C, followed by 50 cycles of 15 s each at 95 °C, 30 s at 55–62 °C (i.e. at the optimal annealing temp. for each primer pair), and 30 s at 72 °C. This was followed by 1 min at 55–62 °C (optimal annealing temp.) and a melting curve with 84 cycles of 10 s at 55°C increased by 0.5°C per cycle. All

experiments were done in duplicates. The measurements where the threshold cycle (Ct) values between the duplicates had a difference of over 0.9 were repeated. A negative control for a given primer pair and a positive control with 25 ng of genomic DNA was included on each plate. The following HKGs were used to define normalization factors: GAPDH, β-tubulin (TUB), ribosomal protein 19 (RPL19), histone H3 (H3), cyclophilin (CYCLO), β-actin (ACT) and succinate dehydrogenase complex, subunit B (SUCB) (as in 19022308). The sequence of the OTr primer pairs were as follows: TTCTTCTGCTGCTCTGCTCGT (fwd) and TCATGCTGAAGATGGCTGAGA (rev).

3.2.12.2. Data analysis and relative expression calculations. The MyiQ software v. 1.04 (Bio-Rad) was used to analyze qPCR data and derive Ct values. Melting curves were analyzed to confirm that one product was amplified and that it was significantly shifted compared to the melting curve for the negative control. The sample Ct values were analyzed further if the difference between those and the negative control exceeded 3; otherwise, the transcript was considered not to be expressed. Normalization factors were calculated with GeNorm. LinRegPCR was employed to calculate PCR efficiencies for each sample. Grubb's test (GraphPad) was used to identify and remove outliers and calculate average PCR efficiencies for each primer pair. Differences in OTr expression between groups were analyzed with a t-test, and $p < 0.05$ was used as the criterion of statistical significance.

3.3. Results

We have found that direct AcbC (Figure 1-2), but not AcbSh (Figure 3.1 E F G), injections of OT affect food intake. Administration of 1 and 3 µg OT in the AcbC decreased deprivation-induced cumulative chow intake (Figure 3.1 A-B) by approximately 35-40% at 2 h (1 µg *P*=0.028; 3 µg *P*=0.019) and 4 h (1 µg P=0.006; 3 µg *P*=0.004) post-injection. There was no effect on food consumption at 24h (data not shown) or water consumption at 2, 4 or 24 h (Table 3.1).

In non-deprived rats stimulated to eat by palatability of a diet, AcbC infusions of 1 µg and 3 µg OT decreased sucrose solution intake by ca 50% (P=0.017 and P=0.045, respectively). Saccharin solution consumption was decreased to a similar degree by OT, however, lower doses were needed to cause the effect: 0.03 µg (P=0.03), 0.1 µg (P=0.026), 0.3 µg (P=0.025), and 1 µg (P=0.027; Figure 3.1 C-D).

Our CTA study showed that AcbC administration of an anorexigenic 1-µg dose of OT before presentation of the novel 0.1% saccharin solution did not produce learned avoidance of saccharin in the subsequent two-bottle choice test (Figure 3.1 H).

The anorexigenic action of AcbC OT was reversed by a pre-treatment with an OTr antagonist. In overnight-deprived rats, 1 and 3 µg of L-368,899 counteracted the effect of OT at 2 h (3 µg, P=0.042) and 4 h (1 µg, P=0.025; 3 µg, P=0.016) post-injection (Figure 3.2 A B). The antagonist abolished the effect of OT on the intakes of the sucrose (P=0.030) and saccharin (P=0.04) solutions (Figure 3.2 C D).

Furthermore, while OT-induced short-term anorexia was apparent in single-housed animals stimulated to eat either by hunger or by palatability, AcbC infusions of OT at doses effective in the aforementioned scenarios did not affect hunger- or reward-driven consumption in rats subjected to the social context of a 1-hour meal (Figure 3.3).

Our c-Fos study revealed that AcbC infusion of 3 µg OT increased activation of the AcbC itself (P=0.008) as well as two other sites belonging to a widespread central network regulating appetite: the PVN (P=0.029) and the SON (0.036; Figure 3.4). Our double staining for c-Fos and OT revealed an AcbC OT-induced increase in the percentage of activated OT cells in both sites (PVN, p=5.6E-5 and SON, p=4.5E-6; Figure 3.5).

Finally, using real-time PCR, we found that expression of the OTr mRNA in the AcbC is significantly higher in food-deprived rats compared to ad libitum-fed animals (P=0.033) and it decreases in rats given 48-h access to a saccharin solution compared to standard diet-fed rats (P=0.045; Figure 3.6)

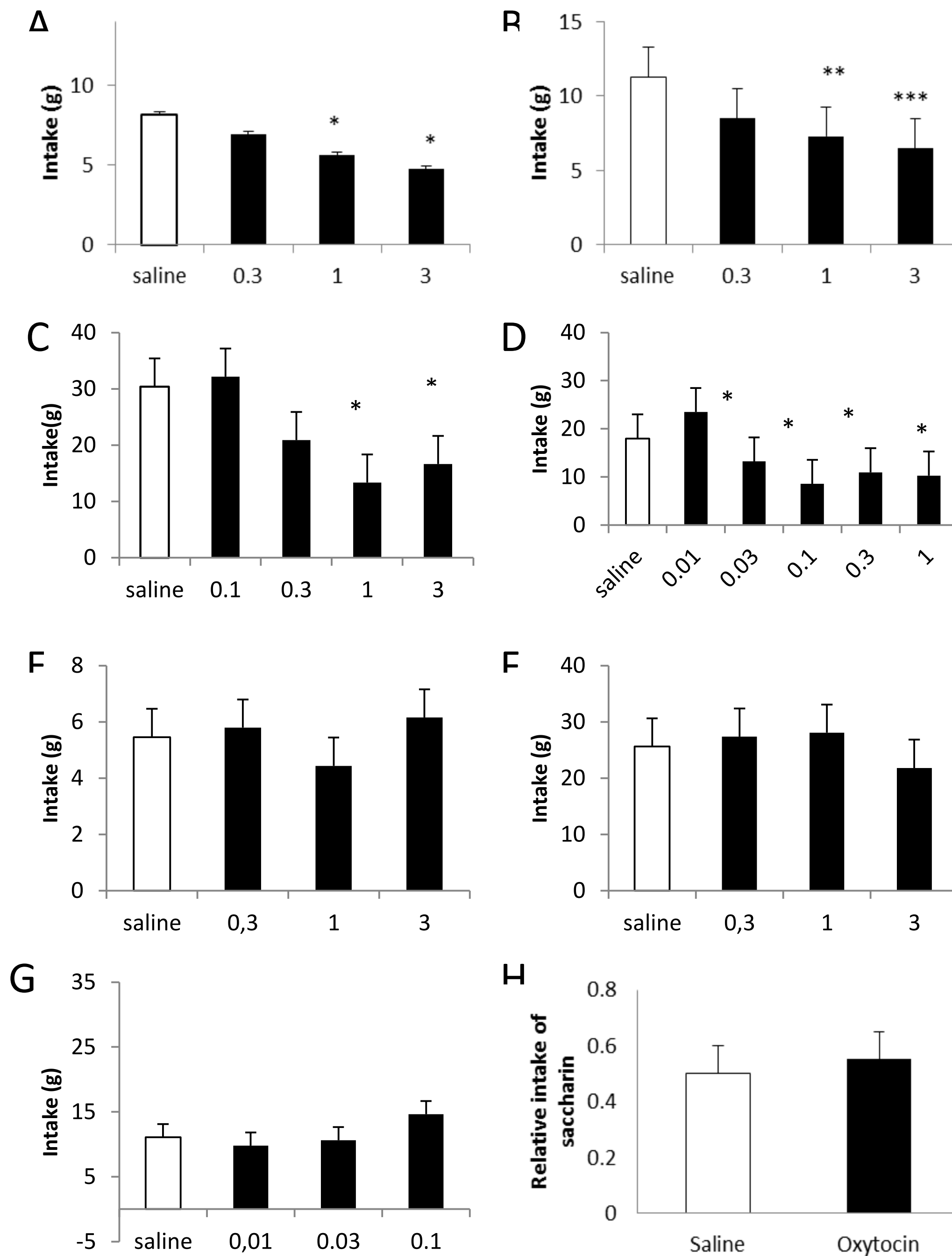

Figure 3.1: The effect of OT injections (doses in µg) in the AcbC and AcbSh on consummatory behavior in rats. (A) The effect of AcbC OT on deprivation-induced chow intake 2h and (B) 4h post-injection. (C) The effect of AcbC OT on the intake of 10% sucrose solution and (D) 0.1% saccharin solution. (E) The effect of AcbSh OT on deprivation-induced chow intake 2h post-injection. (F) The effect of AcbSh OT on the intake of 10% sucrose solution and (G) 0.1% saccharin solution. (H) Preference for a saccharin solution in a two-bottle preference test (saccharin versus water) was assessed to determine whether AcbC OT promotes acquisition of a CTA. * - $p<0.05$; ** - $p<0.01$; ***$p<0.005$.

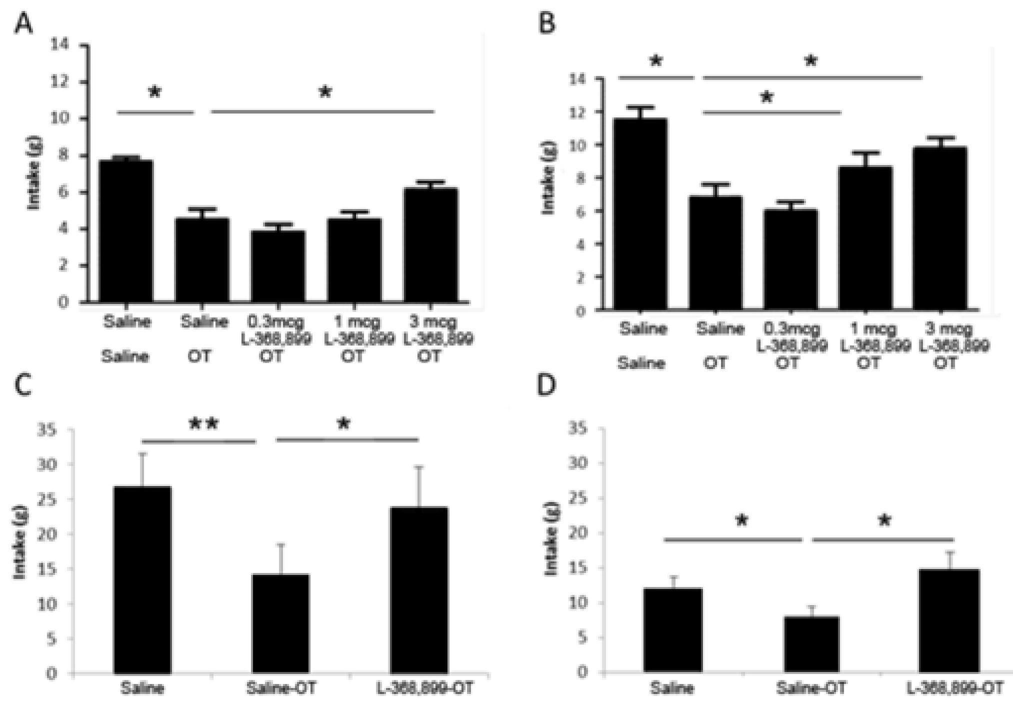

Figure 3.2: The effect of AcbC OTr antagonist (ORA: L-368,899) pretreatment on the ability of AcbC OT to reduce feeding. Deprivation-induced chow intake was measured 2h (A) and 4h (B) after a double injection of saline + saline, saline + 1 µg OT, 0.3,1,3 µg ORA + 1 µg OT. (C) Intake of 10% sucrose solution was determined 2h after AcbC double-injection of saline + saline, saline + 0.3 µg OT, and 1 µg ORA + 0.3 µg OT. (D) Intake of 0. 1% saccharin solution was measured 2h after AcbC double-injection of saline + saline, saline + 0.03 µg OT, 3 µg ORA + 0.03 µg OT. * - p<0.05; ** - p<0.01; ***p<0.005.

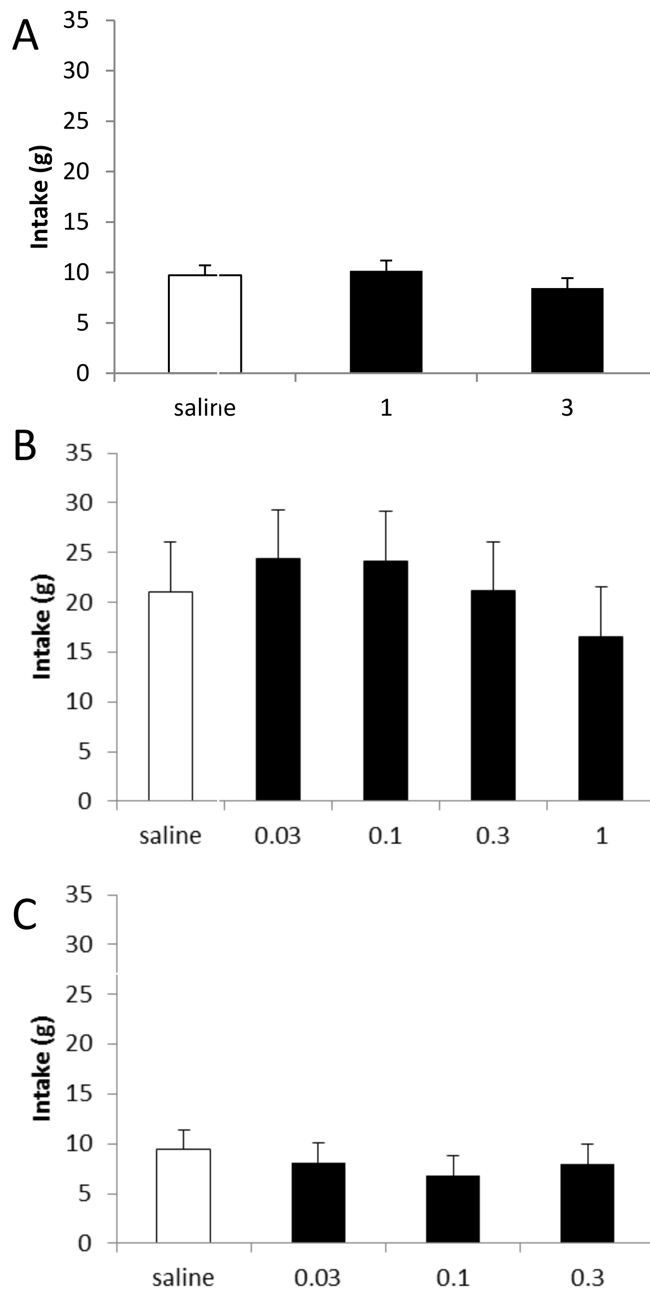

Figure 3.3: The effect of AcbC OT (doses in µg) on consummatory behavior of rats offered a meal in a social context. (A) Deprivation-induced chow intake 1 h post-injection. (B) 10% sucrose solution intake and (C) 0.1% saccharin solution intake 1h post-injection.

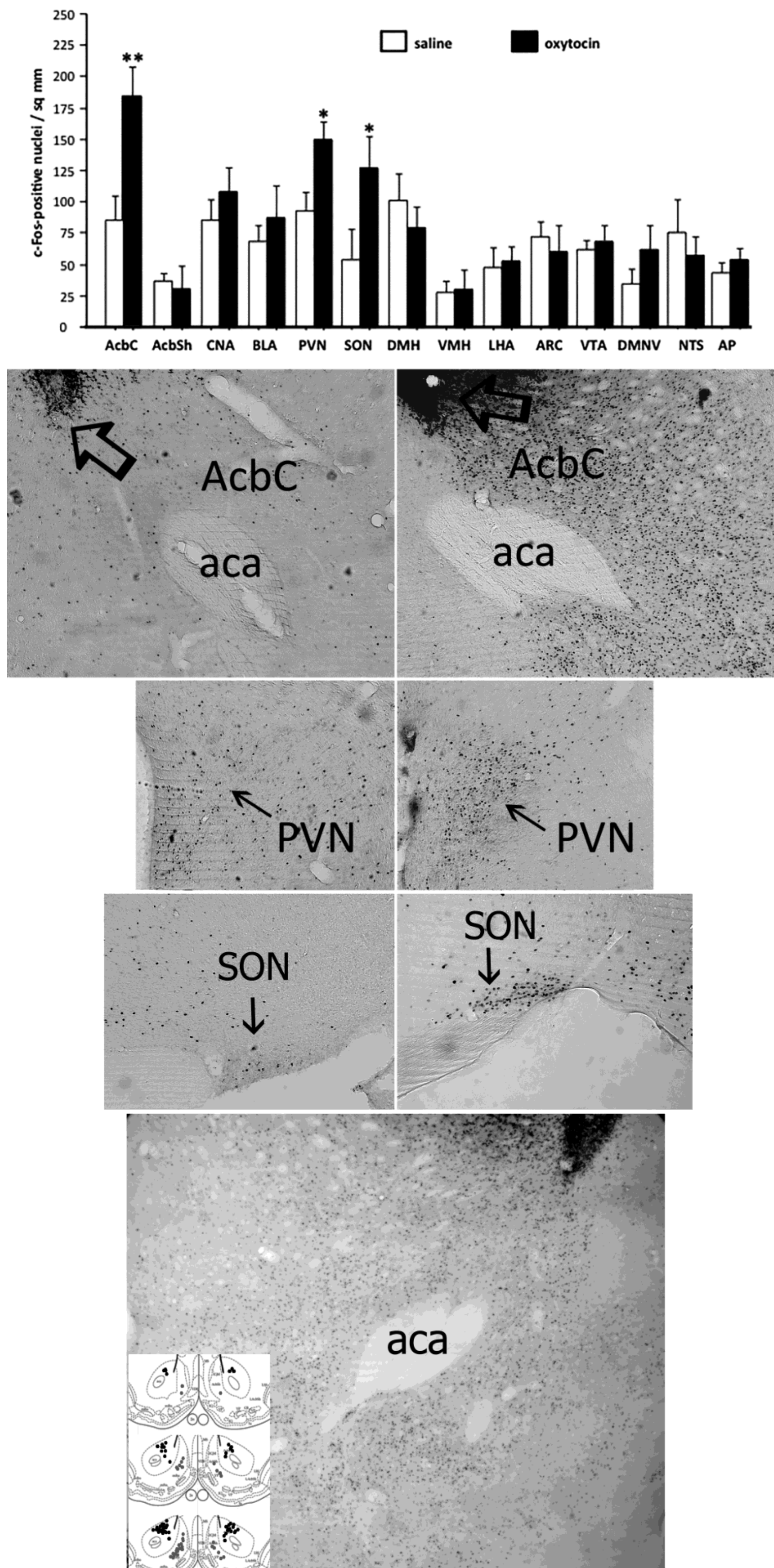

Figure 3.4: c-Fos immunoreactivity in feeding-related brain sites 1 hour after an AcbC injection of OT vs saline. The arrow indicates the injection site. Shell and core of nucleus accumbens, AcbC / AcbS; ventral tegmental area, VTA; central nucleus of the amygdala,

CNA; basolateral amygdala, BLA; dorsal motor nucleus of the vagus, DMNV; nucleus of the solitary tract, NTS; area postrema, AP; paraventricular hypothalamic nucleus, PVN; supraoptic nucleus, SON; dorsomedial nucleus, DMH, ventromedial *nucleus,* VMH; lateral *hypothalamic* area, LHA; arcuate nucleus, ARC; optic tract, otr; 3v, third ventricle. * - p<0.05; ** - p<0.01; scale bar – 0.1 mm.

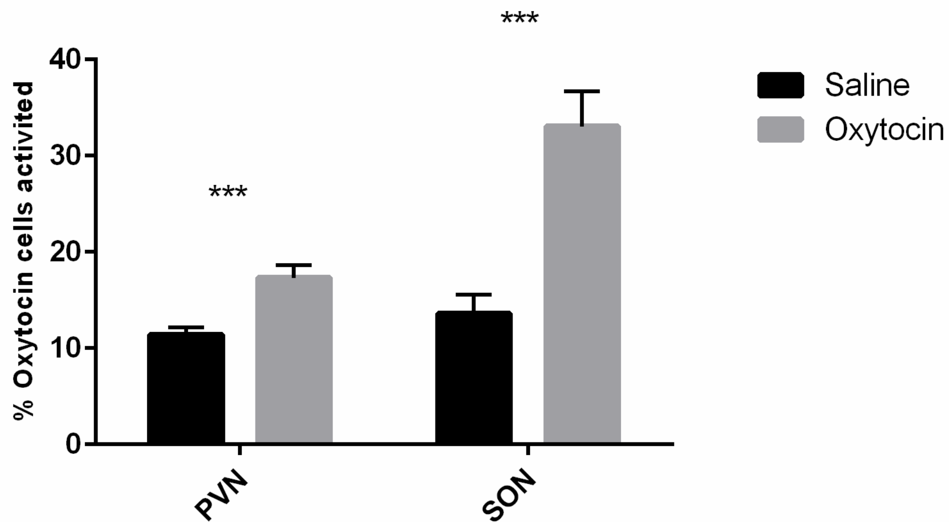

Figure 3.5: Co-localization of c-Fos and OT in the paraventricular (PVN) and supraoptic (SON) nuclei of rats injected in the AcbC with OT vs saline. *** - p<0.001.

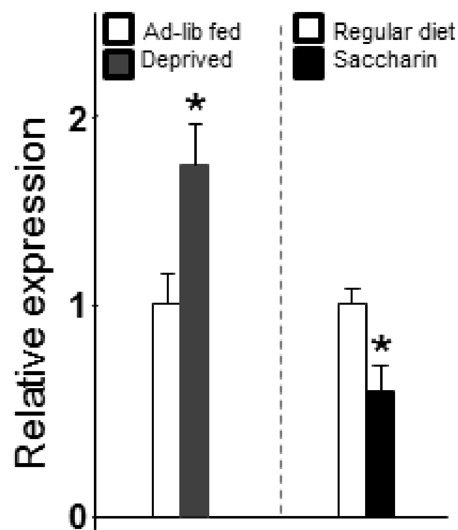

Figure 3.6: Expression of OTr mRNA in the AcbC. (A) The effect of chow deprivation versus ad libitum chow access. (B) The effect of 48-h exposure to 0.1% saccharin solution in addition to standard chow versus water and standard chow. Gene expression was assessed with real-time PCR. $^{*}P < 0.05$.

Table 3.1: The effect of the OT injected in the AcbC at saline (vehicle), 0.3, 1 and 3µg on water intake, 2h, 4h and 24h post injection. The injections were performed after an overnight deprivation of regular chow and just prior to 2-h water and chow exposure period. * - p<0.05; ns: no significant (ANOVA followed by Fisher's test).

	µg	Intake (g)						
		water 2h		***water 4h***		**water 24h**		
OXT		*mean*	*SE*	*mean*	*SE*	*mean*	*SE*	*p Value*
	saline	8.2	± 1.4	15.9	± 2.5	62.8	± 5.9	ns
	0.3	8.1	± 1.1	11.4	± 1.3	56.3	± 6.7	ns
	1	7.4	± 0.4	12.0	± 1.1	51.9	± 6.8	ns
	3	6.5	± 0.7	12.3	± 0.8	61.2	± 8.4	ns

3.4. Discussion

The classical concept of OT's involvement in the regulation of appetite revolves around the phenomena critical for maintaining and rescuing internal milieu. Indeed, OT signaling – especially within the hypothalamic-brainstem circuits -- aids in termination of eating behavior that poses threat to the water-electrolyte balance and to the functioning of the gastrointestinal tract, and that is associated with the ingestion of toxins [3, 4, 25]. The most recent discoveries have expanded upon this view as they strongly suggest that OT decreases appetite for carbohydrates and for palatable sweet tastants [26] by engaging a wider network of neural sites, including the VTA [9]. The current set of studies identifies for the first time the AcbC as a key site integrating OT's involvement in energy homeostasis and feeding reward.

We found that AcbC injections of OT decreased hunger-derived intake of “bland” chow and the palatability-driven consumption of sweet solutions. In both cases, the effect was reversible by the pre-treatment with the OTr antagonist, L-368,899, which suggests that the changes in feeding induced by AcbC OT are mediated by the OTr. The magnitude of the anorexigenic response in overnight-deprived rats to AcbC OT was similar to those observed after lateral ventricular (e.g. [27]) and VMH [28] OT infusions and somewhat smaller than the reduction in feeding after OT administration into the fourth ventricle [29]. It supports the notion that while the hindbrain OTr has a critical role for the regulation of energy balance, the forebrain populations of this receptor, including in the AcbC, should be viewed as an important contributor to central mechanisms governing feeding for calories.

One of the most crucial findings in research on OT’s influence on appetite in the recent years has been the set of discoveries linking OT and appetite for carbohydrates and for sweet taste. OT neuronal activity is particularly high after consumption of a sucrose-rich meal [26]. Generalized knockout of the OT gene in mice results in the phenotype showing sweet and non-sweet carbohydrate and saccharin hyperphagia [30]. Peripheral infusions of the blood-brain barrier penetrant OTr antagonist, L-368,899, in wild-type mice promote intake of mono-, di- and polysaccharides and saccharin [10]. Those findings sparked the debate as to whether the aforementioned effects are particularly relevant to carbohydrates, to sweet taste or to hedonic processing. The latest data as well as the results presented herein point to a key role that OT plays in the regulation of the activity of the reward circuits, including of the AcbC. Carson et al reported

that systemic injection of OT decreases methamphetamine activation of the AcbC [31]. Baracz and colleagues found that pre-treatment with OT administered IP or directly into the AcbC attenuates the formation of a methamphetamine-induced conditioned place preference [32]. The current experiments show reduced consumption of palatable sweet solutions in response to microinjections of OT in the AcbC at doses that were the same or lower than those reported for VTA administration of the peptide [9]. It should be emphasized that the observed change did not stem from altered thirst responsiveness as AcbC OT did not affect water intake in water-deprived rats. Notably, a significant decrease in saccharin solution intake was achieved with the OT dose that was ineffective in reducing the intake of chow or sucrose, which indicates that the presence of energy is not a sole factor in the regulatory mechanisms through which AcbC OT modifies consummatory behavior. This notion is strengthened by the outcome of the real-time PCR experiments in which AcbC OTr transcript levels were affected by the caloric and non-caloric challenge, i.e., by both energy deprivation and exposure to palatable saccharin. This also seems in line with the growing body of evidence suggesting that the relationship between OT and suppression of appetite is independent from energy density of tastants, as OTr ligands modify intake of solid foods (such as chow or chocolate) as well as low-calorie fluids [26, 33, 34]. Accordingly, the AcbC OT signaling appears to represent the intertwined mechanisms of caloric and non-caloric feeding control. The AcbC OT-induced increase in overall c-Fos immunoreactivity in the PVN and SON as well as in the number of activated OT neurons is very much reminiscent of neuronal activity patterns associated with the end of a meal (regardless of whether palatable or "bland" food is offered) [26, 34] and drug treatments that lead to early

termination of hunger- or reward-driven consumption [35, 36]. One should also note the fact that OT acting in the AcbC engages pathways that trigger activation of OT neurons in the hypothalamus. It suggests that AcbC OT might promote positive feedback within the accumbens-hypothalamic OT circuits, though it remains to be elucidated whether there is direct reciprocity between the AcbC-PVN/SON OT pathway components or the effect is mediated via a wider network of sites.

Many authors have reported changes in consumption after direct pharmacologic stimulation of the shell subdivision of the Acb. For example, gamma-aminobutyric acid (GABA) A and GABA B ligands administered in the AcbSh modify a short-term feeding response to food deprivation [37]. Similarly, the blockade of AcbSh glutamate receptors and mu and kappa opioids receptor affects appetite [38], [39]. Importantly, our results add to the growing evidence suggesting that also the core subdivision of the Acb controls meal size after energy deprivation as well as in palatability-driven food intake. Aside from the OTr, the GLP-1 and opioid receptors are involved in AcbC feeding mechanisms [40, 41]. In fact, the change in feeding after the AcbC OT infusion with the lack of behavioral response to the AcbSh OT treatment resembles the effects of AcbC versus AcbSh GLP-1 injections [21]. Interestingly, just as OT, GLP-1 in the Acb affects processing of feeding-related and -unrelated rewards [40, 42, 43].

While the results of our studies define AcbC OT signaling as crucial in energy- and reward-induced consumption, the CTA experiment did not show aversive consequences of the AcbC OT treatment. The lack of the learned avoidance response strongly suggests that the anorexigenic effects of OT do not stem from

undesirable sickness/malaise. It lends us confidence in classifying AcbC OT's role as related to appetite regulation rather than to learning avoidance of potentially dangerous foods. It should be noted that the reason behind assessing CTA effects of AcbC OT was not only to control for drug-induced illness, but also to examine whether the OTr in the AcbC might be physiologically involved in the development of taste aversions: It has been previously shown that OT is released in response to the presence of toxins, contributing to malaise-dependent anorexia [12], and that a systemic OTr antagonist treatment impairs acquisition of a CTA [44].

For a number of reasons, including practicality of an experimental setup, the vast majority of feeding studies reported here and by other authors have been performed on animals housed individually. One should not forget though that food intake is oftentimes a social behavior, in which initiation, termination, dietary choices and meal duration, are influenced by the characteristics of the social environment. This particular issue needs to be considered especially in studies on neural systems that regulate both appetite and affiliative behaviors, including OT.

OT facilitates pair bonding, mother-infant bonding, and social approach/recognition, and diminishes anxiety [45-49]. Peripheral and central OT release has been reported in response to positive social exposure [50]. Importantly, socially stimulated changes in Acb activity have been proposed to influence other concurrent physiological and behavioral parameters. For example, studies on the link between the Acb and drug abuse have shown that preference for cocaine can be modified by social interaction [51]. Lesions of the

AcbC induce preference for the social compartment in the CPP paradigm, whereas lesions of the AcbSh shift it towards the cocaine-associated chamber [52]. The results of the current study indicate that a social context has a profound effect on the ability of OT to affect feeding via the AcbC. Once the animal is placed in the environment in which social interaction occurs, intra-AcbC administration of OT does not diminish food intake driven by hunger or feeding induced by sweet flavor. While at present, it is extremely difficult to speculate on the exact nature of the observed phenomenon, it fits well with the previously reported findings showing that orexigenic effects of peripherally administered OTr antagonist in mice that were offered a meal in a social setting depended on the social status of animals [53]. Therefore, social cues appear to act as a modifying factor in the ability of OTr ligands to affect appetite. It is likely that in the case of a crosslink between social interactions and feeding, OT acts as a secretagogue of behavioral activity in response to complex cues. A similar hypothesis has been proposed in relation to OT facilitating a switch from food intake to affiliative or sexual behavior, thus balancing behaviors that contribute to internal homeostasis and evolutionary success [54, 55].

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Chapter 4

Is there a functional relationship between oxytocin and connexin 36 in the regulation of feeding?

The data present in Chapter 2 and 3 of this thesis as well as the results reported by several laboratories indicate that central OT is involved in the regulation of three key aspects of food intake regulation: (a) it promotes termination of food intake upon consumption of high volume of food and associated increase in salt loading; (b) it decreases intake of palatable carbohydrates and sweet non-carbohydrate, saccharin, thereby reducing feeding for reward; and (c) it brings on an immediate discontinuation of ingestive behavior when food is tainted with toxins (in the laboratory setting, this phenomenon is visualized through a conditioned taste aversion paradigm).

These effects of OT are mediated by a widespread network of feeding-related brain sites that encompass areas classically viewed as involved in feeding for energy homeostasis (and homeostasis in general) and sites controlling consumption for reward. The hypothalamic – brainstem pathways largely

contribute to the homeostatic aspect of OT-driven regulation of appetite, preventing excessive distention of the stomach or abnormal plasma osmolality and promoting avoidance of foods whose ingestion might cause sickness/malaise [1]. On the other hand, the reward system – mainly the nucleus accumbens and ventral tegmental area – mediates OT's inhibitory effects on the consumption of carbohydrates and non-carbohydrate sweet tastants, such as saccharin [1]. Importantly, as evidenced in the previous chapters of this thesis and Mullis et al. [2], the role of the aforementioned sites in particular aspects of feeding control is not rigid: for example, the Acb injections of OT decrease both feeding for reward and deprivation-induced intake of "bland" yet energy-dense chow; and generalized injections of OTr antagonists increase intake of palatable tastants (Chapter 2 and [3]).

The quest for a better understanding of the nature of OT's involvement in feeding regulation has led me to explore a question of whether OT would still affect the key aspects of food intake in a unique animal model characterized by a combined dysregulation of feeding for energy, feeding for reward, and avoidance of toxic foods.

Therefore, **in Part A of this chapter (Section 4.1)**, **I present the ingestive behavior characterization of a novel knockout (KO) mouse strain that does not express a key gap junction protein, connexin 36** (Cx36) and – consequently – shows aberrant intake of energy-dense "bland" food, decreased intake of palatable tastants and hypersensitivity to aversive stimulation. It should be emphasized that Cx36 is ubiquitous in the reward system as well as in the hypothalamus and brain stem – hence, all areas through which OT exerts its

action on homeostatic and reward-driven feeding. Properly functioning gap junctions ensure integrity of the neural circuit by providing a means of communication between adjacent neurons. Therefore, I hypothesize that - in the Cx36-deficient feeding-related brain networks in the Cx36 KO mouse - OT might show diminished effectiveness in modifying most relevant aspects of food intake. **This functional relationship between OT and Cx36 in the feeding context is explored in Part B of this chapter (Section 4.2)**.

4.1. (PART A) Connexin 36 KO mice show overconsumption of high-energy "bland" food, diminished sensitivity to feeding reward and excessive taste aversions.

Abstract

Connexin 36 (Cx36) is a gap junction molecule ubiquitously expressed in central areas that govern energy homeostasis, reward and food aversions. It has been shown that Cx36 deletion is associated with dysregulation of GABA and dopamine in reward pathways and with decreased ethanol intake in Cx36 knockout (KO) mice. Here, we used the Cx36 KO model to investigate whether the lack of Cx36 affects key specific aspects of food intake control: eating for energy, eating for pleasure, and food avoidance resulting from an association between consumption of a given tastant and subsequent gastrointestinal sickness. To study an association between Cx36 and homeostatic control of feeding, we determined intake of standard chow in ad libitum-fed Cx36 KO versus WT mice as well as a magnitude of a conditioned taste aversion (CTA) response to lithium chloride (LiCl). A potential relationship with feeding reward was studied by assessing intake of palatable sweet and fatty tastants in WT vs Cx36 KO mice, by examining expression profile of opioid system genes involved in feeding reward, and sensitivity of Cx36 KOs to feeding reward modifying opioid ligands, naltrexone and butorphanol. Cx36 KOs showed increased intake of energy-dense "bland" chow and decreased consumption of sweet and fatty palatable solutions offered episodically and in an unrestricted manner. Cx36-

deficient mice maintained on a regular diet displayed an altered baseline expression profile of opioid system genes in the hypothalamus and nucleus accumbens. Cx36 KOs exhibited a diminished sensitivity to consumption modifying properties of naltrexone and butorphanol (sucrose) or a lack thereof (saccharin). Finally, Cx36 KOs developed taste aversions even after administration of LiCl at a dose that was subthreshold in the WTs. Overall, the data indicate that Cx36 is involved in the "homeostatic" (energy intake and aversions) and "non-homeostatic (reward) mechanisms governing appetite. Its absence shifts the threat-pleasure continuum by reducing feeding reward and promoting hypersensitivity to negative stimuli associated with a meal. It supports ingestion of highly caloric, "bland" and "safe" foods.

4.1.1. Introduction

Gap junctions (GJs), pores formed by connexin (Cx) subunits, provide a means for intercellular communication in the brain [4]. The passage of molecules through neuronal GJs coordinates cell firing, and metabolic as well as transcriptional events between coupled neurons. Compared to chemical synapses, GJ electrical synapses synchronize outputs of coupled neurons and allow ultra-fast spread of information.

Thus far, 20 connexin genes have been defined [5]. Among the two types of Cxs most ubiquitously expressed in mammalian neurons, only Cx36 is prevalent in the mature CNS [6]. Cx36 positive neurons have been demonstrated in a number of brain areas involved in cognition, learning, memory and emotion-related processing, including the hippocampus, cerebral and piriform cortex, amygdala,

mesencephalon, and thalamus [6]. Cx36 has been shown to facilitate plasticity [7], promote glutamate-mediated cell death post-injury [8], affect motor learning [9, 10] mediate sensitivity to anesthetic drugs [11, 12], and affect learning- and anxiety-related parameters [13].

From the standpoint of food intake regulation, it is extremely important that Cx36 is abundantly expressed in the vast network of central sites affecting appetite, especially the mesolimbic reward system, hypothalamus, and various brain stem nuclei [6]. Cx36 has been shown to be co-expressed in corticotropin releasing hormone (CRH) neurons, which are part of the brainstem-hypothalamic pathways regulating feeding for energy and participating in the generation of emetic responses [14]. A relationship between Cx36 and reward has been underscored by comprehensive electrophysiological studies that established the role of Cx36 in the generation of high-frequency oscillations and synchrony [15], particularly within GABA and dopamine (DA) circuits [16-19]. Behavioral experiments have brought a groundbreaking discovery that Cx36 knockout (KO) mice are less prone to drinking alcohol. The loss of Cx36-dependent electrical coupling within the key reward pathway component, the ventral tegmental area (VTA), leads to the dyssynchrony of the GABA system resulting in disinhibition of DA neurons (hyper-DA state), and consequently decreases the hedonic value of ethanol intake [19].

Surprisingly, despite evidence suggesting a potential link between Cx36 and appetite, thus far, this issue has not been investigated in detail. Therefore, in the current set of studies, we used the Cx36 KO mouse model to investigate whether the lack of Cx36 affects key specific aspects of food intake control: eating for

energy, eating for pleasure, and food avoidance resulting from an association between consumption of a given tastant and subsequent gastrointestinal sickness. The regulation of calorie consumption as well as avoidance of potentially toxic foods can be viewed as “homeostatic” control of appetite, whereas eating for pleasant taste is driven by the rewarding component of gustatory mechanisms. First, in order to establish energy-driven appetite in the absence of Cx36, we measured consumption of standard chow in ad libitum-fed Cx36 KO versus WT mice. Then, in order to determine whether there is a link between Cx36 and feeding reward, we measured the consumption of sweet and lipid solutions in episodic and unrestricted access paradigms in Cx36 KO versus WT mice and substantiated our findings by using a pharmacological blocker of Cx36 gap junctions, quinine, in WT animals. As the consumption of palatable tastants was lower in the KO animals, we hypothesized that the genetic deletion of Cx36 affects baseline expression of opioid system-related genes, which are the key components of central mechanisms governing feeding for pleasure [20] thereby diminishing KO animals’ sensitivity to feeding reward. Hence opioid transcript levels were assessed with real-time PCR in the hypothalamus and Acb of Cx36 KOs and their background strain. Finally, we tested sensitivity of Cx36 KOs versus WTs to feeding inhibitory properties of an opioid receptor antagonist, naltrexone, and feeding stimulatory properties of an agonist, butorphanol, on sugar and saccharin solution intake. Finally, in order to examine whether Cx36 status affects aversive responsiveness, we injected Cx36 KO and WT mice with the same low dose of malaise inducing toxin, LiCl, and determined whether the magnitude of a conditioned taste aversion (CTA) differs between the genotypes.

4.1.2. Materials and Methods

4.1.2.1. Animals

The Cx36 KO model, developed and kindly provided to our laboratories by Prof. David Paul [21], has been used by us and others in earlier studies [22], [23]. Male homozygous Cx36 KO and WT littermates (C57/B6-129SvEv mixed background) were individually housed in conventional cages with a 12:12 LD photoperiod (lights on at 0700) in a temperature-controlled room (21°C). Age-matched animals weighed ca. 26 g ± 3 at the beginning of the studies (there was no difference in b. wt. between the genotypes). Mice had unlimited access to tap water and standard rodent chow (Teklad Global Diet 2018) throughout the studies unless noted otherwise. The procedures described herein were approved by the University of Waikato animal ethics committee.

4.1.2.1.1. Energy-driven intake of standard chow in Cx36vKO vs WT mice

Age-matched Cx36 KO and WT male mice (n=8/genotype; there is no difference in body weight between the strains: 26 g ± 3) had intake of regular ("bland") chow and water measured every 24 h for 3 days. The animal had not been exposed to other non-standard tastants. The amounts of chow and water consumed were calculated per kg of body weight and the means ± SEM were established for each genotype. A Student's t-test was used to establish the difference in consumption between WT and Cx36 KO mice (significant when $p \leq 0.05$).

4.1.2.1.2. Episodic intake of palatable tastant solutions in Cx36 KO vs WT mice

In a series of experiments, we tested the intake of 0.1% saccharin, 5% sucrose, 5% glucose, 5% fructose or 4.1% Intralipid (Fresenius, Sweden) solutions in a single-bottle no-choice paradigm. Cx36 KO and WT mice (n=13-18/tastant) had been pre-exposed to the respective solutions 2 h/day (10:00–12:00) for 5 days. Chow and water were removed from the cages for the 2-h period of palatable tastant presentation. The animals were then given a single bottle containing a palatable tastant. The amount of the solution consumed was calculated and the means were reported in g/kg of b. wt. A Student's t-test was used to assess differences in consumption for each of the palatable solutions between WT and KO animals (significant when $p \leq 0.05$).

As a control study for the ability of Cx36 gap junctions to affect episodic consumption of palatable tastants, we subjected the WT mice to a similar paradigm of saccharin solution availability as described above, injected them with saline or 30 mg/kg b. wt quinine (Cx36 gap junction blocker) 5 minutes before palatable tastant presentation and determined saccharin solution intake in the 2-h test period. The dose of quinine was selected based on previous studies showing antinociceptive and antiepileptic effects of the compound [24], [25]. The amount of consumed saccharin solution was expressed in g/kg b. wt. and the data of saline vs. quinine groups compared with a t-test (significant when $p \leq 0.05$).

4.1.2.1.3. Unrestricted intake of palatable solutions in Cx36vKO vs WT mice

We assessed the intake of 0.1% saccharin, 5% sucrose, 5% glucose, 5% fructose or 4.1% Intralipid when each of these tastants was available ad libitum (along

with standard chow and water) for 3 consecutive days. KO and WT mice were given a single bottle containing a palatable tastant for 3 days (n=8/WT and n=8 KO for each tastant). The amount of the tastants and chow consumed on Day 2 and 3 (data from Day 1 were not included in the analysis to discount the potential effect of novelty) was calculated and the means were reported in g/kg of b. wt. A Student's t-test was used to establish the difference between WT and Cx36 KO consumption in consumption (significant when $p \leq 0.05$).

4.1.2.2. Baseline expression of opioid system genes in the hypothalamus and Acb in Cx36 KO vs. WT mice

Wt and Cx36 KO mice (n=8/group), maintained on ad libitum access to standard chow and water, were decapitated (10:00-11:00) and the hypothalamus and Acb were collected according to the Paxinos and Watson brain atlas. The tissue was placed in RNAlater at room temperature for 2h and then stored at -80°C. RNA was extracted by the Quick-RNA^{TM}kits (Zymo Research) and the absence of DNA was confirmed by PCR. Total RNA concentration was measured with Nanodrop 2000 (Thermo Scientific). For cDNA synthesis, 9 µl of RNA was reverse-transcribed in a final volume of 20 µl containing 10 µl 2X RT Reaction Solution and 1 µl Enzyme Mix (HiSenScript^{TM} RH(-) cDNA Synthesis Kit, iNtRON Biotechnology). The reaction was performed for 1h at 42°C, followed by 5 min at 85°C and 5 min at 4°C, and the presence of cDNA was confirmed by PCR. Each rtPCR, with a total volume of 20 µl, contained 8 µl template cDNA, 2µl Primers Mix (Forward and Reverse primers) and 10 µl of 2X RealMOD^{TM} GH Green Real-time PCR Master Mix (Master Mix Kit, iNtRON). rtPCRs were done in duplicates, and negative controls were included on each plate. Amplification was performed

as follows: denaturation at 94°C for 5 min, 50 cycles of denaturation at 94°C for 10 s, annealing for 15 s, and extension at 62°C for 30 s. Three housekeeping genes were analyzed (Table 4.1). A Rotor-Gene SYBR Green PCR (QIAGEN) was used. Data analyses have been performed by Rotor-Gene 6000 software 1.7 (QIAGEN). Primer efficiencies of 11 genes (Table 4.1) were calculated and samples were corrected for differences in efficiencies. The Pfaffl equation [26] [27] was used to calculate normalization factors based on housekeeping gene expression. Differences in gene expression between groups were analyzed using a t-test (different when $p \leq 0.05$).

4.1.2.3. Effect of opioid receptor agonist and antagonist on consumption of sweet palatable tastants in Cx36 KO vs. WT mice

Several days prior to the beginning of the experiments, Cx36 KO and WT mice had been pre-exposed to 0.1% saccharin or 10% sucrose solutions for 24 h to prevent neophobia. Similar to the episodic exposure experiments described above (section 4.1.2.1.2), animals (n=7-10/group) were accustomed to having access to either 0.1% saccharin or 10% sucrose for 2 h/day (1000–1200 h). Standard food and water were removed for the 2-h period of palatable tastant presentation. Five min prior to palatable solution exposure, mice were injected IP with (a) saline or 0.03, 0.1, 0.3, 1 and 3 mg/kg b. wt. of naltrexone or (b) saline or 0.1, 0.3, 1 and 3 mg/kg b. wt. of butorphanol tartrate. The amount of the palatable tastant consumed during the 2-h period was calculated in g/kg of b. wt. One-way ANOVA followed by Fisher's post-hoc test was used to establish effective doses of naltrexone and butorphanol in saccharin and sucrose consumption for the Cx36 KO and WT strains (different when $p \leq 0.05$).

Table 4.1.1 Forward and reverse real-time PCR primer sequences.

Opioid system genes		
	Forward	Reverse
KOR	CAC CTT GCT GAT CCC AAA	TTC CCA AGT CAC CGT CAG
MOR	CCT GCC GCT CTT CTC TGG	CGG ACT CGG TAG GCT GTA AC
PNOC	AGC ACC TGA AGA GAA TGC CG	CAT CTC GCA CTT GCA CCA AG
DYN	GAC AGG AGA GGA AGC AGA	AGC AGC ACA CAA GTC ACC
ORPL1	ATG ACT AGG CGT GGA CCT GC	GAT GGG CTC TGT GGA CTG ACA
PENK	CGA CAT CAA TTT CCT GGC GT	AGA TCC TTG CAG GTC TCC CA
Housekeeping genes		
B actin	TGG CAC CAC ACC TTC TAC AAT GAG	GGG TCA TCT TTT CAC GGT TGG
Atp5b	GGC ACA ATG CAG GAA AGG	TCA GCA GGC ACA TAG ATA GCC
B tub	CGG AAG GAG GCG GAG AGC	AGG GTG CCC ATG CCA GAG C

4.1.2.4. Effect of lack of Cx36 on acquisition of LiCl-induced CTA

We used a standard CTA protocol in which Wt and KoCx36 mice were accustomed to having access to water for 1 h per day (11:00–13:00, 2d). Standard chow was removed from hoppers for the 1-h period of scheduled fluid presentation. On day 3, mice were given a novel flavored drink (pineapple juice, Golden Circle, Australia; composition: per 100ml, Energy 219kJ, Total carbs 12.2g, Sugars 10.6g) instead of water for sixty minutes and immediately after the end of the drinking session they received an IP injection of LiCl (Sigma; 0.6 mEq/kg b. wt.) or saline (n=8/group). On day 4, a standard two-bottle preference test (flavored solution versus water) was used to assess acquisition of a CTA in Wt vs KoCX36 mice. Bottles were weighed and percentages of the juice solution intake

(out of cumulative, i.e. sweetened solution plus water, intakes) were calculated. A difference in consumption between groups was calculated using a one-way ANOVA followed by Fisher's post-hoc test. A Student's t-test was used to establish the difference consumption between two groups (significant when $p \leq 0.05$).

4.1.3. Results

Mice lacking the functional Cx36 gene showed elevated intake of energy-dense regular ("bland") chow in an ad libitum feeding paradigm. Adult (age-matched), same-weight Cx36 KOs ate more energy-dense chow over a 24-h period ($p=0.0001$) than their WT counterparts (Figure 4.1.1).

On the other hand, the KO animals drank significantly less of the sweet palatable solutions and lipid emulsion (saccharin, $p=0.008$; sucrose, $p=0.009$; glucose, $p=1E-5$; fructose, $p=0.0003$, Intralipid, $p=0.002$) offered in a brief 2-h session (with no chow or water available during that time; Figure 4.1.2A). A similarly diminished intake of the palatable tastants was observed when saccharin ($p=0.02$) sucrose ($p=2E-6$), glucose ($p=2E-6$), fructose ($p=2E-7$) and Intralipid ($p=0.0002$) were given in an unrestricted manner for 48 h (chow/water available ad libitum; Figure 4.1.2B). Importantly, water intake did not differ between KO and WT mice ($p=0.23$).

Cx36 blocker, quinine, administrated IP just before the 1-h period of exposure to the saccharin solution in WT mice, significantly decreased the intake of this sweet tastant ($p=0.004$; Figure 4.1.2C).

In the PCR studies, we found that baseline expression of opioid system genes differs in Cx36KO vs WT mice maintained on a standard diet (Figure 4.1.3). The KOs showed decreased mRNA levels of KOR (p=1E-06), MOR (p=5E-08), PNOC (p=0.0005), ORPL1 (p=6E-05), and increased DYN mRNA content (p=1E-05) in the hypothalamus, whereas PENK transcript expression was higher (p=0.03) in the nucleus accumbens.

NTX or butorphanol injections in animals given access to saccharin or sucrose, produced different dose-response profiles in Cx36 vs WT mice (saccharin: ANOVA F=6 p=2E-4, sucrose ANOVA:F=4 p=0.02) (Figure 4.1.4). While the 0.1-mg and 0.3-mg doses of NTX decreased sucrose consumption in WTs (p=0.02 and p=0.01, respectively), 3 mg NTX had to be used to generate a reduction in sucrose intake in KOs (p=0.007:). NTX at 1 mg (p=0.008) decreased saccharin intake in WT animals, but even the 3-mg dose was ineffective in the KO strain (p=0.6).

Conversely, butorphanol increased sucrose consumption at 0.3 mg (p=0.02); and 1 mg (p=0.004) in WTs, whereas 3 mg (p=0.01) was the lowest effective dose in Kos (ANOVA: F= 6.5 p= 2E-4)(Figure 4.1.4C). Saccharin consumption increased after injection of 0.3 and 1 mg butorphanol in WT mice (p=0.004 and p=0.004, respectively ANOVA F= 11.8 p<1E-4) but it remained unchanged even after the 3-mg butorphanol treatment (Figure 4.1. 4).

LiCl injected at a 0.6 mEq/kg dose was not sufficient to induce a conditioned taste aversion in WT mice: LiCl- and saline-treated WTs showed similar preference for the flavored solution. On the other hand, LiCl at the same dose induced a robust aversive response in Cx36 KO mice (p=8E-5, ANOVA F=13

p=1.7E-7) as their flavored solution intake on the subsequent presentation in a two-bottle test was decreased by 70% (Figure 4.1.5)

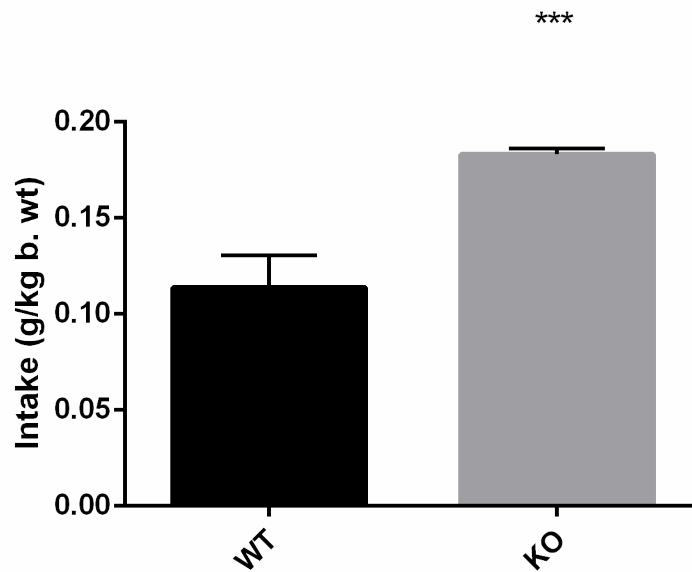

Figure 4.1.1: Connexin 36 knockout (Cx36 KO) mice consume more regular chow in an unrestricted 24-h access paradigm, compared to wild-type (WT) controls. *** - p≤0.001.

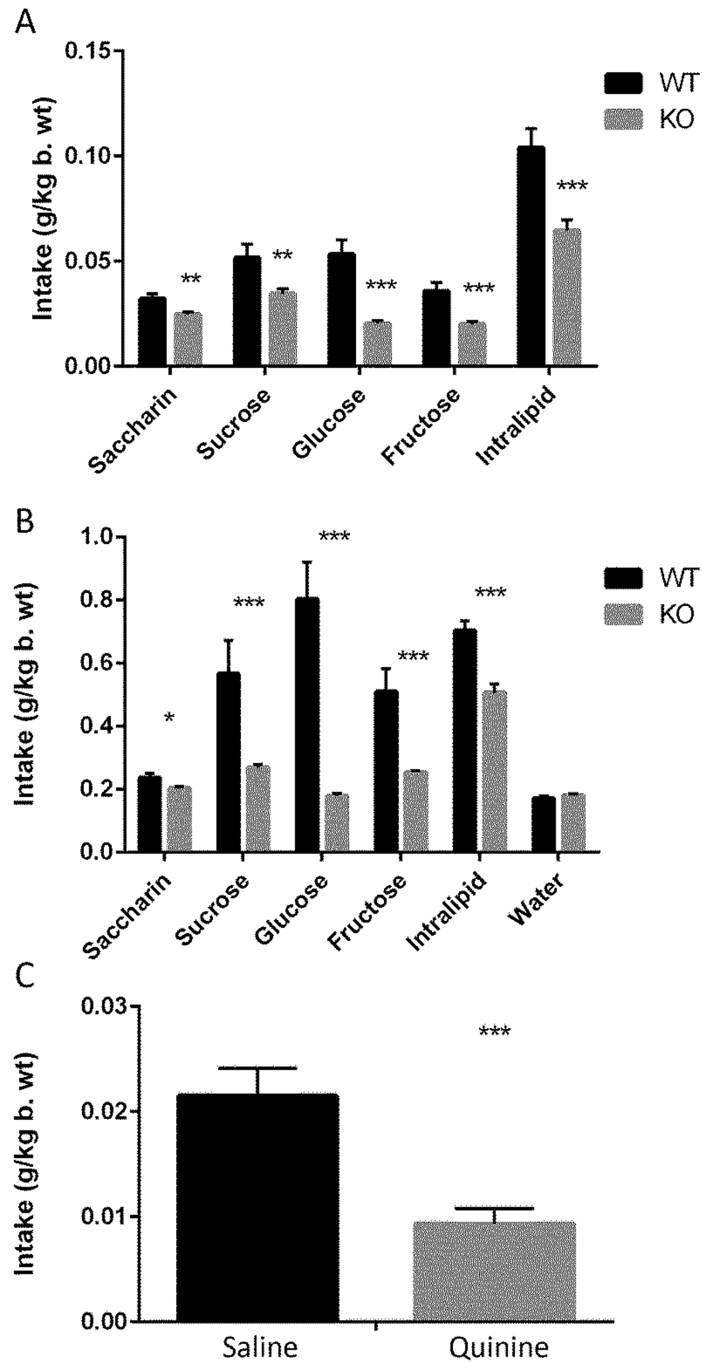

Figure 4.1.2: Connexin 36 knockout (Cx36 KO) mice consume smaller amounts of palatable solutions offered **(A)** episodically for 2 h or **(B)** in an unrestricted 48-h access paradigm, compared to wild-type (WT) controls. A peripheral injection of a pharmacological blocker of Cx36 gap junctions, quinine, reduces saccharin solution intake **(C)**. * - p≤0.05; ** - p≤0.01; *** - p≤0.001.

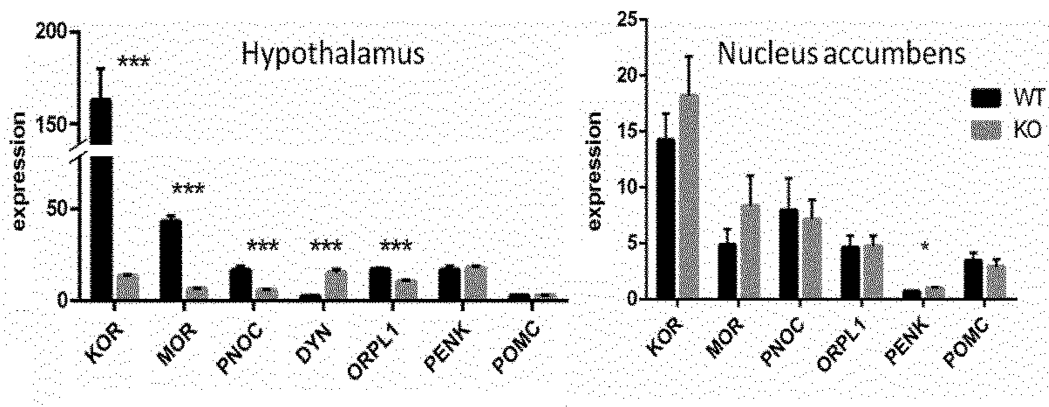

Figure 4.1.3: Connexin 36 knockout (Cx36 KO) mice having unrestricted access to standard food and water display a different baseline mRNA expression profile of select opioid system genes than wild-type (WT) controls. Gene expression levels in the hypothalamus and nucleus accumbens were measured with real-time PCR. KOR, kappa opioid receptor; MOR, mu opioid receptor; PNOC, pronociceptin; ORPL1, nociceptin/orphanin FQ receptor; PENK, proenkephalin; POMC, proopiomelanocortin. * - p≤0.05; ** - p≤0.01; *** - p≤0.001.

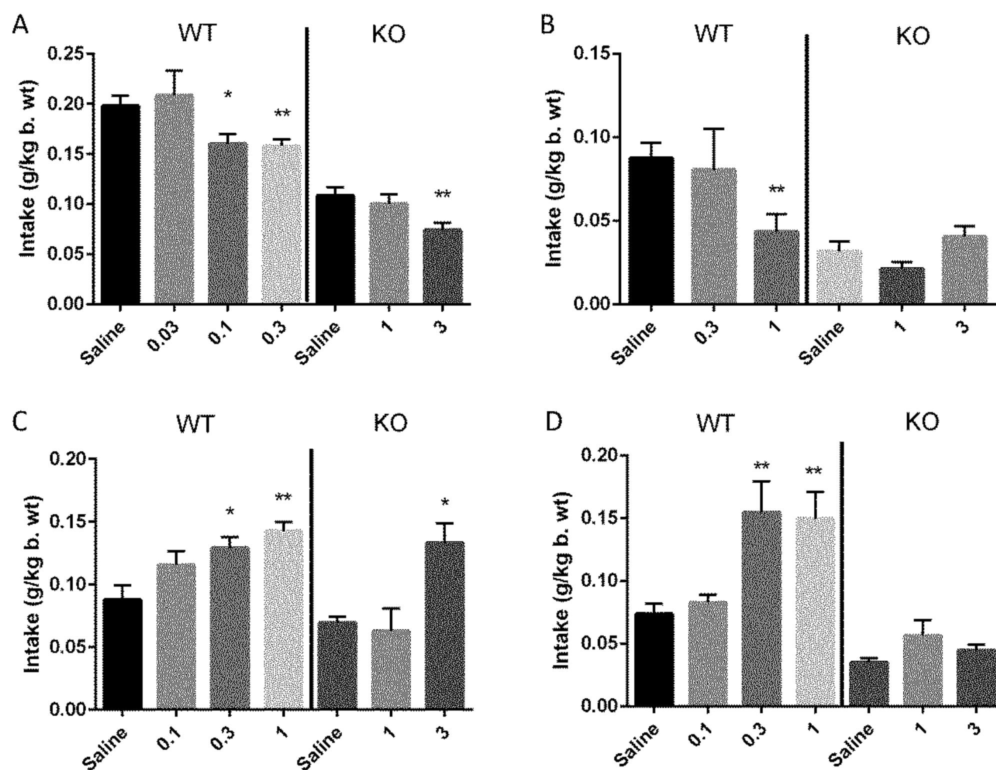

Figure 4.14: Effects of naltrexone **(top: A, B)** and butorphanol tartrate **(bottom: C, D)** on the intake of sucrose (A,C) and saccharin (B, D) solutions offered for 2 h to connexin 36 knockout (Cx36 KO) and wild-type (WT) mice. Doses of naltrexone and butorphanol represent mg/kg b. wt. injected intraperitoneally. * - p≤0.05; ** - p≤0.01; *** - p≤0.001.

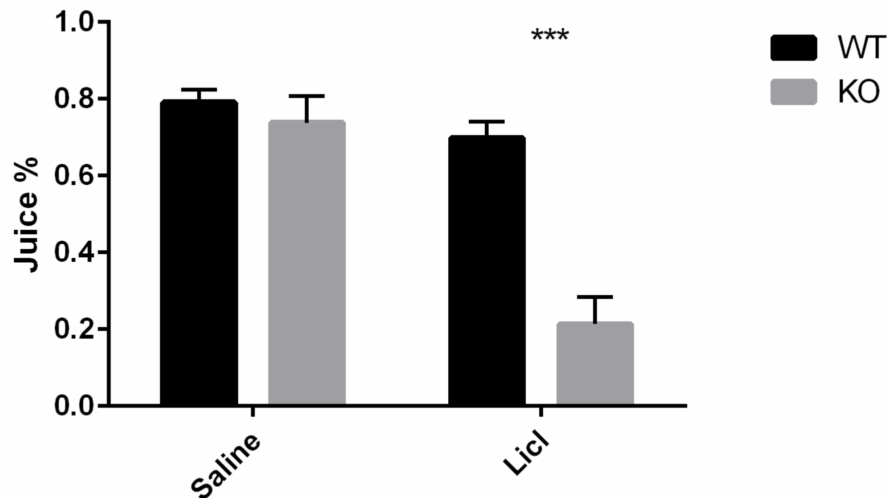

Figure 4.1.5: A CTA response after injection of the same 0.6 mEq dose of LiCl in Cx36 KO vs WT mice. The graph shows relative (%) intake of a flavored solution offered in a two-bottle choice test in water-deprived animals (water was the control fluid in the two-bottle test).*** - p≤0.001.

4.1.4. Discussion

A dynamic balance between numerous mechanisms that govern appetite determines the overall energy consumption, proper macro- and micronutrient intake, as well as avoidance of those foods that – despite their attractive taste or energy content – may provoke unpleasant gastrointestinal sensations [28, 29]. A dysregulation of this intricate system may cause most profound metabolic and energy balance consequences for the organism by, for example, inducing excessive intake of highly palatable tastants, shifting the drive to eat away from foods that bring energy to those of low nutritional yet high gustatory value, and failing to prevent intake of toxic ingestants [30]. The current set of studies shows that Cx36 is critical in central processing of energy homeostasis, reward and aversion, thus, it appears to be part of the molecular mechanisms that serve as the broad foundation of appetite control.

The mesolimbic reward pathways as well as reward-related molecules scattered throughout the widespread network of sites that govern food intake and energy metabolism, promote overconsumption of tasty foods [31, 32]. Cx36, as a key molecular component of gap junctions in the adult CNS, serves a critical role in ensuring functional integrity of brain circuits, including those involved in reward [6, 19]. It has been previously shown that silencing Cx36 expression leads to a disruption of GABA and DA signaling [16-19]. Genetic deletion of Cx36 has also been found to decrease ethanol consumption in mice [19]. The current set of data shows that Cx36 gene knockout and the pharmacological blockade of Cx36 GJs diminish intake of palatable solutions in mice. The dysregulation of the opioid system - a consequence of impaired Cx36 GJ coupling - likely underlies this reduced drive to eat for pleasure.

Animals typically prefer sweet and fatty foods, and they ingest large quantities of solid and liquid diets that contain sugars, non-carbohydrate sweeteners, and/or fats, regardless of overall caloric density of such diets [33, 34]. Importantly, reward-driven consumption of solid and liquid diets occurs regardless of the fact whether the animal has or has not been food- or water-deprived [35, 36]. Non-deprived Cx36 KO mice did consume the palatable liquid diets given episodically and in an unrestricted manner, which suggests that these solutions are not completely devoid of hedonic value to these animals. However, each of the palatable tastants was consumed less avidly by the KOs compared to their WT counterparts, indicating that the processing of rewarding aspects of consumption of these liquid diets was impaired in the Cx36-deficient mice. That water intake does not differ between KOs and WTs makes it unlikely that the effect was cause

by differences in thirst responsiveness. The fact that KOs ingests less of the non-caloric saccharin solution than WT controls rules out a scenario in which diminished energy needs are the culprit underlying lower intake of palatable tastants. It should also be noted that the feeding reward experiments presented herein employed a wide variety of tastants that engage different subsets of taste receptors; therefore, along with the previously reported data on the relationship between Cx36 and alcohol-derived reward [19], our results imply that aberrant processing of reward in Cx36 KOs expands upon feeding for pleasant taste. At the same time, it should be noted that while genetic deletion of Cx36 led to a reduction of feeding for palatability, it was associated with a higher baseline level of "bland" chow consumption. It should be emphasized that the more avid intake or regular chow in the KOs did not result in differences in body weight between the two genotypes, which suggests that metabolic and/or absorptive processes might be affected by Cx36 knockout. Importantly, regardless of the nature of energy conversion efficiency, KO animals' enhanced appetite for high-energy foods indicates a change in feeding control that promotes an increase in energy consumption.

Interestingly, administration of quinine in non-deprived WT mice just prior to their gaining brief episodic access to the saccharin solution generated a decrease in saccharin intake. The parallel effects of gene deletion and pharmacological blockade of the GJ molecule lend us more confidence in defining Cx36 as being involved in feeding reward. Overall, changes in reward processing are evoked not just by a constitutive absence of the Cx36 gene, but also by using a transient Cx36 blocker; hence they can be dependent on acute changes in the Cx36 GJ

functional status as well as on long-term molecular changes associated with the lack of Cx36.

It has been previously reported that Cx36 deficiency in the KO model leads to significant abnormalities in DA and GABA signaling within the VTA-accumbens pathways, thereby contributing to the impaired processing of rewarding stimulation [16-19]. Our real-time PCR data suggest that changes in the molecular content of reward-related circuits are even more profound as the baseline expression profile of genes that give rise to select opioid peptides and receptors is greatly affected. The key role of endogenous opioids in palatability-induced consumption has been shown beyond reasonable doubt. It is well known that opioid receptor agonists induce intake of preferred tastants, while antagonists are effective in decreasing intake of tasty foods [37, 38]. Modifications in access to palatable diets affect opioid peptide/receptor mRNA and protein levels [39-41], whereas constitutive and conditional changes in expression of opioid system genes are associated with an altered drive to ingest rewarding tastants [42-44]. That Cx36 KO animals maintained on standard “bland” chow show different levels of opioid mRNAs indicates that - already at the baseline behavioral state - there is an atypical expression of genes related to feeding reward, most likely being one of the underlying factors in aberrant processing of palatability in Cx36 KOs. It should be noted that opioid system mRNA levels were changed not only in the nucleus accumbens, which is intuitive taking into account its role in reward, but predominantly in the hypothalamus, where the receptor transcripts were greatly affected. While numerous intraparenchymal injection studies have shown that opioid receptors in the

hypothalamus modify palatability-driven feeding [45], they are also though to couple reward system's activity with the homeostatic and neuroendocrine responses of the hypothalamus [37, 46] .

The real-time PCR findings showing an altered expression profile of opioid-related genes are further substantiated by the studies utilizing injections of opioid receptor ligands that are known to stimulate (butorphanol) or reduce (naltrexone) consumption of palatable tastants [37, 38]. We found that WT mice offered either sucrose or saccharin exhibited a typical orexigenic response to butorphanol and hypophagia after naltrexone treatment. On the other hand, Cx36 KOs exhibited a diminished sensitivity to consumption modifying properties of each of the opioid ligands (sucrose) or a lack thereof (saccharin). While it is difficult to speculate whether the marked shifts in the dose-response curves can be directly attributed to changes in opioid system's expression profile or rather to impaired coupling of reward signaling due to DA and GABA disruption (or both), it is clear that Cx36 deletion leads to gross abnormalities in the molecular content, sensitivity and functioning of reward circuits.

Finally, our taste aversion experiments show that Cx36 KO mice exhibit a greater level of sensitivity to unpleasant consequences of LiCl injection. While administration of 0.6 mEq/kg LiCl in WT mice did not produce a CTA, the same dose of the toxin caused a profound CTA in the KOs. Hence, the Cx36 molecule participates also in central processing of stimuli that bring on a challenge to internal milieu. The loss of the Cx36 protein translates into hypersensitivity to aversions, which poses a potential threat for the animal in that it would become

too selective in search for “safe” foods or would avoid tastants even after very benign negative outcomes of consumption.

Overall, the data indicate that Cx36 is involved in the “homeostatic” (energy intake and aversions) and “non-homeostatic (reward) mechanisms governing appetite. Its absence shifts the threat-pleasure continuum of food intake by reducing the rewarding component of eating behavior and promoting abnormally high sensitivity to negative stimuli associated with a meal. It also supports ingestion of highly caloric, “bland” and “safe” foods.

4.2. (PART B) Functional relationship between oxytocin and connexin 36: a preliminary report

Abstract

Connexin 36 (Cx36) is a gap junction molecule expressed in feeding-related neural circuits. The Cx36 knockout (KO) mouse is a unique model that encompasses dysregulation of three major aspects of food intake: eating for energy, for pleasure, and hypophagia in response to toxins. Interestingly, anorexigenic neuropeptide oxytocin (OT) also regulates the three aspects of feeding. As the OT peptide/receptor and Cx36 are coexpressed in feeding networks, OT and Cx36 may be part of common pathways governing appetite. We hypothesized that the presumed OT-Cx36 functional relationship might be reflected by the altered ability of OT to exert its action in brain circuits devoid of Cx36. Effectiveness of injections of OTr ligands, changes in OT gene expression and in OT neuronal activation in Cx36 KOs were determined in the three aspects of consumption. Cx36 KO mice ingest more energy (standard chow) than WTs, and we found that this enhanced appetite for calorie-dense food is associated with changed expression of OT and OTr genes (qPCR). Cx36 KOs exhibit increased sensitivity to taste aversion inducing chemical stimuli. We showed that this is associated with a higher number of Fos-positive OT neurons activated by toxin (LiCl). Finally, to study the link between Cx36 and OT in feeding reward, we injected KO mice with an OT antagonist, L-368,899 and determined that Cx36 deletion impairs effectiveness of OTr blockade on consumption driven by sweet

taste. Overall, the data suggest that Cx36 is a key component of feeding-related circuits through which OT executes its functions.

4.2.1. Introduction

The connexin 36 knockout (Cx36 KO) mouse is a unique model that encompasses dysregulation of three major aspects of food intake control: eating for energy, eating for pleasant taste, and eating in situations when homeostasis can potentially be jeopardized by ingesting tainted foods. Cx36 KO mice show increased "bland" chow intake and decreased intake of palatable solutions (both sweet and fat). Furthermore, these mice are prone to developing conditioned taste aversions (CTA) even when food exposure is paired with low doses of LiCl, a toxin that causes unpleasant gastrointestinal sensations, that are not effective in wild-type (WT) control animals.

Oxytocin (OT) has been hypothesized to play a significant role in all three of the aforementioned aspects of feeding. OT neurons are thought to promote satiation-driven termination of food intake, a decrease in palatability-dependent consumption and – finally – an immediate discontinuation of consummatory behavior once the presence of toxins is recognized by the chemoreceptor neurons localized in the area postrema (AP). Importantly, studies in laboratory animals have shown the presence of gap junctions in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus, where OT perikarya are amassed [14, 47, 48]. Some (but not all – [14]) authors have provided evidence that Cx36-containing gap junctions exist between hypothalamic OT cells [47, 49]. Therefore, both OT and Cx36 may be molecular substrates of common

neural pathways that govern appetite, and this presumed functional relationship – if it indeed takes place - might affect the ability of OT to exert its action in circuits that have to rely on gap junctions devoid of Cx36.

The aim of these experiments was to examine whether there is a functional relationship between OT and Cx36 in the regulation of food intake. The Cx36 KO mouse was used as a Cx36-null animal model that displays aberrant energy intake, reward-driven feeding and taste aversions. Effectiveness of injections of OTr ligands, changes in OT gene expression and in OT neuronal activation in the Cx36 KO animal were determined in key paradigms associated with each of the three aspects of consummatory behavior. Since Cx36 KO mice eat on average more standard ("bland") chow than WT controls, we determined whether this enhanced appetite for food is associated with different levels of expression of the OT gene (established with real-time PCR) in the hypothalamus and nucleus accumbens. A difference in OT gene expression could predispose the KOs to consuming more energy (thus, having delayed satiety). In order to study the link between Cx36 and OT in the feeding reward context, we injected KO mice with a BBB penetrant OTr antagonist, L-368,899 [50] to examine whether the Cx36-null status impairs effectiveness of OTr blockade on consumption driven by sweet taste. Finally, we investigated whether the enhanced CTA responsiveness of Cx36 KO mice is associated with a greater level of OT neuronal activity. Neuronal activation was established by using double immunohistochemistry for c-Fos (an immediate-early gene product) and OT.

4.2.2. Materials and Methods

4.2.2.1. Animals

Male Cx36 KO and WT littermates (C57/B6-129SvEv mixed background) were individually housed in conventional cages with a 12:12 LD photoperiod (lights on at 0700) in a temperature-controlled room (21°C). Age-matched animals weighed ca. 26 g ± 3 at the beginning of the studies (there was no difference in b. wt. between the genotypes). Mice have unlimited access to tap water and standard rodent chow (Teklad Global Diet 2018) throughout the studies unless noted otherwise. The procedures described herein were approved by the University of Waikato animal ethics committee.

4.2.2.2. Baseline expression of OT and OTr genes in the hypothalamus and Acb in Cx36 KO vs. WT mice

Wt and Cx36 KO mice (n=8/group), maintained on ad libitum access to standard chow and water, were decapitated (10:00-11:00) and the hypothalamus and Acb were collected according to the Paxinos and Watson brain atlas. The tissue was placed in RNAlater at room temperature for 2h and then stored at -80°C. RNA was extracted by the Quick-RNA^{TM}kits (Zymo Research) and the absence of DNA was confirmed by PCR. Total RNA concentration was measured with Nanodrop 2000 (Thermo Scientific). For cDNA synthesis, 9 µl of RNA was reverse-transcribed in a final volume of 20 µl containing 10 µl 2X RT Reaction Solution and 1 µl Enzyme Mix (HiSenScript^{TM} RH(-) cDNA Synthesis Kit, iNtRON Biotechnology). The reaction was performed for 1h at 42°C, followed by 5 min at

85°C and 5 min at 4°C, and the presence of cDNA was confirmed by PCR. Each rtPCR, with a total volume of 20 µl, contained 8 µl template cDNA, 2µl Primers Mix (Forward and Reverse primers) and 10 µl of 2X RealMOD^{TM} GH Green Real-time PCR Master Mix (Master Mix Kit, iNtRON). rtPCRs were done in duplicates, and negative controls were included on each plate. Amplification was performed as follows: denaturation at 94°C for 5 min, 50 cycles of denaturation at 94°C for 10 s, annealing for 15 s, and extension at 62°C for 30 s. Three housekeeping genes were analyzed (**Table 1**). A Rotor-Gene SYBR Green PCR (QIAGEN) was used. Data analyses have been performed by Rotor-Gene 6000 software 1.7 (QIAGEN). Primer efficiencies of 2 genes (Table 1) were calculated and samples were corrected for differences in efficiencies. The Pfaffl equation [26] was used to calculate normalization factors based on housekeeping gene expression. Differences in gene expression between groups were analyzed using a t-test (different when P≤0.05).

Table 4.2.1. Real-time PCR primers (all supplied by Integrated DNA Technologies).

Eating behavior genes		
	FORWARD	REVERSE
OXT	CCT ACA GCG GAT CTC AGA CTG A	TCA GAG CCA GTA AGC CAA GCA
OXTR	TCT TCT TCG TGC AGA TGT GG	CCT TCA GGT ACC GAG CAG AG

Housekeeping genes		
	FORWARD	REVERSE
B actin	TGG CAC CAC ACC TTC TAC AAT GAG	GGG TCA TCT TTT CAC GGT TGG
Atp5b	GGC ACA ATG CAG GAA AGG	TCA GCA GGC ACA TAG ATA GCC
B tub	CGG AAG GAG GCG GAG AGC	AGG GTG CCC ATG CCA GAG C

4.2.2.3. Effect of OTr blockade on episodic intake of palatable tastant solutions in Cx36 KO mice.

Cx36 KO mice were pre-exposed to palatable sweet tastants for 24 hours. Animals (n=7) were accustomed to having access to 0.1% saccharin, 10% sucrose, 10% glucose or 10% fructose, given alone, for 1 h (1100–1200 h) per day for 1 day. Chow and water were removed from the cages for the 1h period of palatable tastant presentation. Five min prior to palatable solution exposure, mice were injected IP with (a) saline or 0.1, 0.3, 1 and 3 mg/kg b. wt. of L-368,899 (Tocris) at a dose range known to affect consummatory behavior (based on [3] and Chapter 2). The reference dose of the OTr antagonist was 1 mg/kg b. wt. (vs saline vehicle). The Bottles of palatable tastants were weighed before consumption and 1 hour after L-368,899 and saline administration. The amount of the palatable solution consumed was calculated and reported in g/kg of body weight. The amount of consumed palatable tastant was corrected for spillage. One-way ANOVA followed by Fisher's post-hoc test was used to establish whether L-368,899 at different doses affected saccharin, sucrose, glucose and fructose consumption in the Cx36 Kos and compared to the WT values.

4.2.2.4 OT neuronal activity feeding-related brain sites in response to a CTA in Cx36 KO mice.

Mice were divided into four groups (n=8/group) and standard chow and water were removed from the cage before saline or LiCl (Sigma; 6 mEq/kg body wt; isotonic solution) injection in IP in Wt and Ko Cx36 mice. 60 minutes later, animals were anesthetized with urethane (35%) and perfused with 10 ml of saline followed by 50 ml of 4% paraformaldehyde (PFA) in 0.1 M phosphate

buffer (pH 7.4). Brains were excised and postfixed overnight in PFA at 4°C. Coronal 60-µm Vibratome (Leica) sections were processed for double (c-Fos and oxytocin) immunostaining. The tissue was treated for 10 min in 3% H_2O_2 in 10% methanol (in TBS, pH 7.4–7.6) and incubated overnight at 4°C in the goat anti-c-Fos antibody (1:2000; Santa Cruz). Subsequently sections were incubated for 1h at room temperature in the rabbit-anti-goat antibody (Vector) and then in the avidin-biotin complex (1h; Vector). Peroxidase was visualized with 0.05% diaminobenzidine, 0.01 H2O2, and 0.2% nickel sulfate. All incubations were done in a mixture of 0.25% gelatin and 0.5% Triton X-100 (Sigma) in TBS. Intermediate rinsing was done with TBS. Following the completion of c-Fos staining, sections were further processed for visualization of OT. The procedure was similar to that used to staining for c-Fos. However, rabbit anti-oxytocin was used as the primary antibody (1:15000; Millipore, Temecula, CA), and nickel sulfate was omitted from the DAB solution to obtain brown staining. Sections were mounted on gelatinized slides, dried, dehydrated in ascending concentrations of ethanol, soaked in xylene, and embedded in Entellan. The number of Fos positive nuclear profiles in the regions of interest was counted on 4-5 sections per animal using Scion Image software. In the double staining analysis, the following estimates were assessed per section and then per region: the total number of OT neurons and the number of OT neurons positive for c-Fos. Cells were counted bilaterally, and the percentage of OT neurons containing c-Fos-positive nuclei was tabulated. An ANOVA and t-test was used to show the presence of significant difference between the number of c-Fos positive in oxytocin cells in saline and LiCl groups in WT and KO Cx36 mice.

4.2.3. Results

The experiments show a relationship between the OT system and each aspect of food intake in the feeding-aberrant Cx36 KO model.

The real-time PCR analysis in the feeding-for-energy paradigm revealed that Cx36 KO mice maintained on an ad libitum chow availability schedule (and found to be consuming more chow-derived calories than WT controls) show a significantly lower level of OT expression in the Acb (p=0.03) and an increased OTr mRNA profile (p=3E-07) in the hypothalamus. There was no effect of genotype on OT expression in the hypothalamus (p=0.03) or on the OTr expression in the Acb (p=0.03) (Figure 4.2.1).

In the feeding for reward experimental scenario, an OTr antagonist, L-368,899 (at the effective reference dose of 1 mg/kg b. wt. – as described in detail in Chapter 2), administered just before the 2-h exposure to the palatable solutions, significantly increased intake of palatable sweet solutions (carbohydrates and non-carbohydrates) in WT animals. OTr antagonist-treated WTs drank significantly more sucrose (sal: 0.05±0.012; antagonist: 0.09±0.015; p=0.008), glucose (sal: 0.060.013±; antagonist: 0.08±0.017; p=0.02), fructose (sal: 0.06±0.015; antagonist: 0.07±0.012; p=0.016). On the other hand, in the Cx36 KO mice, L-368,899 was ineffective in increasing appetite for palatable carbohydrates (sucrose: ANOVA F=1.5 p=0.15, glucose: ANOVA F=1 p= 0.74 and fructose: ANOVA F=1 p=0.1) and a sweet non-carbohydrate, saccharin (ANOVA F=0.6 p= 0.39) (Figure 4.2.2).

Finally, in the taste aversion paradigm, LiCl injection induced activation of OT neurons in the SON and PVN of both the Cx36 KO and WT strains (ANOVA F=31 p= 0.009; PVN: (WT/saline vs Wt/LiCl p= 1E-15, Cx36 KO/saline vs Cx36KO/LiCl p= 2E-10; SON: WT/saline vs Wt/LiCl p= 1E-10, Cx36 KO/saline vs Cx36 KO/LiCl p=2E-15). Importantly, the Cx36-deficient animals that display hypersensitivity to aversive stimulation showed a heightened baseline (i.e., saline-induced) PVN OT neuronal activity (p=0.01) and significantly higher LiCl-induced OT neuronal activation in the SON (p=0.01) (Figure 4.2.3)

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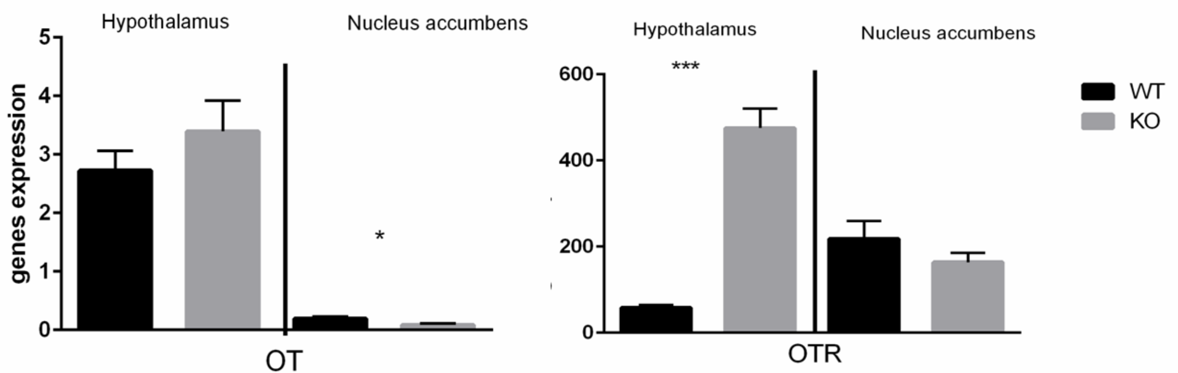

Figure 4.2.1: mRNA levels of OT and OTr in the hypothalamus and Acb of ad libitum chow-fed WT vs Cx36 KO mice. Differences in qPCR-established gene expression between groups were analyzed using a t-test.* - p<0.05; ***-p<0.001.

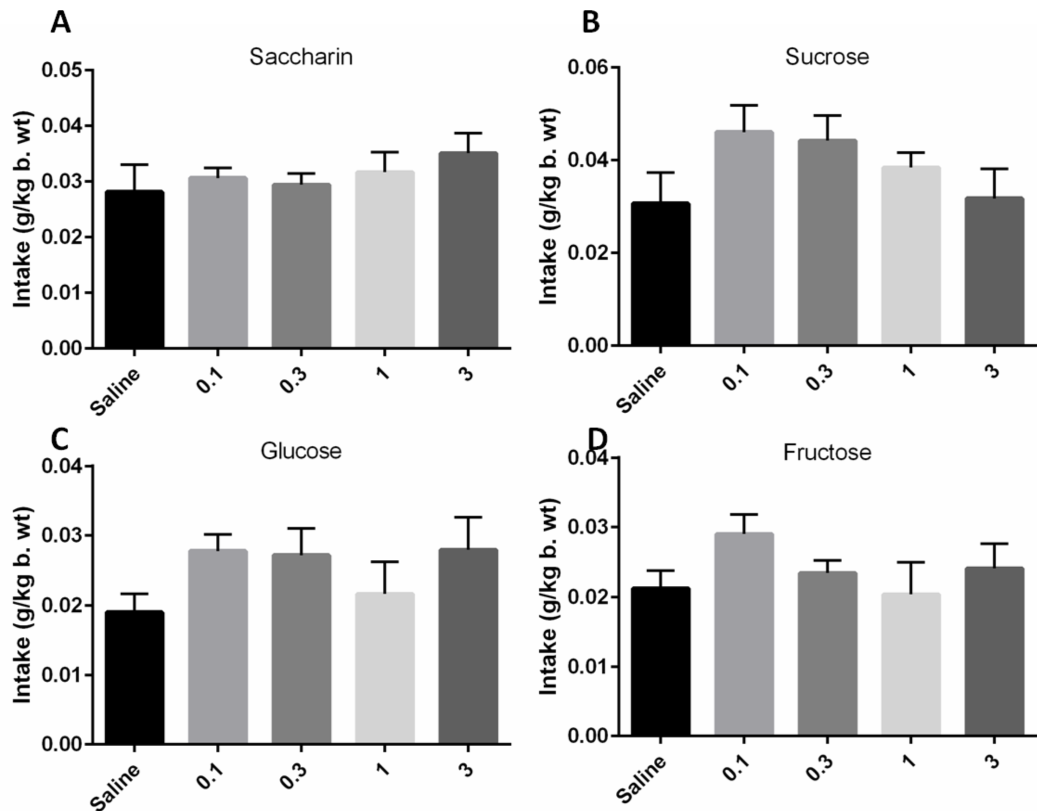

Figure 4.2.2: The effect of the OTr antagonist, L-368,899, at 0 (saline vehicle), 0.1, 0.3, 1 and 3 mg kg/b. wt on the intake of solutions containing 0.1% saccharin (A), 10% sucrose (B), 10% glucose (C) and 10% fructose (D) in Cx36 KO mice. The injections were performed just prior to 2-h tastant exposure period. Regular chow and water were removed from the cage and a single bottle containing one of the solutions was placed in the cage.

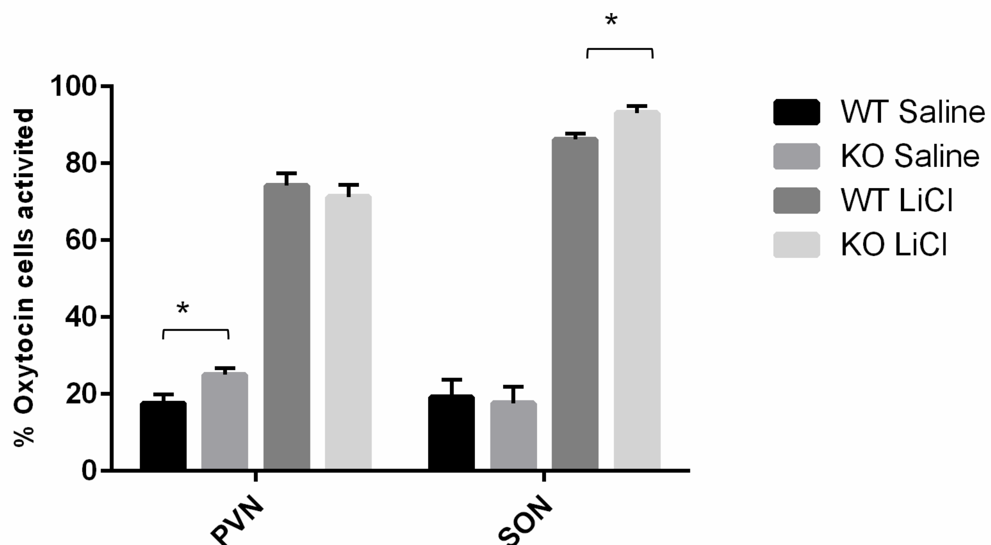

Figure 4.2.3: The percentage of Fos-positive OT cells in the paraventricular (PVN) and supraoptic (SON) nuclei in WT and Cx36 KO mice after an injection of saline or LiCl. OT neuronal activation was compared separately for the effect of saline injection and for the effect of LiCl injection (* - significant difference between WTs and KOs treated with the same injectant: $p<0.05$).

4.2.4. Discussion

The Cx36 KO mouse is a unique genetic model that displays aberrant energy intake, reward-driven feeding and taste aversions. The complexity of food intake dysregulation in this knockout animal parallels the complexity of proposed involvement of OT in appetite control. Cx36-rich gap junctions have been found in feeding neural circuits [14, 16, 51], thus, in those brain pathways through which OT modifies appetite. Therefore, the presumed functional relationship between OT and Cx36 in feeding control would have to be based on an altered ability of OT to affect relevant aspects of food intake via the Cx36-deficient brain networks in the knockout mouse. In other words, an impairment in gap junction-based integrity of feeding-related neural networks, would intuitively have to be reflected – endogenously - by an abnormal profile of the OT and OTr transcript levels and OT neuronal activity in response to feeding challenges, and – with exogenous neuroactive chemicals – by aberrant sensitivity (or lack thereof) to OTr ligands administered before feeding challenges.

The current set of experiments provides data that support the hypothesis that endogenous responses of the OT/OTr system as well as behavioral responsiveness to OTr antagonist treatment are significantly affected in the animal whose brain circuits are devoid of Cx36. Importantly, this is evident in three key aspects of feeding control exerted by OT: energy-driven termination of food intake, feeding for pleasant taste and aversion-induced hypophagia [3, 36, 52, 53].

When Cx36 KO mice are maintained on freely accessible “bland” chow, thus, their ingestive behavior is driven mainly by calorie needs, they show abnormally high level of energy intake. There are obviously many possible factors that may underlie this overconsumption of energy in the KO model (e.g. higher energy expenditure or impaired absorptive processes), but even if the elevated feeding activity is caused by some other parameters, still satiation mechanisms have to be blunted in order to allow the animal to consume more food (despite, e.g., significant stomach distension or raised plasma osmolality). The real-time PCR experiment shows that ad libitum-fed Cx36 KO mice display a decreased level of OT mRNA in the Acb compared to their WT counterparts, which is in line with the general chow hyperphagia observed in the KO strain. Although it may seem somewhat surprising that hypothalamic OT expression is unaffected, one should note that our Chapter 3 studies on OT and Acb point to the fact that non-hypothalamic OT also affects appetite for calories. Simultaneously, OTr mRNA levels in the hypothalamus are higher in the KOs, which indicates that OT signaling in the hypothalamus is imbalanced in the hyperphagic KO model.

While the Cx36 KO mouse exhibited increased appetite for energy-dense “bland” chow, it also showed striking sensitivity to meal-associated toxicity. In the CTA paradigm (described in detail in Part 3.1 of this chapter), the KO mice acquired aversion even with very low doses of LiCl that did not cause a CTA in WT controls. OT is known to play a critical role in the process of development of aversive responses, both in producing an immediate termination of ingestive behavior once the toxin is detected by the brainstem chemoreceptors as well as in retrieval of aversion – once a potentially tainted ingestant is presented again [36,

53]. The immunohistochemical analysis of the OT system's response provided herein sheds some light on the nature of the CTA phenomenon in Cx36 KOs. First, the percentage of PVN OT neurons is already higher in saline-treated (control) KO mice, which potentially underpins heightened sensitivity of these animals to even mild perturbations in internal milieu. Second, the SON OT neuronal activation is higher in the LiCl-injected Cx36 KOs than in similarly treated WTs, most likely contributing to a greater magnitude of an aversive response to the same dose of a given toxin (in this case, LiCl) of the knockout animals.

Finally, the feeding reward paradigm, in which animals were given episodic access to palatable tastants provides evidence pertaining to atypical feeding responses to OTr antagonist administration in Cx36 KOs. It has been shown by our laboratory as well as by others ([3] and Chapters 2 and 3) that the OTr blocker, L-368,899, increases consumption of tasty carbohydrate and non-carbohydrate (but sweet) solutions in mice and rats. The current set of studies employing a variety of highly preferred sweet tastants (containing saccharin or mono- or disaccharides) showed complete inability to enhance intake of these ingestants by blocking the OTr with the IP-injected BBB-penetrant OTr antagonist.

Obviously, it should be emphasized that OT is clearly not the only peptide whose function within the Cx36-null feeding circuit is affected: Part 3.1 of this Chapter has identified opioids as another candidate group of molecules. In the network whose integrity is challenged by the absence of a key gap junction molecule, it is very much likely that some other feeding-related peptides are influenced in certain feeding scenarios as well. However, the fact that -in the Cx36 KO - the dysregulation of the OT system and of the three distinct aspects of appetite

controlled by OT go hand-in-hand, strongly suggests that the Cx36 molecule is a key component of feeding-related neural circuits through which OT executes its functions.

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Chapter 5

Discussion and Perspectives

In the obesogenic environment, many individuals see their energy intake exceed energy expenditure and, in result, energy balance becomes dysregulated. In such environment, the usual reason to initiate and maintain consumption is not dictated by the necessity to replenish lacking calories, but rather it is driven by a pleasant taste of food. Therefore, identification of molecules and systems within the CNS that alter feeding for reward is an essential step in conceptualizing pharmacological treatment strategies that could assist in curbing excessive consumption of highly palatable foods.

Upon undertaking the experimental work included in this thesis, it was clear that a neurohormone OT promotes termination of consumption in order to protect internal milieu (i.e., related mainly to energy needs, stomach distension and osmolality) [1-5]. At that time, some evidence had already been gathered pointing to a possible role of OT in reducing consumption of select palatable tastants [6-8]. For example, it had been shown that OT KO mice consume sweet carbohydrates and non-caloric saccharin less avidly than wild-type controls [9]. Pharmacological blockade of the OTr was known to elevate intake of sucrose over fat in sated mice [7], whereas OT administration reduced intake of glucose in food-deprived rats [6]. Opioid receptor antagonist injections that decrease palatable sucrose intake had been found to activate OT neurons [7], and OT gene

expression had been reported to increase after a high-sugar diet intake in rats [8]. Finally, long-term habitual consumption of sugar had been associated with diminished OT neuronal activity in response to a food load, suggesting that OT dysregulation leads to reward-driven overeating [10]. The studies included in this thesis as well as most recent reports by other authors (see the following publications as recent milestones in our understanding of the link between OT and feeding for palatability: [11, 12]) strongly suggest that OT serves as a cross-link between mechanisms that bring on termination of consumption due to "homeostatic" and palatability-related (i.e., flavor- and macronutrient-specific) satiation.

Prior to taking up this project and the most recent surge in interest in the functional relationship between OT and ingestive behavior, one of the greatest conundrums was associated with defining the role of OT in feeding for palatability. The data presented herein (as well as reports by others) demonstrate that OT is particularly effective in reducing intake of carbohydrates (regardless of whether sweet or non-sweet) as well as a non-carbohydrate sweetener, saccharin. Peripheral injections of the BBB-penetrant OT receptor antagonist, L-368,899 (Chapter 2), in mice led to an increase in the consumption of all the carbohydrates offered in that experiment. The wide range of carbohydrate-rich liquid diets included sweet sucrose (at several concentrations), fructose, glucose, and Polycose, as well as non-sweet cornstarch. Thus, L-368,899 stimulated intake of all the carbohydrates independent of a subtype, sweetness and relative rewarding value of a molecule. It should be mentioned that the effective dose that was 10 times lower in sucrose tests than in trials involving the

remaining tastants. Saccharin solution intakes showed a strong trend towards an increase in L-368,899-treated mice, approaching significance (P=0.08). The latter was somewhat surprising considering the convincing data obtained by other authors in, e.g., OT knockout mice that overconsume saccharin [9, 13], strongly suggested the link between OT and appetite for this sweetener. It is possible that the episodic, mid-day schedule of saccharin presentation might have modified the response to saccharin in IP OT antagonist-injected mice, especially considering that the peripheral OT energy metabolism-related mechanisms come into play with an IP antagonist infusion [14, 15] and that saccharin was the only non-caloric palatable tastant offered, whereas sucrose, fructose, sucrose, and Polycose – albeit dilute – are the sources of energy.

The notion that OT terminates consumption of carbohydrates and saccharin was strengthened by the outcome of Acb injection studies described in Chapter 3. Importantly, the data defined the AcbC, an integral part of the reward system, as a key site mediating the effects of OT on appetite. In that set of experiments, AcbC OT decreased the intake of sucrose in energy non-deprived rats. Interestingly, when OT was injected directly in this reward-related site, very low doses of OT were effective in reducing appetite for non-caloric and highly palatable saccharin. Therefore, once the OT ligand-targeted receptor population is limited to the AcbC, the effectiveness of an OT receptor ligand to modify consumption of the non-caloric, palatable sweetener is greater. Notably, all anorexigenic effects of Acb OT were abolished by a pretreatment with an OTr antagonist, L-368,899, injected in the same site, which underscores the

involvement of the OT receptor in this process rather than of other non-selective peptide-receptor interactions.

The link between OT acting in the reward system and the consumption of palatable tastants has also been suggested in studies utilizing intra-VTA administration of OT ligands. Mellis et al found that VTA-infused OT dose-dependently promotes a decrease in sucrose consumption, whereas VTA acting OT receptor antagonists support enhanced appetite for sugar [12]. Furthermore, OT's ability to modify intake for reward is abolished in the Cx36 KO strain (Part B of Chapter 4), in which reward processing is abnormal (Chapter 4, Part A). In this unique mouse model of aberrant Cx36 gap junction connectivity (mainly) within the reward and neuroendocrine systems of the CNS, OT receptor antagonist L-368,899 fails to increase intake of sweet carbohydrate solutions (sucrose, fructose and glucose) and saccharin. It is therefore apparent that when the reward system's functioning is impaired, OT ligands do not affect feeding for palatability. Thus, it is likely that the observed effects of OT on the intake of palatable tastants directly involve actual reward mechanisms. This is in concert with the fact that both OT and Cx36 have been found to affect other parallel aspects of reward, including alcohol abuse [16-26].

The changes in sensitivity to appetite modifying effects of OTr ligands are reflected by differences in the OT peptide and receptor mRNA content in the aforementioned key paradigms. In rats exposed to the saccharin solution (Chapter 3), OTr mRNA is downregulated in the AcbC. The hypothalamic OT mRNA levels in mice consuming diets enriched with carbohydrates or saccharin are particularly enhanced by carbohydrate intake (Chapter 2).

One should note, however, that the role of OT acting within the reward system is pleiotropic and it involves – among others - rewarding aspects of sociality (for example, see: [27-29]). Moreover, various aspects of social interactions (from the antagonistic nature of social exposure to bond formation), change OT tone in the CNS and the periphery (e.g., [30-34]). Thus, while studying effectiveness of OT receptor ligands in the control of feeding for reward, one should not neglect the fact that the type, frequency and quality of social behaviors may have a profound influence on the effectiveness of OT ligand treatment on appetite. As evidenced by the data presented in Chapter 3, AcbC OT did not change consummatory behavior in animals that were offered a rewarding meal in a social setting. This is in stark contrast with the results obtained in single-housed animals. However, this outcome is in agreement with the recently published data in the mouse model of sociality of feeding, in which it was shown that once a social environment of a palatable meal was introduced, only animals of a particular hierarchy status would express hyperphagia in response to the OTr antagonist IP treatment [35]. In that study, all animals – regardless of the hierarchy – displayed elevated appetite for sugar when a meal was given in a setting devoid of social cues.

The results presented herein as well as by other authors [9,24,36-39] substantiate the claim that OT serves as a neuroregulator of carbohydrate-specific satiety and inhibitor of consumption driven by sweet taste and that OT's effects are – at least to some extent – mediated via the reward pathways; but these data also expand on our understanding of OT's involvement in "homeostatic" meal control. The classical approach to defining the role of OT in

appetite has revolved almost entirely around homeostatic factors. Most notably, an increased level of OT neuronal activity and OT release have been measured at the end of a meal and in response to excessive plasma osmolality and stomach distension, as well as after ingestion of toxic foods whose consumption needs to be avoided in the future (CTA context) [3, 4]. In all these cases, the hypothalamic – brainstem pathways that encompass OT neurons and receptors have been typically identified as key neural mechanisms whose activation leads to termination of consummatory behavior [2,40,41]. Functional relationships between OT and other CNS peptides that affect consumption have also allow researchers to identify more precisely networks through which OT terminates consumption in order to maintain internal milieu. GLP-1 and alpha-MSH are two appetite suppressing neuropeptidergic systems whose direct input into the OT pathways has been described in most detail [42,43].

The current set of data provides additional evidence supporting the involvement of OT in the regulation of homeostatic aspects of feeding. OTr antagonist, L-368,899, administered peripherally increased energy deprivation-induced intake of relatively "bland" carbohydrate, cornstarch in mice (Chapter 2). In the Cx36 KO model – which shows increased consumption of highly caloric bland chow in an ad libitum access setting (Chapter 4, Part A) – this propensity to overconsume energy was associated with a significantly higher baseline level of the hypothalamic OTr mRNA content and a lower OT mRNA in the Acb (Chapter 4, Part B). While it is clear that dysregulation of many other genes (aside from OT and – obviously considering the KO status – Cx36) may accompany this voracious eating phenotype, it is also critical that in the context of increased intake of

energy, OT system's expression is changed, most likely being one of the factors facilitating this increased and sustained appetite for calories.

It is extremely interesting that injections of OT in energy-deprived rats directly into the key site of the reward system, the AcbC, caused a marked decrease in the amount of standard laboratory chow eaten by the animals (Chapter 3). In fact, the results of AcbC OT injections mirror those obtained in VTA infusions of OT [12]. One explanation of this phenomenon is obviously that there is some element of palatability associated with the consumption of standard chow, hence changes in the reward system's activity affect chow intake. However, one can propose also an alternative explanation, which – in any case – does not negate the aforementioned hypothesis, but rather enriches it. It is possible that OT acting in the AcbC and VTA serves as a component of molecular mechanisms that bridge intake for energy and intake for pleasure. While we typically envision pleasant taste as the main source of feeding reward, in the state of energy deprivation, the pleasure of consumption is derived largely from ingesting caloric food rather than from this food's taste alone [44-46]. Hence, OT in the reward system may be reducing intake of energy (thus acting as a "homeostatic" satiety mediator) by diminishing reward stemming from consumption of calories (thus serving as an inhibitor of feeding reward). The fact that OT stimulation of the AcbC results in the activity of wider brain circuits that include hypothalamic OT neurons (multisynaptic pathways are most likely involved) suggests that OT-derived signaling within the AcbC leads to further increases in hypothalamic OT neuronal activation, possibly ensuring that consummatory behavior does not continue. Finally, it should be mentioned that OT is not the only neuropeptide

that fits this description: also AcbC-infused GLP-1 affects both meal size and rewarding aspects of food consumption [47-49].

Overall, the findings presented in this thesis strongly suggest that OT acts as a cross-link molecule that bridges termination of consumption due to "homeostatic" and palatability-related (i.e., flavor- and macronutrient-specific) satiation. Aside from providing additional impetus on purely neurobiological and appetite-related basic research on OT, the complexity of OT's role in ingestive behavior suggested here (and by other authors) might have a profound influence on whether OT-based pharmaceuticals can be successfully tried in obese patients.

In the light of obesity "epidemic", significant efforts have been made to introduce drugs that could alleviate at least some negative factors contributing to the development of excessive BMI; obviously, overeating is recognized as one of the most critical contributors. The general industry rules imposed by the US Federal Drug Administration on Developing Products for Weight Management [50] stipulate that medicinal products have to be scrutinized under very specific scenarios in order to reach the market. One of the key rules is that the anti-obesity drug should be tested in patients with BMI > 30 kg/m^2 or > 27 kg/m^2 if co-morbidities are present (sleep apnea; cardiovascular disease of diverse etiologies; type 2 diabetes; dyslipidemia; hypertension). The difference in mean weight loss between the drug and placebo groups should at least 5% (and statistically significant). The proportion of subjects who lose > 5% of body weight in the active- product group is at least 35%. The proportion of subjects who lose greater than or equal to 5% of baseline body weight in the active- product group is at least 35%, is approximately double the proportion in the placebo-treated

group. Considering the many circumstances under which OT does not affect appetite, these guidelines might prove to be a very difficult obstacle for any OT-based anti-obesity (anti-overeating) product. One of the major issues is that OT reduces intake of only some macronutrients and flavors: modern diet is extremely diverse and patients taking a drug that curbs overeating of only some foods might simply have their food preferences shifted by OT, and this shift will not be associated in any desirable weight loss. Furthermore, many people eat food in a social setting (e.g., with family members), hence the fact that OT's effectiveness is reduced when a meal is offered in a social context, makes it a less likely candidate to successfully go through clinical trials.

On the other hand, the fact that a neuropeptide affects only certain aspects of appetite makes it an attractive pharmaceutical whose role is aimed exclusively at overeating only specific tastants. Taking into account an anxiolytic effect of OT [51, 52], perhaps OT ligands could be used in those anxiety-prone or eating disorder patients whose overeating seems limited to sweet/high-carbohydrate diets.

Finally, OT's involvement in several unrelated behaviors suggests that this neuropeptide, may act as a behavioral "switch" between food intake and other competing behaviors/processes. OT might facilitate a choice between food intake and other behaviors/processes (e.g., social or sexual), in order to balance behaviors and contribute to the transient and long-term survival of the individual [35,53,54].

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Conclusions

The overarching aim of the current thesis was to examine the hypothesis that OT suppresses appetite for sweet tastants by reducing palatability-driven reward. The findings of the studies are:

- OT is involved in energy intake and this role is facilitated not only via circuits classically seen as "homeostatic", but also via the nucleus accumbens, a reward-related site.
- OT reduces appetite for carbohydrates; sucrose consumption appears to be particularly affected by OT.
- OT tends to decrease appetite for non-carbohydrate and non-caloric sweetener, saccharin.
- OT may serve as a key neuroregulator of carbohydrate-specific satiety and inhibitor of consumption driven by sweet taste.
- OT acting via the AcbC (but not shell) decreases food intake driven by hunger and by reward.
- Anorexigenic effects of AcbC OT do not stem from sickness/malaise.
- A social environment in which a meal is presented reduces effectiveness of OT as an inhibitor of appetite.
- OT in the AcbC affects activation of the AcbC itself as well as of hypothalamic sites involved in feeding control.
- AcbC OTr mRNA levels change in response to both energy deprivation and exposure to palatable tastants.

- Cx36 is involved in the "homeostatic" and reward mechanisms governing appetite: (a) Cx36 deletion promotes overconsumption of highly caloric food; (b) Cx36 deletion promotes hypersensitivity to taste aversion; and (c) Cx36 deletion reduces feeding for reward.
- Enhanced appetite for calorie-dense food in Cx36 knockouts is associated with changed expression of OT and OTr genes.
- Cx36 knockouts' increased sensitivity to taste aversion is associated with a higher number of activated OT neurons in toxin-treated animals.
- Cx36 deletion impairs effectiveness of OTr blockade on consumption driven by sweet taste.

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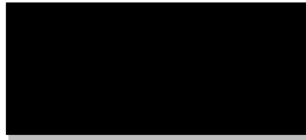
Appendix

Paper I



Contribution statement:

Herisson FM: data collection, analysis, interpretation and drafted the manuscript

Brooks LL: helped with data collection

Waas JR: critical revision of the article

Levine AS: data analysis and critical revision of the article

Olszewski PK: conceptualised the studies, helped draft the manuscript

All authors read and approved the final manuscript

Functional relationship between oxytocin and appetite for carbohydrates versus saccharin

Florence M. Herisson[a], Lydia L. Brooks[a], Joseph R. Waas[a], Allen S. Levine[b,c] and Pawel K. Olszewski[a,c]

Centrally acting oxytocin (OT) inhibits feeding. Recent evidence suggests a link between OT and control of carbohydrate and saccharin intake, but it is unclear whether OT affects appetite for only carbohydrates, especially sweet ones, or sweet tastants irrespective of their carbohydrate content. Therefore, a blood–brain barrier penetrant OT receptor antagonist, L-368,899, was administered in mice and intake of liquid diets containing carbohydrates sucrose, glucose, fructose, polycose, or cornstarch (CS) or the noncarbohydrate, noncaloric sweetener saccharin was studied in episodic intake paradigms: one in which only one tastant was available and the other in which a choice between a carbohydrate (sucrose, glucose, or fructose) and saccharin was provided. We also used real-time PCR to examine hypothalamic OT mRNA levels in mice provided short-term access to sucrose, CS, or saccharin. In the no-choice paradigm, L-368,899 increased the intake of all carbohydrates, whereas its effect on saccharin consumption showed only a trend. A 10 times lower dose (0.3 mg/kg) stimulated intake of sucrose than other carbohydrates. In the choice test, a very low 0.1 mg/kg dose of L-368,899 doubled the proportion of sucrose consumption relative to saccharin, but did not affect fructose or glucose preference. OT gene expression increased after sucrose and CS, but not saccharin exposure compared with the controls; however, a higher level of significance was detected in the sucrose group. We conclude that OT inhibits appetite for carbohydrates. Sucrose consumption considerably enhances OT gene expression and is particularly sensitive to OT receptor blockade, suggesting a special functional relationship between OT and sugar intake. *NeuroReport* 00:000–000
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Introduction

A neurohormone oxytocin (OT) produced mainly in the hypothalamic supraoptic and paraventricular nuclei inhibits food consumption. Central infusions of OT reduce chow intake and OT neurons are activated at meal termination [1,2]. Large food loads [3–5], an increase in plasma osmolality [6], treatments with emetic agents [7], and injections with satiety-regulating neuroactive endogenous substances [3,8,9] increase OT neuronal activity. Genetic deletion of the OT receptor (OTr) gene underlies obesity in the murine KO strain [10]. Although the role of OT in consumption-related homeostasis is well established, most recent evidence suggests that OT is involved in feeding reward. OT appears to serve as a cross-link bridging homeostatic and reward-related satiety. OT neuronal activity is particularly robust in response to manipulations that reduce intake of rewarding foods, for example to naloxone injections [11], and it is considerably reduced by orexigenic doses of opioid receptor agonists [12]. Long-term habitual consumption of sugar is associated with reduced OT neuronal activity in response to a food load, suggesting that OT dysregulation leads to reward-driven overeating [13]. Importantly, initial studies suggest that OT seems to affect preferentially intake of those rewarding tastants that are sweet and/or high in carbohydrates. OT KO mice show enhanced preference for sugar and other carbohydrates and OT gene deletion is also associated with increased appetite for saccharin [13,14]. OTr blockade enhances appetite for sucrose [5,14,15]. Central injections of OT reduce the intake of the monosaccharide glucose, but only in food-deprived rats [4]. To add to the confusion, OTr KOs have unchanged preference for sucrose [16]. Furthermore, OT KOs drink increased amounts of saccharin, but OTr blockade in thirsty animals does not increase preference for saccharin in the saccharinversus water two-bottle test [17].

Overall, it is still unclear whether OT affects appetite for only carbohydrates, especially sweet ones, or sweet tastants irrespective of their carbohydrate content. Therefore, we administered a blood–brain barrier penetrant, potent and selective antagonist of the mouse OTr, L-368,899 [18] to examine its effect on the intake of solutions containing sucrose, glucose, fructose, polycose, cornstarch (CS), or saccharin. Two paradigms were used: (a) only one tastant was available and the dose–response

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curves were established and (b) a choice between two sweet ingestants, a carbohydrate (sucrose, glucose or fructose) versus noncarbohydrate saccharin, was studied in mice injected with a very low dose of the antagonist. We also used real-time PCR (RT-PCR) to examine changes in hypothalamic OT mRNA levels in mice provided short-term access to sucrose, CS, or saccharin solutions.

Methods

Animals

Male C57BL/6J mice (26 ± 3 g; AgResearch, New Zealand), housed individually (LD 12 : 12; lights on at 0700) at 21–22°C, had unlimited access to chow (Teklad) and water unless noted otherwise. The procedures were approved by the University of Waikato animal ethics committee.

Effect of OTr blockade on consumption in the no-choice single-bottle paradigm

Animals ($n = 8$/group) were accustomed to having access to palatable 0.1% saccharin, 10% sucrose, 30% sucrose, 10% fructose, 10% polycose, or 10% glucose, administered alone, for 2 h (1000–1200 h)/day for 3 days. In the CS study, to stimulate intake of the carbohydrate, mice were food-deprived overnight and then they received access for 2 h (1000–1200) to a single bottle containing a 10% CS suspension (as CS is insoluble in water, 0.3% xanthan gum was added to the liquids in this experiment as described previously in [19]). Chow and water were removed for the 2-h period. Animals started drinking right after the solution was presented and finished within 50 min. On day 4, 5 min before solution exposure, mice were injected intraperitoneally with saline or 0.1, 0.3, 1, and 3 mg/kg body weight L-368,899 (Tocris). At this dose range, L-368,899 increases intake of and preference for a 10% sucrose solution without affecting thirst [5]. Bottles were weighed; the amount of the solution consumed was corrected for spillage. To account for in-group variability in body weights, the amount of ingested solution was reported in g/kg of body weight. One-way analysis of variance, followed by Fisher's post-hoc test was used to establish the effect of L-368,899. P values up to 0.05 were considered significantly different.

Effect of OTr blockade on sugars versus saccharin preference

Mice had been pre-exposed to 0.1% saccharin, 10% sucrose, 10% fructose, or 10% glucose for 24 h several days before the beginning of the trials to avoid neophobia. Animals ($n = 6$/group) were accustomed to having access to two bottles for 3 days: one containing saccharin and the other containing (a) sucrose or (b) fructose or (c) glucose for 2 h (1000–1200). Food was removed from the hoppers for the 2-h period of tastant presentation. Five minutes before the presentation of the bottles, mice were injected intraperitoneally with saline or 0.1 mg/kg L-368,899, and the dose of the antagonist was selected as a low, subthreshold dose insufficient to increase the intake of any carbohydrate tested in a single-bottle paradigm in the no-choice paradigm described above and in previous studies (e.g. Olszewski *et al.* [5]). The amount of the solution consumed corrected for spillage was calculated in g/kg of body weight and the data were expressed as percentage of sweet carbohydrate solution (e.g. sucrose) intake in the total fluid (e.g. sucrose + saccharin) intake. A t-test was used to establish whether L-368,899 affected preference for carbohydrate versus noncarbohydrate. P values up to 0.05 were considered significantly different.

OT mRNA levels in mice consuming sucrose, CS, or saccharin

We assessed whether consumption of the same amount of sucrose, CS, or saccharin on 2 subsequent days affects OT gene expression. To ensure a high and similar level of consumption of the solutions (particularly crucial in the CS group, which would otherwise have to be deprived of chow to stimulate avid intake of CS), we chose to supply the carbohydrate solutions instead of water during the initial 5 h of the dark phase. Therefore, on the 2 experimental days, water bottles were removed 2 h before the beginning of the dark phase, and – at lights off – the animals ($n = 7$–8/group) were provided access to 3 ml of water (control) or the 10% CS, 10% sucrose, or 0.1% saccharin solutions/suspensions (prepared as in 2.2): 3 ml represented ca 40% of their night-time water intake. The solutions were the only source of liquid available in the cages at that time. The total amount of the fluid was consumed by all except two mice (one from the water and one from the CS group – excluded from the study) between 3 and 4 h after presentation. At 5 h, water bottles were placed back in the cages to preclude dehydration. We did not observe differences in night-time chow (4.3–4.8 g range) and water (4.0–4.7 ml range) intakes between the groups. The animals were decapitated after the second night of tastant exposure (decapitation time: 7:00–8:00), and hypothalami were dissected and placed in RNAlater (Ambion) overnight (4°C). A standard protocol of sample preparation and RT-PCR was followed (details in the study by Olszewski *et al.* [5]) and for reasons of brevity, only the main elements are described here. Samples were homogenized in TRIzol (Ambion); RNA was extracted with chloroform and precipitated in isopropanol. After centrifuging, the pellet was washed, air dried, and dissolved in the DNase buffer (NEB). The samples were treated with RNase-free DNase I (37°C, 1 h; Merck) and the absence of genomic DNA was established by PCR of a 5% template in the PCR mix ($MgCl_2$-free buffer, 50 mM $MgCl_2$, Tween, 20 mM dNTP, forward and reverse primers, Taq polymerase, and MilliQ H_2O; 10 μl total volume). A volume of 0.5 μl 100 ng/μl genomic DNA was added as a positive control and 0.5 μl MilliQ H_2O as a negative one. The product

was analyzed by electrophoresis. To synthesize cDNA, 5 μg RNA samples (concentration determined spectrophotometrically) were diluted with MilliQ H_2O to 12 μl. RNA was reverse transcribed in the master mix (Promega; 20 μl). Samples were incubated for 1 h (37°C), followed by PCR to confirm cDNA synthesis. RT-PCR reactions were performed in duplicate; negative controls were included. Sample cDNA template (25 ng) was used per primer [OT primer sequences: cggtggatctcggactgaac (forward) and tagcaggcggaggtcagag (reverse)]. Each RT-PCR (20 μl total volume) contained 2 μl $MgCl_2$-free buffer 10×, 0.2 μl 20 mM dNTP, 1.6 μl 50 mM $MgCl_2$, 0.05 μl forward and reverse primer (100 pmol/μl), 1 μl DMSO, 0.5 μl Sybr Green (1 : 50 000), 0.08 μl Taq polymerase, and 9.52 μl MilliQ H_2O. The amplification step included denaturation (95°C, 3 min), and 40 cycles of denaturing (95°C, 20 s), annealing (30 s), and elongation (72°C, 30 s). Expression of four housekeeping genes, β-actin, β-tubulin, glyceraldehyde-3-phosphate-dehydrogenase, and ribosomal protein, was used to calculate normalization factors (GeNorm). Primer efficiencies were calculated with LinRegPCR and C_t values were corrected for differences in primer efficiencies. Differences between groups were analyzed using analysis of variance, followed by Fisher's test, with significance set at P value up to 0.05.

Results

In the no-choice paradigm, L-368,899 increased the intake of all carbohydrates (Fig. 1), including the CS, whereas its effect on saccharin consumption only approached significance. A dose as low as 0.3 mg/kg elevated 10 and 30% sucrose consumption (10%: 0.3 mg/kg body weight $P=0.025$; 1 mg/kg body weight $P=0.008$; 30%: 0.3 mg/kg body weight $P=0.012$; 1 mg/kg body weight $P=0.027$). Fructose, glucose, polycose, and CS intakes were also increased; however, the lowest effective dose of L-368,899 (3 mg/kg) was 10 times higher (fructose: $P=0.016$; glucose: $P=0.020$; polycose: $P=0.031$; CS: $P=0.039$). L-368,899 did not affect appetite for saccharin, although there was a trend at 3 mg/kg ($P=0.088$).

In a two-bottle test, mice provided access to a sugar (sucrose, glucose, or fructose) and saccharin were pretreated with a 0.1 mg/kg dose of L-368,899, subthreshold in the no-choice experiment. L-368,899 doubled the proportion of sucrose consumption relative to saccharin ($P=0.006$), but had no effect on the preference for fructose or glucose versus saccharin (Fig. 2).

Hypothalamic OT gene expression (Fig. 3) was higher in animals exposed to sucrose and CS compared with controls; however, a higher level of significance was detected in the group fed sucrose ($P=0.008$) than CS ($P=0.036$). Saccharin exposure did not affect OT mRNA levels.

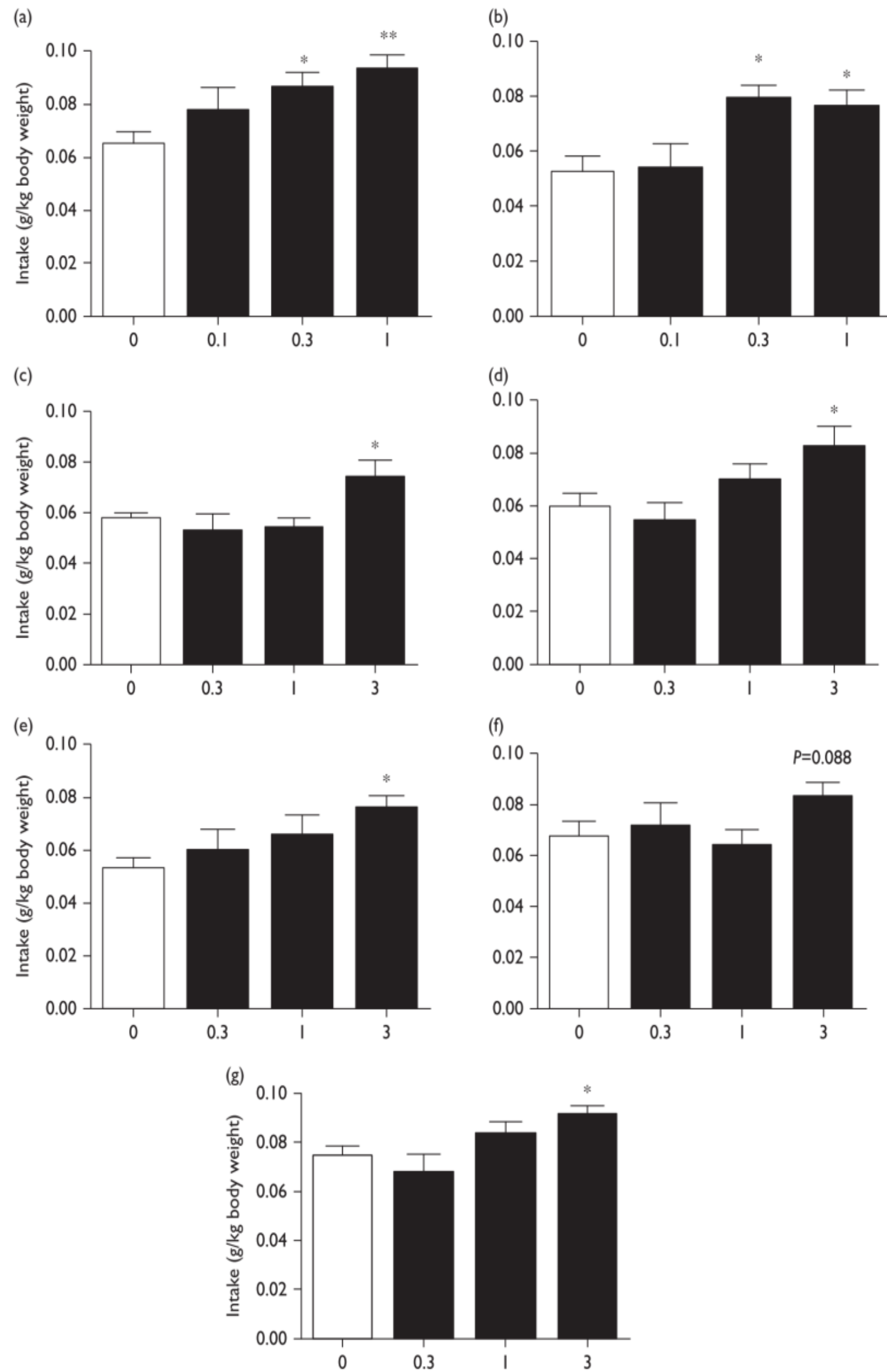
Discussion

The current set of experiments confirms that OT affects appetite for carbohydrates and it points to a special functional relationship between OT and sucrose intake. Antagonism of the OTr in the no-choice paradigm led to an increase in the intake of all carbohydrates, that is, sucrose, glucose and fructose, polycose, and CS, but it should be emphasized that the consumption of sucrose was induced by the antagonist dose that was 10 times lower than that needed to affect intake of the remaining carbohydrates. Importantly, blockade of the OTr stimulated appetite for carbohydrates independent of their sweetness and rewarding value: bland CS and palatable sweet carbohydrates were ingested more avidly after the treatment. This is in concert with the KO animal data in which the genetic deletion of the OT gene caused overconsumption of a variety of carbohydrates, including starch [14]. Therefore, a sweet flavor does not seem to be a prerequisite underlying the effectiveness of OT in regulating carbohydrate-specific satiety and neither is the complexity of their structure (i.e. monosaccharide, disaccharide, and polysaccharide). Furthermore, the effect of L-368,899 on sucrose intake remained even after the three-fold increase in the concentration of sucrose in the solution; hence, the link between carbohydrate consumption and OT does not appear to be modified by the caloric density of the tastant.

Besides the episodic intake of carbohydrates being decreased by OTr antagonism, we found that short-term enrichment of the diet with carbohydrates (either sucrose or CS) caused upregulation of the OT transcript in the hypothalamus compared with controls. This is in line with the OT mRNA findings in sucrose-exposed versus regular diet-exposed rats [20]. It is noteworthy that a greater increase in our RT-PCR study was found in sucrose-fed than in CS-fed mice despite the same amount of the carbohydrate-containing fluid consumed by the respective groups.

Unlike appetite for carbohydrates, saccharin intake was not elevated by OTr blockade, although the highest dose of the compound induced the consummatory response that showed a trend approaching significance. Also, our RT-PCR experiment showed that hypothalamic OT gene expression was not affected by short-term saccharin exposure. Although these data are in contrast to OT KO murine studies in which saccharin overconsumption was noted [14], they are in line with antagonist injection experiments that showed the lack of the effect of L-368,899 on preference for saccharin in water-deprived mice provided a choice between the saccharin solution and water [17]. Overall, this suggests that the involvement of the OTr in saccharin intake regulation is not as critical as in appetite for carbohydrates, but obviously considering the trend in saccharin consumption reported here and the positive KO mouse data [14], it cannot be negated. Importantly, it appears that it is not the sweet

Fig. 1

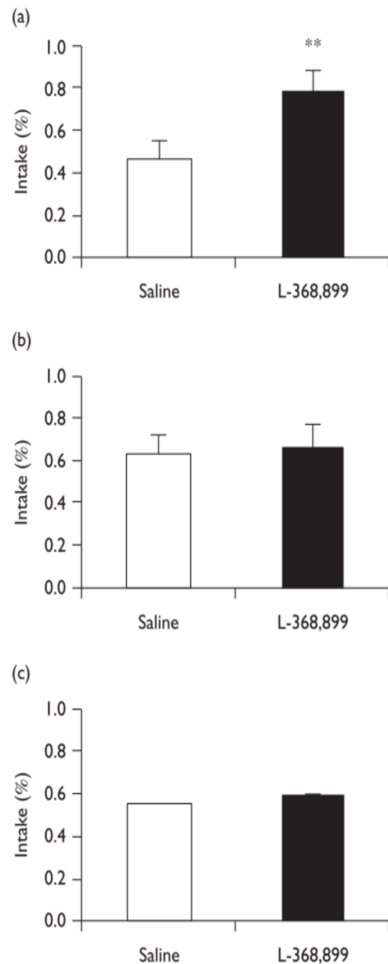


The effect of the OTr antagonist, L-368,899, at 0 (saline vehicle), 0.1, 0.3, 1, and 3 mg/kg body weight on the intake of solutions containing (a) 10% sucrose, (b) 30% sucrose, (c) 10% fructose, (d) 10% glucose, (e) 10% polycose, (f) 0.1% saccharin, and (g) 10% CS. *$P < 0.05$; **$P < 0.01$. CS, cornstarch; OTr, oxytocin receptor.

flavor (note saccharin vs. CS intakes), but rather the carbohydrate content of an ingestant that defines the link between OT and feeding control.

The two-bottle preference tests confirmed the proposed special relationship between OT and the control of sucrose intake. The very low dose of L-368,899 that was

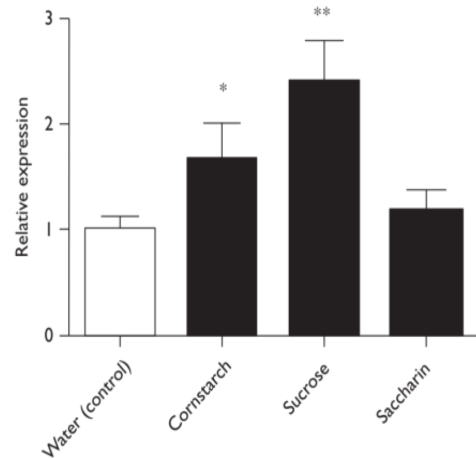
Fig. 2



The effect of the OTr antagonist, L-368,899, at 0 (saline vehicle), 0.1 mg/kg/body weight on the intake of solutions containing (a) 10% sucrose, (b) 10% fructose, and (c) 10% glucose (carbohydrate) versus 0.1% saccharin (noncarbohydrate) expressed as the percentage of carbohydrate solution consumed in the total volume of ingested tastants. $^{**}P < 0.01$. OTr, oxytocin receptor.

found to be insufficient to affect the intake of any of the carbohydrates in the no-choice paradigm increased the preference for sucrose versus saccharin in the two-bottle test. It did not affect the relative preference for the other two carbohydrates, fructose and glucose, which strengthens the claim that the OTr plays a role in sucrose-specific satiety. The fact that there was no change in preference for fructose and glucose versus saccharin implies that calories, at least in the relatively energy-dilute liquid diets, are not the causative factor behind the shift in preference in the sucrose versus saccharin scenario.

Fig. 3



Hypothalamic OT gene expression established with RT-PCR. On two consecutive nights, mice were provided access to 3 ml of 10% sucrose, 10% cornstarch, or 0.1% saccharin solutions; controls were given water. $^{*}P < 0.05$; $^{**}P < 0.01$. OT, oxytocin; RT-PCR, real-time PCR.

Our understanding of the role of OT in the regulation of food intake and food preferences exemplified by the data presented here is also crucial considering the fundamental function of OT in control of physiological and social processes that are intertwined with changes in eating behavior. For example, OT reduces anxiety, stimulates social interactions, pair-bond formation, in-group trust, maternal behavior, and lactation [21]. As all these phenomena affect various aspects of feeding regulation, from the amount of food eaten to food preferences, data on the link between OT and macronutrient/flavor preferences are a step toward defining neuroendocrine bases of appetite control in specific behavioral and (patho) physiological conditions.

In summary, the present study shows that OT inhibits appetite for carbohydrates and this role is independent of sweet flavor. Sucrose consumption considerably enhances OT gene expression and appears to be particularly sensitive to the OTr blockade, which strengthens the notion that there is a special functional relationship between OT and sucrose intake, and suggests that OT may serve as a key neuroregulator of sucrose-specific satiety.

Acknowledgements

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Conflicts of interest
There are no conflicts of interest.

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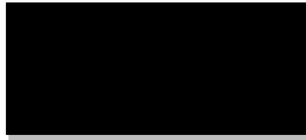
Paper II



Contribution statement:

Herisson FM: data collection, analysis, interpretation; drafted the manuscript

Waas JR: critical revision of the article

Frediksson R: help with data collection and analysis

Schiöth HB: help with PCR data collection and analysis

Levine AS: help with data analysis and critical revision of the article

Olszewski PK: designed the studies, helped draft the manuscript

All authors read and approved the final manuscript

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ORIGINAL ARTICLE

Oxytocin Acting in the Nucleus Accumbens Core Decreases Food Intake

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Central oxytocin (OT) promotes feeding termination in response to homeostatic challenges, such as excessive stomach distension, salt loading and toxicity. OT has also been proposed to affect feeding reward by decreasing the consumption of palatable carbohydrates and sweet tastants. Because the OT receptor (OTR) is expressed in the nucleus accumbens core (AcbC) and shell (AcbSh), a site regulating diverse aspects of eating behaviour, we investigated whether OT acts there to affect appetite in rats. First, we examined whether direct AcbC and AcbSh OT injections affect hunger- and palatability-driven consumption. We found that only AcbC OT infusions decrease deprivation-induced chow intake and reduce the consumption of palatable sucrose and saccharin solutions in nondeprived animals. These effects were abolished by pretreatment with an OTR antagonist, L-368,899, injected in the same site. AcbC OT at an anorexigenic dose did not induce a conditioned taste aversion, which indicates that AcbC OT-driven anorexia is not caused by sickness/malaise. The appetite-specific effect of AcbC OT is supported by the real-time polymerase chain reaction analysis of OTR mRNA in the AcbC, which revealed that food deprivation elevates OTR mRNA expression, whereas saccharin solution intake decreases OTR transcript levels. We also used c-Fos immunohistochemistry as a marker of neuronal activation and found that AcbC OT injection increases activation of the AcbC itself, as well as of two feeding-related sites: the hypothalamic paraventricular and supraoptic nuclei. Finally, considering the fact that OT plays a significant role in social behaviour, we examined whether offering animals a meal in a social setting would modify their hypophagic response to AcbC OT injections. We found that a social context abolishes the anorexigenic effects of AcbC OT. We conclude that OT acting via the AcbC decreases food intake driven by hunger and reward in rats offered a meal in a nonsocial setting.

Key words: oxytocin, food intake, reward, hunger, satiety

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Oxytocin (OT) promotes feeding termination mainly via central mechanisms (1,2). An increased level of OT neuronal activity and OT release occur at the end of a meal and in response to excessive plasma osmolality and stomach distension (3,4). In 1990, Arletti *et al.* (5) showed that i.c.v. OT injections cause a marked reduction in food intake. Subsequent to that initial study, other studies have confirmed the anorexigenic effect of OT and have begun identifying brain sites mediating OT-driven food termination (6). Much of the work has focused on the brainstem component of the circuitry: OT infusions in the nucleus of the solitary tract (NTS) (2) inhibit feeding, and the activity within the OT NTS–paraventricular nucleus (PVN) pathway has been functionally linked with neuroendocrine regulators of energy homeostasis (especially those involved in gastrointestinal and osmotic control), including cholecystokinin, glucagon-like peptide-1 (GLP-1) and peptide YY (7). Furthermore, OT administration in the ventromedial hypothalamic nucleus acutely decreases chow intake and increases energy expenditure in rats (7,8).

OT acting outside the brainstem–hypothalamic pathways also promotes hypophagia. Mullis *et al.* (9) found that OT administered in the ventral tegmental area (VTA) in rats causes a dose-dependent decrease in deprivation-induced chow intake and affects sucrose consumption. Their findings were in agreement with other reports suggesting that OT appears to serve as a cross-link between mechanisms bridging the termination of consumption as a result of 'homeostatic' (i.e. related mainly to stomach distension and osmolality) and palatability-specific (i.e. related to sweet flavour

and carbohydrate) satiety. For example, OT knockout mice consume sweet carbohydrates and saccharin less avidly than wild-type controls. Pharmacological blockade of the OT receptor (OTR) with a peripherally injected blood–brain barrier penetrant OTR antagonist L-368,899 elevates the intake of sweet tastants in sated mice (10), whereas i.c.v. OT administration reduces the intake of glucose in food-deprived rats (11). Opioid receptor antagonist injections that decrease palatable sucrose intake also activate OT neurones (12), and OT gene expression is increased after a high-sugar diet intake in rats (13).

Despite the presence of the OTR in the core (C) and shell (Sh) subdivisions (2,14,15) of the nucleus accumbens (Acb), another key extrahypothalamic appetite regulating area, there have been no reports delineating potential involvement of this region in mediating the effects of OT on food intake. Extensive studies on the relationship between AcbSh and feeding control have shown that opioid and dopamine signalling in the AcbSh particularly affects the hedonics of consumption (15,16), whereas glutamate and GABA in this subdivision also regulate energy intake (17,18). Less is known about AcbC, although several studies report that GLP-1 in the AcbC affects meal size and the rewarding aspects of food consumption (19–21). Katsuura *et al.* (22) have found that AcbC mu opioid receptor activation increases the rewarding value of a palatable lipid emulsion, Intralipid (Baxter Healthcare Corp., Deerfield, IL, USA), and attenuates inhibitory post-ingestive feedback. Also dopamine signalling in the AcbC appears to modify feeding reward because it affects the need-based motivational value of food-paired cues (23).

The present study investigated whether OT acting in the Acb affects appetite in rats. First, we examined whether AcbC and/or AcbSh injections of OT decrease hunger- and palatability-driven consumption. Once it was established that only the AcbC injections produce hypophagia, we focused on providing a more detailed characterisation of the role of the AcbC OT in feeding regulation. A conditioned taste aversion (CTA) paradigm was used to examine whether AcbC OT-driven anorexia is related to malaise. Considering that OT plays a significant role in social behaviour (24–26), we also examined how OT injected in the AcbC affects feeding in a social setting. Finally, we determined changes in c-Fos immunoreactivity in feeding-related brain sites after AcbC infusion of an anorexigenic dose of OT, and we analysed changes in OTR mRNA expression in rats exposed to food deprivation and to a palatable diet. *In vivo* pharmacology studies employed a native OT molecule [Ki (nM): hOTR, 0.8. h V1a, 120; hV1b > 1000; hr2, 3500; rOTR, 1.0; rV1a, 71; rV1b, 294; rV2, 89; mOTR, 0.6; mV1a, 46.1; mV1b′ 494] and L-368,899 [Ki (nM): hOTR, 7.6; rOTR, 570; rV1a, 370; rV1b, 8.9; rV2, 89] with an approximate half-life of OT of 3 min the blood and 20 min in the central nervous system, whereas the half-life of the antagonist is approximately 2 h (27–29).

Materials and methods

Animals

Adult male Sprague-Dawley rats weighing approximately 335 g at the beginning of the experiment were housed individually in plastic cages under a 12 : 12 h light/dark cycle (lights on 07.00 h) at 20–22 °C with tap water and standard laboratory chow (Teklad; Harlan, Indianapolis, IN, USA) available *ad lib.* unless noted otherwise. All procedures received prior approval from the University of Waikato ethics committee.

Surgeries

Rats used for intracranial infusion studies were anaesthetised with i.p. ketamine (100 mg/kg)/xylazine (20 mg/kg) and equipped unilaterally with a 7.5-mm stainless steel cannula (23-gauge; Plastics One Inc., Roanoke, VA, USA) aimed at the AcbC or AcbSh. Stereotaxic coordinates for the AcbC were: 1.5 mm anteroposterior; −1.4 mm mediolateral to bregma; −5.5 mm dorsoventral to skull surface; and, for the AcbSh: 1.5 mm, −0.8 mm; −7 mm, respectively. The injector protruded 0.5 mm below the tip of the cannula. Dental cement was used to secure the cannula to three screws inserted in the skull. Rats received the nonsteroidal anti-inflammatory drug caprofen (5 mg/ml, s.c.) immediately following the surgery. They were allowed to recover for at least 7 days. After the completion of all behavioural experiments, rats were sacrificed and cannula placement was assessed in immunohistochemically processed brains (see below on c-Fos immunoreactivity in feeding-related brain sites in response to AcbC OT injection).

Injections

OT (Phoenix Pharmaceuticals, Inc. Burlingame, CA) and OT receptor antagonist (L-368,899; Tocris Bioscience, St Louis, MO, USA) were injected with Hamilton syringes in a volume of 0.5 μl over 1 min. Drugs were dissolved in isotonic saline. Time intervals between an injection and food presentation or another drug administration given in each experimental session are counted from the moment of completion of the first injection. Additional injection studies aimed at validating the experimental approaches described here are presented in the Supporting information (Table S1).

Effect of OT in the AcbC versus AcbSh on deprivation-induced chow intake

Rats were deprived of chow overnight. Five minutes before food presentation (10.00 h), they were injected in the AcbC or AcbSh with 0 (saline), 0.3, 1 and 3 μg of OT (n = 8 or 9 per group; n = 9 only in saline controls). Chow was weighed before and 2, 4 and 24 h after the treatment.

Effect of OT in the AcbC or AcbSh on the intake of sweet palatable solutions

We followed a protocol similar to that described previously (10). In brief, animals were accustomed to having access to a bottle of a 10% sucrose solution for 2 h/day (10.00–12.00 h) for 3 days (chow and water were removed for the 2 h). On day 4, just before sucrose presentation, rats were injected in the AcbC or AcbSh with 0 (saline), 0.1, 0.3, 1 and 3 μg of OT (n = 8 per group in the AcbSh experiments and n = 12 per group in AcbC experiments) and the amount of consumed solution was established 2 h post-injection. The same approach was employed to study the effect of OT on 0.1% saccharin intake; however, doses of OT of 0, 0.01, 0.03, 0.1, 0.3 and 3 μg were used.

Effect of AcbC OTR antagonist pre-treatment on the ability of AcbC OT to decrease deprivation-induced feeding

Rats were deprived of chow overnight. Just prior to regaining access to chow (10.00 h), they received two AcbC injections spaced 10 min apart:

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(i) saline followed by saline; (ii) saline followed by 1 μg of OT (lowest effective OT dose based on the deprivation-induced chow intake experiment); (iii) 0.3 μl of L-368,899 followed by 1 μg of OT; (iv) 1 μl of L-368,899 followed by 1 μg of OT; and (e) 3 μl of L-368,899 followed by 1 μg of OT (n = 7 per group). Food intake was measured 2 and 4 h post-injection. L-368,899 doses were chosen based on previous studies (10).

Effect of AcbC OTR antagonist pre-treatment on the ability of AcbC OT to decrease consumption of sucrose and saccharin solutions

As described for the experiment involving intake of sweet palatable solutions, animals were accustomed to having access to a bottle of a 10% sucrose (or 0.1% saccharin) solution for 2 h/day (10.00–12.00 h) for 3 days. On day 4, just before sucrose presentation, rats were double-injected (10 min apart) in the AcbC with (i) saline followed by saline; (ii) saline followed 0.3 μg of OT; and (iii) 1 μl of L-368,899 followed by 0.3 μg of OT. Before the 0.1% saccharin presentation, rats were treated in the same manner except for the doses of OT and L-368,899, which were 0.03 μg and 3 μl, respectively (n = 7 or 8 per group; n = 7 only in saline–saline controls). Consumption was measured 2 h post-injection.

Effect of AcbC OT on deprivation-induced and palatability-induced consumption in the social context

To assess the effect of AcbC OT on food intake in the social context, single-housed rats were episodically placed in a predictable social setting in which food intake occurred. We designed an apparatus that was a subdivided standard Makrolon cage (Tecniplast, Buguggiate, Italy)**).** with a metal grid lid (with an overhead food hopper and bottle holder on one side of the cage). It was subdivided into two identical chambers (length 48 cm, width 19 cm) by a transparent, Plexiglas partition wall, containing multiple round openings (diameter 1.5 cm), which allowed the rats placed simultaneously in each chamber for incomplete socialisation (visual, auditory, olfactory and partial tactile) devoid of major direct interactions (especially antagonistic ones) that could impair the ability of an animal to access food. All animals used in these studies were accustomed to being in the apparatus and having food presented there for at least eight separate 1-h training sessions (starting between 10.00 and 12.00 h). The chambers were wiped with ethanol after each use. During the pharmacological studies, only one of the two animals simultaneously present in the apparatus received an injection of saline or OT. The dose ranges were chosen based on the results obtained in single-housed animals indicating that higher doses of AcbC OT are needed to diminish deprivation-induced chow intake, whereas lower doses are sufficient to affect palatability-driven feeding.

To examine the effect of OT on deprivation-induced feeding in the social setting, animals had chow removed overnight. Afterwards, two rats were placed simultaneously in the social context apparatus (one rat per chamber) where pre-weighed chow was put in the hopper. Just prior to the session, one of the two rats received an injection of 0 (saline), 1 or 3 μg of OT (n = 8–10 per group; n = 8 only in the 3 μg treatment) in the AcbC, and food intake was measured 1 h later.

To assess the effect of OT on intake of sweet palatable tastants in the social setting, rats were accustomed to having episodic access to a single bottle of 10% sucrose or 0.1% saccharin solution similarly to the nonsocial scenario described above for the effect of OT in the AcbC or AcbSh on the intake of sweet palatable solutions. On the experimental day, two rats were placed simultaneously in the social context apparatus (one rat per chamber) where they gained access to individual pre-weighed bottles containing a sweet solution. Just prior to the session, one of the two rats received an injection of saline) or OT. Doses of OT of 0.03, 0.1, 0.3 and 1 μg were injected in the sucrose-exposed animals, whereas doses of OT of 0.03, 0.1 and 0.3 μg were used in rats given the saccharin solution (n = 6 animals were injected with the 0.03 μg dose; n = 8 for all the other treatments). Bottles were collected and weighed before and after 1 h post-injection.

Does social exposure affect OT receptor expression in the AcbC?

To better understand the effect of a social context on the effectiveness of the OT treatment as an anorexigen, we repeated the social exposure paradigm described above; however, no dietary modification was introduced. Every day for 8 days, animals were placed for 2 h (starting between 10.00 and 12.00 h) with the same conspecific in the apparatus. Animals were not food-deprived; hence, no food was given during those 2 h. Animals placed singly in the socialisation apparatus served as controls (n = 8 per group). Immediately after the 2-h exposure to the conspecific, the rats were decapitated (10.00–12.00 h) and the AcbC was collected, excised, immersed in RNAlater (Ambion, Austin, TX, USA), kept at room temperature for 2 h and subsequently stored at −80 °C, as described previously (13). The tissue was analysed by a real-time polymerase chain reaction (PCR) for the presence of the OT receptor mRNA (a detailed description of the procedure is given below regarding RNA isolation, cDNA synthesis and real-time PCR).

AcbC OT and CTA development

Rats were accustomed to having access to water for 2 h (11.00–13.00 h) per day for 3 days. Food was removed from hoppers for the 2-h period of scheduled fluid presentation. On day 4, rats were given a novel 0.1% saccharin solution instead of water for 60 min. Afterwards they received an AcbC injection of saline or 1 μg of OT (an effective anorexigenic dose based on feeding experiments) (n = 5 per group). On day 5, a two-bottle preference test (saccharin versus water) was used to assess the acquisition of a CTA to the saccharin solution. Bottles were weighed and percentages of the saccharin solution intake (out of cumulative, i.e. saccharin plus water, intake) were calculated.

Consummatory behaviour data analysis

All food intake experiments utilising single OT injections were analysed by one-way ANOVA followed by Fisher's post-hoc test. In the double injection experiments, first, the effect of OT against saline control was confirmed with a Student's *t*-test and then the effect of the antagonist pretreatment on OT-induced anorexia was determined by one-way ANOVA followed by Fisher's post-hoc test. Post-hoc tests were only applied when the F-ratio was significant. The CTA OT versus saline two group comparison was performed using Student's *t*-test. $P < 0.05$ was considered statistically significant.

c-Fos immunoreactivity in feeding-related brain sites in response to AcbC OT injection

Rats received a single injection of saline (n = 5) or 3 μg of OT in the AcbC (n = 6). Injections were performed between 10.00 and 12.00 h. Food and water were removed immediately after the drug treatment. Sixty minutes later, animals were anaesthetised and perfused with 50 ml of saline followed by 500 ml of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Brains were excised and postfixed overnight in PFA (4 °C). Coronal 60-μm vibratome (Leica Microsystems, Wetzlar, Germany) sections were processed for c-Fos immunostaining. The tissue was treated for 10 min in 3% H_2O_2 in 10% methanol [in Tris-buffered saline (TBS), pH 7.4] and incubated overnight at 4 °C in the goat anti-c-Fos antibody (dilution 1:2000; Santa

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Cruz Biotechnology, Santa Cruz, CA, USA). Subsequently, sections were incubated for 1 h in the rabbit-anti-goat antibody (Vector Laboratories, Inc., Burlingame, CA, USA) and then in the avidin-biotin complex (1 h; Vector Laboratories, Inc.). Peroxidase was visualised with 0.05% diaminobenzidine, 0.01 H_2O_2 and 0.2% nickel sulfate (Sigma, St Louis, MO, USA). All incubations were conducted in a mixture of 0.25% gelatin and 0.5% Triton X-100 (Sigma) in TBS. Intermediate rinsing was performed with TBS. Sections were mounted on gelatinised slides, dried, dehydrated in ethanol, soaked in xylene and embedded in Entellan (Merck, Darmstadt, Germany). The number of c-Fos positive nuclei was counted bilaterally in all regions of interest (four to five sections containing a given site per animal), except for the AcbC where c-Fos IR was assessed ipsilaterally to the cannula, using SCION IMAGE (Scion Corp., Frederick, MD, USA). Densities of Fos positive nuclei (per mm^2) were averaged per rat and then per group. A *t*-test with the Bonferroni correction for multiple comparisons was used to determine differences between the two groups.

Effect of regular diet versus sweet diet and regular diet versus food deprivation on OTR gene expression levels in the AcbC

To assess the effect of sweet palatable solution exposure on AcbC OTR mRNA levels, rats were given access to either chow and water (control) or to chow and 0.1% saccharin solution (instead of water) for 48 h (n = 8 per group). In a separate experiment assessing the effect of food deprivation on OTR mRNA levels, rats were either maintained on *ad lib.* access to chow and water (control) or chow was removed for 24 h prior to decapitation (n = 8 per group).

The animals were decapitated (10.00–12.00 h) and the AcbC was collected excised, immersed in RNAlater (Ambion), kept at room temperature for 2 h and subsequently stored at −80 °C, as described prevously (13).

RNA isolation, cDNA synthesis and real-time PCR

Samples were sonicated in TRIzol (Invitrogen, Carlsbad, CA, USA), chloroform was added to the homogenate, which was then centrifuged at 10 000 **g** at 4 °C for 15 min. The water phase was transferred to a new tube, and RNA was precipitated with isopropanol. The pellets were washed with 75% ethanol, air dried and dissolved in RNAse-free water. DNA was removed with DNAse I treatment (Roche, Basel, Switzerland; 4 h, 37 °C) and the enzyme was inactivated by heating the samples at 75 °C for 15 min. The absence of genomic DNA was determined by PCR analysis with primers for the RNA extractions with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on the DNAse-treated RNA. RNA concentration was measured with an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). cDNA was synthesised with Moloney murine leukemia virus reverse transcriptase (GE Healthcare, Little Chalfont, UK) using random hexamers as primers in accordance with the manufacturer's instructions. cDNA was analysed with a MyIQ thermal cycler (Bio-Rad, Hercules, CA, USA). Each real-time PCR reaction with a total volume of 20 μl contained cDNA synthesised from 25 ng of total RNA, 0.25 M each primer, 20 mM Tris/HCl (pH 8.4), 50 nM KCl, 4 mM $MgCl_2$, 0.2 mM dNTP and SYBR Green (dilution 1 : 50000). Reactions were performed with 0.02 U/l Taq DNA polymerase (Invitrogen) under the conditions: initial denaturation for 4 min at 95 °C, followed by 50 cycles of 15 s each at 95 °C, 30 s at 55–62 °C (i.e. at the optimal annealing temp. for each primer pair) and 30 s at 72 °C. This was followed by 1 min at 55–62 °C (optimal annealing temperature) and a melting curve with 84 cycles of 10 s at 55 °C increased by 0.5 °C per cycle. All experiments were performed in duplicate. The measurements where the threshold cycle (Ct) values between the duplicates had a difference of over 0.9 were repeated. A negative control for a given primer pair and a positive control with 25 ng of genomic DNA was included on each plate. The HKGs used to define normalisation factors were: GAPDH (forward: ACATGCCGCCTGGAGAAACCT; reverse: GCCCAGGATGCCCTTTAGTGG), β-tubulin (forward: CGGAAGGAGGCG GAGAGC; reverse: AGGGTGCCCATGCCAGAGC), ribosomal protein 19 (forward: TCGCCAATGCCAACTCTCGTC; reverse: AGCCCGGGAATGGACAGTCAC), histone H3 (forward: ATTCGCAAGCTCCCCTTTCAG; reverse: TGGAAGCGCA GGTCTGTTTTG), cyclophilin (forward: GAGCGTTTTGGGTCCAGGAAT; reverse: AATGCCCGCAAGTCAAAGAAA), β-actin (forward: CACTGCCGCATCCTCTTCCT; reverse: AACCGCTCATTGCCGATAGTG) and succinate dehydrogenase complex, subunit B (forward: GGGAGTGCCGTGGTGTCATTG; reverse: TTCGCCCATA GCCCCCAGTAG). The sequences of the OTR primer pairs were: TTCTTCTGCTGCTCTGCTCGT (forward) and TCATGCTGAAGATGGCTGAGA (reverse).

Data analysis and relative expression calculations

MYIQ, version 1.04 (Bio-Rad) was used to analyse quantitative PCR data and derive Ct values. Melting curves were analysed to confirm that one product was amplified and that it was significantly shifted compared to the melting curve for the negative control. The sample Ct values were analysed further if the difference between those and the negative control exceeded 3; otherwise, the transcript was considered not to be expressed. Normalisation factors were calculated with GENORM (https://genorm.cmgg.be). LinRegPCR (http://www.hartfaalcentrum.nl) was employed to calculate PCR efficiencies for each sample. Grubb's test (GraphPad Software Inc., San Diego, CA, USA) was used to identify and remove outliers and calculate average PCR efficiencies for each primer pair. Differences in OTR expression between groups were analysed with a Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

We have found that direct AcbC (Figs 1 and 2) but not AcbSh (Fig. 1E,F,G) injections of OT affect food intake. Administration of 1 and 3 μg of OT in the AcbC decreased deprivation-induced cumulative chow intake (Fig. 1A,B) by approximately 35–40% at 2 h (1 μg, P = 0.028; 3 μg, P = 0.019) and 4 h (1 μg, P = 0.006; 3 μg, P = 0.004) post-injection. There was no effect on food consumption at 24 h (data not shown). Water consumption remained unchanged (Table 1), although, at 4 h post-injection, there was a trend towards decreased water intake (0.3 μg, P = 0.068; 1 μg, P = 0.092; 3 μg, 0.084).

In nondeprived rats stimulated to eat by palatability of a diet, AcbC infusions of 1 μg and 3 μg of OT decreased sucrose solution intake by approximately 50% (P = 0.017 and P = 0.045, respectively). Saccharin solution consumption was decreased to a similar degree by OT; however, lower doses were needed to achive this effect: 0.03 μg (P = 0.03), 0.1 μg (P = 0.026), 0.3 μg (P = 0.025) and 1 μg (P = 0.027) (Fig. 1C,D).

Our CTA study showed that AcbC administration of an anorexigenic 1-μg dose of OT after presentation of the novel 0.1% saccharin solution did not produce learned avoidance of saccharin in the subsequent two-bottle choice test (Fig. 1H).

The anorexigenic action of AcbC OT was reversed by pre-treatment with an OTR antagonist. In overnight-deprived rats, 1 and 3 μg of L-368,899 counteracted the effect of OT at 2 h (3 μg, P = 0.042) and 4 h (1 μg, P = 0.025; 3 μg, P = 0.016) post-injection (Fig. 2A,B). The antagonist abolished the effect of OT on the

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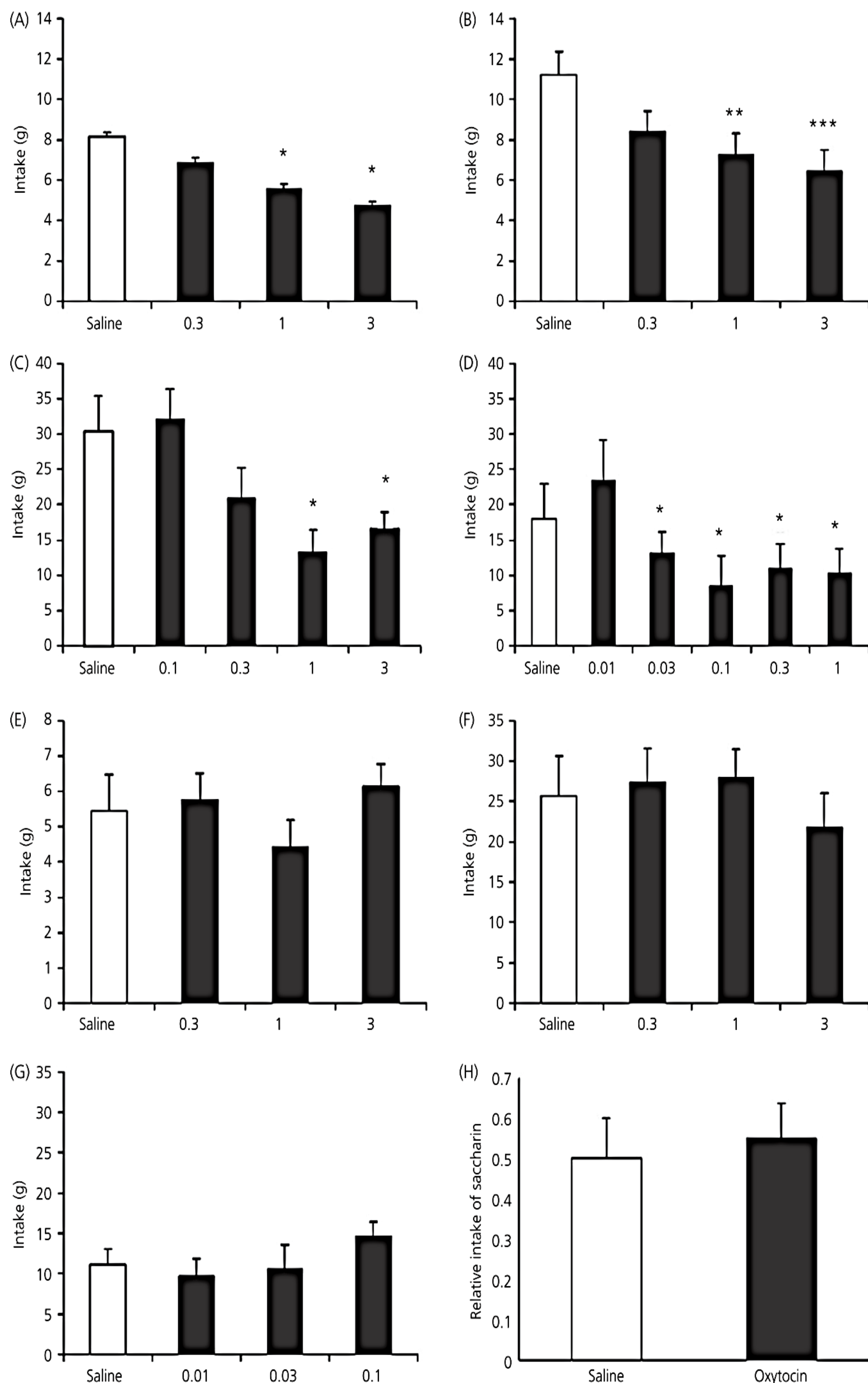

Fig. 1. The effect of oxytocin (OT) injections (doses in µg) in the nucleus accumbens core (AcbC) and nucleus accumbens shell (AcbSh) on consummatory behaviour in rats. (A) The effect of AcbC OT on deprivation-induced chow intake 2 h and (B) 4 h post-injection. (C) The effect of AcbC OT on the intake of 10% sucrose solution and (D) 0.1% saccharin solution. (E) The effect of AcbSh OT on deprivation-induced chow intake 2 h post-injection. (F) The effect of AcbSh OT on the intake of 10% sucrose solution and (G) 0.1% saccharin solution. (H) Preference for a saccharin solution in a two-bottle preference test (saccharin versus water) was assessed to determine whether AcbC OT promotes acquisition of a conditioned taste aversion (CTA). *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$.

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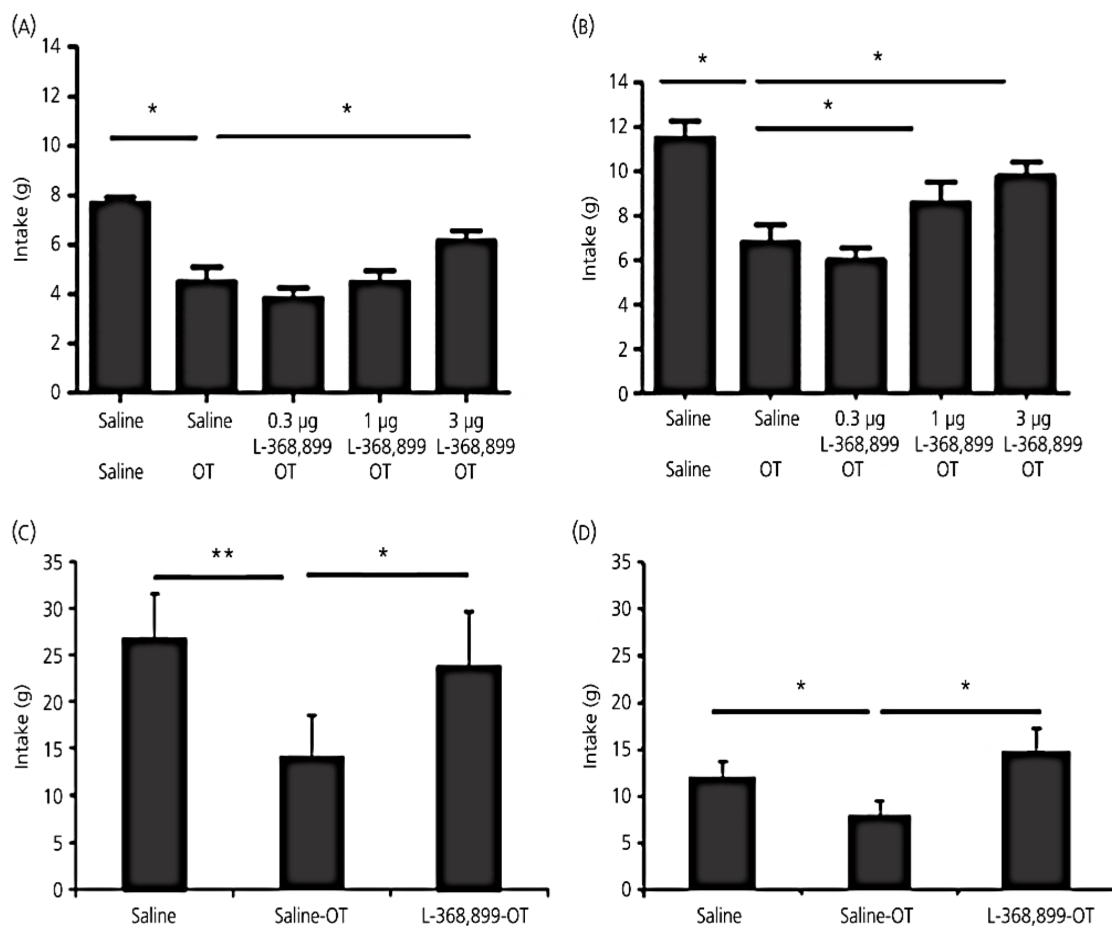

Fig. 2. The effect of nucleus accumbens core (AcbC) oxytocin (OT) receptor antagonist (L-368,899) pretreatment on the ability of AcbC OT to reduce feeding. Deprivation-induced chow intake was measured 2 h (A) and 4 h (B) after a double injection of saline + saline, saline + 1 µg of OT, 0.3,1,3 µg of L-368,899 + 1 µg of OT. (C) Intake of 10% sucrose solution was determined 2 h after AcbC double-injection of saline + saline, saline + 0.3 µg OT, and 1 µg of L-368,899 + 0.3 µg of OT. (D) Intake of 0. 1% saccharin solution was measured 2 h after AcbC double-injection of saline + saline, saline + 0.03 µg of OT, 3 µg of L-368,899 + 0.03 µg of OT. *P < 0.05; **P < 0.01.

Table 1. The Effect of Oxytocin (OT) Injected in the Nucleus Accumbens Core (AcbC) at 0 (Saline), 0.3, 1 and 3 µg, on Water Intake, 2, 4 and 24 h Post-Injection.

	Water intake (g)		
OT (µg)	2 h	4 h	24 h
0 (saline)	8.2 ± 1.4	15.9 ± 2.5	62.8 ± 5.9
0.3	8.1 ± 1.1	11.4 ± 1.3	56.3 ± 6.7
1	7.4 ± 0.4	12.0 ± 1.1	51.9 ± 6.8
3	6.5 ± 0.7	12.3 ± 0.8	61.2 ± 8.4

intakes of the sucrose (P = 0.030) and saccharin (P = 0.04) solutions (Fig. 2C,D).

Furthermore, although OT-induced short-term anorexia was apparent in single-housed animals stimulated to eat either by hunger or by palatability, AcbC infusions of OT at doses effective in the aforementioned scenarios did not affect hunger- or reward-driven consumption in rats subjected to the social context of a 1-h meal (Fig. 3). Rats subjected to the social condition (without any dietary modification) showed a lower level of OT receptor mRNA in the AcbC than nonsocialised controls (normalised OT mRNA levels in nonsocialised rats: 1.0 ± 0.16; in socialised rats: 0.56 ± 0.12; P = 0.014).

Our c-Fos study revealed that AcbC infusion of 3 µg of OT increased activation of the AcbC itself (P = 0.008), as well as two other sites belonging to a widespread central network regulating appetite: the PVN (P = 0.029) and the supraoptic nucleus (SON) (P = 0.036) (Fig. 4).

Finally, using real-time PCR, we found that expression of the OTR mRNA in the AcbC is significantly higher in food-deprived rats compared to *ad lib.*-fed animals (P = 0.033) and expression decreases in rats given 48-h access to a saccharin solution compared to standard diet-fed rats (P = 0.045) (Fig. 5).

Discussion

The classical concept of the involvement of OT in the regulation of appetite revolves around the phenomena critical for maintaining

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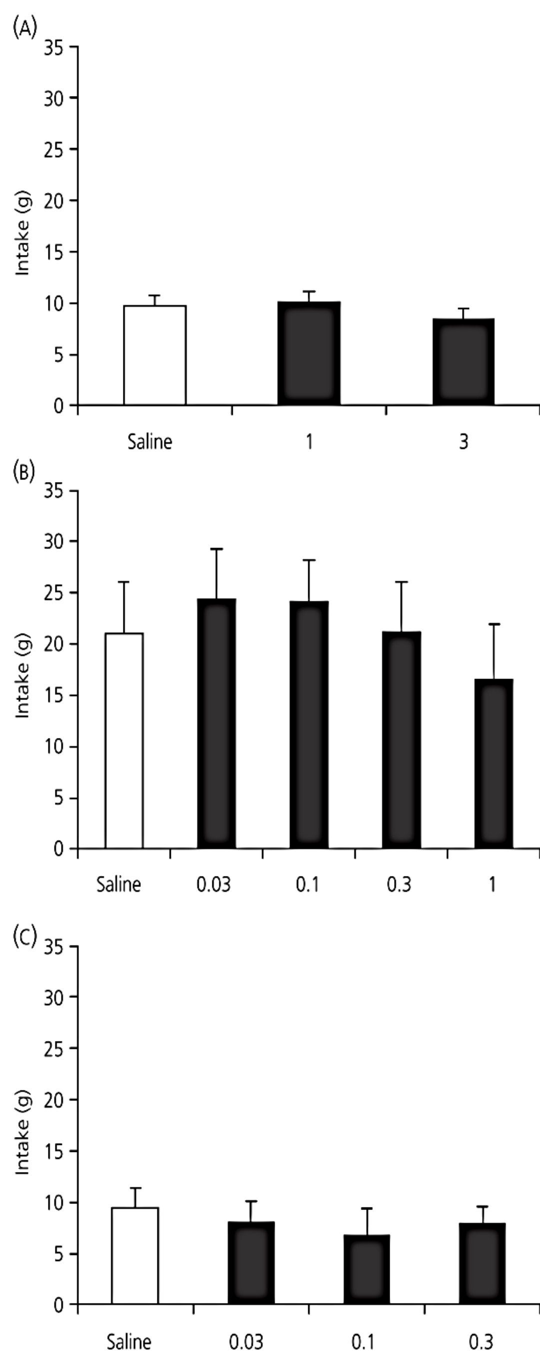

Fig. 3. The effect of nucleus accumbens core (AcbC) oxytocin (OT) (doses in μg) on consummatory behaviour of rats offered a meal in a social context. (A) Deprivation-induced chow intake 1 h post-injection. (B) 10% sucrose solution intake and (C) 0.1% saccharin solution intake 1 h post-injection.

and rescuing internal milieu. Indeed, OT signalling, especially within the hypothalamic-brainstem circuits, aids in the termination of eating behaviour that poses a threat to the water-electrolyte balance and to the functioning of the gastrointestinal tract, and also is associated with the ingestion of toxins (3,4,30). The most recent discoveries have expanded upon this view because they strongly suggest that OT decreases appetite for carbohydrates and for palatable sweet tastants (31) by engaging a wider network of neural sites, including the VTA (9). The current set of studies identifies, for the first time, the AcbC as a key site integrating the involvement of OT in energy homeostasis and the feeding reward.

We found that AcbC injections of OT decreased hunger-derived intake of 'bland' chow and the palatability-driven consumption of sweet solutions. In both cases, the effect was reversible by the pre-treatment with the OTR antagonist, L-368,899, which suggests that the changes in feeding induced by AcbC OT are mediated by the OTR. The magnitude of the anorexigenic response in overnight-deprived rats to AcbC OT was similar to those observed after lateral ventricular (32) and ventromedial hypothalamic nucleus (33) OT infusions and somewhat smaller than the reduction in feeding observed after OT administration into the fourth ventricle (34). This supports the notion that, although the hindbrain OTR has a critical role for the regulation of energy balance, the forebrain populations of this receptor, including in the AcbC, should be viewed as comprising an important contributor to the central mechanisms governing feeding for calories. It should be noted that our experiments utilised unilateral injections of OT; therefore, it is likely that bilateral infusions of OT might produce anorexigenic effects even at a lower dose range.

One of the most crucial findings in research on the influence of OT on appetite in the recent years has been the set of discoveries linking OT and appetite for carbohydrates and for sweet taste. OT neuronal activity is particularly high after the consumption of a sucrose-rich meal (31). Generalised knockout of the OT gene in mice results in the phenotype showing sweet and nonsweet carbohydrate and saccharin hyperphagia (35). Peripheral infusions of the blood-brain barrier penetrant OTR antagonist, L-368,899, in wild-type mice promote the intake of mono-, di- and polysaccharides and saccharin (10). Those findings started the debate regarding whether such effects are particularly relevant to carbohydrates, to sweet taste or to hedonic processing. The latest data, as well as the results reported in the present study, demonstrate the key role that OT plays in the regulation of the activity of the reward circuits, including the AcbC. Carson *et al.* (36) reported that systemic injection of OT decreases methamphetamine activation of the AcbC. Baracz *et al.* (37) found that pre-treatment with OT administered i.p. or directly into the AcbC attenuates the formation of a methamphetamine-induced conditioned place preference. The present study shows a reduced consumption of palatable sweet solutions in response to microinjections of OT in the AcbC at doses that were the same or lower than those reported for VTA administration of the peptide (9). It should be emphasised that the observed change did not stem from altered thirst responsiveness because AcbC OT did not affect water intake in water-deprived rats. Notably, a significant decrease in saccharin solution intake was achieved with the OT dose that was ineffective in reducing the intake of chow or sucrose, which indicates that the presence of energy is not a sole factor in the regulatory mechanisms by which AcbC OT modifies consummatory behaviour. This notion is strengthened by the outcome of the real-time PCR experiments in which AcbC OTR transcript levels were affected by the caloric and noncaloric challenge (i.e. by both energy deprivation and exposure to palatable saccharin). This also appears to be in line with the growing body of

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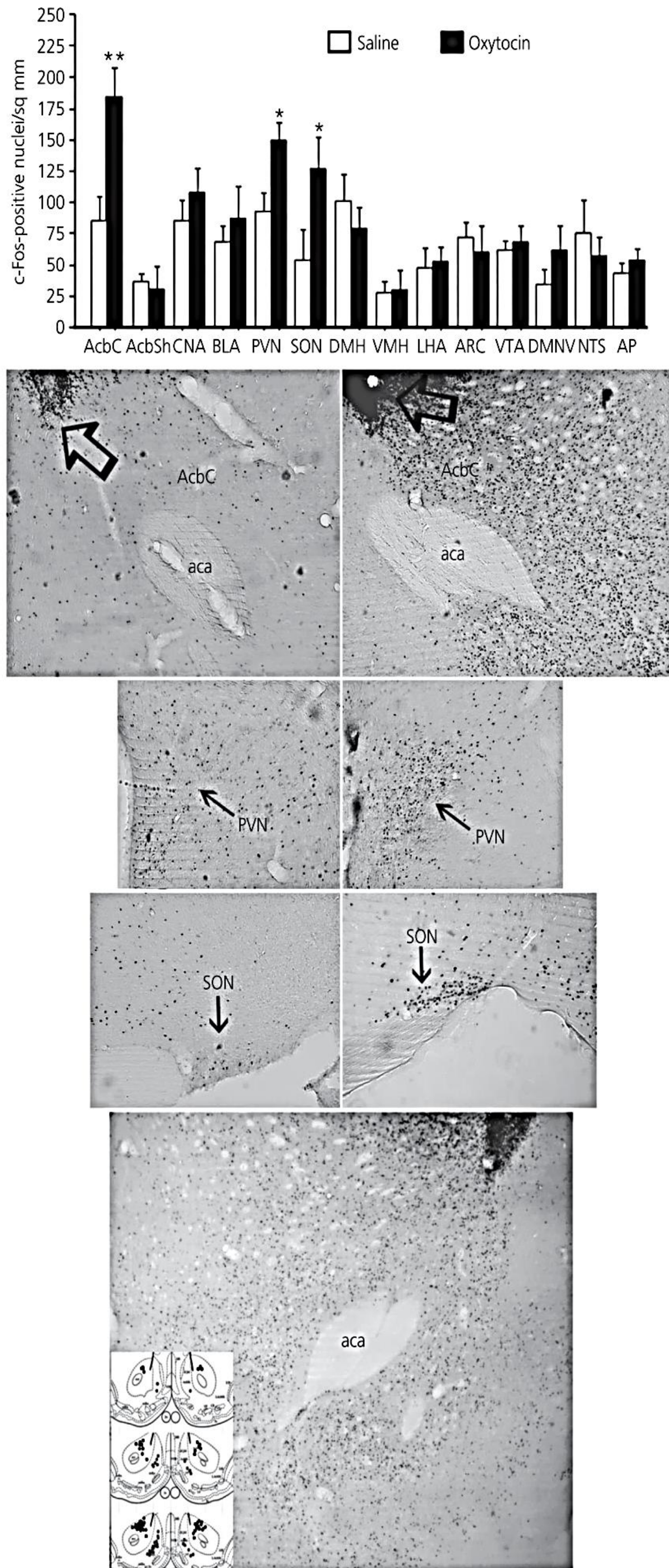

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Fig. 4. c-Fos immunoreactivity in feeding-related brain sites 1 h after an nucleus accumbens core (AcbC) injection of oxytocin (OT) versus saline. The arrow indicates the injection site. AcbSh, nucleus accumbens shell; VTA, ventral tegmental area; CNA, central nucleus of the amygdala; BLA, basolateral amygdala; DMNV, dorsal motor nucleus of the vagus; NTS, nucleus of the solitary tract; AP, area postrema; PVN, paraventricular hypothalamic nucleus; SON, supraoptic nucleus; DMH, dorsomedial nucleus; VMH, ventromedial nucleus; LHA, lateral hypothalamic area; ARC, arcuate nucleus; aca, anterior commissure. *P < 0.05; **P < 0.01. Microscope images on the left show brain sections of animals injected with saline, whereas those on the right brain sections of animals injected with OT. The bottom photomicrograph depicts a large-area view of distribution of c-Fos in the AcbC and AcbSh area in an OT-injected rat. Insert: map of estimated targets of intraparenchymal injection.

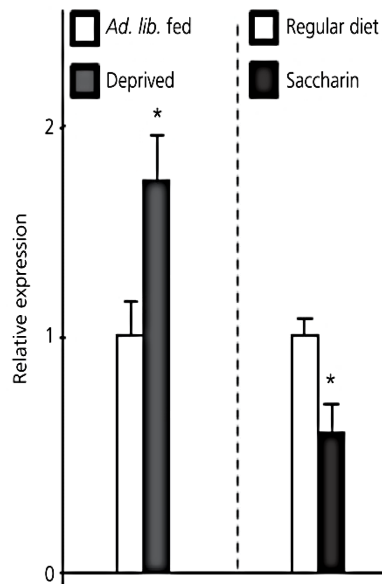

Fig. 5. Expression of oxytocin (OT) receptor mRNA in the nucleus accumbens core (AcbC). (A) The effect of chow deprivation versus *ad lib.* chow access. (B) The effect of 48-h exposure to 0.1% saccharin solution in addition to standard chow (Saccharin) versus water and standard chow (Regular diet). Gene expression was assessed by a real-time polymerase chain reaction. *P < 0.05.

evidence suggesting that the relationship between OT and suppression of appetite is independent of the energy density of tastants because OTR ligands modify intake of solid foods (such as chow or chocolate), as well as low-calorie fluids (31,38,39). Accordingly, the AcbC OT signalling appears to represent the intertwined mechanisms of caloric and noncaloric feeding control. The AcbC OT-induced increase in c-Fos immunoreactivity in the PVN and SON is very much reminiscent of neuronal activation patterns associated with the end of a meal, regardless of whether palatable or 'bland' (31,39), as well as drug treatments that lead to early termination of hunger- or reward-driven consumption (40,41). It is also in agreement with the suggestion that the Acb and PVN are part of the limbic-pituitary-hypothalamic pathways and that the monoaminergic ascending hypothalamic pathways play a major role in stress, emotion and reward (42). Although the data related to the core subdivision of the Acb are lacking, intra-AcbSh injections that affect appetite (e.g. with endocannabinoids) increase the activity of hypothalamic areas (43). Obviously, an increase in Fos levels in the hypothalamus observed in the present study may be an outcome of activation within a larger network (not via direct projections) because it has been shown, for example, that intrahypothalamic pathways modulate the PVN activity locally.

One of the most striking observations related to *in vivo* pharmacology experiments utilising OT is that site-specific injections of OT in reward-related areas require a very high dose of the ligand to be used. Indeed, in some paradigms (e.g. in deprivation-induced food intake in the present study), AcbC OT had to be infused at a dose that would be sufficient if delivered i.c.v. This finding is not unique for this set of studies because, for example, Mullis *et al.* (9) were unable to affect even the intake of sugar by intra-VTA administration of OT at doses lower than 1 μg. The fact that, in the present study, saccharin intake was reduced by 0.03 μg of OT suggests that some effects of OT within the reward sites on feeding may be strictly pharmacological, whereas the actual physiological involvement might be more intricate and limited to very specific aspects of food intake (from palatability to macronutrient composition to sociality).

Many studies have reported changes in consumption after direct pharmacological stimulation of the shell subdivision of the Acb. For example, $GABA_A$ and $GABA_B$ ligands administered in the AcbSh modify a short-term feeding response to food deprivation (44). Similarly, the blockade of AcbSh glutamate receptors and mu and kappa opioid receptors affects appetite (45,46). Importantly, our results add to the growing evidence suggesting that the core subdivision of the Acb also controls meal size after energy deprivation, as well as in palatability-driven food intake. Aside from the OTR, the GLP-1 and opioid receptors are involved in AcbC feeding mechanisms (47,48). Indeed, the change in feeding after AcbC OT infusion with the lack of a behavioural response to AcbSh OT treatment resembles the effects of AcbC versus AcbSh GLP-1 injections (21). Interestingly, similar to OT, GLP-1 in the Acb affects the processing of feeding-related and -unrelated rewards (47,49,50).

Although the results of our studies define AcbC OT signalling as being crucial in energy- and reward-induced consumption, the CTA experiment did not show any aversive consequences of AcbC OT treatment. The lack of the learned avoidance response strongly suggests that the anorexigenic effects of OT do not stem from undesirable sickness/malaise. This allows confidence when classifying the roles of AcbC OT as being related to appetite regulation rather than to learning avoidance of potentially dangerous foods. It should be noted that the reason behind assessing the CTA effects of AcbC OT was not only to control for drug-induced illness, but also to examine whether the OTR in the AcbC might be physiologically involved in the development of taste aversions. It has been reported previously that OT is released in response to the presence of toxins, contributing to malaise-dependent anorexia (12), and that a systemic OTR antagonist treatment impairs the acquisition of a CTA (51).

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For a number of reasons, including the practicality of an experimental set-up, the vast majority of feeding studies, as reported in the present study as well as previously, have been performed on animals housed individually. It should not be forgotten that food intake is often a social behaviour, in which the initiation, termination, dietary choices and meal duration, are influenced by characteristics of the social environment. This particular issue needs to be considered, especially in studies on neural systems that regulate both appetite and affiliative behaviours, including OT.

OT facilitates pair bonding, mother–infant bonding and social approach/recognition, and also diminishes anxiety (52–56). Peripheral and central OT release has been reported in response to positive social exposure (57). Importantly, socially stimulated changes in Acb activity have been proposed to influence other concurrent physiological and behavioural parameters. For example, studies on the link between the Acb and drug abuse have shown that preference for cocaine can be modified by social interaction (58). Lesions of the AcbC induce preference for the social compartment in the conditioned place preference paradigm, whereas lesions of the AcbSh shift it towards the cocaine-associated chamber (59). The results of the present study indicate that a social context has a profound effect on the ability of OT to affect feeding via the AcbC. Once the animal is placed in the environment in which social interaction occurs, intra-AcbC administration of OT does not diminish food intake driven by hunger or feeding induced by sweet flavour. Our additional study in which we assessed only the effect of social versus nonsocial settings on OT receptor mRNA levels in the AcbC helps to clarify the possible nature of the observed phenomenon. We found that social interaction alone leads to down-regulation of OT receptor expression, which suggests that a social environment is conducive to stimulating OT release in the AcbC, thereby contributing to a down-regulation of the receptor in that site. Consequently, the decreased ability of AcbC-injected OT to affect consumption is hampered likely because of a lower availability of the OT receptor. Certainly, the Acb participates in processing social rewards and the perception of socially driven stimulation (both positive and negative). For example, Kohls *et al.* (60) reported that dynamic social incentives elicit activation of the Acb in humans and that this increase in accumbal activity likely translates to an avoidance of social punishment. Furthermore, variation in the Acb and amygdala activity appears to underlie individual differences in prosocial and Individualistic economic choices (61). Obviously, in laboratory animals, 'economic' choices are more likely to be associated with the access to resources (and one of those resources is food). Although it is still unknown whether and, if so, to what extent OT might participate in the integration of social and nonsocial behavioural processes, there are data highlighting the neuroanatomical link between the OT system and the Acb: Knobloch *et al.* (62) found that magnocellular OT neurones innervate the Acb. This fits well with the previously reported findings showing that orexigenic effects of peripherally administered OTR antagonist in mice that were offered a meal in a social setting depended on the social status of animals (63). Therefore, social cues appear to act as a modifying factor in the ability of OTR ligands to affect appetite. It is likely that, in the case of a cross-link between social interactions and feeding, OT acts as a neuroendocrine regulator of behavioural activity in response to complex cues. A similar hypothesis has been proposed in relation to OT facilitating a switch from food intake to affiliative or sexual behaviour, thus balancing behaviours that contribute to internal homeostasis and evolutionary success (64,65). These findings should be interpretated with caution because the data available thus far are very fragmented. It appears that one of the most important steps is to define whether the cross-link between OT and social and nonsocial behaviours is facilitated by shared pathways or rather by independent circuits whose activity sums up to produce a certain behavioural response.

In summary, we conclude that OT acting via the AcbC decreases food intake driven by hunger and by sweet flavour-derived reward in rats offered a meal in a nonsocial setting, and also that this anorexigenic effect does not stem from malaise. Injection of OT in the AcbC affects activity of the AcbC itself, as well as the hypothalamic sites involved in feeding control. Finally, AcbC OTR mRNA levels change in response to food deprivation and after the consumption of palatable saccharin.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Effect of saline versus 1 µg of OT (n = 7) injected in the nucleus accumbens core (AcbC) versus nucleus accumbens shell (AcbSh) on deprivation-induced chow intake (4 h post-injection).

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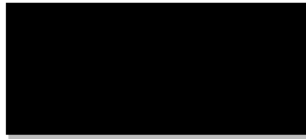
Paper III



Contribution statement:

Herisson FM: data collection, analysis; drafted the manuscript

Bird S: helped with the PCR work, contributed regents/materials/analysis tools

Sleigh JW: critical revision of the article

Levine AS: data analysis and critical revision of the article

Olszewski PK: design of the studies; helped draft the manuscript; approved the final version

All authors read and approved the final manuscript

Connexin 36 deletion diminishes feeding for reward

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Abstract

OBJECTIVES: Connexin 36 (Cx36) is a gap junction molecule ubiquitously expressed in the brain, especially in the reward circuit. Cx36 deletion is associated with dysregulation of GABA and dopamine in reward pathways and with decreased ethanol intake in Cx36 KO mice. Here, we used the Cx36 KOs to investigate whether the link between Cx36 and reward expands onto palatability-induced feeding. We determined intake of palatable tastants, expression profile of opioid system genes involved in feeding reward, and sensitivity of Cx36 KOs to feeding reward modifying opioid ligands, naltrexone and butorphanol. METHODS: Consumption of sweet (sucrose, glucose, fructose, saccharin) and lipid solutions during episodic (2h) and unrestricted access was measured in Cx36 KO versus wild-type (WT) mice. WTs were treated with a pharmacological Cx36 blocker, quinine, to substantiate the findings. Opioid transcript levels were assessed with qPCR. Finally, naltrexone or butorphanol were administered in KOs and WTs prior to sucrose or saccharin solution presentation and dose-response curves were established. RESULTS: Cx36 KOs showed decreased consumption of all palatable solutions offered in a brief and unrestricted manner. Quinine in WTs decreased palatability-driven feeding. Cx36-deficient mice maintained on a regular diet displayed an altered baseline expression profile of opioid system genes in the hypothalamus and nucleus accumbens. Cx36 KOs exhibited a diminished sensitivity to consumption modifying properties of naltrexone and butorphanol (sucrose) or a lack thereof (saccharin). CONCLUSIONS: Cx36 is essential in generating feeding for reward. The lack of Cx36 is associated with impaired functioning of the reward system, including its opioid-dependent component.

Highlights

Cx36 knockout mice show diminished consumption of palatable tastants.

Pharmacological blockade of Cx36 gap junctions decreases saccharin intake.

Connexin 36 knockout mice display altered expression of opioid system genes.

Connexin 36 knockouts show diminished feeding responses to opioid receptor ligands.

1. Introduction

Gap junctions (GJs), pores formed by connexin (Cx) subunits, provide a means for intercellular communication in the brain [1]. The passage of molecules through neuronal GJs coordinates cell firing, and metabolic as well as transcriptional events between coupled neurons. Compared to chemical synapses, GJ electrical synapses synchronize outputs of coupled neurons and allow ultra-fast spread of information.

Thus far, 20 connexin genes have been defined [2]. Among the two types of Cxs most ubiquitously expressed in mammalian neurons, only Cx36 is prevalent in the mature CNS [3]. Cx36 positive neurons have been demonstrated in the hippocampus, cerebral and piriform cortex, striatum, amygdala, cerebellum, mesencephalon, thalamus, hypothalamus, and various brain stem nuclei [3]. Cx36 has been shown to facilitate plasticity [4], promote glutamate-mediated cell death post-injury [5], affect motor learning [6, 7] mediate sensitivity to anesthetic drugs [8, 9], and affect learning- and anxiety-related parameters [10].

Comprehensive electrophysiological studies have established the role of Cx36 in the generation of high-frequency oscillations and synchrony [11], particularly within GABA and dopamine (DA) circuits [12][13][14][15]. The relationship between Cx36 and GABA/dopamine prompted studies on a possible link between Cx36 and reward. These experiments brought a groundbreaking discovery that Cx36 knockout (KO) mice are less prone to drinking alcohol. The loss of Cx36-dependent electrical coupling within the key reward pathway component, the ventral tegmental area (VTA), leads to the dyssynchrony of the GABA system resulting in disinhibition of DA neurons (hyper-DA state), and consequently decreases the hedonic value of ethanol intake [15].

In the current set of studies, we used the Cx36 KO mouse model to investigate whether the link between Cx36 and reward expands onto the pleasure of consumption of palatable tastants. First, we measured consumption of sweet and lipid solutions in episodic and unrestricted access paradigms in Cx36 KO versus WT mice and substantiated our findings by using a pharmacological blocker of Cx36 gap junctions, quinine, in WT animals. As the consumption of palatable tastants was lower in the KO animals, we hypothesized that the genetic deletion of Cx36 affects baseline expression of opioid system-related genes, which are the key components of central mechanisms governing feeding for pleasure [16] thereby diminishing KO animals' sensitivity to feeding reward. Hence opioid transcript levels were assessed with real-time PCR in the hypothalamus and nucleus accumbens of Cx36 KOs and their background strain. Finally, we tested sensitivity of Cx36 KOs versus WTs to feeding inhibitory properties of an opioid receptor antagonist, naltrexone, and feeding stimulatory properties of an agonist, butorphanol, on sugar and saccharin solution intake.

2. Materials and Methods

2.1. Animals

The Cx36 KO model, developed and kindly provided to our laboratories by Prof. David Paul [17], has been used by us and others in earlier studies [18], [19]. Male homozygous Cx36 KO and WT littermates (C57/B6-129SvEv mixed background) were individually housed in conventional cages with a 12:12 LD photoperiod (lights on at 0700) in a temperature-controlled room (21°C). Age-matched animals weighed ca. 26 g ± 3 at the beginning of the studies (there was no difference in b. wt. between the genotypes). Mice had unlimited access to tap water and standard rodent chow (Teklad Global Diet 2018) throughout the studies unless noted otherwise. The procedures described herein were approved by the University of Waikato animal ethics committee.

2.2. Ingestive behavior assessment

Prior to the beginning of exposing animals to non-standard tastants, we determined that despite the same body weight as WT counterparts, age-matched Cx36 KO mice eat more regular chow over a 24-h period with water and food available ad libitum (KOs: 0.18±0.003 g/kg b. wt; WTs: 0.11±0.01; p=0.01 in t-test), which suggests energy metabolism changes in the KO strain.

2.2.1. Episodic intake of palatable tastant solutions in Cx36 KO vs WT mice

In a series of experiments, we tested the intake of 0.1% saccharin, 5% sucrose, 5% glucose, 5% fructose or 4.1% Intralipid (Fresenius, Sweden) solutions in a single-bottle no-choice paradigm. Cx36 KO and WT mice (n=13-18/tastant) had been pre-exposed to the respective solutions 2 h/day (10:00–12:00) for 5 days. Chow and water were removed from the cages for the 2-h period of palatable tastant presentation. The animals were then given a single bottle containing a palatable tastant. The amount of

the solution consumed was calculated and the means were reported in g/kg of b. wt. A Student's t-test was used to assess differences in consumption for each of the palatable solutions between WT and KO animals (significant when $p \leq 0.05$).

As a control study for the ability of Cx36 gap junctions to affect episodic consumption of palatable tastants, we subjected the WT mice to a similar paradigm of saccharin solution availability as described above, injected them with saline or 30 mg/kg b. wt quinine (Cx36 gap junction blocker) 5 minutes before palatable tastant presentation and determined saccharin solution intake in the 2-h test period. The dose of quinine was selected based on previous studies showing antinociceptive and antiepileptic effects of the compound [20], [21]. The amount of consumed saccharin solution was expressed in g/kg b. wt. and the data of saline vs. quinine groups compared with a t-test (significant when $p \leq 0.05$).

2.2.2. Unrestricted intake of palatable solutions in Cx36vKO vs WT mice

We assessed the intake of 0.1% saccharin, 5% sucrose, 5% glucose, 5% fructose or 4.1% Intralipid when each of these tastants was available ad libitum (along with standard chow and water) for 3 consecutive days. KO and WT mice were given a single bottle containing a palatable tastant for 3 days (n=8/WT and n=8 KO for each tastant). The amount of the tastants and chow consumed on Day 2 and 3 (data from Day 1 were not included in the analysis to discount the potential effect of novelty) was calculated and the means were reported in g/kg of b. wt. A Student's t-test was used to establish the difference between WT and Cx36 KO consumption in consumption (significant when $p \leq 0.05$).

2.3. Baseline expression of opioid system genes in the hypothalamus and nucleus accumbens in Cx36 KO vs. WT mice

Wt and Cx36 KO mice (n=8/group), maintained on ad libitum access to standard chow and water, were decapitated (10:00-11:00) and the hypothalamus and nucleus accumbens were collected according to the Paxinos and Watson brain atlas. The tissue was placed in RNAlater at room temperature for 2h and then stored at -80°C. RNA was extracted by the Quick-RNA™kits (Zymo Research) and the absence of DNA was confirmed by PCR. Total RNA concentration was measured with Nanodrop 2000 (Thermo Scientific). For cDNA synthesis, 9 µl of RNA was reverse-transcribed in a final volume of 20 µl containing 10 µl 2X RT Reaction Solution and 1 µl Enzyme Mix (HiSenScript™ RH(-) cDNA Synthesis Kit, iNtRON Biotechnology). The reaction was performed for 1h at 42°C, followed by 5 min at 85°C and 5 min at 4°C, and the presence of cDNA was confirmed by PCR. Each rtPCR, with a total volume of 20 µl, contained 8 µl template cDNA, 2µl Primers Mix (Forward and Reverse primers) and 10 µl of 2X RealMOD™ GH Green Real-time PCR Master Mix (Master Mix Kit, iNtRON). rtPCRs were done in duplicates, and negative controls were included on each plate. Amplification was performed as follows: denaturation at 94°C for 5 min, 50 cycles of denaturation at 94°C for 10 s, annealing for 15 s, and extension at 62°C for 30 s. Three housekeeping genes were analyzed (Table 1). A Rotor-Gene SYBR Green PCR (QIAGEN) was used. Data analyses have been performed by Rotor-Gene 6000 software 1.7 (QIAGEN). Primer efficiencies of 11 genes (Table 1) were calculated and samples were corrected for differences in efficiencies. The Pfaffl equation [22] [23] was used to calculate normalization factors based on housekeeping gene expression. Differences in gene expression between groups were analyzed using a t-test (different when $p \leq 0.05$).

2.5. Effect of opioid receptor agonist and antagonist on consumption of sweet palatable tastants in Cx36 KO vs. WT mice

Several days prior to the beginning of the experiments, Cx36 KO and WT mice had been pre-exposed to 0.1% saccharin or 10% sucrose solutions for 24 h to prevent

neophobia. Similar to the episodic exposure experiments described above (section 2.2.1), animals (n=7-10/group) were accustomed to having access to either 0.1% saccharin or 10% sucrose for 2 h/day (1000–1200 h). Standard food and water were removed for the 2-h period of palatable tastant presentation. Five min prior to palatable solution exposure, mice were injected IP with (a) saline or 0.03,0.1, 0.3, 1 and 3 mg/kg b. wt. of naltrexone or (b) saline or 0.1, 0.3, 1 and 3 mg/kg b. wt. of butorphanol tartrate. The amount of the palatable tastant consumed during the 2-h period was calculated in g/kg of b. wt. One-way ANOVA followed by Fisher's post-hoc test was used to establish effective doses of naltrexone and butorphanol in saccharin and sucrose consumption for the Cx36 KO and WT strains (different when $p \leq 0.05$).

3. Results

Mice lacking the functional Cx36 gene display diminished intake of palatable tastants in both episodic and unrestricted access paradigms. The KO animals drank significantly less of the sweet palatable solutions and lipid emulsion (saccharin: p=0.008,; sucrose, p=0.009; glucose, p=1E-5; fructose, p=0.0003, Intralipid, p=0.002) offered in a brief 2-h session (with no chow or water available during that time; Fig.1A). A similarly diminished intake of the palatable tastants was observed when saccharin (p=0.02) sucrose (p=2E-6), glucose (p=2E-6), fructose (p=2E-7) and Intralipid (p=0.0002) were given in an unrestricted manner for 48 h (chow/water available ad libitum; Fig.1B). Importantly, water intake did not differ between KO and WT mice (p=0.23).

Cx36 blocker, quinine, administrated IP just before the 1-h exposure to the saccharin solution in WT mice, significantly decreased the intake of this sweet tastant (p=0.004; Fig. 1C).

In the PCR studies, we found that baseline expression of opioid system genes differs in Cx36KO vs WT mice maintained on a standard diet (Fig.2). The KOs showed decreased mRNA levels of KOR (p=1E-06), MOR (p=5E-08), PNOC (p=0.0005), ORPL1 (p=6E-05), and increased DYN mRNA content (p=1E-05) in the hypothalamus, whereas PENK transcript expression was higher (p=0.03) in the nucleus accumbens.

NTX or butorphanol injections in animals given access to saccharin or sucrose, produced diiferent dose-response profiles in Cx36 vs WT mice (Fig 3). While the 0.1-mg and 0.3-mg doses of NTX decreased sucrose consumption in WTs (p=0.02 and p=0.01, respectively), 3 mg NTX had to be used to generate a reduction in sucrose intake in KOs (p=0.007). NTX at 1 mg (p=0.008) decreased saccharin intake in WT animals, but even the 3-mg dose was ineffective in the KO strain.

Conversely, butorphanol increased sucrose consumption at 0.3 mg (p=0.02; and 1 mg (p=0.004) in WTs, whereas 3 mg (p=0.01) was the lowest effective dose in KOs (Fig. 3C). Saccharin consumption increased after injection of 0.3 and 1 mg butorphanol in WT mice (p=0.004 and p=0.004, respectively) but it remained unchanged even after the 3-mg butorphanol treatment (Fig. 3).

4. Discussion

Excessive intake of highly palatable tastants brings on most profound metabolic and energy balance consequences for the organism [24]. The mesolimbic reward pathways as well as reward-related molecules scattered throughout the widespread network of sites that govern food intake and energy metabolism, promote overconsumption of tasty foods [25, 26]. Cx36, as a key molecular component of gap junctions in the adult CNS, serves a critical role in ensuring functional integrity of brain circuits, including those involved in reward [3, 15]. It has been previously shown that silencing Cx36 expression leads to a disruption of GABA and DA signaling [12-15]. Genetic deletion of Cx36 has also been found to decrease ethanol consumption in mice [15]. The current set of data shows for the first time that Cx36 gene knockout and the pharmacological blockade of Cx36 GJs diminish intake of palatable solutions in mice, and that dysregulation of the opioid system - a consequence of impaired Cx36 GJ coupling - likely underlies this reduced drive to eat for pleasure.

Animals typically prefer sweet and fatty foods, and they ingest large quantities of solid and liquid diets that contain sugars, non-carbohydrate sweeteners, and/or fats, regardless of overall caloric density of such diets [27, 28]. Importantly, reward-driven consumption of solid and liquid diets occurs regardless of the fact whether the animal has or has not been food- or water-deprived [29, 30]. Non-deprived Cx36 KO mice did consume the palatable liquid diets given episodically and in an unrestricted manner, which suggests that these solutions are not completely devoid of hedonic value to these animals. However, each of the palatable tastants was consumed less avidly by the KOs compared to their WT counterparts, indicating that the processing of rewarding aspects of consumption of these liquid diets was impaired in the Cx36-deficient mice. That water intake does not differ between KOs and WTs makes it unlikely that the effect was cause by differences in thirst responsiveness. The fact that KOs ingest less of the non-caloric

saccharin solution than WT controls rules out a scenario in which diminished energy needs are the culprit underlying lower intake of palatable tastants. It should also be noted that the feeding reward experiments presented herein employed a wide variety of tastants that engage different subsets of taste receptors; therefore, along with the previously reported data on the relationship between Cx36 and alcohol-derived reward [15], our results imply that aberrant processing of reward in Cx36 deficiency expands upon pleasure-derived consummatory behavior.

Interestingly, administration of quinine in non-deprived WT mice just prior to their gaining brief episodic access to the saccharin solution generated a decrease in saccharin intake. The parallel effects of gene deletion and pharmacological blockade of the GJ molecule lend us more confidence in defining Cx36 as being involved in feeding reward. Overall, changes in reward processing are evoked not just by a constitutive absence of the Cx36 gene, but also by using a transient Cx36 blocker; hence they can be dependent on acute changes in the Cx36 GJ functional status as well as on long-term molecular changes associated with the the lack of Cx36.

It has been previously reported that Cx36 deficiency in the KO model leads to significant abnormalities in DA and GABA signaling within the VTA-accumbens pathways, thereby contributing to the impaired processing of rewarding stimulation [12-15]. Our real-time PCR data suggest that changes in the molecular content of reward-related circuits are even more profound as the baseline expression profile of genes that give rise to select opioid peptides and receptors is greatly affected. The key role of endogenous opioids in palatability-induced consumption has been shown beyond reasonable doubt. It is well known that opioid receptor agonists induce intake of preferred tastants, while antagonists are effective in decreasing intake of tasty foods [31][32]. Modifications in access to palatable diets affect opioid peptide/receptor mRNA and protein levels [33-35], whereas constitutive and conditional changes in expression of opioid system genes are

associated with an altered drive to ingest rewarding tastants [36-38]. That Cx36 KO animals maintained on standard “bland” chow show different levels of opioid mRNAs indicates that - already at the baseline behavioral state - there is an atypical expression of genes related to feeding reward, most likely being one of the underlying factors in aberrant processing of palatability in Cx36 KOs. It should be noted that opioid system mRNA levels were changed not only in the nucleus accumbens, which is intuitive taking into account its role in reward, but predominantly in the hypothalamus, where the receptor transcripts were greatly affected. While numerous intraparenchymal injection studies have shown that opioid receptors in the hypothalamus modify palatability-driven feeding [39], they are also though to couple reward system’s activity with the homeostatic and neuroendocrine responses of the hypothalamus [31, 40] .

The real-time PCR findings showing an altered expression profile of opioid-related genes are further substantiated by the studies utilizing injections of opioid receptor ligands that are known to stimulate (butorphanol) or reduce (naltrexone) consumption of palatable tastants [31][32]. We found that WT mice offered either sucrose or saccharin exhibited a typical orexigenic response to butorphanol and hypophagia after naltrexone treatment. On the other hand, Cx36 KOs exhibited a diminished sensitivity to consumption modifying properties of each of the opioid ligands (sucrose) or a lack thereof (saccharin). While it is difficult to speculate whether the marked shifts in the dose-response curves can be directly attributed to changes in opioid system’s expression profile or rather to impaired coupling of reward signaling due to DA and GABA disruption (or both), it is clear that Cx36 deletion leads to gross abnormalities in the molecular content, sensitivity and functioning of reward circuits.

In sum, we conclude that Cx36 is essential in generating avid consumption of palatable tastants. The lack of Cx36 is associated with impaired functioning of the reward system, including its opioid-dependent component.

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Table 1. Forward and reverse real-time PCR primer sequences.

Opioid system genes	Forward	Reverse
KOR	CAC CTT GCT GAT CCC AAA	TTC CCA AGT CAC CGT CAG
MOR	CCT GCC GCT CTT CTC TGG	CGG ACT CGG TAG GCT GTA AC
PNOC	AGC ACC TGA AGA GAA TGC CG	CAT CTC GCA CTT GCA CCA AG
DYN	GAC AGG AGA GGA AGC AGA	AGC AGC ACA CAA GTC ACC
ORPL1	ATG ACT AGG CGT GGA CCT GC	GAT GGG CTC TGT GGA CTG ACA
PENK	CGA CAT CAA TTT CCT GGC GT	AGA TCC TTG CAG GTC TCC CA
Housekeeping genes		
B actin	TGG CAC CAC ACC TTC TAC AAT GAG	GGG TCA TCT TTT CAC GGT TGG
Atp5b	GGC ACA ATG CAG GAA AGG	TCA GCA GGC ACA TAG ATA GCC
B tub	CGG AAG GAG GCG GAG AGC	AGG GTG CCC ATG CCA GAG C

Figure legends

Fig. 1. Connexin 36 knockout (Cx36 KO) mice consume smaller amounts of palatable solutions offered **(A)** episodically for 2 h or **(B)** in an unrestricted 48-h access paradigm, compared to wild-type (WT) controls. A peripheral injection of a pharmacological blocker of Cx36 gap junctions, quinine, reduces saccharin solution intake **(C)**. * - $p \leq 0.05$; ** - $p \leq 0.01$; *** - $p \leq 0.001$.

Fig. 2. Connexin 36 knockout (Cx36 KO) mice having unrestricted access to standard food and water display a different baseline mRNA expression profile of select opioid system genes than wild-type (WT) controls. Gene expression levels in the hypothalamus and nucleus accumbens were measured with real-time PCR. KOR, kappa opioid receptor; MOR, mu opioid receptor; PNOC, pronociceptin; ORPL1, nociceptin/orphanin FQ receptor; PENK, proenkephalin; POMC, proopiomelanocortin. * - $p \leq 0.05$; ** - $p \leq 0.01$; *** - $p \leq 0.001$.

Fig. 3. Effects of naltrexone **(top: A, B)** and butorphanol tartrate **(bottom: C, D)** on the intake of sucrose (A,C) and saccharin (B, D) solutions offered for 2 h to connexin 36 knockout (Cx36 KO) and wild-type (WT) mice. Doses of naltrexone and butorphanol represent mg/kg b. wt. injected intraperitoneally. * - $p \leq 0.05$; ** - $p \leq 0.01$; *** - $p \leq 0.001$.

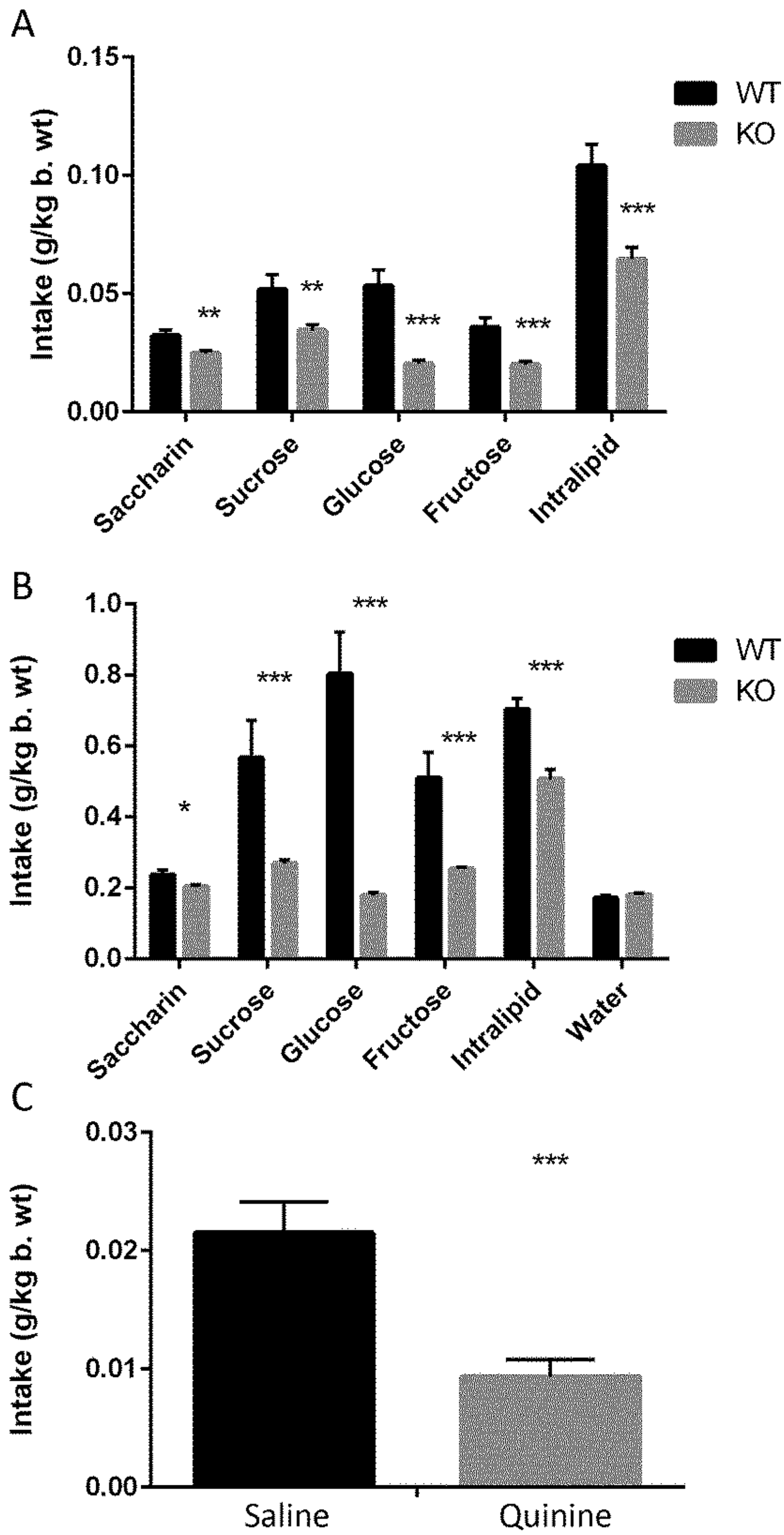


Fig. 1.

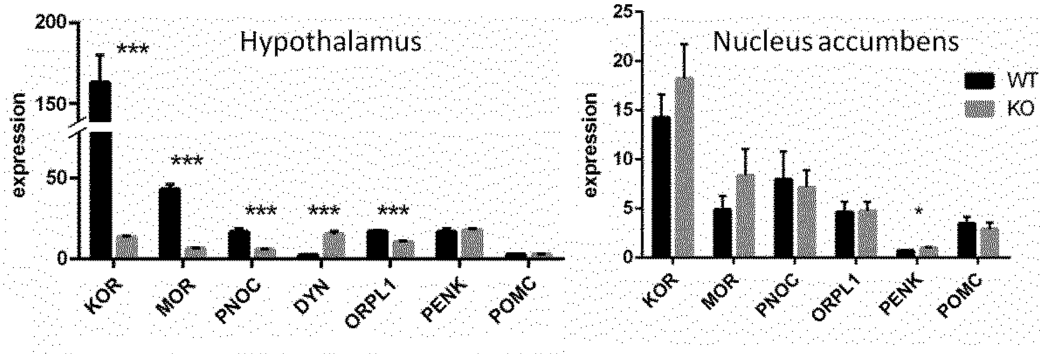


Fig. 2.

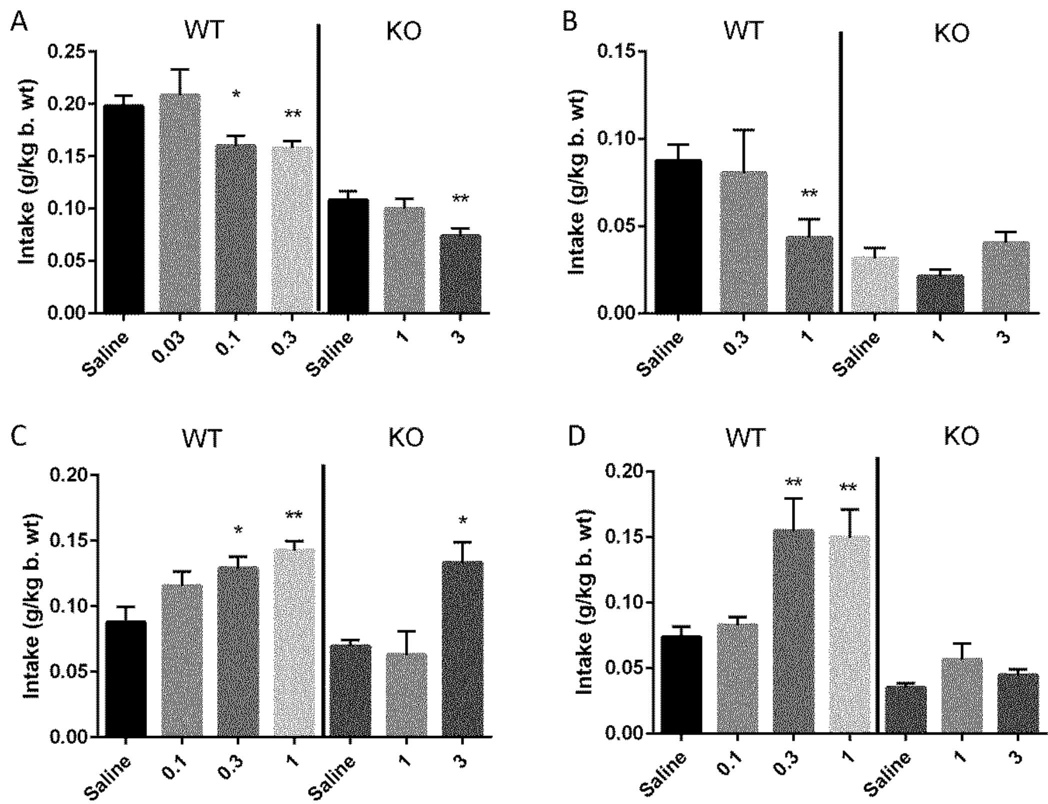


Fig. 3.

A general power calculation method to determine animal numbers in individual experiments

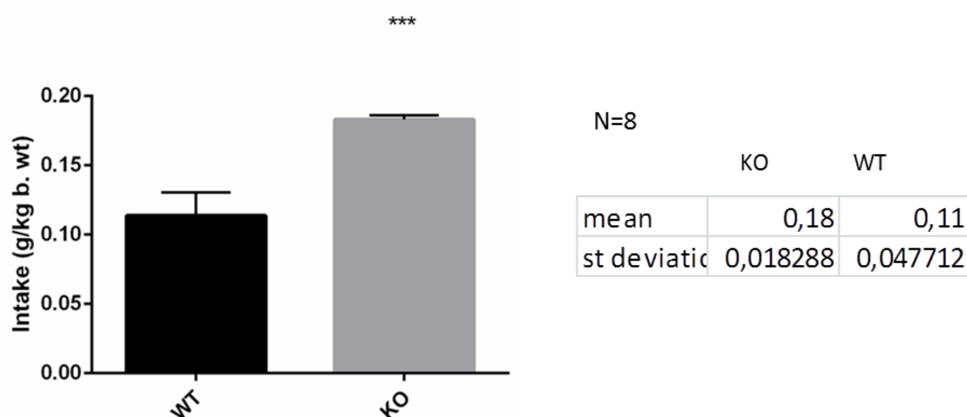
Power calculations to determine sampling:

Power calculation:

- *before data collection :* to decide the sample size needed for the study
- *after data analysis:* to verify whether the non-significant result is due to really no relation in the sample or due to a lack of statistical power.

Stat significance can be affected by 4 main parameters:

- Effect size
- Sample size
- Probability errors: Alpha significance ; 0.05 Beta: 0.2
- Statistical power: 0.80



	KO	WT
mean	0,18	0,11
st deviatio	0,018288	0,047712

Find sample size:

If you can estimate group means and standard deviation, use this form to find the number of subjects you need.

Group 1 mean: 0.11

Group 2 mean: 0.18

Standard deviation: 0.04

Click here for sample size: Result

You will need 7 subjects in Group 1

You will need 7 subjects in Group 2

For different power or significance level, change the fields below:

Alpha: Prob(reject H_0 when H_0 is true) 0.05

Power: Prob(reject H_0 when H_1 is true) 0.80

For unequal sample sizes, change the ratio below:

Group2/Group1: 1.0

http://www.sample-size.net