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IDENTIFICATION OF CENTRAL CIRCUITRY COMPONENTS MEDIATING ANOREXIGENIC PROPERTIES OF OXYTOCIN

A thesis
submitted in fulfilment
of the requirements for the degree
of
Doctor of Philosophy in Biological Sciences
at
The University of Waikato
by
OSCAR ANDREAS KLOCKARS



THE UNIVERSITY OF
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Foreword to the thesis

by Dr. Pawel K. Olszewski, Chief Supervisor of Oscar A. Klockars' doctoral project

Oscar Klockars was a PhD student at the Faculty of Science and Engineering at the University of Waikato from September 2014 until his untimely passing in September 2018. This doctoral thesis represents the first draft of the document that Oscar provided to the supervisory team.

Prior to joining the University of Waikato as a PhD student, Oscar graduated from the Örebro University in Sweden with a Masters degree in medicine. In Sweden, he acquired broad theoretical knowledge of molecular processes that underlie proper physiological functioning of the organism as well as those that lead to pathological states. He gained significant research experience by performing his Masters thesis work in the clinical molecular microbiology laboratory (that project led to his being a co-author of a scientific report entitled 'Pharmacodynamic studies of nitrofurantoin against common uropathogens' published in Journal of Antimicrobial Chemotherapy), and by doing a long-term internship with Prof. Dan Larhammar's neuropharmacology group at Uppsala University.

Oscar's substantial expertise was crucial in his being able to jumpstart a series of successful experimental research projects at the University of Waikato. He was independent in formulating hypotheses and in devising practical strategies to tackle scientific questions. Over a relatively short time period, not only did Oscar master the advanced skills involving intracranial surgeries, in vivo pharmacology, and brain

activation analyses, but he also incorporated a number of previously learned methodologies, and single-handedly developed approaches allowing him to record select complex behaviors of laboratory animals.

Oscar generated a large body of data during his doctoral work. These data are a part of this PhD thesis, and have been the basis of three research publications. The first report, 'Central oxytocin receptor stimulation attenuates the orexigenic effects of butorphanol tartrate' (Olszewski PK, Klockars OA, Klockars A, Levine AS; *Neuroreport*; 2016; 4 citations) showed that a satiety mediator, oxytocin, is able to decrease extreme overeating induced pharmacologically by a potent, mixed opioid receptor ligand, butorphanol. It was an important discovery from the standpoint of expanding limited tools to alleviate excessive appetite induced by agonism of opioid receptors. In the second paper, 'Neural basis of ventromedial hypothalamic oxytocin-driven decrease in appetite' (Klockars OA, Waas JR, Klockars A, Olszewski PK; *Neuroscience*, 2017; 5 citations), Oscar determined that oxytocin acting via a discrete hypothalamic site regulating energy metabolism, the ventromedial hypothalamic nucleus, decreases interest in consumption of highly caloric diets (although this effect is unrelated to the flavor of these foods). He defined widespread changes in brain activation patterns following this pharmacological treatment and identified relevant gene expression changes. In the most recent publication (Oxytocin administration in the basolateral and central nuclei of amygdala moderately suppresses food intake. Klockars OA, Klockars A, Levine AS, Olszewski PK. *Neuroreport*. 2018; 3 citations), Oscar reported for the very first time that two specific portions of the amygdala, its basolateral and central nuclei, mediate satiating properties of oxytocin in the context

of terminating consumption of high-energy foods. He also determined that the basolateral nucleus, which belongs to the network of sites that regulate emotional processing of behaviors, including eating behavior, promotes cessation of consumption of non-caloric and low-calorie palatable fluids via its oxytocin receptor. Overall, Oscar's findings make a tremendous contribution to our understanding of neural mechanisms through which oxytocin reduces appetite and of aspects of food intake (eating for energy versus eating for pleasant taste) affected by oxytocin acting via distinct brain areas.

Oscar's accomplishments were possible thanks to his fantastic rapport with staff and fellow students at the University. His respectful and kind attitude, his willingness to discuss research progress, offer and accept advice, being a team player unafraid of taking responsibility upon himself, made him a truly valued member of our community and the person with whom we enjoyed working and interacting daily.

I would like to take this opportunity and thank the members of the supervisory team, Prof. Joseph Waas and Dr. Steve Bird. In my conversations with Oscar, it was certain that he felt genuine support from both Joe and Steve, and he greatly appreciated their willingness to share professional expertise and provide guidance and advice not just on research, but also on personal matters.

In the lab, Oscar was surrounded by colleagues and friends who shared his passion for science and contributed alongside him to creating the pleasant and supportive atmosphere: Anica Klockars, Kerry Allen, Sarah Gartner, Erin Wood, Mitch Head, Florence Herisson, Laura McColl, Fraser Aidney, Kathryn Laloli, Kiriana Isgrove, Cushla

Moscricp, Moh Arafat, Chloe Brunton, and Tiffany Fehlmann. Thank you all for accompanying Oscar on his journey toward completing his doctoral research.

Finally, I would like to express my gratitude to Oscar's family. There is no surprise that two wonderful, loving children, Heidi and Rocky, were the most important 'project' that Oscar and Anica shared. The love for the children helped Oscar tackle all the challenges encountered on his research path, it served as the most welcomed and endearing distraction, and it filled his life with purpose, hope and joy. Oscar's family in Sweden, his Mom, Dad and siblings, were always the source of support, willing to talk to Oscar, listen to him, and provide him with advice and loving words whenever needed.

Abstract

Recent years have brought exciting discoveries showing that a neurohormone, oxytocin (OT), acts as an appetite suppressant. Importantly, OT decreases energy needs-driven consumption of high-calorie foods by promoting early satiation, and it terminates intake motivated by palatability related to sweet taste. OT's anorexigenic effects on both aspects of ingestive behavior are mediated by the OT receptor (OTr) expressed broadly in the brain, however, our understanding of which specific sites relay anorexigenic actions of OT is limited. Thus far, only the nucleus accumbens and ventral tegmental area of the mesolimbic system, and the brainstem's dorsal vagal complex, have been directly implicated in relaying OT-induced hypophagia. Thus, the overarching goal of this thesis was to examine which discrete components of circuitry expressing the OTr mediate OT's anorexigenic effects on energy- versus reward-driven feeding. The research approaches included intracranial drug administration, analyses of region-specific expression of genes, neuronal activation mapping, and feeding behavior testing in rats.

The first set of studies explored whether extreme overeating induced by a powerful orexigen, butorphanol tartrate (BT), can be alleviated by pharmacologically stimulating the OT receptor in the forebrain versus the hindbrain (via lateral (LV) and fourth ventricular (4V) OT injections, respectively). I established effective doses of BT and LV / 4V OT. Then, I determined doses of LV and 4V OT that reduce hyperphagia produced by BT in sated and deprived rats. Finally, I assessed whether OT's effects on BT-induced feeding can be suppressed by an OTr antagonist. 4 mg/kg BT increased

intake in fed and in deprived rats, whereas LV and 4V OT at 1 μ g caused a decrease in deprived rats. BT-induced chow intake in hungry and sated animals was suppressed by a very low, 0.1- μ g dose of 4V OT, whereas 1 μ g OT was effective LV. The effect of OT was attenuated by OTr antagonist. The data strongly suggest that while both the forebrain and hindbrain populations of the OTr promote hypophagia, the hindbrain component of the circuitry is particularly sensitive to appetite reducing properties of OT in animals motivated to eat by a potent orexigen, BT.

In the second set of studies, I examined whether either of the two populations of the hypothalamic OTr, in the medial preoptic area (MPOA) or the ventromedial hypothalamic nucleus (VMH), mediates OT-driven hypophagia in energy- and reward-related ingestive behaviors. I provide insights into mechanisms underlying OT-driven anorexia mediated by the hypothalamus by (a) defining whether OT MPOA or VMH administration affects feeding for energy versus palatability; (b) identifying feeding-related sites activated by VMH OT injection; (c) measuring VMH OTr mRNA changes in response to hunger and palatability; and (d) examining how VMH OT affects sweet solution intake in rats. MPOA had no effect on intake of energy-dense chow in deprived rats nor did it decrease intake of calorie-dilute palatable solutions. VMH OT decreased chow intake and the effect was reversed by the antagonist. OT did not affect intakes of saccharin and sucrose solutions. Fos immunoreactivity, a marker of neuronal activation, was elevated in the VMH and energy balance-related paraventricular and arcuate nuclei, but not reward areas. VMH OT receptor expression was higher in hungry than sated rats; saccharin intake had no effect. In

sum, MPOA OTr is not involved in OT-driven hypophagia mediated by hypothalamic networks. VMH OT decreases intake driven by energy not by palatability.

Finally, I assessed whether the OTr present in the basolateral (BLA) and central amygdala (CNA), sites implicated in emotional/pleasure processing of food intake, is involved in appetite control by OT. I injected OT in the BLA or CNA and assessed intake of chow induced by energy deprivation and intake of sweet solutions in non-deprived rats. I examined whether these effects are reversible by OTr blockade. I determined the effect of energy deprivation and exposure to saccharin on BLA and CNA expression of OTr mRNA. BLA OT at 0.3 μg and CNA OT at 1 μg reduced chow intake after deprivation. Only BLA OT was effective at suppressing consumption of sucrose and saccharin. The anorexigenic effects of BLA and CNA OT were attenuated by an OTr antagonist. BLA OTr mRNA expression was affected by exposure to saccharin, whereas that of CNA OTr, by energy deprivation. The relationship between amygdalar OT and energy- vs palatability-driven intake depends on the discrete localization of the OTr in this complex structure.

Overall, these findings shed light on the specific elements of brain circuitry mediating anorexigenic properties of OT. Both the forebrain and hindbrain OTr populations are relevant to feeding control. OT's inhibitory effects on feeding for energy are mediated by a broader network of sites that includes, aside from the previously reported nucleus accumbens and dorsal vagal complex, the VMH, BLA, and CNA. Only the BLA OT modifies eating for reward. Surprisingly, the OTr in the MPOA is not involved in feeding regulation.

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1. Chapter One - Introduction and specific aims

Ingestion of energy is one of the most fundamental functions of all living organisms. The vast majority of species have evolved in the environment where sources of energy are scarce and, therefore, any opportunity that arises to replenish lacking calories should be undertaken. The vast majority of species have not thus evolved to be protected from the 'environment of plenty'. The consequence of our evolutionarily conserved adaptation to energy scarcity is that hormonal and neural mechanisms that regulate food intake favor avid consumption over feeding termination. In fact, the act of feeding termination appears to be quite unique and it seems to be reserved to situations in which continued consumption would likely result in jeopardized homeostasis. One such circumstance can be ingestion of toxic or tainted foods. Neuroendocrine signals that mediate inhibition of food intake are activated immediately after a toxin has been detected by chemoreceptors. Similarly, hyperosmolality or extreme levels of stomach distention also lead to cessation of eating behavior. Genotypes that have been evolutionarily deemed successful are those that are capable of fast acquisition of non-toxic sources of calories, boosting energy utilization, and allowing for efficient storage of energy that can be engaged upon challenges stemming from low food supplies. Consequently, molecules (and their respective genes) that facilitate feeding control are well conserved, to the extent that the evolutionary predecessors of many feeding-relevant peptidergic systems in mammals, arose early in the vertebrate evolution and their orthologs can be found even in algae and bacteria (Schioth, Haitina et al. 2005, Scherag, Dina et al. 2010).

The hypothalamus is strategically equipped to integrate neural and endocrine signaling related to food intake and to execute dynamic changes to energy intake and expenditure. Already in the mid-twentieth century, disruption of the ventromedial hypothalamic nucleus (VMH) was shown to promote abnormally high feeding (and lead to excessive weight gain in a long-term) in rodent models. On the other hand, ablations of the lateral hypothalamic (LH) induced anorexia and a decrease in body weight (Mayer and Thomas 1967). Disruption of the early development of the hypothalamus, including the arcuate nucleus (ARC), in mice by administration of monosodium glutamate, led to dysregulated neuroendocrine responses throughout the lifespan of these animals and one of the observed phenotypic features was hyperphagia and obesity (Olney 1969). During those early years of research on hypothalamic control of energy balance, many investigators found that lesions of the paraventricular nucleus of the hypothalamus (PVN) and the disruption of the hypothalamic-brainstem reciprocal circuit prevented laboratory animals to execute termination of feeding that would protect them from pathophysiological consequences of extreme overeating (Leibowitz, Hammer et al. 1981, Shor-Posner, Azar et al. 1985, Sims and Lorden 1986, Kirchgessner and Sclafani 1988).

It was several years later that eventually a neurohormone, oxytocin (OT), was found to be a crucial feeding-regulatory component of the cytoarchitecture of the PVN as well as of the supraoptic nucleus (SON), another hypothalamic site whose projections target the neurohypophysis. The broadening array of techniques that could be applied to study the functional importance of molecular pathways in behavioral

processes, allowed us to better understand that the relationship between the hypothalamus (including its OT neuronal populations) and multiple peripheral and central mechanisms. It has since become clear that inter- and intra-organ communication is the foundation allowing the recognition of the current feeding/energy balance status by the organism and shaping a proper consummatory response.

In this introductory chapter, I will first describe integration of peripheral (gut- and adiposity-derived) signaling by the hypothalamus, key hypothalamic molecular systems involved in energy balance control, and – finally – I will focus on the currently available information on the role of hypothalamic OT in feeding control.

1.1. Input from the white adipose tissue into the hypothalamus

The discovery of the adipocyte-released hormone, leptin (Lep), encoded by the *ob* gene, was one of the first indicators of how important white fat (and, peripheral, in general) signaling is to the ‘assessment’ of the organism’s energy status by the hypothalamus (Friedman and Halaas 1998) (though, one should not neglect the fact that the current body of evidence implicates also other tissue types in contributing to the leptin levels, e.g., the gastrointestinal (GI) tract and urogenital system (Sobhani, Bado et al. 2000, Challier, Galtier et al. 2003)).

The amount of leptin secreted by the fat tissue is equivalent to adiposity (Murphy and Bloom 2004). Mice (*db*) with loss-of-function mutations in the leptin receptor (*Lepr*) gene synthesize are resistant to leptin (Maffei, Fei et al. 1995, Farooqi,

Wangensteen et al. 2007). Animals with a knocked-out Lep gene mice are deficient in leptin due to the loss-of-function mutation (Zhang, Proenca et al. 1994, Friedman and Halaas 1998) and administration of leptin rescues them from overeating and excessive body weight (Friedman and Halaas 1998). Peripheral and brain infusions of Lepr agonists diminish appetite in wild-type animals (Ahima, Prabakaran et al. 1996, Friedman and Halaas 1998). Rodents subjected to food deprivation and restriction show a suppressed Lep expression; refeeding or administration of insulin (which serves as the signal for a recently completed meal) reverses this effect on Lep receptor levels (Frederich, Lollmann et al. 1995). Finally, knock-in of additional copies of the Lepr in the hypothalamus (including in the ARC, PVN and VMH) produces a hypophagic phenotype in transgenic animals (Bagnasco, Dube et al. 2002).

Though for many years it remained a controversial issue, it has now been well documented that leptin crosses the blood-brain barrier (BBB) and, upon reaching its target neurons, it triggers the JAK2-STAT3 and PI3K-PDE3B-cAMP cellular pathways (Mori, Hanada et al. 2004). Distribution of the Lepr in the hypothalamic ARC, VMN, DMH and LH is consistent with its role in energy metabolism. Importantly, the Lepr is expressed on ARC neurons synthesizing peptides involved in initiation of food intake, such as neuropeptide Y (NPY)/Agouti-related protein (AgRP), as well as on those that supply anorexigens, for example, cocaine and amphetamine related transcript (CART) and proopiomelanocortin (POMC)-derived alpha-melanocyte stimulating hormone (alpha-MSH). In vivo pharmacology studies have found that alpha-MSH/POMC neurons are stimulated by leptin, whereas AgRP/NPY cells,

inhibited by this hormone, which underpins a dual mode of action through which leptin suppresses consummatory behavior (Cowley, Smart et al. 2001).

Adipose tissue synthesizes and releases also another molecule that acts in an endocrine fashion at the CNS, adiponectin. The adiponectin receptor is expressed in the neurohypophysis, and the hypothalamus, especially in the PVN and ARC (Rodriguez-Pacheco, Martinez-Fuentes et al. 2007, Wilkinson, Brown et al. 2007). Peripheral administration of this molecule result in an increase in adiponectin concentration in the cerebral ventricles, which indicates the ability of the peptide to get into the brain tissue (Kubota, Yano et al. 2007). Circulating glucose levels determine whether adiponectin excites or inhibits ARC POMC neuronal activity and whether the behavioral outcome is associated with initiation or termination of food intake (Suyama, Maekawa et al. 2016). ICV co-administration of adiponectin with glucose causes hyperphagia, whereas the lack of glucose or its low dose, facilitates androgenic action of this adipocyte-derived hormone. Somewhat counterintuitively, in vitro studies showed that adiponectin diminishes activity of cells expressing POMC when glucose is present, whereas an opposite effect was detected upon removal of glucose (Suyama, Maekawa et al. 2016). However, one should note that POMC gives rise to not only anorexigens, such as alpha-MSH, but also orexigens, for example, beta-endorphin. Furthermore, activity of ARC neurons producing AgRP and NPY is inhibited by adiponectin (Sun, Gao et al. 2016), and adiponectin strengthens inhibitory postsynaptic input onto ARC NPY cells to attenuate action potential firing in a glucose-independent fashion (Suyama, Lei et al. 2017).

One should note that insulin, despite being derived from pancreatic beta cells, is a brain-targeting signaling molecule relevant to adiposity. The insulin receptor is expressed at high levels in the PVN, ARC, and DMH (Marks, Porte et al. 1990). Fasting levels of this hormone parallel the amount of fat tissue (Rocha, Barata et al. 2011). Insulin receptor knockout mice display excessive food intake, especially in the presence of highly caloric and palatable ingestants, which leads to diet-induced obesity. This overeating and obesity are associated with elevated secretion of leptin and insulin and with resistance to insulin (Bruning, Gautam et al. 2000). ICV and intraparenchymal infusions of this hormone generate cessation of food intake in animals under restriction/ deprivation regimens and in sated rodents during the natural cycle of night-time consumption (McGowan, Andrews et al. 1992, McGowan, Andrews et al. 1992). Third ventricular insulin administration in deprived rats causes hypophagia and this outcome was alleviated by subthreshold doses of the synthetic antagonist of the MC3/4 receptor, SHU-9119 (thus, a molecule that mimics the effects of endogenous AgRP). ARC POMC expression studied with qPCR is also elevated after ventricular insulin injections (Benoit, Air et al. 2002). Finally, injections of antisense oligodeoxynucleotides against the insulin receptor in the cerebral ventricle in rats generate overeating and elevated body weight, and upregulate NPY mRNA levels in the ARC, which strongly suggests a functional relationship between insulin and NPY circuits in feeding control (Obici, Feng et al. 2002).

1.2. Key gastrointestinal hormonal input into the hypothalamus

Fat tissue-derived signaling tends to pair feeding behavior to the organism's energy state (especially, long-term), whereas the endocrine input into the hypothalamus that originates in the GI tract corresponds to feeding/nutritional status of a more transient and immediate nature.

The first identified GI hormone was cholecystokinin (CCK), the endogenous ligand of CCK1 and CCK2 receptors (Dufresne, Seva et al. 2006). It is released into the general circulation shortly after food reaches the small intestine (Gibbs, Young et al. 1973, Buffa, Solcia et al. 1976, Liddle, Goldfine et al. 1985). Peripheral and ICV injections of CCK produce undereating in a dose-dependent fashion by reducing meal size (Kissileff, Pi-Sunyer et al. 1981, West, Fey et al. 1984) and by inhibiting gastric emptying (Castillo, Delgado-Aros et al. 2004, Cummings and Overduin 2007, Jordan, Greenway et al. 2008). CCK binds the CCK1 receptor present on hindbrain vagal afferents (Noetzel, Stengel et al. 2009), leading to activation of the nucleus of the solitary tract (NTS) and dorsal motor nucleus of the vagus (DMNV). Those brainstem sites convey this signal into the forebrain, including the hypothalamic PVN and DMH (Monnikes, Lauer et al. 1997, Kobelt, Paulitsch et al. 2006, Noetzel, Stengel et al. 2009). OLETF rats lacking the CCK1 receptor exhibit extreme hyperphagia (Moran, Katz et al. 1998, Covasa and Ritter 2001), young OLETF rats that have not yet developed obesity, express elevated NPY mRNA levels in the DMH (Moran and Bi 2006).

Enteroendocrine L cells of the intestine release preproglucagon gene products, glucagon-like peptides 1/2 (GLP-1/2) and oxyntomodulin, between 10 and 60 minutes after completion of a meal (Holst 2007). The amount of these hormones secreted into the circulation corresponds to the amount of ingested food (Cohen, Ellis et al. 2003, Dakin, Small et al. 2004). Injections of these hormones into the brain as well as in the periphery produces early termination of feeding (Dakin, Gunn et al. 2001, Cohen, Ellis et al. 2003). GLP-1 and oxyntomodulin act at ARC cells that express the GLP-1 receptor by blocking ghrelin-induced neuronal activation that would lead to hyperphagia (Riediger, Eisele et al. 2010). Both peptides induce c-Fos expression in the PVN and NTS, and in the PVN, they activate the anorexigenic corticotropin releasing hormone (CRH) OT populations (Bojanowska and Stempniak 2000, Katsurada, Maejima et al. 2014).

Intestinal L cells also synthesize peptide YY (PYY), an endogenous ligand of the Y2 receptor (Halatchev and Cone 2005). The increase in PYY blood levels occurs at the end of a meal and it is proportional to the number of consumed calories (Grandt, Schimiczek et al. 1994, Batterham, Cowley et al. 2002). Intraperitoneal PYY(3-36) increases Fos immunoreactivity in the ARC and downregulates NPY expression in the hypothalamus (Halatchev, Ellacott et al. 2004). Unlike peripheral PYY, centrally injected molecule inhibits activity of POMC neurons, which may explain disparate feeding effects in animals treated peripherally versus centrally with this peptide (Boggiano, Chandler et al. 2005, Ghamari-Langroudi, Colmers et al. 2005). PYY produces hypophagia in animals with inactivated MC4 receptor, which suggests that

POMC neurons do not mediate the effects of this hormone on appetite and that other hypothalamic populations should be considered functionally relevant (Halatchev, Ellacott et al. 2004).

The upper part of the small intestine contains cells that release a recently identified anorexigenic hormone, oleoylethanolamide (OEA). The OEA secretory response is extremely specific in terms of macronutrient content of food present in the intestine as OEA levels rise only upon the ingestion of lipids (Fu, Oveisi et al. 2005, Fu, Astarita et al. 2007). OEA's action is mediated via the nuclear receptor peroxisome proliferator-activated receptor alpha (PPAR-alpha) (Fu, Gaetani et al. 2003, Guzman, Lo Verme et al. 2004, Yang, Chen et al. 2007). It influences energy homeostasis through regulation of lipolysis (Guzman, Lo Verme et al. 2004), as well as through triggering central mechanisms that underpin satiety processing. Intraduodenal administration of the vector that causes overexpression of N-acylphosphatidylethanolamine (NAPE)-phospholipase D (PLD), an enzyme catalyzing the NAPE → OEA hydrolysis thereby increasing OEA production, decreased calorie consumption by elevating feeding latency and postmeal interval (Fu, Kim et al. 2008). OEA diminishes appetite in mice and rats (Rodriguez de Fonseca, Navarro et al. 2001, Gaetani, Oveisi et al. 2003), and this effect is thought to be mediated via the NTS and PVN (Umehara, Fabbri et al. 2016). Importantly, histamine and OT have been proposed to be necessary components of hypothalamic circuits responding to OEA (Provensi, Coccorello et al. 2014).

While the aforementioned gut and adipose hormones (with very few exceptions related to, e.g., the aforementioned PYY and concurrent glucose levels) generate termination of food intake and – in a long-term – a reduction of body weight, an agonist of the growth hormone secretagogue (GHS) receptor, ghrelin acts as an orexigen. Ghrelin is synthesized chiefly by the stomach, although one should note that brainstem neurons also express this peptide (Kojima, Hosoda et al. 1999). Peripheral and CNS injections of this molecule generate avid consumption whose magnitude resembles only that induced by NPY (Bailey, Giles et al. 1999, Wren, Small et al. 2001). Not surprisingly, therefore, the GHS receptor is mainly expressed in the hypothalamus, particularly in the sites that underpin eating for energy, such as the ARC, PVN and VMH, as well as in the dorsovagal complex in the brainstem (Zigman, Jones et al. 2006). Administration of ghrelin produces c-Fos expression in the ARC, PVN, NTS, and DMNV (Hewson and Dickson 2000, Ruter, Kobelt et al. 2003, Takayama, Johno et al. 2007) and it induces NPY release from hypothalamic explants (Wren, Small et al. 2002). At the CNS level, ghrelin upregulates ARC NPY and AgRP mRNA profiles (Kamegai, Tamura et al. 2001) and postnatal deletion of AgRP/NPY cells abolishes hyperphagic effects of peripheral ghrelin (Luquet, Phillips et al. 2007). Ghrelin inactivation by specific antibodies prevents 2-DG-induced hyperphagia, and concomitant changes in activation of LH orexin neurons (Solomon, De Fanti et al. 2007).

The overall summary of endocrine and neural communication relevant to the hypothalamus and to hypothalamic OT is shown in Figure 1 below.

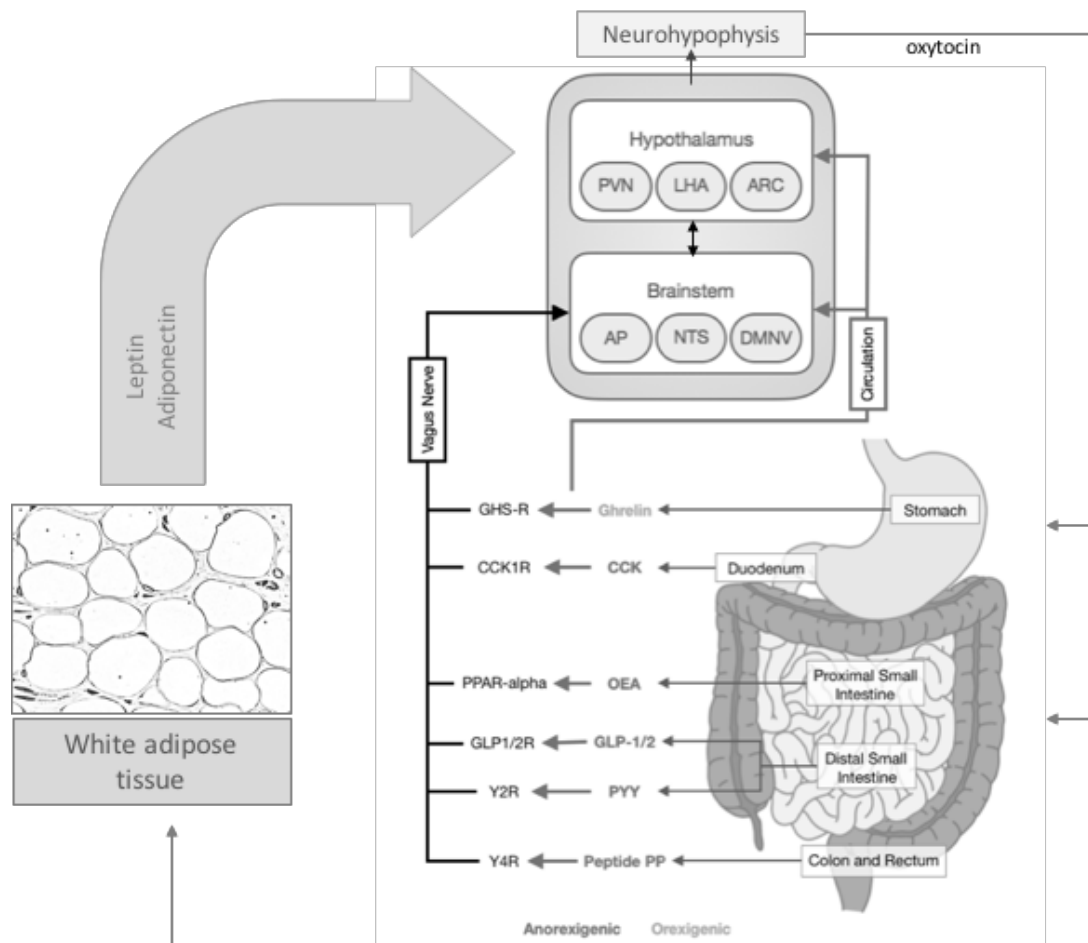


Figure 1. Schematic representation of neural and endocrine communication within the peripheral and central pathways that regulate appetite. AP – area postrema; ARC – arcuate nucleus; CCK – cholecystokinin; DMNV – dorsal motor nucleus of the vagus; GHS-R – growth hormone secretagogue receptor; GLP – glucagon-like peptide; LHA – lateral hypothalamus; NTS – nucleus of the solitary tract; OEA – oleoylethanolamide; PVN – paraventricular nucleus of the hypothalamus; PPAR-alpha; peroxisome proliferator-activated receptor alpha; PYY – peptide YY.

1.3. Oxytocin peptide and its receptor as anorexigenic components of central pathways regulating appetite

The peripheral input originating either in the GI tract or in the adipose tissue, needs to be integrated at the CNS level in order to trigger central processes responsible for adjusting consumption to energy contents of food/energy requirements of the organism, to the pleasure of consumption stemming from palatability of the food, and to the overall pathophysiological state of the body. Studies strongly suggest that hypothalamic OT neurons serve as a crucial element of pathways that integrate the diverse routes of feeding-related peripheral information flow into the CNS.

OT is a neurohormone produced in the CNS, primarily in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus, although other sites of OT expression (for example, the brain stem) have also been identified (Choy and Watkins 1977). Both the PVN and SON contain so-called magnocellular populations of OT neurons, whereas the PVN also contains parvocellular OT cells. Magnocellular neurons release OT mainly to the general circulation via the posterior portion of the pituitary gland, thereby contributing to the peripheral pool of OT. On the other hand, parvocellular OT neurons send their projections to a variety of brain areas, such as the dorsomedial hypothalamic nucleus, medial preoptic area (MPOA), several thalamic nuclei, the dorsal and ventral hippocampus, amygdala, olfactory bulbs, and the nucleus of the solitary tract, as well as to the pituitary. As such, centrally derived OT affects its receptors scattered throughout the brain as well as in the peripheral

tissues (Sawchenko and Swanson 1983, Yamashita, Okuya et al. 1987, Moos and Richard 1989, Renaud and Bourque 1991, Morris and Ludwig 2004).

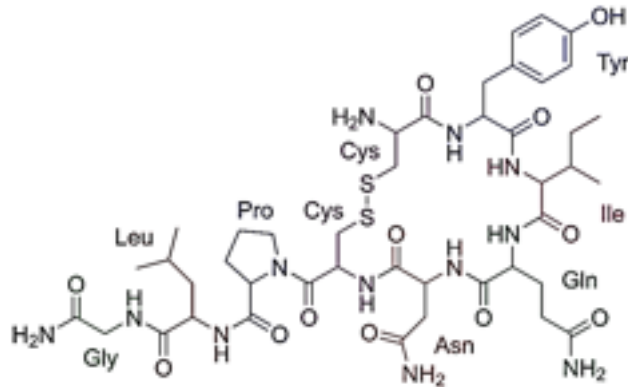


Figure 2. The structure of the oxytocin molecule.

OT is a nonapeptide (Cys–Tyr–Ile–Gln–Asn–Cys–Pro–Leu–Gly(NH₂)) (Fig. 2) with a sulphur bridge between the two cysteines. This neuropeptide is evolutionarily conserved across species and is thought to have originated ca 500 million years ago (Acher, Chauvet et al. 1995). Consequently, virtually all vertebrates carry the genes for a variation of the OT-like peptides (the classification depends on the amino acid present at the eight position (Table 1.1)) (Gimpl and Fahrenholz 2001). The oxytocin receptor (OTr) belongs to the G protein-coupled receptor (GPCR) superfamily, having seven transmembrane domains and the alpha-helix (Fig 3). Its activation results in an increase of intracellular calcium (Ca²⁺) and Protein Kinase C (PKC) levels (Fig 3). Roughly 80% of OTrs are present in the CNS. The OTr is highly expressed in multiple brain regions, for example, in select cortical areas, the olfactory system, the basal ganglia, the limbic system, the thalamus, the hypothalamus, the brain stem, and the spinal cord, and this broad distribution reflects the plethora of physiological and

behavioral o (Rozen, Russo et al. 1995, Breton, Neculcea et al. 1996, Arpin-Bott, Waltisperger et al. 1997). OTr mRNA distribution mostly overlaps with OT binding sites defined through radiolabeled ligand methodologies (Table 1).

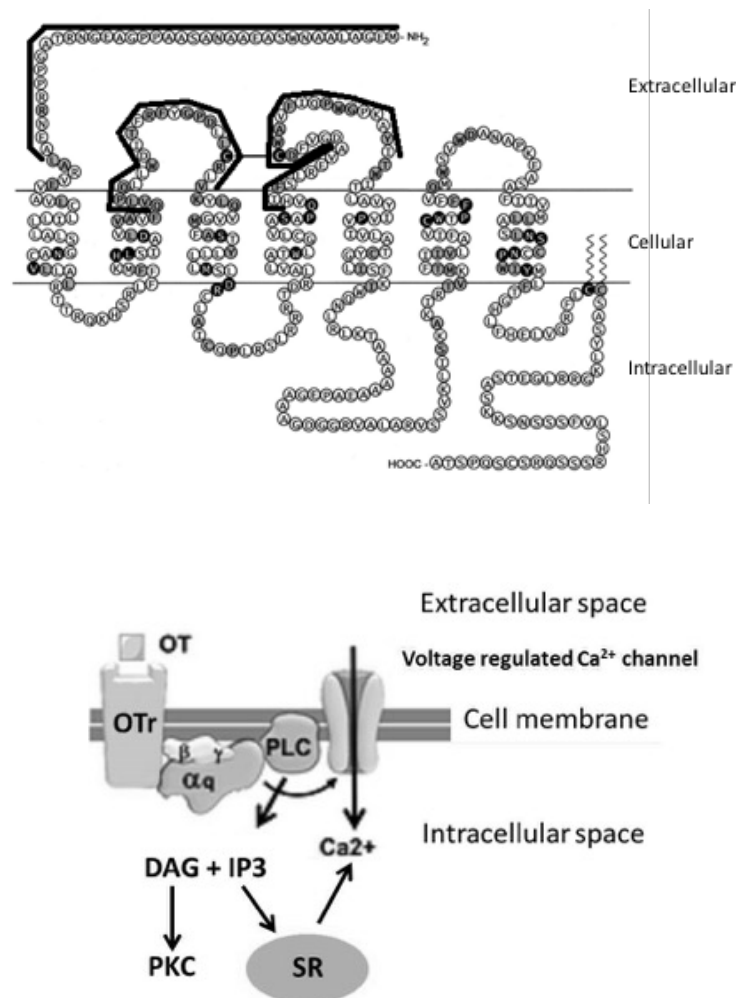


Figure 3. The oxytocin receptor is a 7-transmembrane domain G protein-coupled receptor. Its elements that interact with the natural ligand molecule are marked with the black line (top). The bottom portion of the figure summarizes the cascade of cellular responses induced by activation of this receptor. Binding of OT to the OTr activates Gα q/11 and then phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) to inositol 1,4,5-trisphosphate (IP₃) and

diacylglycerol (DAG). IP₃ causes release of Ca²⁺ from the sarcoplasmic reticulum (SR), while DAG activates PKC. Gα_{q/11} also causes activation of voltage-regulated Ca²⁺ channels and Ca²⁺ entry into the cell.

Table 1. Amino acid configuration of oxytocin and oxytocin-like peptides (modified from (Gimpl and Fahrenholz 2001))

	1	2	3	4	5	6	7	8	9
Oxytocin	Cys	Tyr	Ile	Gln	Asn	Cys	Pro	Leu	Gly(NH ₂)
Mesotocin	*	*	*	*	*	*	*	Ile	*
Isotocin	*	*	*	Ser	*	*	*	Ile	*
Glumitocin	*	*	*	Ser	*	*	*	Gln	*
Valitocin	*	*	*	*	*	*	*	Val	*
Aspargtocin	*	*	*	Asn	*	*	*	*	*
Asvatocin	*	*	*	Asn	*	*	*	Val	*
Phasvatocin	*	*	Phe	Asn	*	*	*	Val	*
Cephalotocin	*	*	Phe	Arg	*	*	*	Ile	*
Annetocin	*	Phe	Val	Arg	*	*	*	Thr	*
Vasotocin	*	*	*	*	*	*	*	Arg	*
Vasopressin	*	*	Phe	*	*	*	*	Arg	*
Lysipressin	*	*	Phe	*	*	*	*	Lys	*
Phenypressin	*	Phe	Phe	*	*	*	*	Arg	*
Locupressin	*	Leu	*	Thr	*	*	*	Arg	*
Arg-conopressin	*	Ile	*	Arg	*	*	*	Arg	*
Lys-conopressin	*	Phe	*	Arg	*	*	*	Lys	*

Mechanisms regulating food intake ensure that organisms maintain a proper energy balance. Physiological systems relevant to feeding continuously facilitate adaptation to changes in the internal Localization of neurons localization at this(understood as the biochemical milieu) and external environment. There are two major mechanisms defining feeding regulation: homeostatic (feeding for hunger/satiety and protection from risks due to excessive stomach distension, osmolality and toxicity) controlled mainly by the hypothalamus-brain stem pathways, and non-homeostatic (for

example, feeding for pleasure) controlled by the reward network of which the two main components are the nucleus accumbens (NAcc) and ventral tegmental area (VTA) (Benelli, Bertolini et al. 1995, Blevins, Eakin et al. 2003). Importantly, PVN and SON OT neurons receive brain stem-derived innervation critical for homeostatic control of consumption and they are part of reciprocal circuit with the reward-related NAcc and VTA (Bagnol, Lu et al. 1999, Arora and Anubhuti 2006, Hayes, Skibicka et al. 2008, Hayes, Bradley et al. 2009). Consequently, the OT system components have been found in brain areas relevant to homeostatic and non-homeostatic appetite control: the OT, in the hypothalamus, and the OTr, in the DVC and the hypothalamic PVN, SON, VMH and LH.

Arletti and colleagues were first to report that IP and ICV OT generates hypophagia related to energy consumption. They found that OT decreases chow intake, increases the latency to start a meal and reduces time spent eating in energy-deprived as well as in unrestricted rats. The effects of OT on feeding are reversed by antagonism of the OTr. Since that initial report, a number of authors have confirmed the functional link between termination of calorie intake and the OT system. For example, OT release from the neurohypophysis coincides with cessation of eating behavior (Arletti, Benelli et al. 1989). The percentage of activated PVN and SON OT neurons is elevated at the end of a meal (Olszewski and Levine 2007). IP, ICV and PVN injections of agents that decrease appetite, such as alpha-MSH and GLP-1, produce robust activation of OT cells (Olszewski, Wirth et al. 2001, Katsurada, Maejima et al. 2014). Interestingly, also peripheral injections of OT decrease feeding (Arletti, Benelli et al.

1990). While IP OT decreases appetite, OTr antagonism in the brain stem does not completely prevent IP OT-induced hypophagia, but only somewhat dampens it (Ho, Anekonda et al. 2014). The effects of peripheral OT might be secondary to processes related to energy metabolism and adiposity. In line with that notion, IP OT in mice reduces visceral fat mass and improves glucose metabolism (Maejima, Iwasaki et al. 2011). It elevates the content of N-oleoyl-phosphatidylethanolamine, the OEA precursor, in white fat cells (Deblon, Veyrat-Durebex et al. 2011).

Aside from its involvement in energy intake control, OT protects the organism by terminating consumption that can disrupt key homeostatic parameters. For example, OT release and neuronal activation occur upon excessive stomach distension when large food loads have been consumed (Qin, Feng et al. 2009). This GI mechanoreceptor-detected information is relayed into the brain by the vagus nerve, eventually reaching OT cells via multisynaptic pathways (Berthoud 2008). ICV OT reduces gastric motility in a dose-dependent fashion (Dubois-Dauphin, Raggenbass et al. 1992). Elevated sodium levels increase OT plasma concentration leading to feeding termination (Flanagan, Blackburn et al. 1992). ICV OT decreases salt intake (Stricker and Verbalis 1996), whereas genetic deletion of the OTr increases sodium consumption (Puryear, Rigatto et al. 2001, Rigatto, Puryear et al. 2003)(Verbalis, Mangione et al. 1991)]. Finally, exposure to toxins, such as LiCl and CuSO₄, that cause GI discomfort and an immediate anorexic response, produces a dramatic increase in the number of c-Fospositive OT neurons in the hypothalamus and a surge in

circulating OT (Flanagan, Verbalis et al. 1988, McCann, Verbalis et al. 1989, Billig, Yates et al. 2001).

The past several years have brought new discoveries that indicate that brain OT controls also a key non-homeostatic aspect of consumption, feeding reward. These discoveries define OT as a neuroregulator of complex dietary choice and consummatory behavioral processes.

PVN OT neuronal projections form somatic and axodendritic synapses with mesolimbic neurons (Sofroniew 1980, Succu, Sanna et al. 2008). Data from human and laboratory animal studies link OT receptor activation with modifications in non-feeding rewards, from natural rewards, such as social and reproductive behaviors to administration of drugs of abuse. For example, cocaine treatment changes OTr binding density in the bed nucleus of the stria terminalis in female rats (Johns, Lubin et al. 2004, McMurray, Cox et al. 2008). Baracz et al. reported that administration of OT in the core of the nucleus accumbens decreases methamphetamine seeking (Baracz, Everett et al. 2014). The same group of investigators found also that OT attenuates methamphetamine induced conditioned place preference in rats (Baracz, Rourke et al. 2012). In a recently published set of experiments with OTr ligand injections in the nucleus accumbens and lentiviral-mediated overexpression of the OTr in this site, Bahi showed that OT attenuates ethanol-induced conditioned place preference (Bahi 2015). Intracranial infusions of OT in female mice promote the development of a conditioned social preference (Kent, Arientyl et al. 2013). Damiano et al. showed with fMRI that some single nucleotide polymorphisms in the OTr gene

are associated with a differential response of the mesolimbic system during anticipation of money rewards in humans (Damiano, Aloï et al. 2014). Neurochemical studies have found a relationship between OT and dopamine in perceiving rewards. For example, central administration of OT reduced methamphetamine-induced dopamine release in the striatum and nucleus accumbens (Qi, Yang et al. 2008), decreased glutamate release and increased γ -aminobutyric acid (GABA) in the medial prefrontal cortex (Qi, Han et al. 2012). Pioneering studies on feeding reward and OT were performed on OT knockout (KO) mice: genetic deletion of OT leads to the enhanced initial and sustained intake of palatable sucrose in the KO compared to the wild-type (WT) mice (Amico, Vollmer et al. 2005). The effect of the OT KO on sucrose consumption occurred in both dark and light phase of the 24-hour cycle and it persisted even in animals subjected to periods of repetitive stress (Billings, Spero et al. 2006). OT KO and WT mice tested in a progressive ratio operant licking paradigm show a similar motivation to consume sucrose (Sclafani, Rinaman et al. 2007). OT KOs given a choice between two tastants (water served as a control), exhibit a higher preference not only for sucrose, but also for palatable carbohydrate solutions in general (e.g., Polycose and cornstarch). A non-caloric non-carbohydrate sweetener, saccharin, was also overconsumed by the KOs (Billings, Spero et al. 2006). The overconsumption of palatable tastants in OT KO mice does not generalize to fat. Two-bottle tests in which mice could choose between water and a lipid emulsion showed a similar fat preference in KOs and WTs (Sclafani, Rinaman et al. 2007). To further examine the issue of preference to fat, Miedlar et al. used a similar paradigm as the one used in the initial study on sucrose intake in OT KOs, however, instead of the

sugar water, the animals were given Intralipid. OT KO mice drank more Intralipid during the first day of having access to the tastants (which may be related to altered neophobic or stress-related processing), but on subsequent days they consumed the same amount of Intralipid as WTs (Miedlar, Rinaman et al. 2007). The OT KO findings are largely in agreement with the results of experiments on laboratory animals without genetic modifications. Real-time PCR showed upregulation of OT mRNA in the hypothalami of rats eating scheduled, unrestricted, sugar diet compared to standard food (Olszewski, Shaw et al. 2009). An increase in OT expression has been found in mice given 48-h ad libitum access to a 10% sucrose solution versus animals consuming Intralipid (Olszewski, Klockars et al. 2010). Herisson et al. studied hypothalamic OT gene expression in mice given short-term access to sucrose, cornstarch, or saccharin and found that exposure to carbohydrates but not to saccharin elevated OT mRNA above control values; notably, a higher level of significance was detected after sucrose intake (Herisson, Brooks et al. 2014). The comparison of OT neuronal activity induced by consumption of sucrose or Intralipid shows a greater number of Fos positive OT cells in the sucrose group. Injection studies using a blood-brain barrier (BBB) penetrating OTr antagonist, L-368,899, in choice and no-choice feeding paradigms (Singru, Wittmann et al. 2012), has caused increase in carbohydrate intake, but consumption of Intralipid has not been affected (Olszewski, Klockars et al. 2010, Herisson, Brooks et al. 2014). When a choice between carbohydrates is given, L-368,899 induces preferentially sucrose consumption. Mullis et al. have recently reported an important piece of evidence linking OT to feeding reward (Mullis, Kay et al. 2013). They implanted rats with a

cannula aimed at the ventral tegmental area and found that OT infusion in this site decreases deprivation-induced chow intake as well as palatability-driven sucrose consumption. These effects are abolished by L-368,899. When L-368,899 or another OTr antagonist, (d(CH₂)₅(1),Tyr(Me)(2),Orn(8))-Oxytocin, were injected alone in the ventral tegmental area, they stimulated sugar intake, but they did not change chow consumption (Mullis, Kay et al. 2013). This is in agreement with the earlier findings showing that when animals are given a choice between sucrose and fat diets, systemic administration of an OTr blocker shifts preference towards sugar without affecting total energy consumption (Olszewski, Klockars et al. 2010). There have been very few studies done in humans. Ott et al (Ott, Finlayson et al. 2013) studied the effects of intranasally administered OT on ingestive behavior, with special emphasis on rewarding aspects of consumption. OT decrease snack intake (chocolate cookies, rice waffles and salt crackers were offered to the subjects) during the snack test administered shortly after a full buffet-style breakfast. Total snack intake was reduced mainly by restraining by 25% the consumption of high-sugar chocolate cookies.

Orexigenic opioid receptor ligands (and possibly also other neuromediators of feeding for pleasure) diminish meal-end activity of hypothalamic OT neurons. For example, butorphanol tartrate at a dose that promotes overeating of sugary foods dampens OT PVN neuronal activity in rats that have consumed the amount of high-sucrose powder diet that is satiating for saline-treated controls (Olszewski and Levine 2007). Opioid receptor agonists have been shown to decrease OT neuronal activity

to noxious stimuli, whereas an antagonist, naloxone, potentiates anorexigenic effects of vomiting inducing agents (Flanagan, Verbalis et al. 1988, Olszewski, Shi et al. 2000). Mitra et al. showed that daily intake of sucrose in rats reduces c-Fos expression in OT neurons after a high-sucrose or low-sucrose meal compared to rats receiving daily low-sugar food (Mitra, Gosnell et al. 2010).

1.4. Devising aims of the project by identifying critical gaps in knowledge: focus on contribution of site-specific populations of the OT receptor to appetite control

The localization of neurons which synthesize a given peptide is certainly a good predictor of this peptide's action or role. One should not, however, neglect of the fact that the peptides exerted their actions via intricate networks of receptors that are scattered throughout the brain. Therefore, the assessment of the function of the signaling molecule should rely to a large extent on the distribution of its receptor in discrete central sites.

This is precisely the case with the pleiotropic neurohormone, OT. Based solely on the localization of OT neurons in the hypothalamic PVN and SON, it could be stipulated that the maintenance of homeostasis via endocrine control is the primary role of this neuropeptide. However, as mentioned in the earlier sections of this chapter, OT appears to be involved also in processing a pleasure-related value of consumption. Furthermore, oxytocin's effect on food intake seems to be dependent on the social status of the animal and that it might also be modified by gender (Olszewski, Klockars et al. 2016). Surprisingly, despite this vast array of functions and despite the broad expression of the OT receptor throughout the CNS (Table 2), our understanding of

which sites relay hypophagic actions of oxytocin is very limited. In fact, only singular reports exist that implicate specific reward, hypothalamic and hindbrain elements of circuitry in regulating select facets of ingestive behavior.

Table 2. Feeding-relevant distribution of the OTr in the central nervous system in rats (modified from (Gimpl and Fahrenholz 2001))

Brain Regions	mRNA	OT binding
Nucleus accumbens	+	+
Bed nucleus of stria terminalis (BNST)	+++	+++
Central amygdaloid nucleus	+++	+++
Medial amygdaloid nucleus	++	+
Basolateral amygdaloid nucleus	+++	+
Paraventricular thalamic nucleus	++	+
Ventromedial hypothalamic nucleus	+++	++
Anterior medial preoptic area	+++	-
Supraoptic nucleus (SON)	+++	(+)
Paraventricular nucleus (PVN)	++	(+)
Ventral and dorsal tegmental area	++	-
Nucleus of the solitary tract	-	(+)
Dorsal motor nucleus of the vagus nerve	+++	+
Medial preoptic area	++	+

Considering the great interest of the scientific community to utilize oxytocin as a potential pharmaceutical agent to combat obesity and excessive food intake, it is extremely important to define the map of brain areas that are obligatory in facilitating OT-induced early termination of consumption.

The **overarching goal of this thesis** was to examine whether select circuits expressing the OT receptor participate in mediating specific anorexigenic responses to OT. This overarching goal was pursued through the following **specific aims**:

Specific aim 1. To determine whether extreme overeating induced by a powerful orexigen, butorphanol tartrate, can be alleviated by pharmacologically stimulating the OT receptor in the forebrain versus hindbrain.

Specific aim 2. To examine whether either of two specific subpopulations of the hypothalamic OT receptor, located in the medial preoptic area or the ventromedial hypothalamic nucleus, mediates OT-driven hypophagia in energy- and reward-related facets of ingestive behavior.

Specific aim 3. To assess whether the OT receptor present in the basolateral and central amygdala, sites implicated in emotional processing of food intake, is involved in appetite control by OT.

The studies utilized laboratory rats as an animal model for eating behavior. The experimental approaches encompassed intracranial surgeries and drug administration, analyses of region-specific expression of genes, brain circuitry-wide mapping of immunohistochemically detected immediate-early gene products, and examination of feeding behavior parameters related to eating for energy, eating for reward, and developing avoidance mechanisms to diets whose consumption jeopardizes internal milieu.

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2. Chapter Two - Effects of lateral versus fourth ventricular administration of oxytocin receptor ligands on potent orexigenic effects of butorphanol tartrate

Abstract

Butorphanol tartrate (BT), a mixed mu/kappa/delta opioid receptor agonist, is one of the most potent orexigens known to date. Interestingly, BT suppresses meal-end activation of neurons synthesizing anorexigenic neuropeptide, oxytocin (OT), which suggests that BT promotes hyperphagia by silencing OT-derived satiety signaling. Since OT terminates consumption by acting via distinct hindbrain and forebrain circuits, I investigated whether stimulation of the OT receptor in the forebrain or hindbrain (via lateral (LV) and fourth ventricular (4V) OT injections) leads to termination of food intake induced by BT. I established effective doses of BT on chow intake in ad libitum-fed and overnight-deprived rats as well as effective doses of LV and 4V OT in deprived animals. Then, I determined doses of LV and 4V OT that reduce hyperphagia produced by BT in sated and deprived rats. Finally, I assessed whether OT's effects on BT-induced feeding can be suppressed by an OT receptor antagonist. 4 mg/kg BT increased intake in ad libitum-fed and overnight deprived rats, whereas LV and 4V OT at 1 μ g caused a decrease in deprived rats. BT-induced chow intake in hungry and sated animals was suppressed by a very low, 0.1- μ g dose of 4V OT, whereas 1 μ g OT was effective LV. The effect of OT was attenuated by OT receptor antagonist, L-368,899. Reduced activity of the OT circuit, especially its hindbrain component, is a critical factor in shaping the magnitude of consumption in response to the potent BT treatment.

Keywords: oxytocin, opioids, food intake, paraventricular nucleus, butorphanol

2.1. Introduction

Opioid peptide-induced hyperphagia is a well-described phenomenon. All subtypes of opioid receptors contribute to increases in feeding. Opioid receptor agonists elevate consumption of palatable tastants, promote meal maintenance, and are able to stimulate intake of “bland” foods (Gosnell, Levine et al. 1986, Gosnell and Levine 2009). Conversely, antagonists reduce eating for pleasure, decrease deprivation-induced feeding and support discontinuation of a meal (Bodnar, Glass et al. 1995).

Butorphanol tartrate (BT), a synthetic opioid ligand, is one of the most potent orexigens known to date (Morley, Parker et al. 1985, Levine, Grace et al. 1994). Yet, our knowledge of mechanisms that mediate its effects on food intake is extremely limited. Boggiano et al. (2005) suggested that BT acts chiefly as a kappa and mu agonist (Boggiano, Chandler et al. 2005), whereas earlier studies pointed to the compound’s affinity for the delta receptor (Chang, Hazum et al. 1981, Lahti, Mickelson et al. 1985). In some paradigms, BT has been shown mixed agonist/antagonist-like properties (Craft and McNiel 2003). In feeding studies, BT administration has consistently generated robust feeding. Peripheral injections of BT elevate intake of standard laboratory chow in non-deprived rats, and the magnitude of the orexigenic responses to BT is comparable to those induced by

neuropeptide Y (Levine and Morley 1983, Morley, Levine et al. 1983, Jewett, Grace et al. 2001, Mitra, Kotz et al. 2012). BT administered centrally, including in the paraventricular nucleus of the hypothalamus (PVN), promotes moderate hyperphagia, which suggests that peripheral and central mechanisms contribute to BT-driven overfeeding.

It has recently been shown that subcutaneous (SC) administration of BT at an orexigenic dose causes a meal-end reduction in the activation of oxytocin (OT) neurons in the PVN. Since OT is an anorexigen, that initial finding allowed us to coin a hypothesis that BT promotes hyperphagia by suppressing OT-derived satiety signaling. In the current set of studies, I further explored this hypothesis by focusing on the fact that OT terminates food intake by acting via distinct hindbrain and forebrain circuits. Thus, I investigated whether stimulation of the OT receptor in the forebrain or hindbrain (via lateral (LV) and fourth ventricular (4V) OT injections) leads to termination of food intake induced by BT. I first established the effective doses of SC BT on chow intake in ad libitum-fed and overnight-deprived rats as well as effective doses of LV and 4V OT in deprived animals. Then, I determined doses of LV and 4V OT that attenuate hyperphagia produced by SC BT in sated and deprived rats. Finally, I assessed whether OT's effects on BT-induced feeding can be suppressed by an OT receptor antagonist delivered via the same routes.

2. 2. Materials and Methods

Animals. Adult male Sprague–Dawley rats (AgResearch, Hamilton, NZ) weighing approximately 320g at the beginning of the studies were housed individually in Plexiglas cages in a temperature-controlled vivarium (21°C) with LD 12:12 (lights on

at 0800). Tap water and standard laboratory chow (Teklad) were available ad libitum unless noted otherwise. The University of Waikato animal ethics committee had approved the procedures, and they are compliant with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publ., no. 80–23, rev. 1996). A different set of animals (n=7-9/study) was used for each of the experiments described below.

Cannula implantation. Animals were anesthetized with the mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg) and implanted with a stainless steel cannula aimed at the lateral ventricle (LV) or the fourth ventricle (4V). Stereotaxic coordinates (Paxinos 1982) for an LV 26-gauge cannula (Plastics One) were 1.5 mm lateral to the midline, 1.0 mm posterior to bregma and 3 mm below the surface of the skull, whereas a 4V cannula was positioned on the midline 3.2 mm caudal to lambda and 7.2 mm below the skull surface. The injector needle extended 1 mm beyond the tip of the guide cannula. Dental acrylic was used to secure the cannula to two screws inserted in the skull.

Cannula placement was verified after a 9-12-day recovery period and again, following the completion of feeding experiments, via LV injections of 100 ng angiotensin II (those rats that drank <6 ml of water within 30 min of the treatment were excluded from the study) (Mitra, Klockars et al. 2012) and 4V injections of 30 pmol bombesin (animals showing more than 25% decrease in food intake were kept in the study) (Flynn 1989).

Drugs and injections. BT was acquired from MSD (NZ), and OT and L-368,899 were purchased from Tocris Bioscience (UK), Intracranial injections were performed using Hamilton syringes in a volume of 2 µl. Drugs were dissolved in isotonic saline. I

employed a repeated measures paradigm, i.e., each animal within a given experiment received each dose of the peptide in a counterbalanced manner. Hence, each study was divided into sessions spaced 3 days apart. Injections were performed between 9:30 and 10:30 in a random fashion.

Experiment 1: Determining effective orexigenic doses of SC BT in ad libitum-fed and overnight-deprived rats. Ad libitum-fed or overnight-deprived rats (in all deprivation studies reported here, food was taken away 1-2 h before lights off) were injected SC with 0 (saline), 1, 2 and 4 mg/kg b. wt. and pre-weighed chow pellets were placed in food hoppers immediately after the drug treatment. Food intake was measured 1, 2, 4, 6 and 24 h post-injection. In this and remaining experiments, food intake was corrected for spillage.

Experiment 2: Determining effective anorexigenic doses of LV -and 4V-injected OT in overnight-deprived rats. Animals deprived of food overnight were injected in the LV or in the 4V with 0 (saline), 0.1, 0.3 or 1 µg OT and chow was returned to hoppers just after the drug administration. Food intake was measured 1, 2, 4, and 24 h post-injection.

Experiment 3: Effect of LV OT on BT-induced feeding. Ad libitum-fed or overnight-deprived rats treated SC with an orexigenic dose of BT (4 mg/kg; SC saline-treated animals served as controls for BT-induced hyperphagia) were injected 5 minutes earlier in the LV or 4V with 0 (saline), 0.1, 0.3 or 1 µg OT. Pre-weighed chow was placed in cages immediately after the second (i.e. SC BT or SC saline) injection. Food intake was measured 1, 2, 4, and 24 h post-injection.

Experiment 4: Effect of 4V OT on BT-induced feeding. I used the same double-injection protocol in ad libitum-fed and overnight-deprived rats as described in Experiment 3 (section 2.6); however, OT was administered in the 4V at the doses of 0 (saline), 0.03, 0.1 and 0.3 μg . Food intake was measured 1, 2, 4 and 24 h post-injection.

Experiment 5: Effect of OTA pretreatment on the ability of OT to affect BT-induced feeding. In order to assess whether OT receptor blockade would prevent the ability of OT to affect BT-induced feeding, I employed the same protocol as in Experiments 3 and 4 (intracranial OT ligand administration followed by SC BT), however, 5 minutes prior to injecting animals with effective doses of LV and 4V OT (1 and 0.1 μg , respectively), I administered via the same routes 1 (LV) or 0.1 μg (4V) of an oxytocin receptor antagonist (OTA), L-368,899. Food was placed in hoppers immediately after the SC (BT/vehicle) treatment and consumption was measured 4 h post-injection.

Data analysis. Interval intakes were analyzed in all experiments. In Experiments 1 and 2, analysis was performed with one-factor ANOVA with repeated measures and means were compared using Dunnet's post-hoc test. In experiment 3-4, for a two-group comparison (SC BT vs saline) a Student's t-test was used. To determine the effect of OT on BT-dependent feeding, I used one-factor ANOVA with repeated measures followed by Dunnet's post-hoc test with correction for multiple comparisons. The effect of OTA on OT-induced changes in feeding in BT-treated animals was established using Student's t-test. Values were considered significantly different when $P < 0.05$. Data are presented as a means \pm SEM.

2.3. Results

BT increased food intake in both ad libitum-fed and overnight-deprived rats (Fig. 1). In ad libitum-fed animals, 2 (P=0.019; F(3,21)= 2.21) and 4 mg (P=0.00043) doses of BT were effective during the 1-2 h period, whereas at 2-4 h post-injections, animals maintained a significantly higher level of consumption (P=0.00082; F(3,21)=7.81). In overnight-deprived rats, only the 4-mg dose of BT produced hyperphagia during the 0-1 (P=0.021; F(3,18)=2.64), 1-2 (P=0.004; F(3,18)=5.27) and 2-4 h (P=0.005; F(3,18)=4.03) timeframes. There was no effect of BT on 24-h food intake (data not shown).

Overnight-fasted rats treated with 1 µg OT LV or 4V (Fig. 2) showed a reduction in food intake during the first hour of re-feeding (P=0.026, F(2,14)=5.02 and P=0.005, F(2,14)=8.45, respectively).

0.1 µg OT administered in the 4V decreased BT-induced chow intake in ad libitum-fed animals from 2-4 h post-injection (P=0.012; F(4,28)=16.43), whereas the 0.3-µg dose was effective during the 1-2 and 2-4 h periods (P=0.020, F(4,28)=5.35 and P<0.001, F(4,28)=16.43, respectively). In overnight-deprived BT-treated rats, 0.3 µg 4V OT reduced consumption at 1-2 h (P=0.002; F(4,24)=9.38) and 2-4 h (P=0.038; F(4,24)=4.89) post-injection. A lower dose – 0.1 µg OT – was effective at 1-2 h (P=0.028; F(4,24)=9.38).

Only the 1-nmol dose of LV OT decreased BT-induced food intake in ad libitum-fed rats at 1-2 h (P=0.001; F(4,28)=23.34) and 2-4 h post injection (P=0.027; F(4,28)=7.02) and in overnight-deprived animals during 0-1 h (P=0.027; F(4,24)=7.05), 1-2 h (P=0.034; F(4,24)=7.84), and 2-4 h (P=0.001; F(4,24)=6.41) of

re-feeding. LV and 4V OTA reduced the effect of OT on BT-driven food intake in ad libitum-fed and fasted rats (Table 4).

2.4. Discussion

It has been shown beyond reasonable doubt that opioid peptides increase feeding, and that their action via reward-related brain sites, including the nucleus accumbens and ventral tegmental area, is of particular importance for the orexigenic effects to occur. However, the reward circuit cannot be considered a sole player in mechanisms that facilitate voracious food intake induced by opioid agonists, such as BT, and a set of more complex neuroendocrine changes is likely needed to permit ingestion of large food loads. This is crucial considering the fact that opioids increase consumption beyond actual calorie needs of the animal (thus, past the point of satiation), and oftentimes also despite the risks to internal milieu brought upon by excessive osmolality, stomach distension or potential toxicity.

Our previously published data suggest that BT may be silencing satiety signaling provided by the OT system: animals injected with SC BT and pair-fed to saline controls display a lower level of PVN OT neuronal activation at the end of a meal (Kim, Shi et al. 2001). Notably, PVN OT neurons project to a vast variety of forebrain and brainstem sites through which they modify consummatory responsiveness, but that initial study did not explore which subpopulations of PVN OT cells are affected. The current results further substantiate and expand on those early findings, as they indicate that BT's effect on feeding can be attenuated by OT at doses that are equivalent to or even much lower than those that reduce energy-driven

consumption, but that the hindbrain OT stimulation appears more critical in shaping the magnitude of BT-driven hyperphagia.

The ability of central OT to abolish BT-induced overeating was tested in two BT treatment paradigms that differed in energy needs at the onset of the experiment, i.e., ad libitum feeding and post-deprivation. I found that BT was effective not only in promoting food intake in sated rats, but also in prolonging consumption in animals already motivated to eat by overnight fast. Regardless of the paradigm, LV and 4V OT suppressed BT-induced feeding. LV OT was effective in BT-injected animals at a 1- μ g dose, which is minimal to diminish deprivation-induced chow intake in BT-untreated rats, as evidenced in our control experiment here as well as in previous studies on OT's effects on appetite after starvation (Arletti, Benelli et al. 1990, Olson, Drutarosky et al. 1991, Schwartz, Woods et al. 2000). 4V OT also reduced appetite in BT-treated rats in both paradigms. Both LV and 4V OT showed a similar time course of action in suppressing BT's hyperphagia: their effect was significant during the 1-2 h and 2-4 h period post-injection.

Importantly, the 0.1- μ g dose of 4V OT effective against BT-induced feeding was 10 times lower than the one needed to decrease deprivation-driven ingestive behavior in non-BT animals. This great sensitivity of BT-derived hyperphagia to hindbrain OT receptor stimulation strongly suggests that the functional relationship between BT and OT in feeding control involves the OT input into the brain stem. While the forebrain sites targeted by the OT system, including the dorsomedial nucleus and nucleus accumbens, mediate anorexigenic action of OT, the dorsal vagal complex (DVC) plays a critical and most comprehensive role in integrating feeding-related

peripheral signals (from toxicity to stomach distension and osmotic changes to meal size) and OT's action (Blevins, Eakin et al. 2003). Not surprisingly, therefore, BT - whose effects include promoting overconsumption in a non-hungry state and post-meal as well as preventing food avoidance despite unpleasant gastrointestinal sensation - might depend on the activation of the descending OT pathways into the brain stem. In line with that, it has been shown that orexigenic doses of opioid receptor agonists, including BT, simultaneously decrease LiCl-induced activation of OT cells in the PVN and unidentified nucleus of the solitary tract neurons (Olszewski, Shi et al. 2000). Conversely, naloxone has been found to elevate the release of OT induced by administration of cholecystinin and LiCl and potentiate DVC-mediated changes in gastrointestinal motility (Flanagan, Verbalis et al. 1988, Leng, Dyball et al. 1992).

Furthermore, in order to address the question whether the effects of OT injections are specific to the OT receptor, I determined that a decrease in BT-induced feeding caused by OT can be abolished by a pre-treatment with an OTA delivered via the same routes. As OTAs, including L-368,899 used herein, do not increase chow intake (Mullis, Kay et al. 2013), it strengthens the notion that the observed effects are indeed mediated by the OT receptor.

2.5. Conclusions

Overall, our data add to the evidence that BT's effects on food intake stem, at least to some extent, from blockade of tonic feeding-inhibitory pathways. The current studies indicate that reduced activity of OT circuit, especially its hindbrain

component, is a critical factor in shaping the magnitude of excessive consumption in response to BT treatment.

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2.7. Figures and tables

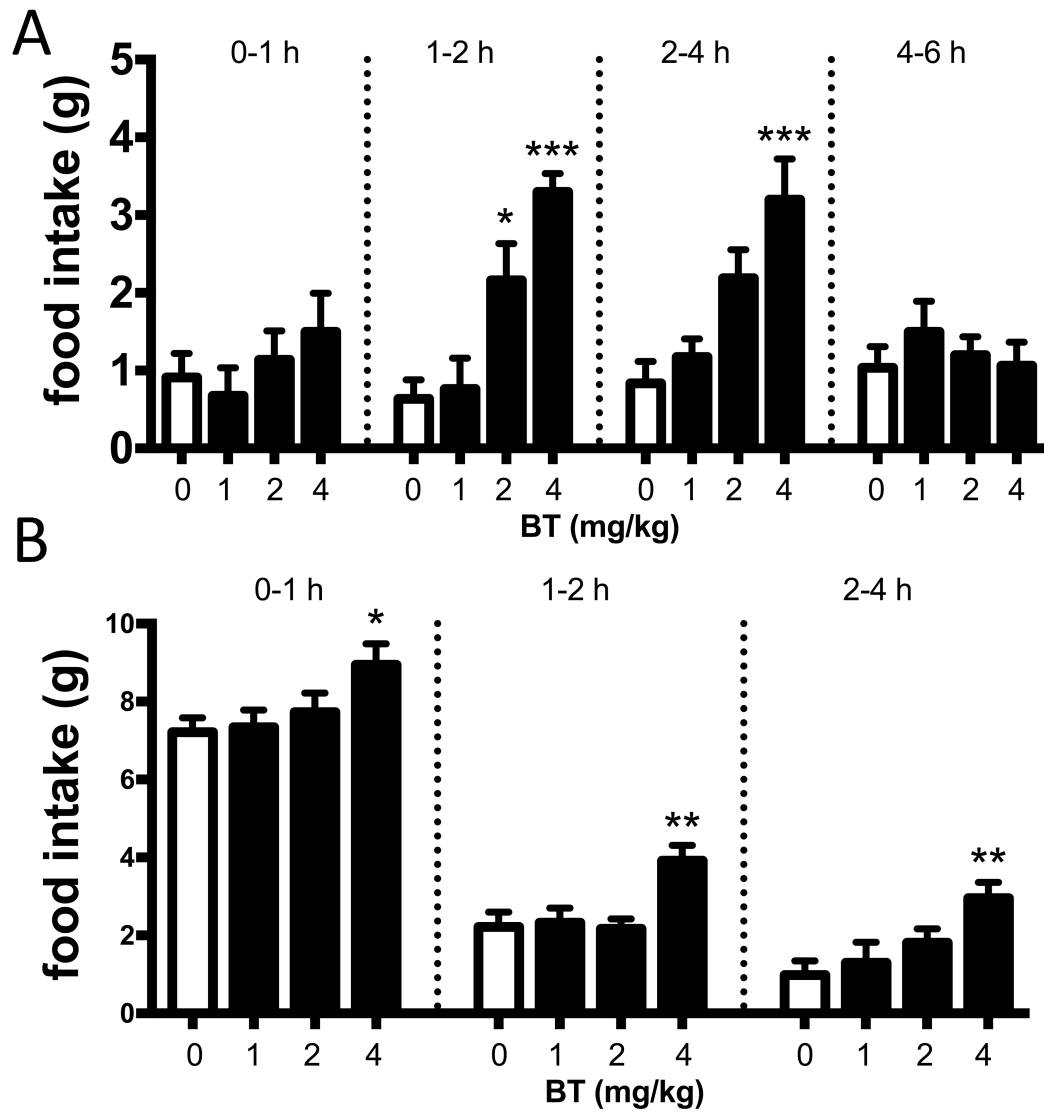


Figure 1. Effect of SC BT on feeding in rats fed ad libitum (A) and following overnight deprivation (B). */**/** - significantly different from saline-injected controls, $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

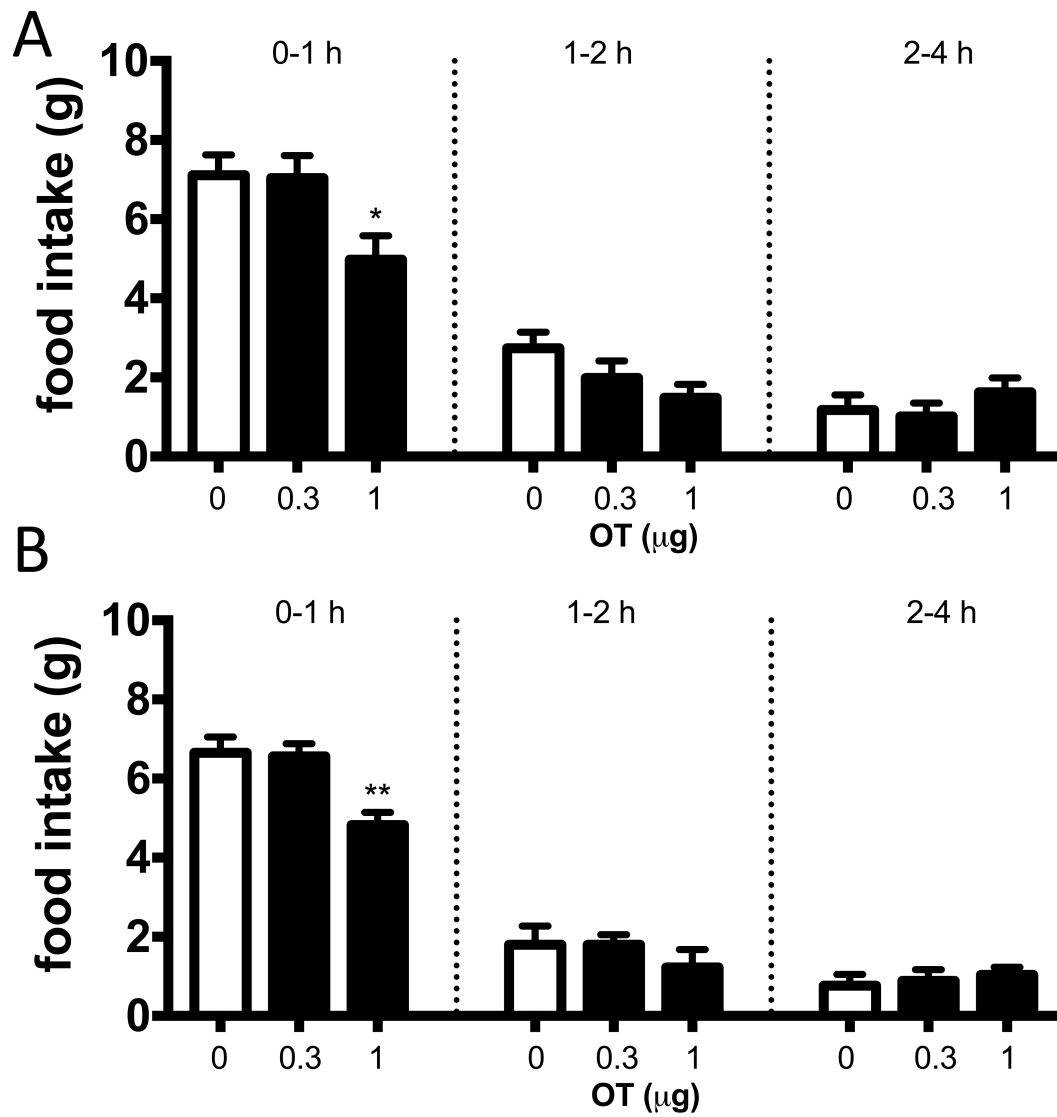


Figure 2. Effect of LV (A) and 4V (B) OT on feeding induced by overnight deprivation. */** - significantly different from saline-injected controls, $P < 0.05$ and $P < 0.01$, respectively.

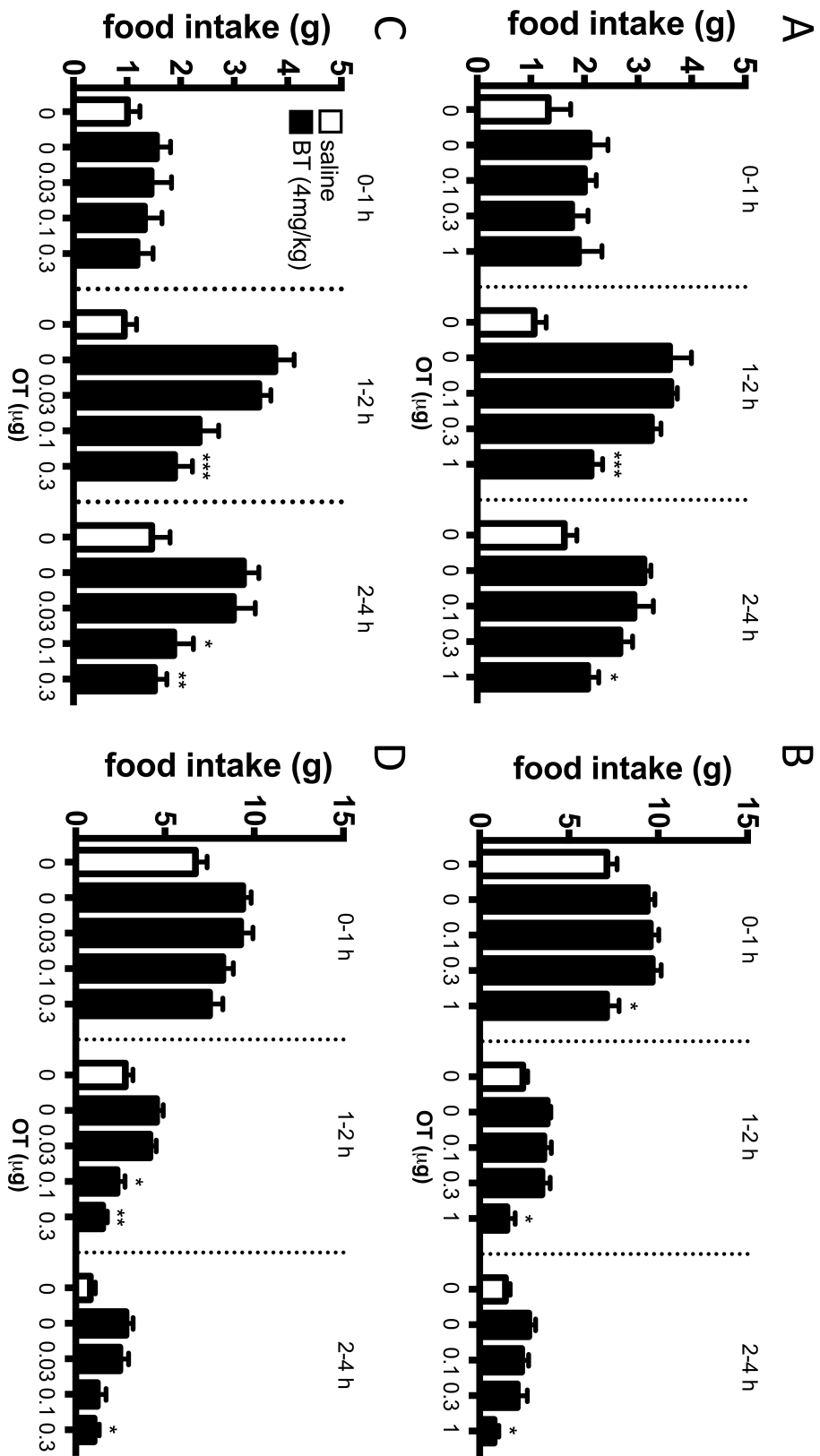


Figure 3. Effect of LV (A, B) and 4V (C, D) OT on BT-induced feeding in rats fed ad libitum (A, C) and following overnight deprivation (B, D).

Table 1. Effect of OT receptor antagonist (OTA) pretreatment on the ability of OT to suppress 4 mg/kg BT-induced food intake as established in a 4-hour meal in ad libitum-fed (left side) and overnight deprived (right side) rats.

Injection		Ad libitum-fed rats	Injection		Overnight deprived rats
LV	SC		LV	SC	
Saline/saline	Saline	3.26 ± 0.72	Saline/saline	Saline	11.04 ± 0.71
Saline/saline	BT	7.76 ± 0.46 [^]	Saline/saline	BT	16.59 ± 0.54 [^]
Saline/OT	BT	5.04 ± 0.20 [*]	Saline/OT	BT	11.45 ± 0.86 ^{**}
OTA/OT	BT	7.06 ± 0.76	OTA/OT	BT	15.89 ± 0.64
4V	SC	Ad libitum-fed rats	4V	SC	Overnight deprived rats
Saline/saline	Saline	3.5 ± 0.65	Saline/saline	Saline	10.54 ± 0.59
Saline/saline	BT	8.3 ± 0.74 [^]	Saline/saline	BT	14.99 ± 0.96 [^]
Saline/OT	BT	5.66 ± 0.45 [*]	Saline/OT	BT	11.54 ± 0.55 ^{**}
OTA/OT	BT	7.77 ± 0.62	OTA/OT	BT	13.31 ± 0.71

[^] - significantly different from SC saline (P<0.05); ^{*}/^{**} - significantly different from animals treated intracranially with saline/saline and SC with BT (P<0.05 and P<0.01, respectively);

3. Chapter Three - Anorexigenic potential of oxytocin acting directly in the ventromedial hypothalamic nucleus and medial preoptic area

Abstract

It remains to be elucidated whether oxytocin (OT) reduces various aspects of food intake by acting directly at hypothalamic circuits, either via the ventromedial hypothalamic nucleus (VMH) or the medial preoptic area (MPOA). Here I provide insight into neural mechanisms underlying OT-driven anorexia mediated by the hypothalamus by (a) defining whether MPOA or VMH administration of OT affects feeding driven by energy needs versus palatability; (b) identifying feeding-related brain sites activated by VMH OT injection; (c) measuring VMH OT receptor (OTr) mRNA changes in response to hunger and palatability; and (d) examining how VMH OT affects episodic sweet solution intake in sated and hungry rats. MPOA had no effect on intake of energy-dense standard chow in overnight-deprived rats nor did it decrease intake of calorie-dilute palatable solutions that differed in macronutrient composition (sucrose- or saccharin-sweetened; or Intralipid-enriched). On the other hand, VMH OT decreased intake of chow and the effect was reversed by the antagonist, though the antagonist alone was not orexigenic. OT did not affect intakes of energy-dilute saccharin and sucrose solutions in sated or hungry rats. Fos immunoreactivity, a marker of neuronal activation, was elevated in the VMH and energy balance-related paraventricular and arcuate nuclei, but not reward areas. VMH OTr expression was higher in hungry rats than in sated controls;

saccharin intake had no effect. MPOA OT receptor is not involved in OT-driven hypophagia mediated by hypothalamic networks. OT acting in the VMH decreases intake driven by energy not by palatability, and it stimulates activity of hypothalamic sites controlling energy balance.

Keywords: oxytocin, VMH, reward, energy

3.1. Introduction

Arletti and colleagues reported for the first time that intracerebroventricularly (ICV) injected oxytocin (OT), a neuropeptide synthesized mainly in the paraventricular (PVN) and supraoptic (SON) nuclei and released throughout CNS targets and into the general circulation, reduces energy intake (Arletti, Benelli et al. 1989). Subsequently, the PVN-brainstem pathway was found to be critical in facilitating OT-driven hypophagia (Ho, Anekonda et al. 2014, Olszewski, Klockars et al. 2016). Notably, the OT receptor (OTr) is highly expressed also outside the brain stem, including in a number of sites that regulate appetite. Among those areas, the nucleus accumbens (Acb) and ventral tegmental area (VTA) OTr populations have been implicated in feeding control as well (Mullis, Kay et al. 2013, Herisson, Waas et al. 2016, Olszewski, Klockars et al. 2016). Importantly, OT appears to be very effective in reducing the intake of energy-dense foods (regardless of macronutrient composition) and – as shown in several knockout and injection animal model experiments – decreasing consumption of sweet solutions (Miedlar, Rinaman et al. 2007, Mullis, Kay et al. 2013, Herisson, Brooks et al. 2014, Herisson, Waas et al. 2016).

The quest to identify hypothalamic sites that integrate OT's influence on feeding has recently led Noble et al. (Noble, Billington et al. 2014) to note that the ventromedial hypothalamic nucleus (VMH) – an area whose malfunctioning leads to reduced sympathetic nervous system activity and delayed satiety (Sakaguchi, Arase et al. 1988, Tokunaga, Fukushima et al. 1989) - is particularly rich in the OTr and it exhibits enhanced neuronal activation after systemic OT treatment (Zhang and Cai 2011). Consequently, they performed the initial set of experiments showing that OT injections in this site reduce energy-driven chow intake at the beginning of the night phase and after deprivation, and elevate energy expenditure as well as spontaneous physical activity in rats (Noble, Billington et al. 2014). Unfortunately, to date, there has been no additional attempt to further characterize the nature of the anorexigenic action of VMH OT.

On the other hand, a potential involvement of the medial preoptic area (MPOA) in mediating OT-driven hypophagia has never been examined. This is particularly surprising considering that OT receptors are expressed at a high level in the MPOA and that MPOA controls adaptive responses to ambient temperature changes, including adjustments in energy expenditure and food intake to compensate for the external environmental challenge (Yu, Qualls-Creekmore et al. 2016).

The current report provides insight into neural mechanisms that underlie VMH OT-driven anorexia by identifying a network of feeding-related brain sites whose activation (determined through c-Fos immunoreactivity) is changed by administration of OT in the VMH. I further substantiate the proposed involvement of the VMH OTr in feeding control by measuring OTr mRNA changes in this area in

response to hunger as well as palatability. I present a series of experiments examining the effect of VMH OT on sweet solution intake (episodic in sated and hungry animals) and use energy-driven ingestion paradigms (deprivation- and daily scheduled intake of standard chow) as control for anorexigenic action of VMH OT. Finally, I provide the very first set of data on the ability of OT infused directly in the MPOA to affect consumption of standard laboratory chow induced by energy deprivation, as well as of calorie-dilute yet highly palatable solutions in non-deprived rats.

3.2. Materials and Methods

Animals and surgeries. Male Sprague–Dawley rats (AgResearch, NZ), aged 9 weeks and weighing between 265.1-280.7 g at the beginning of the studies were single-housed in Plexiglas cages (21°C; 12:12 LD, lights on: 08:00). They had unrestricted access to water and standard chow (Teklad) unless noted otherwise. The University of Waikato ethics committee had approved the procedures, and they are compliant with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publ., no. 80–23, rev. 1996).

Animals in injection studies were anesthetized with ketamine-xylazine (100mg/kg + 20mg/kg) and unilateral 28-gauge stainless-steel guide VMH cannulas (Plastics One, Roanoke, VA) were implanted according to the following stereotaxic coordinates: +0.5mm lateral, 2.5mm posterior to bregma, and 8.6mm below the skull surface (the injector extended 1 mm beyond the tip) (Paxinos 1982). Two stainless steel screws inserted in the skull and dental cement were used to secure the cannula. Animals were allowed to recover for 8-9 days and afterwards they were subjected

to 3 days of handling and sham injections. After completion of the studies, histological verification of placement was performed.

We used two separate main cohorts of cannulated rats: one cohort was used in studies on deprivation-induced and scheduled chow intake (Experiments 1, 2), and the other, in studies involving palatable liquids and in the c-Fos experiment (Experiments 3, 4, 6). The map of correct placements for individual animals belonging to these main cohorts is shown in Fig. 1.

Drugs and injections. OT and L-368,899 (Tocris Bioscience, UK) were dissolved in isotonic saline. Injections were done with Hamilton syringes (0.5 μ l; 30 s) between 9:00 and 10:00.

Experiment 1: Effect of VMH OT and OTr antagonist on deprivation-induced chow intake

Out of 30 rats that were initially equipped with a VMH cannula, 25 had a correct placement (Fig. 1). Animals (n=6-7/group) were food-deprived overnight and injected in the VMH with either OT (0.3 and 1 μ g) or saline prior to re-feeding (food returned at 10:00). Food intake was measured 1 and 2 h post-injection. After a 'wash-out' period of 4 days, by applying the same deprivation scenario, we examined whether VMH L-368,899 (1 and 3 μ g) by itself changes chow intake during re-feeding (saline was used as vehicle; n=6-7/group).

To assess whether the effect of OT can be blocked with the antagonist, animals were double-injected with (a) saline-saline, (b) saline-OT (1 μ g), (c) L-368,899 (1 μ g) – OT (1 μ g) (n=6/group). The two injections were spaced 15 min apart. Food was

returned to cages 5-10 min after the second injection and consumption was measured 2 h later.

The validity of the 0.5- μ l injection volume is based on diffusion coefficients (Nicholson 1985), as well as on previous empirical data, showing ,e.g., the diffusion radius of 0.5 μ l of 0.5% pontamine blue dye from the PVN to be approximately 0.25 mm (Wang, Bomberg et al. 2007). It is also the same as the volume used in the previous VMH OT study by Noble et al (Noble, Billington et al. 2014) as well in the works of other groups that performed targeted intraparenchymal injections (e.g., (Fekete, Inoue et al. 2007, Beckman, Shi et al. 2009, Vogel, Wolf et al. 2016)). However, to ensure that the injectant volume does not exacerbate the effect of VMH OT on deprivation-induced chow intake, in a pilot study, we implanted an additional cohort of rats with a VMH cannula. After recovery, these rats were subjected to overnight food deprivation and – prior to refeeding – injected with only 0.3 μ l (instead of 0.5 μ l) of 1 μ g OT (n=7) or saline (n=5). We analyzed the data with a t-test and found a decrease in 2-h chow consumption from 13.56 \pm 1.71g in controls to 8.81 \pm 0.9g in VMH OT-treated rats (P=0.0119), thus the response mirrored the one observed after 0.5 μ l injections.

Experiment 2: Effect of VMH OT on scheduled intake of chow

Following the completion of Experiment 1, animals were accustomed to receiving chow only once a day for 2 h (10:00-12:00) over 14 days. VMH injections of OT (0.3 and 1 μ g) or saline (n=7/group) were done 10-15 min prior to returning food to hoppers and consumption was measured 1 and 2 h later.

Experiment 3: Effect of VMH OT and OTr antagonist on episodic intake of sweet solutions

We initially implanted cannulas in 29 rats, however, in one case, the cannula became dislodged, and in additional 6 cases, the placement was deemed incorrect and the data were discarded. The animals were used in Experiments 3, 4 and 6 (3-4 days between experiments were given as a 'wash-out'). We based the protocol on previous OT injection studies (Olszewski, Klockars et al. 2010, Herisson, Brooks et al. 2014, Herisson, Waas et al. 2016). Animals were accustomed to receiving one of the sweet solutions (10% sucrose, 0.1% saccharin) for 2 h/day on 2 days (10:00-12:00) prior to the injection experiments to avoid neophobia. First, studies involving sucrose (preceded by sucrose solution pre-exposure) were performed. On the experimental day, animals were injected with 1 μ g OT or saline in the VMH. After a 2-day break, rats were given the 2-h sucrose access again and just prior to it, they were injected in the VMH with 1 μ g L-368,899 or saline (n=11/group).

Three days without any treatment elapsed. Saccharin pre-exposure was done on two consecutive days, followed – 2 days later – by a 2-h experimental session in which 1 μ g OT or saline was injected in the VMH before presentation of 0.1% saccharin (n=6/group). In the antagonist study, after a 2-day treatment break, rats were given the 2-h saccharin access again and just prior to it, they were injected in the VMH with 1 μ g L-368,899 or saline (n=6/group).

Palatable solution intake was measured 1 and 2 h post-injection. Chow and water were removed during the time of sweet tastant presentation.

Experiment 4: Effect of VMH OT on intake of a calorie-dilute palatable sucrose solution and energy-dense standard chow after a period of overnight food deprivation.

Prior to injections, animals were accustomed on three different occasions (once a week) to receiving 2-h simultaneous access to standard chow and 10% sucrose solution (no water) during a re-feeding session after overnight deprivation (from 10:00 to 12:00). Rats received VMH injections of OT (1 μ g) or saline just prior to re-feeding (n=8/group). Consumption was measured after 2 h.

Feeding data analysis. In experiments involving more than two groups (Experiments 1, 2), one-factor ANOVA followed by Fisher's post-hoc test was used. For two-group comparisons (Experiments 3 and 4), a t-test was used. Values were considered significantly different when $P < 0.05$. Data are presented as a means \pm SEM.

Experiment 5: Changes in VMH OTr expression in rats exposed to food deprivation or to a sweet saccharin solution compared to ad libitum chow-fed controls.

While the control rats had chow and water available ad libitum, the remaining groups were either (a) deprived of chow overnight (water remained in the cages) or (b) given overnight access to a palatable 0.1% saccharin solution (instead of water; chow remained in the cages; n=8/group). The animals were decapitated within the first hour after the onset of light, the VMH was dissected and placed in RNAlater (Ambion, USA). A previously used standard protocol of sample preparation and rtPCR was followed and, for brevity, only the main elements are described (see [5] for details). Samples were homogenized in TRIzol (Ambion, USA); RNA was extracted with chloroform and precipitated in isopropanol. After centrifuging, the pellet was washed, dried, and dissolved in the DNase buffer (NEB, USA). The samples were treated with RNase-free DNase I (Merck, Germany) and the absence

of genomic DNA was established by PCR of a 5% template. 100ng/ μ l genomic DNA served as a positive control and MilliQ H₂O as a negative one. The product was analyzed by electrophoresis. 5 μ g RNA samples were diluted with MilliQ H₂O. RNA was reverse-transcribed in the master mix (Promega, Australia; 20 μ l). Samples were incubated for 1 h (37°C), followed by PCR to confirm cDNA synthesis. RtPCR reactions were performed in duplicates. Sample cDNA template (25ng) was used per primer [OTr primer sequences: CGGTGGATCTCGGACTGAAC (fwd) and TAGCAGGCGGAGGTCAGAG (rev)]. Expression of three housekeeping genes (mRPL19, mCyclo and GAPDH) was used to calculate normalization factors (GeNorm, CMG, Belgium). Primer efficiencies were calculated with LinRegPCR (HFRC, Netherlands) and Ct values were corrected for differences in primer efficiencies. Differences between groups were analyzed with ANOVA, followed by Fisher's test (significant when $P < 0.05$).

Experiment 6: Effect of VMH OT on Fos IR in feeding-related brain sites

Rats received a VMH injection of saline or 1 μ g OT (n=5/group; 4 were excluded due to either an incorrect cannula placement (2) or poor staining quality (2)). Food and water were then removed. One hour later, animals were perfused with 50 ml saline followed by 500 ml 4% paraformaldehyde (PFA). Brains were excised and postfixed in PFA. Coronal 60- μ m vibratome (Leica, Germany) sections were processed for Fos. The tissue was treated for 10 min in 3% H₂O₂/10% methanol in TBS and incubated overnight in the goat c-Fos antibody (4°C; 1:2000; Santa Cruz Biotechnology, USA), then in the rabbit-anti-goat antibody (1 h; Vector, USA) and finally in avidin/biotin (1 h; Vector). Peroxidase was visualized with 0.05% DAB, 0.01% H₂O₂ and 0.2%

nickel sulfate (Sigma, USA). A mixture of 0.25% gelatin and 0.5% Triton X-100 (Sigma) in TBS was used for incubations. Sections were mounted on slides and the number of Fos positive nuclei was counted bilaterally (3-4 sections containing a given site per animal), using SCION IMAGE (Scion Corp., USA). Densities of Fos-IR nuclei (per mm²) were averaged per rat and group. Differences between the two groups were determined with a t-test.

Experiment 7: Effect of MPOA OT on chow intake after overnight food deprivation. Overnight food-deprived rats were injected in the MPOA with 0 (saline) or 1 µg OT (n=8/group). Pre-weighed chow pellets were placed in food hoppers immediately after the drug treatment. Food intake was measured 2 h post-injection and corrected for spillage.

Experiment 8: Effect of MPOA OT on episodic intake of palatable solutions. We followed a similar protocol as in (Herisson, Brooks et al. 2014). Animals were accustomed to having access to a bottle of a 10% sucrose/0.1% saccharin/4.1% Intralipid solution for 2 h/day (10:00–12:00; separate experimental sessions for each tastant) for 3 days (chow and water were removed for the 2 h). On day 4, just before solution presentation, rats were injected in the MPOA with 0 (saline) or 1 µg OT (n=6-7/group) and the amount of consumed solution was established 2 h post-injection.

3.3. Results

Direct VMH OT administration decreased deprivation-induced chow consumption 1 h (1µg, P=0.0027) and 2 h (0.3µg, P=0.0324; 1µg, P=0.003) post-injection (Fig. 2A).

In schedule-fed animals, 1 μ g VMH OT reduced consumption at the 2-h time point (P=0.0014; Fig. 2B).

While the effect of 1 μ g VMH OT on hunger-driven feeding (P=0.0027) was reversed by a pre-treatment with 1 μ g OTr antagonist, L-368,899 (Fig. 2C), the antagonist itself even at 3 μ g did not increase chow intake after overnight deprivation (Fig. 2D). Daily monitoring of animals did not reveal any effect of OT or the antagonist on 24-h chow intake (data not shown), similarly to the data presented in the previous OT VMH report as well as other site-specific and generalized OT ligand injection studies (Herisson, Brooks et al. 2014, Noble, Billington et al. 2014, Herisson, Waas et al. 2016).

Consumption of calorie-dilute/non-caloric 10% sucrose and 0.1% saccharin remained unchanged even after 1 μ g VMH OT (Fig. 3A left panel) and 1 μ g L-368,899 (Fig. 3A right panel) in energy non-deprived rats. In animals subjected to overnight deprivation offered standard chow and 10% sucrose water during re-feeding, 1 μ g VMH OT reduced ingestion of chow (P=0.0105) without affecting sugar solution intake (Fig. 3B). RtPCR revealed that VMH OTr expression was elevated in energy-deprived animals compared to ad libitum-fed controls (P=0.0404), but it was unaffected in rats ingesting palatable saccharin (Fig. 3C).

Finally, direct infusion of OT in the VMH produced an increase in Fos IR in the VMH itself (P=0.0221) as well as in the ARC and PVN (P=0.035 and 0.0197, respectively; Fig. 4).

There was no effect of MPOA OT injected in deprived animals prior to chow consumption, nor was MPOA OT effective in reducing intake of palatable solutions (Fig. 5).

3.4. Discussion

Evidence implicates the VMH in the control of energy intake and expenditure. Excessive food intake and obesity are typically observed consequences of VMH lesions in laboratory animals, as VMH neurons promote satiety (thereby, directly affecting short-term consummatory behavioral responses during a meal) and increase energy expenditure by enhancing sympathetic tone leading to negative energy balance (Sakaguchi, Arase et al. 1988, Tokunaga, Fukushima et al. 1989). Anorexigenic peptides, such as cholecystokinin and leptin, elevate VMH activation (Niimi, Sato et al. 1999, Zeeni, Nadkarni et al. 2010). Glucose administration increases brain-derived neurotrophic factor (BDNF) expression in the VMH and genetic deletion of the BDNF promoter in this site leads to obesity (Unger, Calderon et al. 2007). Finally, starvation causes an increase in VMH opioid receptor expression (Barnes, Primeaux et al. 2008). In this context, the initial findings of Noble et al. showing that VMH OT infusion decreases chow intake and increases energy expenditure and spontaneous physical activity, fit in well with the presumed role of the VMH in energy balance (Noble, Billington et al. 2014). The current studies further substantiate the notion that the VMH OTr plays a crucial role in the regulation of feeding for energy, and – notably - they strongly suggest that consumption of sweet palatable solutions is not regulated by VMH OT.

On the other hand, despite a substantial array of tastants (calorie-dense and – dilute, as well as non-caloric), I did not observe any change in short-term consummatory behavior in MPOA-injected rats. The lack of a relationship between MPOA OT and appetite is further underscored by the fact that the dose of OT injected in this area is sufficient to reduce all of the tested aspects of feeding upon OT administration in the lateral ventricle and the third ventricle. As both lateral and third ventricular OT is likely able to access multiple hypothalamic areas, including the MPOA, that the activation of the MPOA OT receptor alone does not result in changes in appetite, it strongly suggests that this part of the OT hypothalamic circuit does not mediate hypophagia. It is in contrast to the MPOA pool of other orexigenic and anorexigenic receptors, including galanin-like peptide, orexin A, or estrogen (Dagnault and Richard 1997, Patterson, Murphy et al. 2006, Sarihi, Emam et al. 2015).

In our studies, VMH OT, aside from decreasing food intake induced by overnight deprivation, was effective in reducing the consumption of chow in a scheduled feeding paradigm, in which access to food was restricted to 2 h/day. In that scenario, all the calories have to be ingested in the short time frame leading to consumption of food in quantities exceeding those in occasional deprivation paradigms, as animals are highly motivated by energy needs and entrainment serves as an additional factor driving ingestive behavior. It is therefore not surprising that, oftentimes, consumption induced by such schedule is not as easily modified by pharmacological treatment as in other feeding regimens (Murphy and Mercer 2014). Remarkably, VMH OT did reduce the amount of food eaten, with the hypophagic effect becoming apparent in the second hour post-injection (of note is

the fact that in deprived rats, OT more reliably reduced consumption at 2 h of re-feeding as well). It underscores the importance of the VMH as a relay site in OT-driven meal termination, especially in the context of OT promoting early satiety. It should be noted that, while we have not measured non-feeding parameters, other authors have reported that VMH administration of OT causes a short-lasting increase in wheel running activity (30 min) and spontaneous physical activity (1h), which indicates that motor impairments do not underlie VMH OT-driven hypophagia (Noble, Billington et al. 2014, Narita, Murata et al. 2016). Also, since energy expenditure increases briefly in response to VMH OT administration (Noble, Billington et al. 2014), therefore, lower energy intake after OT injection cannot be interpreted as a compensatory mechanism in energy balance control. The lack of effect of VMH OT on the consumption of liquids that contain energy (albeit, dilute), further strengthens this argument.

That the VMH OTr antagonist abolishes the effect of OT on deprivation-induced feeding indicates that the anorexigenic action of VMH OT is indeed mediated via the OTr. The lack of an increase in chow consumption in the paradigm in which L-368,899 was administered alone is not surprising: previous studies have shown that while the antagonist is very effective in elevating intake of palatable solutions, it does not increase energy-dense chow consumption or – in the case of simultaneous presentation of two energy-dilute solutions - overall calorie intake (Herisson, Waas et al. 2016, Olszewski, Klockars et al. 2016). However, one should remain cautious about the possibility that bilateral antagonism of the VMH OT receptor might produce hyperphagia. While activating an area unilaterally is certainly enough to see the behavioral effects, inhibiting activation may under some circumstances

require the contralateral area to be blocked as well, otherwise it will vicariate the function.

Importantly, some authors have pointed to a possible link between the VMH and reward-related processing in feeding and non-feeding scenarios. For example, long-term exposure to cocaine modifies endocannabinoid 1r binding in the VMH and direct administration of naloxone in this area blocks conditioned place preference induced by paced mating behavior (Garcia-Horsman, Agmo et al. 2008). Figlewicz et al. found that VMH Fos levels are higher in rats performing a progressive ration operant task for sucrose pellets than in controls (Figlewicz, Bennett-Jay et al. 2011). Furthermore, mice chronically maintained on a palatable, high-fat diet show a changed VMH dopamine receptor expression pattern (Huang, Yu et al. 2005). On the other hand, mounting evidence suggests that OT is involved in the regulation of feeding for reward, especially associated with sweet taste. Rats injected an opioid agonist, butorphanol, display a lower number of Fos-IR PVN OT neurons at the end of a high-sugar meal than saline controls (Kim, Shi et al. 2001). OT abolishes orexigenic action of opioid treatment (Olszewski, Klockars et al. 2016). OT knockout mice overconsume saccharin and sucrose solutions, but not fat emulsions (Miedlar, Rinaman et al. 2007). Herisson et al. reported that saccharin and sucrose intakes are diminished by intra-AcbC OT administration, whereas Mullis and colleagues observed a reduction in sugar water consumption after VTA injections of the peptide (Mullis, Kay et al. 2013, Herisson, Waas et al. 2016). Peripheral administration of a blood-brain barrier penetrant OTr antagonist, L-368,899, increase intake of sweet carbohydrate solutions (Herisson, Brooks et al. 2014).

Despite the relationship between OT and reward-driven feeding (Olszewski, Klockars et al. 2016) and evidence that suggests a link between the VMH and reward processing, the data presented herein strongly indicate that OT acting via the VMH does not modify intake of palatable sweet solutions. We did not see any effect of OT at doses effective at reducing deprivation- and schedule-induced energy-dense chow intake on the episodic consumption of non-caloric saccharin and calorie-dilute 10% sucrose solution in sated rats (and, as mentioned before, the antagonist did not elevate intake of the palatable tastants). Introducing the context of energy deprivation prior to giving animals simultaneous access to energy-dense standard chow and palatable (though low-energy) sugar water generated vigorous consumption of both tastants. However, also in this case, VMH OT diminished the intake of chow and the total number of calories, but it did not decrease appetite for sucrose.

The feeding data are in agreement with our Fos and rtPCR findings. Direct administration of OT in the VMH leads to an increase in neuronal activity of the VMH itself and it engages a wider network of sites controlling mainly hunger-satiety responses, including the hypothalamic PVN and ARC. Though the change in Fos-IR might be associated with indirect and direct VMH-derived downstream input, the activation of the PVN and ARC is aligned with previously proposed functional pathways related to feeding control. For example, Sternson et al. found that ARC proopiomelanocortin neurons receive strong excitatory input from the medial VMH and its strength is diminished by hunger (Sternson, Shepherd et al. 2005). Canteras and colleagues reported that all subdivisions of the VMH supply dense projections to the PVN and it has been suggested that BDNF-PVN corticotropin releasing

hormone pathway might be essential in satiety responsiveness (Canteras, Simerly et al. 1994). Finally, the rtPCR data showing upregulation of the OTr mRNA in the VMH of fasted rats indicate that with the diminished OT tone due to the lack of consummatory activity, the VMH OTr expression increases; whereas the lack of change after exposure to a palatable saccharin solution suggests that the VMH OTr population is not affected by fluctuations in OT tone in response to feeding reward. Overall, our results define the VMH with the OTr as a circuit that is dynamically regulated by energy status in a manner consistent with the known role of the VMH and OT in satiety control.

3.5. Conclusions

Overall, our data add to the evidence that OTr in the VMH is involved in the termination of consumption driven by energy needs not by reward derived from consuming palatable yet calorie-dilute solutions. OT action at the VMH activates hypothalamic sites involved mainly in energy balance control, and it is not associated with an altered Fos IR in sites directly linked with pleasure-driven consumption. MPOA OT receptor is unlikely to be involved in the regulation of food intake.

3.6. References

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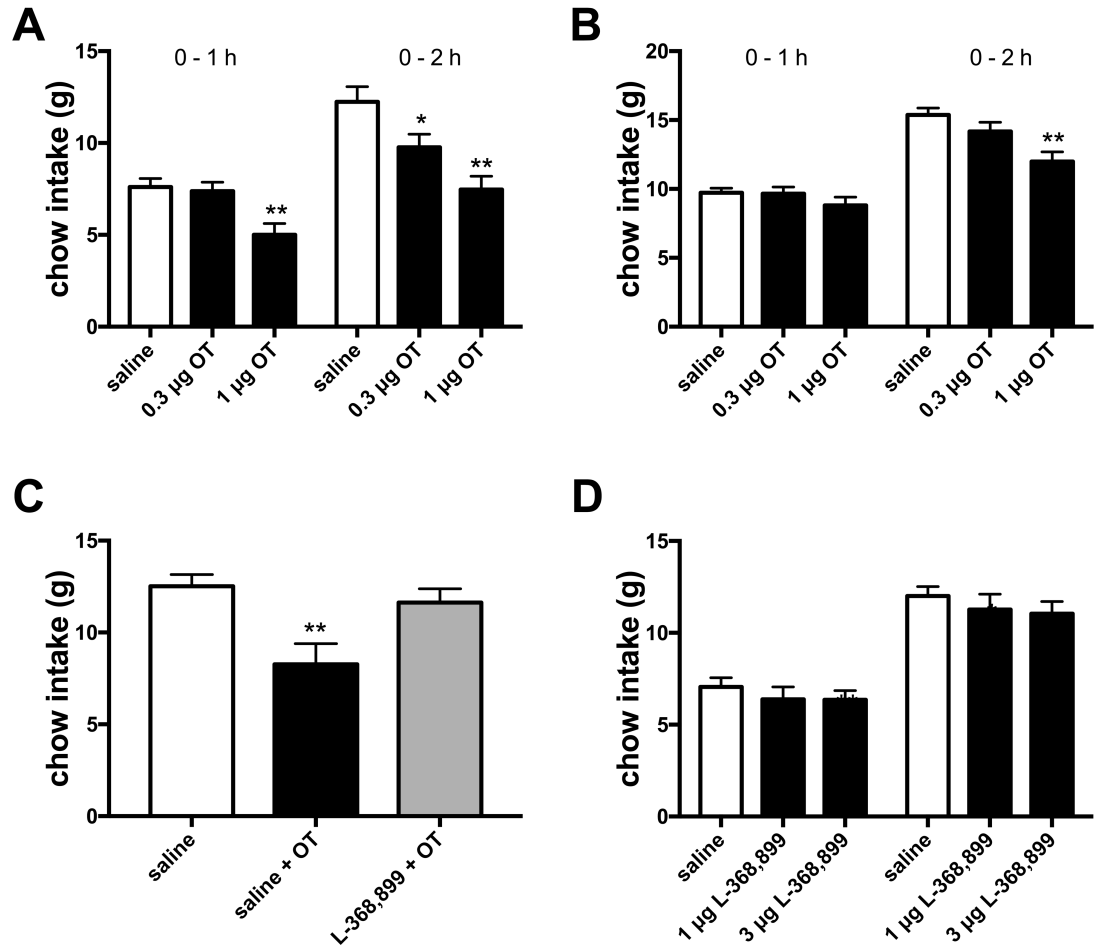


Figure 2. VMH OT and standard chow intake. Effect of (A) VMH OT on deprivation-induced chow intake (n=6-7/group), (B) VMH OT on scheduled chow intake (n=7/group), (C) VMH OTr antagonist, L-368,899, on VMH OT-driven reduction in deprivation-induced chow intake at 2 h of consumption (n=6/group), and (D) VMH L-368,899 on chow consumption after deprivation (n=6-7/group). */** - different from saline-injected controls, P<0.05 and P<0.01, respectively.

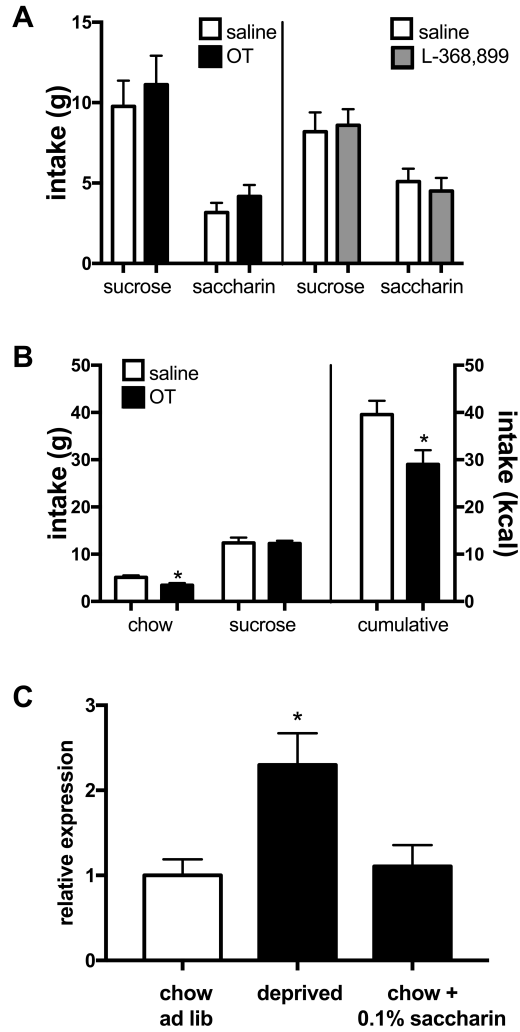


Figure 3. Sweet tastant intake and VMH OTr. Effect of direct VMH injection of (A) OT or OTr antagonist, L-368,899, on episodic 2-h intake of 10% sucrose (n=11/group) and 0.1% saccharin solution (n=6/group) in non-deprived rats (chow taken away during the 2-h sweet solution presentation), (B) OT on 2-hour intake of simultaneously presented standard chow and palatable 10% sucrose solution after overnight deprivation (n=8/group). (C) Effect of overnight energy deprivation and saccharin exposure (instead of water) vs ad libitum chow-fed controls on relative expression of VMH OTr mRNA (n=8/group). * - significantly different, P<0.05.

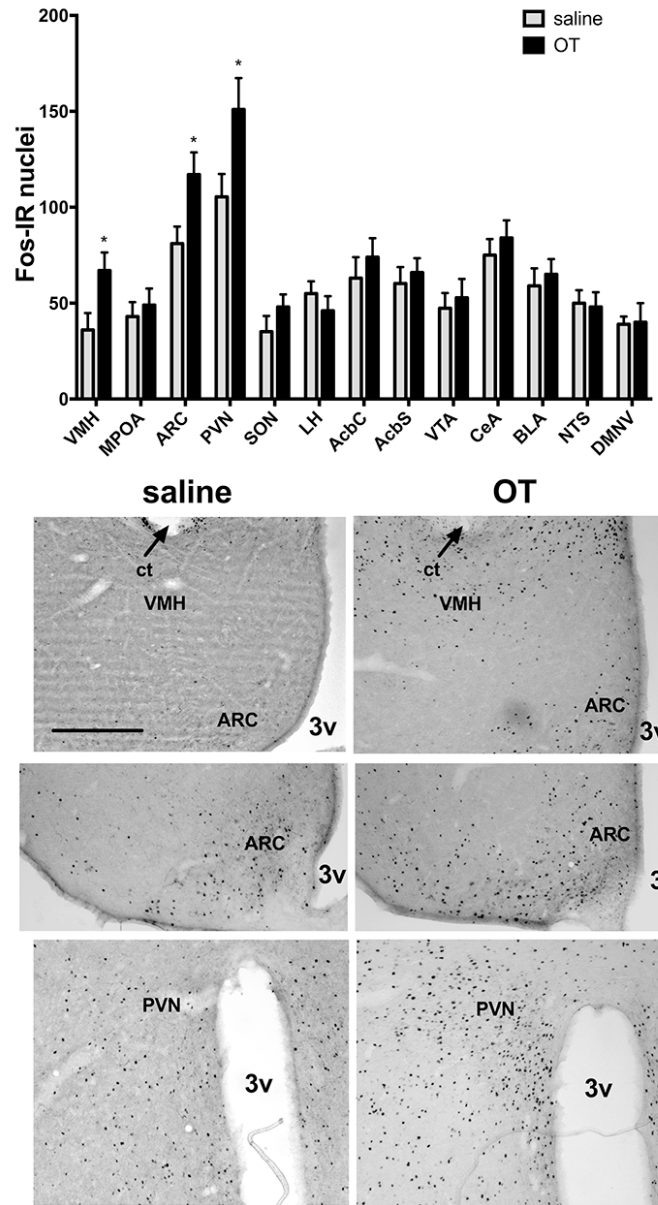


Figure 4. Effect of VMH OT (1 ug) vs saline on Fos immunoreactivity in feeding-related brain sites. Photomicrographs depict the ventromedial (VMH: top – cannula tract shown: ct), arcuate (ARC, middle) and paraventricular (PVN) nuclei. * - significantly different, $P < 0.05$. MPOA, medial preoptic area; SON, supraoptic n.; LH, lateral hypothalamus; AcbC/S, n. accumbens core/shell; VTA, ventral tegmental area; CeA, central n. of the amygdala; BLA, basolateral amygdala; NTS, n. of the solitary tract; DMNV, dorsal motor n. of the vagus. Scale bar: 0.6mm; $n=5$ /group.

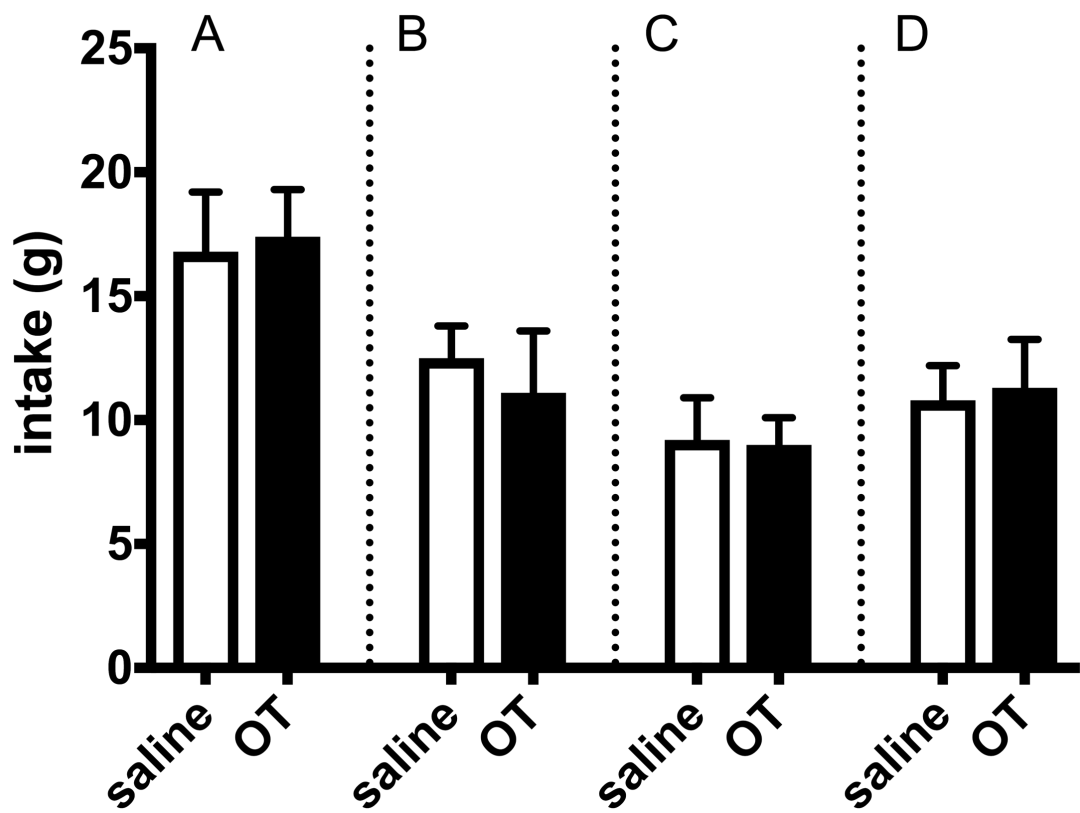


Figure 5. MPOA OT and food consumption. Effect of direct MPOA injection of 1 ug OT on (A) deprivation-induced chow intake 2 h post-administration (n=8/group); (B) episodic 2-h intake of 10% sucrose (n=6/group), (C) 0.1% saccharin solution (n=7/group) and (D) 4.1% Intralipid (n=7/group) in non-deprived rats (chow taken away during the 2-h solution presentation).

4. Chapter Four - Basolateral and central-amygdalar oxytocin moderately suppresses food intake

Abstract

Central oxytocin (OT) decreases meal size and reduces intake of palatable sweet solutions. It remains largely unclear as to which brain sites mediate OT's effect on palatability versus energy or the combination of those aspects of consumption. In the project described in this chapter of the thesis, I expanded the search for sites that mediate anorexigenic properties of OT by focusing on two subdivisions of the amygdala, its central (CNA) and basolateral (BLA) nuclei. I injected OT directly in the BLA or CNA in rats and assessed intake of standard chow induced by energy deprivation and intake of sweet solutions in non-deprived animals. I examined whether these effects are reversible by OT receptor (OTr) antagonism and whether BLA or CNA OT induces taste aversion. I also determined the effect of energy deprivation and exposure to sweet saccharin on BLA and CNA expression of OTr mRNA. BLA OT at 0.3 μg and CNA OT at 1 μg reduced standard chow intake after deprivation by ~25%. Only BLA OT was effective at suppressing consumption of sucrose and saccharin solutions. The anorexigenic effects of BLA and CNA OT were attenuated by OTr antagonist, L-368,899, pretreatment. OT at anorexigenic doses did not promote acquisition of taste aversion. BLA OTr mRNA expression was affected by exposure to palatable saccharin, whereas that of CNA OTr, by energy deprivation. OT in the amygdala moderately decreases food intake. The functional relationship between amygdalar OT and energy intake versus palatability-driven

intake depends on the discrete localization of the OTr within this complex structure.

Keywords: oxytocin; satiety; reward; sugar; appetite

4.1. Introduction

The early discovery by Arletti et al. showing a decrease in food consumption after intracerebroventricular (ICV) administration of oxytocin (OT) in rats (Arletti, Benelli et al. 1990), sparked interest in defining mechanisms that underlie OT-driven hypophagia. The presence of reciprocal hypothalamic OT – brainstem pathways, particularly those incorporating dorsal vagal complex, served as an anatomical basis linking OT with the regulation of meal size as a means to protect internal milieu (Blevins, Eakin et al. 2003). OT release is associated with stomach distension, elevated plasma osmolality and toxicity-derived gastrointestinal (GI) discomfort (Renaud, Tang et al. 1987 , Olson, Drutarosky et al. 1991, Olszewski, Waas et al. 2013). OT neuronal activity coincides with an end of a meal and occurs upon administration of GI and central mediators of satiety, including cholecystokinin (CCK) and glucagon-like peptide (Renaud, Tang et al. 1987, Bojanowska and Stempniak 2001). Direct hindbrain and hypothalamic infusions of OT reduce energy intake (Arletti, Benelli et al. 1990, Klockars, Waas et al. 2017).

More recent studies indicate that central OT regulates another key aspect of appetite, eating for palatability. The OT receptor (OTr) is expressed in the reward circuit and changes in OTr levels have been reported in rats drinking sweet solutions (Herisson, Waas et al. 2016). A blood-brain barrier-penetrant OTr antagonist, L-368,899, while ineffective in altering consumption of standard chow,

stimulates sugar water intake in non-choice and choice scenarios (Olszewski, Klockars et al. 2010). In wild-type mice, it promotes consumption of sweet calorie-dilute solutions. OT knockout (KO) mice show greater preference for sucrose and saccharin (Billings, Spero et al. 2006, Miedlar, Rinaman et al. 2007, Herisson, Brooks et al. 2014).

It remains largely unclear as to which brain sites mediate OT's effect on palatability versus energy (or the combination of those). Thus far, microinjection studies have shown that OT administered in two key components of the reward system, the ventral tegmental area (VTA) and nucleus accumbens (Acb), is highly effective in decreasing episodic consumption of calorie-dilute and non-caloric palatable solutions sweetened with sugar and saccharin, even in the absence of hunger (Mullis, Kay et al. 2013, Herisson, Waas et al. 2016). Conversely, hypothalamic OT reduces only intake of energy-dense tastants regardless of their palatability (Klockars, Waas et al. 2017).

In the current investigation, I expand the search for sites that mediate anorexigenic properties of OT by focusing on two subdivisions of the amygdala, its central (CNA) and basolateral (BLA) nuclei. The amygdala expresses the OTr and its activity in response to food cues is exacerbated by energy needs (Sun, Kroemer et al. 2015). I infused OT directly in these areas in rats and examined the effect on intake of standard chow induced by energy deprivation and on the intake of palatable sweet solutions (or water) in energy non-deprived animals. I assessed whether the anorexigenic properties of BLA and CNA OT stem from aversion and whether they can be reversed by antagonism of the OTr. Finally, I determined the effect of energy

deprivation as well as of exposure to palatable sweet saccharin on the expression of the OTr in the BLA and CNA.

4.2. Materials and Methods

Animals. Adult male Sprague–Dawley rats (AgResearch, Hamilton, NZ) weighing 320 ± 11 g at the beginning of the studies were single-housed in Plexiglas cages (21°C ; LD 12:12, lights on at 07:00). Rats had unrestricted access to water and standard chow (Teklad) unless stated otherwise. The UoW ethics committee had approved the procedures, and they are compliant with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publ., no. 80–23, rev. 1996).

Surgeries. Animals were anesthetized with the ketamine–xylazine mixture (100 mg/kg + 20 mg/kg) and stereotaxically equipped with a cannula (Plastics One) aimed at the BLA or CNA. The cannula was secured with dental acrylic to two screws inserted in the skull. The following placement coordinates (Paxinos 1982) were used: (a) BLA: 5.1 mm lateral to the midline, 2.4 mm posterior to bregma and 7.5 mm below the surface of the skull; (b) CNA: 4.3 mm lateral to the midline, 2.64 mm posterior to bregma and 7.0 mm below the skull surface. The injector extended 1 mm beyond the tip of the guide cannula. Rats were allowed to recover for 9 days. Placement was verified histologically after completion of the studies.

Drugs and injections. OT and L-368,899 were purchased from Tocris (UK). Intracranial injections were performed using Hamilton syringes in a volume of 0.2 μl . OT was delivered within the range previously reported to affect appetite with site-specific administration (0.03 μg = 29.8 pmol; 0.1 μg = 99.3 pmol; 0.3 μg = 297.9 pmol and 1 μg = 993.1 pmol) (Mullis, Kay et al. 2013, Herisson, Waas et al. 2016,

Klockars, Waas et al. 2017). Drugs were dissolved in isotonic saline. In Experiment 1, a repeated measures paradigm was used, in which each animal received each dose of the drug in a counterbalanced manner. Hence, this study was divided into four sessions spaced 3 days apart. The remaining studies included a single experimental session.

Experiment 1: Effect of BLA and CNA OT on chow intake after overnight food deprivation. Overnight food-deprived rats were injected in the BLA (n=11 – repeated measures) or CNA with 0 (saline), 0.1, 0.3 or 1 µg OT (n=10 – repeated measures). Pre-weighed chow pellets were placed in food hoppers immediately after the drug treatment. Food intake was measured 2, 4 and 24 h post-injection and corrected for spillage.

Experiment 2: Effect of BLA and CNA OT on episodic intake of palatable sweet solutions. We followed a similar protocol as in (Herisson, Brooks et al. 2014). Animals 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 (a) in the BLA with 0 (saline), 0.03, 0.1 or 0.3 µg OT (n=8-10/group) or (b) in the CNA with 0 or 1 µg 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, in the case of BLA injections, 0, 0.1, 0.3, or 1 µg OT was used (n=8-9/group), whereas CNA animals were treated with 0 or 1 µg OT (n=8/group).

Experiment 3: Effect of BLA and CNA OT on deprivation-induced water intake.

Rats were water-deprived overnight and injected in the BLA or CNA with either 0 (saline) or 1 μg OT (BLA, n=8-9/group; CNA, n=7/group) prior to regaining access to water. At 10:30, water bottles were returned to the cages and intake was measured 2 h post-injection. Chow was removed from hoppers during the time of the drinking test.

Experiment 4: Effect of OT antagonist pretreatment on hypophagic properties of BLA and CNA OT.

We used those paradigms in which OT was found to produce hypophagia (for BLA: deprivation-induced chow intake and intake of sweet solutions; for CNA: deprivation-induced chow intake). Just prior to gaining access to either chow (deprivation paradigm), a sucrose or saccharin solution (sweet solutions paradigm), the animals received two intra-amygdalar (BLA or CNA) injections spaced 10 min apart: (a) saline followed by saline; (b) saline followed 1 μg OT (effective OT dose); (c) 1 μl L-368,899 followed by 1 μg OT; (d) 1 μl L-368,899 followed by saline (n=9/group in BLA and CNA injections). Intake of chow was measured at 4 h after the second injection and of palatable solutions at 2 h.

Experiment 5: Effect of BLA and CNA OT on the acquisition of a conditioned taste aversion (CTA).

Animals were accustomed to receiving water for 2 h (10:00–12:00) per day for 3 days. Chow was removed for the 2 h of water presentation. On day 4, rats were given a novel cherry KoolAid solution (Kraft Foods; prepared as per manufacturer's recommendation) instead of water for 2 h. Afterwards they received a BLA or CNA injection of saline or 1 μg OT (an effective anorexigenic dose based on chow experiments; n=5/group). Rats treated with IP 6 mEq LiCl (versus IP

saline) served as a positive control for a CTA (n=5/group). On day 5, a two-bottle preference test between KoolAid and water was used to assess acquisition of a CTA to KoolAid. Bottles were weighed and percentages of KoolAid intake (out of cumulative, i.e. KoolAid plus water, intake) were calculated.

Ingestive behavior data analysis. Experiment 1 data were analyzed with repeated measures ANOVA followed by Dunnet's post-hoc test with correction for multiple comparisons. Data from the remaining studies utilizing single injections were processed with a Student's t-test (two-group comparisons) or one-way ANOVA followed by Dunnet's test (multiple-group comparisons). Values are shown as means \pm SEM and they were deemed significantly different when $p \leq 0.05$.

Experiment 6: Relative expression of OTr mRNA in the BLA and CNA in animals subjected to food deprivation or exposed to palatable saccharin. In order to assess the effect of sweet palatable saccharin solution exposure on BLA and CNA OTr mRNA levels, rats were given chow and 0.1% saccharin (instead of water) for 48h, whereas assessing the effect of food deprivation was done in animals that did not have access to standard chow (water available at all times) for 24 h prior to decapitation. Rats maintained on chow and water ad libitum served as controls. The rats (n=6-8/group) were decapitated (9:00-10:00), coronal brain slices were made using the matrix, the BLA and CNA were excised and placed in RNAlater (Ambion) at 21°C for 2 h and then at 4°C overnight. For brevity reasons, only the main steps of the standard protocol are described below (for details, see (Olszewski, Klockars et al. 2010)). Samples were homogenized in TRIzol (Ambion). Extractions were done with chloroform, and isopropanol was used to precipitate RNA. Samples were

centrifuged, pellets rinsed, dried, and dissolved in the DNase buffer (NEB). The samples were treated with RNase-free DNase I (Merck) at 37°C for 1 h. The absence of genomic DNA was determined by PCR of 5% template in the PCR mix [MgCl₂-free buffer, 50mM MgCl₂, Tween, 20mM dNTP, fwd. and rev. primers, Taq polymerase, and MQ H₂O; 10 µl volume]. 0.5µl 100ng/µl genomic DNA was a positive control, whereas 0.5µl MilliQ H₂O served as a negative one. The product was analyzed with electrophoresis. For cDNA synthesis, 5-µg RNA samples (concentration determined with Nanodrop) were diluted with MQ H₂O to 12 µl. RNA was reverse-transcribed in Master Mix (Promega). Samples were incubated for 1 h at 37°C, followed by PCR to establish cDNA synthesis. Reactions were performed in duplicates; negative controls were included. 25 ng of sample cDNA template was used per primer (OTr primers: ttcttctgctgctctgctcgt (fwd) and tcatgctgaagatggctgaga (rev)). Each rtPCR contained 2µl 10×MgCl₂-free buffer, 0.2 µl 20mM dNTP, 1.6 µl 50 mM MgCl₂, forward and reverse primers (0.05 µl; 100 pmol/µl), 1 µl DMSO, 0.5 µl Sybr Green (1:50000), 0.08 µl Taq polymerase, and 9.52 µl MQ H₂O. Amplification consisted of denaturation (95°C, 3min), and 40 cycles of denaturing (95°C, 20s), annealing (30s), and elongation (72°C, 30s). Normalization factors were established by analyzing expression of three HKGs: GAPDH (fwd. acatgccgcctggagaaacct; rev. gcccgatgcccttagtgg), cyclophilin (fwd. gagcgtttgggtccaggaat; rev. aatgcccgaagtcaaagaaa), and ribosomal protein 19 (fwd. tcgccaatgccaactctcgtc; rev. agccgggaatggacagtcac) (GeNorm). Primer efficiencies were calculated with LinRegPCR, and Ct values were corrected for differences in efficiencies. Grubb's test was used to calculate average PCR efficiencies for primer pairs. Differences in OTr

expression between groups were analyzed with ANOVA followed by Bonferroni's test (significant when $p \leq 0.05$).

4.3. Results

OT decreased deprivation-induced chow intake in BLA- and CNA-treated animals (Fig.1C,D). BLA OT was effective at 0.3 and 1 μg after 2 h ($F(3, 30)=5.575$; $p=0.0012$, and $p=0.022$, respectively) and 4 h post-injection ($F(3, 30)=4.73$; $p=0.011$, and $p=0.046$, respectively). CNA OT reduced chow consumption only at the 1 μg dose at 2 and 4 h ($F(3, 27)=5.41$, $p=0.0042$; and $F(3, 27)=3.787$, $p=0.015$, respectively), although there was a trend ($p=0.0559$) with 0.3 μg at 4 h. We did not observe any differences after 24 h (data not shown).

CNA OT was ineffective at diminishing sugar or saccharin solution consumption, however, BLA OT decreased episodic intake of the sweet fluids (Fig 1E,F). Intake of sucrose was affected by 0.1 and 0.3 μg OT ($F(3, 32)=4.985$; $p=0.0478$, and $p=0.0462$, respectively), whereas 1 μg was needed to reduce the amount of ingested saccharin ($F(3, 21)=5.271$, $p=0.0076$). Neither BLA nor CNA OT changed water intake in thirsty rats (Fig. 1G,H).

In the antagonist studies, 1 μg OT reduced intake of chow (BLA, $F(3, 32)=2.514$, $p=0.0323$; CNA, $F(3, 32)=3.13$, $p=0.0484$) at 4 h in re-fed rats as well as - in BLA injections - intake of sugar ($F(3, 32)=2.446$, $p=0.033$) and saccharin ($F(3, 32)=4.443$, $p=0.0219$). Rats pretreated with the antagonist, L-368,899 (1 μg) prior to OT administration ingested similar amounts of either chow or sweet solutions as relevant controls (Fig. 2 A, B). Unlike IP LiCl used as a positive control for a CTA ($p<0.0001$), neither BLA nor CNA OT induced a CTA to a KoolAid solution (Fig. 2 C).

Finally, saccharin consuming animals showed a marked decrease in BLA OTr mRNA levels ($F(2, 18)=5.594$; $p=0.007$), whereas food deprivation upregulated CNA OTr expression ($F(2, 19)=7.614$; $p=0.0443$).

4.4. Discussion

While obtaining energy is the main reason that underlies food intake, energy balance is not a sole factor that affects consumption. Additional controls shaping the magnitude of ingestive behavior, such as emotional processing or memory, have evolved in order to maximize consumption of physiologically beneficial foods and to adjust food intake to a dynamic state of the organism.

The amygdala is proposed as a key structure linking emotional responsiveness and hunger-driven eating behavior. Activity in the amygdala is elevated in people subjected to visual food cues, and it further increases by fasting (Sun, Kroemer et al. 2015). Anatomically separable into two groups, one that includes the BLA whose internal circuitry is more cortical, and another that is more striatal, involving the CNA, the amygdala is synaptically interconnected with the hypothalamus, striatum, and limbic system. (Swanson 2003). Furthermore, it expresses a plethora of receptors known to mediate hypo- and hyperphagia, including those for opioids and ghrelin (Beckman, Shi et al. 2009, Alvarez-Crespo, Skibicka et al. 2012). Despite the fact that amygdala neurons belong to a feeding-related circuit, our understanding of their precise involvement in food intake is limited. Here, we show for the first time that OT acting in the BLA and CNA, moderately suppresses appetite. The functional relationship between amygdalar OT and feeding depends on the discrete localization of the OTr within this complex structure.

Both the BLA and CNA injections of OT caused a transient ~25% decrease in deprivation-induced intake of energy-dense 'bland' chow. That neither BLA nor CNA OT induced a CTA indicates that the anorexigenic properties of intra-amygdalar OT do not stem from malaise. This short-lived decrease in hunger-driven consumption was similar in magnitude to what had been previously reported for OT administered in the reward-related Acb, and somewhat less pronounced than either after ICV (which leads to simultaneous targeting of the OTr in multiple sites) or after energy balance-linked ventromedial hypothalamic nucleus (VMH) injections (Arletti, Benelli et al. 1990, Herisson, Waas et al. 2016, Klockars, Waas et al. 2017). The doses used here have been typically found effective in intraparenchymal OT infusion experiments published to date, though our understanding of how these doses relate to physiological levels of the peptide is limited (Mullis, Kay et al. 2013, Herisson, Waas et al. 2016). Importantly, hypophagia caused by BLA and CNA OT was abolished by a pre-treatment with an antagonist delivered via the same routes. It strengthens the notion that the observed effects of BLA and CNA OT on deprivation-induced feeding are indeed mediated by the OTr and it implicates intra-amygdalar OT in the regulation of consummatory responses to energy needs. This outcome is in concert with the proposed function of OT in 'homeostatic' control of meal size. It is also in agreement with the growing body of evidence showing that targeting with selective ligands those subsets of amygdala neurons that express receptors for orexigenic and anorexigenic peptides (e.g., galanin, ghrelin and NPY) produces changes in the intake of food irrespective of palatability (e.g., see (Beckman, Shi et al. 2009, Alvarez-Crespo, Skibicka et al. 2012)). Notably, the amygdala (especially, CNA) has been also linked to glucose metabolism and

adiposity, which further underscores its sensitivity to energy states of the organism (Mendes, Castro et al. 2017).

Even if one considered the amygdala to be involved primarily in emotional aspects of feeding control, the fact that amygdalar OT affects the intake of standard chow in hungry animals is not surprising as reward and energy needs are intertwined. In line with that, changes in hunger-satiety responses to 'bland' diets in deprived animals have been shown upon manipulating neural circuits primarily recognized for reward processing (as there is a rewarding component of replenishing calories). It should be noted, though, that the results of our palatable sweet tastant studies suggest that a palatability-OT relationship exists only in the context of BLA injections. We found that intakes of a calorie-dilute sucrose solution and non-caloric saccharin are reduced by BLA OT (0.1 and 1 μg were effective), but not by even the highest dose of CNA OT. The intra-amygdalar OT does not affect water intake, and thus the effects of BLA OT on drinking sweet solutions are unrelated to thirst. These data write well into the existing literature indicating the role of BLA as a neural integrator of reward value (Wassum and Izquierdo 2015). BLA is involved in encoding emotional events with reference to their particular sensory-specific features (Balleine and Killcross 2006). Activation of BLA cells after an unconditioned stimulus is necessary for the expression of both an innate and a conditioned response (Gore, Schwartz et al. 2015). Intuitively, therefore, exposure to rewarding sugar has been found to attenuate BLA output and increase expression of genes driving plasticity (Packard, Di et al. 2017).

Importantly, parallel to the disparate effects of CNA vs BLA OT on ingestion of sweet solutions, the CNA and BLA have been proposed to play distinct (albeit, yet not fully understood) roles in reward processing. For example, BLA but not CNA lesions interfere with conditioning and reinforcer devaluation effects (Hatfield, Han et al. 1996). On the other hand, inactivation of the CNA but not BLA disrupts learning in response to over-expectation of reward (Haney, Calu et al. 2010). Furthermore, though the BLA and CNA receive gustatory input, this overlap is only partial (Swanson 2003). Hence, in the context of appetite regulation, it is quite plausible that while BLA OT affects the portion of feeding driven by reward, CNA OT plays a more profound role in the 'homeostatic' regulation of a meal size. This notion is supported by the outcome of the OTr gene expression study. In the CNA, deprivation-induced changes in the OTr mRNA levels (and, simultaneously, no effect of saccharin consumption) mirror those observed in the VMH (VMH OT does not affect sucrose or saccharin intake). Conversely, OTr expression changes in the BLA after saccharin exposure are similar to what has been reported for the Acb, a key reward site.

We conclude that OT acting in the BLA and CNA decreases deprivation-induced intake of chow and the anorexigenic properties of BLA OT extend onto sweet palatable solutions. Thus, the functional relationship between amygdalar OT and appetite depends on the discrete localization of the OTr within this complex structure.

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4.6. Figures

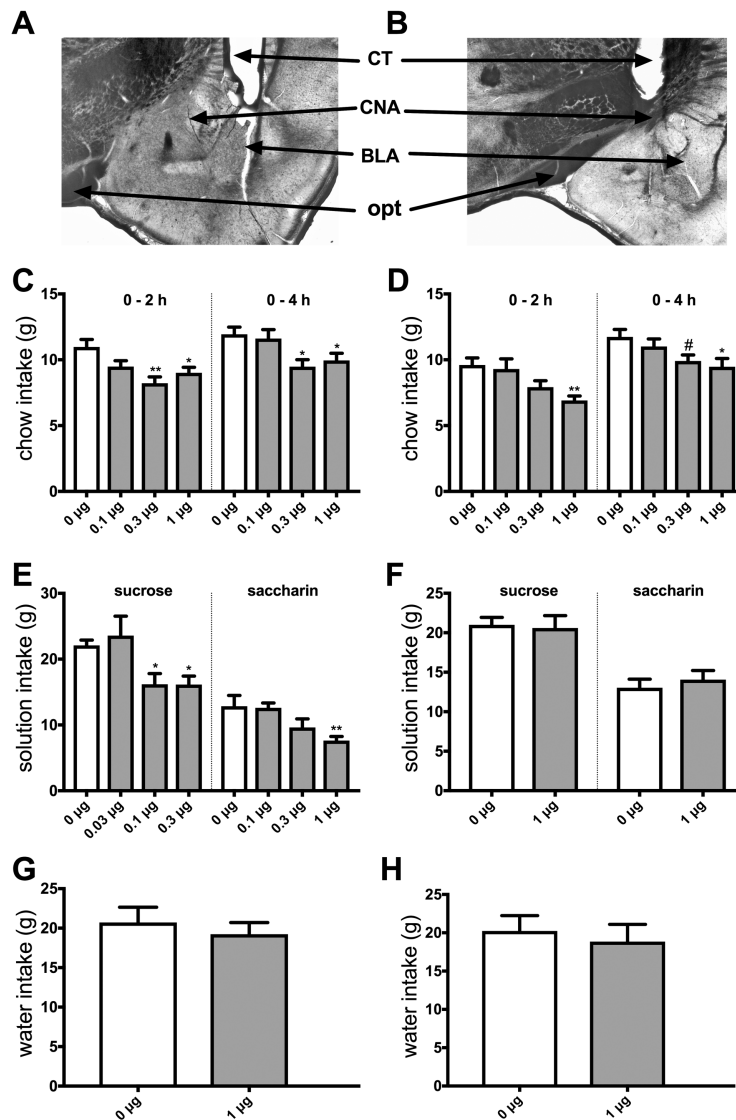


Figure 1. Effect of BLA (left panel; cannula placement shown in A) and CNA (right panel; cannula placement shown in B) administration of OT on standard chow intake induced by overnight deprivation (C, D), on consumption of palatable sweet 10% sucrose or 0.1% saccharin solutions in non-deprived animals (E, F), and on intake of water in thirsty rats (G, H). OT doses shown in μg (0 μg indicates saline control). */** - significantly different from saline-injected controls, $P < 0.05$ and $P < 0.01$, respectively. # - $p = 0.0559$. CT, cannula tract; opt, optic tract.

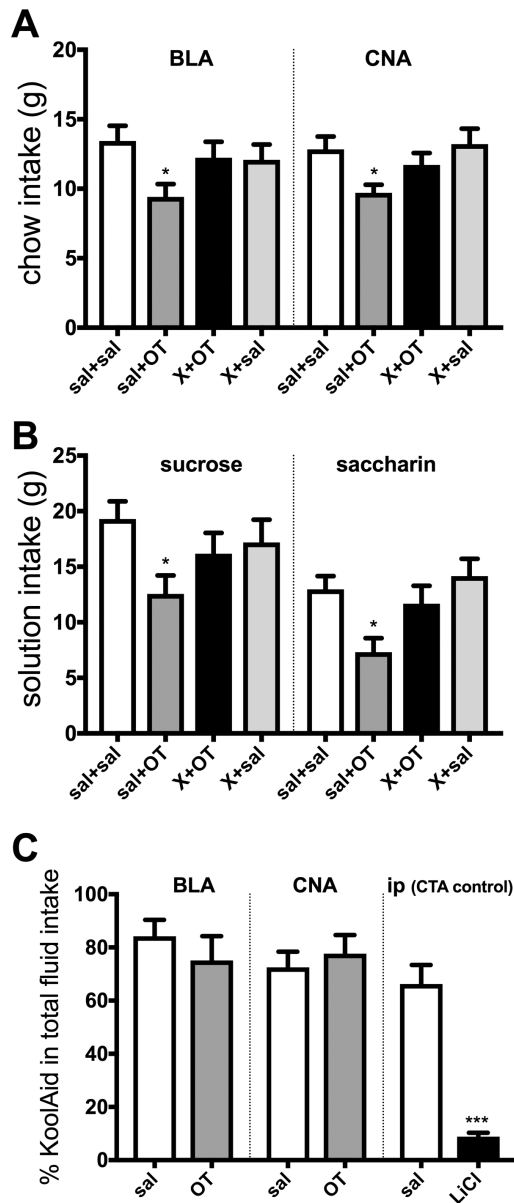


Figure 2. Effect of site-specific OTr blockade with 1 μ g OTr antagonist, L-369,899 (X), on (A) BLA and CNA OT-induced reduction in chow intake after deprivation, and on (B) BLA OT-induced reduction in palatable 10% sucrose or 0.1% saccharin solution intake. (C) Effect of BLA or CNA OT or IP 6 mEq LiCl (positive control) on the development of a conditioned taste aversion (CTA) to a novel KoolAid solution. OT injected at 1 μ g in all applicable studies. */*** - significantly different from saline-injected controls, $P < 0.05$ and $P < 0.001$, respectively.

A: BLA



B: CNA

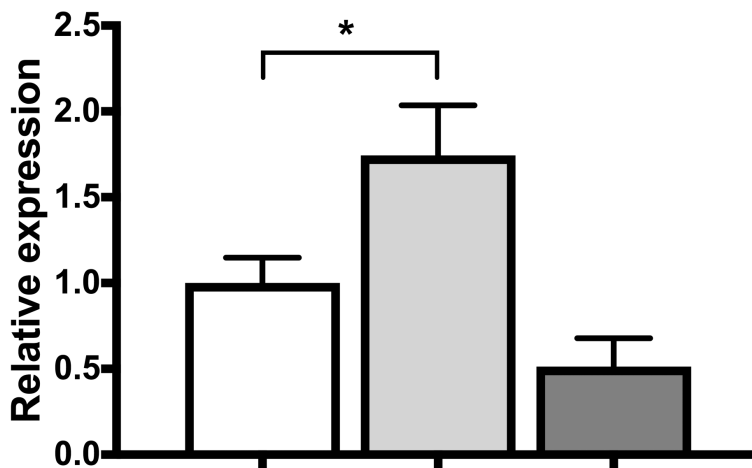


Figure 3. Relative expression of OTr mRNA in the BLA (A) and CNA (B) of rats subjected to food deprivation or given access to a 0.1% saccharin solution (with chow available ad libitum) versus controls maintained on unrestricted access to chow. */** - significantly different from controls, $P < 0.05$ and $P < 0.01$, respectively.

5. Chapter Five - Concluding remarks

The plethora of anorexigenic and anti-obesogenic effects associated with the action of oxytocin (OT) have generated significant interest in exploring potential therapeutic properties of this molecule in preclinical animal models as well as in human trials. Yet, in order to maximize the beneficial properties of OT in reducing excessive consumption and understand the occasionally conflicting data stemming from studies that employ distinct methodologies and research approaches, one has to take into account the diverse aspects of appetite affected by OT and the likely disparate central pathways that mediate these very specific effects.

The ample evidence gathered in the current set of experiments confirms that OT promotes termination of 'homeostatic' aspect of consumption, i.e., intake for energy (Arletti, Benelli et al. 1989, Olson, Drutarosky et al. 1991, Olszewski, Wirth et al. 2001, Romano, Cassano et al. 2013, Katsurada, Maejima et al. 2014, Balazova, Krskova et al. 2016, Klockars, Brunton et al. 2017), and it also acts as satiety factor specific to sweet taste derived from carbohydrates as well as from non-carbohydrate sweetener, saccharin (Miedlar, Rinaman et al. 2007).

I have shown here that LV, 4V, VMH, CNA and BLA administration of OT reduces standard chow-based meal size. This is very much in concert with the previous findings that lesioning of hypothalamic sites, including the PVN (the site of OT synthesis), and the interference in the functioning of bidirectional pathways between the hypothalamus and the hindbrain (where the OT receptor is highly expressed) lead to voracious feeding and obesity (Leibowitz, Hammer et al. 1981, Shor-Posner, Azar et al. 1985, Sims and Lorden 1986). Similarly, reversal of the obese and hyperphagic

phenotype caused by OT synthesis deficiencies, for example, due to genetic deletion of the OT encoding gene or due to developmental abnormalities in SIM-1 mutants, can be accomplished by supplementation of exogenous OT (Kublaoui, Gemelli et al. 2008).

It should be emphasized that OT protects internal milieu not only by controlling calorie in-out balance, but most importantly, by ensuring that physiological parameters arising from feeding, such as stomach distension, salt loading and chemical composition of plasma, do not exceed safe values (Brimble, Dyball et al. 1978, Renaud, Tang et al. 1987, Olszewski, Waas et al. 2013). OT's role is therefore far more critical for the survival of the organism than merely participating in the cessation of food intake once the energy needs have been met. In this context, it seems intuitive that the regulation of the 'homeostatic' aspects of energy intake would be the common outcome of activation of the OT receptor in all brain sites involved in appetite regulation. Indeed, the present data as well as studies published by other authors indicate that while OT decreases eating for palatability only through a subset of the brain regions that mediate OT's effect on standard food, all areas associated with non-caloric control of feeding (particularly, reward), also regulate intake of homeostatically-relevant food loads regardless of their rewarding component (Olszewski, Klockars et al. 2010, Klockars, Levine et al. 2015, Olszewski, Klockars et al. 2016). Furthermore, activation of the OT receptor is essential for the development of proper behavioral avoidance mechanisms toward foods that have been identified as potentially tainted. This involvement of the OT receptor is especially significant in the process of achieving a threshold magnitude of activity in the CNA neuronal populations relevant to taste aversions (Olszewski, Waas et al.

2013). Thus, the functional importance of the OT receptor appears to be shifted toward mechanisms that guarantee safety of ingestive behavior.

It was interesting to see that the hindbrain circuits are particularly sensitive to OT administration in terms of relaying the hypophagic action of this molecule. It is quite likely that hindbrain-acting OT targets those pathways that are reciprocal. Hence, it might simultaneously be enhancing activation of brainstem neurons that innervate the satiety systems in the hypothalamus as well as those brainstem cells that receive hypothalamic feedback aimed at supporting inhibition of food intake. In line with that, the generalized forebrain administration of OT (in the LV, as shown in Chapter 2, and – previously – in the third ventricle (Arletti, Benelli et al. 1989, Blevins, Thompson et al. 2016)) and direct microinjections of the peptide in hypothalamic and extra-hypothalamic forebrain areas (VMH, CNA, and BLA, as shown in Chapters 3 and 4) produce a slightly weaker hypophagic response than that observed after fourth ventricular injections (Chapter 2).

Despite OT being effective in reducing consumption for energy in the vast majority of target areas studied in this project, it is clear that the presence of the OT receptor in a region linked with food intake control does not guarantee that this site indeed mediates OT-driven hypophagia. It was quite surprising to observe the lack of changes in feeding after administration of OT in the MPOA. After all, stimulation of other orexigenic and anorexigenic receptors in the MPOA, including those for galanin-like peptide, orexin A, or estrogen, results in changes in food intake (Dagnault and Richard 1997, Patterson, Murphy et al. 2006, Sarihi, Emam et al. 2015)

Also, the MPOA is strongly involved in the regulation of energy balance and metabolism, most notably via processes related to thermoregulation and adjusting

energy intake/metabolism to challenges stemming from ambient environmental changes (Yu, Qualls-Creekmore et al. 2016).

There is also one more reason why the lack of a functional relationship between MPOA OT and feeding comes as a surprise. Namely, the fact that the MPOA is a part of circuit responsible for affiliative and caregiving behaviors (a schematic representation of the network that incorporates the MPOA is shown in Figure 1) (Cittern and Edalat 2017). Importantly, it parallels one the key functions of OT. As a pleiotropic hormone, OT is also known to arbitrate affiliative and altruistic actions that might support food sharing (Gimpl and Fahrenholz 2001, Lee, Macbeth et al. 2009). Smith et al. reported that intranasal OT administration amplifies huddling behavior between primate (*Callithrix penicillata*) partners while blockade of the OTr by an OTr antagonist, L-368,899 causes a substantial reduction in physical contact and prevented food sharing (Smith, Agmo et al. 2010). Other studies reveal that OT administration induces life-long pair bonding independent of mating in monogamous prairie voles (*Microtus ochrogaster*), reduces negligence and increases mother-infant bonding in prairie voles, humans, mice, rats, sheep and rhesus monkeys (*Macaca mullata*) (Olazábal and Young 2006)(Kendrick 2000, Liu and Wang 2003, Winslow, Noble et al. 2003, Galbally, Lewis et al. 2011). Although still very little is known as to whether OT has an ability to initiate early feeding termination in order to facilitate the act of sharing food with other individuals, we do know that the social status in a group and the social environment per se, do modify the magnitude of anorexigenic responses to OT in rats and mice (Olszewski, Allen et al. 2015, Herisson, Waas et al. 2016, Olszewski, Klockars et al. 2016). Therefore, a complete insensitivity of feeding behaviors to activation of the OT receptor in the MPOA is quite puzzling. While it is

possible that the choice of male rats for our experiments might have affected the outcome, one should note that the OT receptor is present in this brain area in both sexes. Also, a decrease in the magnitude of OT-induced hypophagia in socially vs non-socially fed animals in earlier studies was typically associated with group housing, hence, individuals tested alone (such as in the current experiments) should in fact be more sensitive to OT administration.

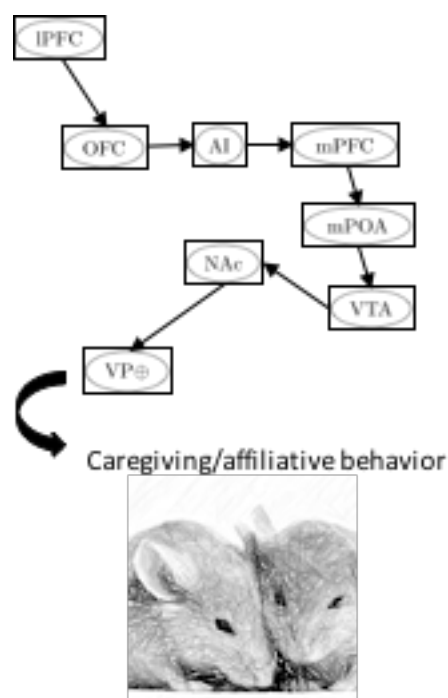


Figure 1. A schematic representation of the place of the MPOA in the immediate circuit responsible for triggering affiliative and caregiving behaviors. IPFC – inferior prefrontal cortex; mPFC – medial prefrontal cortex; Nac – nucleus accumbens; OFC – orbitofrontal cortex; VP – ventral pallidum; VTA – ventral tegmental area;

Unlike the MPOA, another OT receptor expressing hypothalamic area studied in this project, the VMH, mediated a decrease in consumption for energy upon direct OT administration and this effect was reversed by a pre-treatment with an OT receptor

antagonist, L-368,899 (Chapter 3). Aside from the data generated in our laboratory, there is currently one other report that substantiates our findings (albeit by employing different food intake assessment scenarios) and it expands them onto the involvement of the VMH OT receptor system in the regulation of energy expenditure in voluntary exercise models (Noble, Billington et al. 2014). Combined, these independently generated sets of data strongly suggest that the VMH should be viewed as a site that integrates both feeding- and metabolism-related facets of OT's action.

The fact that intra-amygdalar OT (both in the CNA and BLA) suppressed deprivation-induced intake of energy-dense standard chow (Chapter 4) is in agreement with the growing body of evidence showing that amygdala neurons not only express receptors for orexigenic and anorexigenic peptides (including, galanin, ghrelin and NPY), but also that their stimulation affects consumption of food even if this food is unpalatable (Beckman, Shi et al. 2009, Alvarez-Crespo, Skibicka et al. 2012). The amygdala, particularly the CNA, regulates glucose metabolism and adiposity, which is also in line with the known metabolic consequences of the generalized OT receptor stimulation (Mendes, Castro et al. 2017).

While the results of my experiments elucidating the effects of site-specific OT on energy-driven ('homeostatic') aspects of consumption produced relatively uniform hypophagic responses, the studies focused on feeding for reward showed that only a small subset of consumption-related regions mediates macronutrient- and flavor-dependent satiation. As mentioned above, MPOA OT did not affect any aspect of

consummatory behavior, but neither did OT acting at the VMH or CNA affect ingestion of palatable solutions.

It seems somewhat intuitive that the stimulation of the OT receptor in the VMH, the site closely tied to the regulation of the metabolic state of the organism, does not modify intake of fluids whose calorie density is very low (sucrose, Intralipid) or null (saccharin), thus whose contribution to energy balance is minimal. However, that CNA OT (Chapter 4) does not affect consumption of tastants that produce a strong response of the mesolimbic reward system (and the amygdala is closely associated with it) clearly indicates that macronutrient- and flavor-specific satiety promoted by OT is the outcome facilitated by activity of relatively narrow subpopulations of the OT receptor that do not even span the entire reward system. This obviously does not negate the involvement of OT in decreasing appetite for sweets and carbohydrates (or reward, in general), but it rather points to an urgent need to identify discrete components of the reward circuit that are sensitive to OT stimulation in the context of feeding control. In fact, evidence linking OT and reward is substantial. OT terminals are localized in the proximity of reward-related neuroregulators such as dopamine and opioids (Sofroniew 1980, Bunzow, Zhang et al. 1995, Peris, MacFadyen et al. 2017), Activation of the OT receptor in the NAcc core decreases methamphetamine-seeking (Baracz, Everett et al. 2016) and attenuates methamphetamine- and alcohol-induced conditioned place preference in rats (Baracz, Rourke et al. 2012, Bahi 2015). Food intake studies suggest that centrally acting OT decreases palatability- and macronutrient-dependent aspects of consumption, including opioid-induced overeating (Klockars, Levine et al. 2015). In particular, appetite for carbohydrates, as well as for saccharin, seems to be affected. Injections of OT into the VTA and NAcc

are effective in reducing intake of sweetened liquids (Melis, Melis et al. 2007, Herisson, Waas et al. 2016). Mice treated IP with a blood-brain barrier-penetrating OT receptor blocker, L-368,899, elevate intake of carbohydrate and saccharin fluids, but not lipid-containing solutions (Herisson, Brooks et al. 2014). This result parallels increased preference for carbohydrates and saccharin in OT knockout animals (Miedlar, Rinaman et al. 2007). The effect of OT on palatability-driven ingestive behavior may be modified by caloric density of food, and therefore, by the energy-related aspect of feeding. For example, OT treatments suppress ingestion of sugar-free yet palatable high-fat laboratory pellets and genetic deletion of OT promotes overweight in rodents given access to a calorie-dense, high-fat diet (Wu, Xu et al. 2012, Blevins, Thompson et al. 2016).

In this project, however, only the BLA was found to be directly involved in the mediation of OT's action on the intake for reward (Chapter 4). This is in line with the previously published reports defining the BLA as a site that integrates a complex rewarding value of stimuli (Wassum and Izquierdo 2015) and encodes emotional events characterized by sensory experiences (Balleine and Killcross 2006). BLA activation ensures the manifestation of conditioned responses (thus, responses that are based on either positive or negative sensory outcomes) (Gore, Schwartz et al. 2015). Sugar exposure is known to affect BLA output (Packard, Di et al. 2017). The results presented herein allow us to expand our understanding of the breadth of the reward network that relies on the OT receptor in modifying consummatory responses to palatable tastants. Aside from the previously defined VTA and NAcc core (Melis, Melis et al. 2007, Herisson, Waas et al. 2016), we now can include the BLA in this circuit. This is much narrower than the aforementioned OT receptor-

dependent CNS circuit that regulates eating for energy that includes the NTS, DMNV, VMH, NAcc, CNA, and BLA.

Below are the key **CONCLUSIONS** stemming from the current doctoral research project:

- Both lateral and fourth ventricular OT decreases consumption induced by one of the most potent orexigens known to date, butorphanol, but much lower OT doses are sufficient in the fourth ventricle.
- Ventromedial hypothalamic OT receptor is involved in suppressing food intake motivated by energy needs, but does not affect consumption driven by reward.
- The OT receptor in the medial preoptic area is unlikely to be involved in feeding control.
- OT acting in the basolateral amygdala and central amygdala reduces appetite for energy.
- Among the OT receptor populations expressed by the amygdala complex neurons, only the one present in the basolateral nucleus promotes suppression of palatability-driven ingestive behavior.

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