Characterisation of an acute anorexigenic action of l-tryptophan: a link with neural mechanisms regulating appetite

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Abstract

Recent evidence suggests that individual amino acids may promote hypophagia, however, our understanding of processes underlying their anorexigenic action is extremely limited. The few human and laboratory animal studies performed to date suggest that a free essential amino acid, l-tryptophan (TRP) reduces food intake. It is unclear, however, whether TRP acutely affects consumption driven by energy needs or by palatability, and whether central mechanisms underlie TRP-induced hypophagia. This thesis provides a detailed characterisation of ingestive behavioural effects induced by TRP administered intragastrically or (in order to bypass the gut) via an intraperitoneal (IP) injection in laboratory rodents prior to a meal as well as relevant neural processes triggered by TRP.

The first set of experiments explored whether an intragastric preload of TRP affects energy- and palatability-induced feeding in mice. A conditioned taste aversion (CTA) test was used to assess whether hypophagia is unrelated to sickness. Finally, c-Fos immunohistochemistry was employed to detect changes in activation of feeding-related brain sites induced by an anorexigenic dose of intragastric TRP. TRP generated a short-lasting reduction in the intake of energy-dense standard chow in deprived animals and energy-dense palatable chow in sated mice. Anorexigenic doses of TRP did not cause a CTA. TRP failed to affect intake of palatable yet calorie-dilute or noncaloric solutions (10% sucrose, 4.1% Intralipid or 0.1% saccharin) even for higher TRP doses that decreased water intake in thirsty mice. c-Fos analysis revealed an increase in activation of key feeding-related brain areas, especially in the brainstem and hypothalamus. Overall, intragastric TRP was

found to diminish energy-driven food consumption as well as (at high doses) thirst-induced water intake and affect activation of relevant central circuits.

In the second set of experiments, I investigated whether anorexigenic and brain activation effects persist when TRP bypasses the gut, i.e., TRP is injected directly in the IP cavity in rats. The effect of IP TRP was examined in both energy- and palatability-induced feeding. 30 and 100 mg/kg IP TRP suppressed chow intake in energy deprived rats. Only a higher 100 mg/kg IP TRP dose reduced consumption of palatable chow and palatable sucrose, saccharin and Intralipid solutions in sated animals. Thirst-driven water intake was reduced after 30 and 100 mg/kg IP TRP. Neither 30 nor 100 mg/kg IP TRP caused a CTA. c-Fos analysis showed the most pronounced effect of IP TRP on hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei and the nucleus of the solitary tract in the brainstem. It can be concluded that IP TRP suppresses energy deprivation-induced intake of chow and thirst-induced water intake within the same dose range, whereas higher doses are necessary to reduce consumption of palatable solutions in sated animals. IP TRP hypophagia is accompanied by activation of central circuitry that encompasses brainstem and hypothalamic sites, though changes are not as prominent as with intragastric TRP.

Intragastric and IP TRP administered at hypophagic doses increases c-Fos immunoreactivity in the hypothalamic PVN and SON. As these sites host neurons that synthesise a key satiety mediator, oxytocin (OT), the third and final set of studies explored a hypothesis that TRP's anorexigenic action relies on enhanced activity of the OT system. By employing double-immunohistochemistry, I

determined that the lowest effective dose of intragastric and IP TRP increased the activation of both PVN and SON OT neurons, although the effect was more pronounced after intragastric administration. Intragastric TRP upregulated OT mRNA in the hypothalamus. A blood-brain barrier-penetrant OT receptor antagonist, L-368,899, reversed hypophagia induced by TRP administered via both routes. Unlike the lowest effective dose of intragastric TRP which decreases only feeding, IP TRP at the lowest effective dose diminishes both food and water intake. Hence it was confirmed that L-368,899 pre-treatment does not affect TRP-driven reduction in drinking. The findings suggest therefore a functional relationship between TRP and OT in the regulation of energy-driven food intake.

Overall, the findings presented herein shed more light on the nature of anorexigenic properties of TRP. It appears that TRP acutely suppresses ingestive behaviour driven by energy, but it is a very weak inhibitor of palatability-motivated consumption. While both intragastric and IP TRP also decreased water intake, the fact that a low dose of the intragastric amino acid was sufficient to decrease feeding but not drinking, strongly suggests that a direct interaction of TRP with the gastric mucosa is a crucial contributor to reduced energy intake. TRP engages broad brain circuits relevant to feeding, especially the hypothalamus and brainstem. OT appears to be a candidate molecule potentially mediating TRP's effect on food intake.

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Abbreviations

3v	Third ventricle
4v	Fourth ventricle
5HIAA	5-hydroxylindoleacetic acid
5-HT	Serotonin
aca	Anterior commissure
Acb	Nucleus accumbens
AcbC	Nucleus accumbens core
AcbS	Nucleus accumbens shell
AD	Alzheimer’s disease
AGRP	Agouti-related protein
α-MSH	Alpha-melanocyte-stimulating hormone
AMY	Amygdala
AP	Area postrema
ARC	Arcuate nucleus
ATD	Acute tryptophan depletion
BBB	Blood-brain barrier

BCAA	Branched chain amino acids
BLA	Basolateral nucleus of the amygdala
BMI	Body mass index
BNST	Bed nucleus of the stria terminalis
CeA	Central nucleus of the amygdala
CCK	Cholecystokinin
CNS	Central nervous system
CTA	Conditioned taste aversion
CVS	Cardiovascular disease
DAB	3,3'-diaminobenzidine
DMH	Dorsomedial hypothalamic nucleus
DMNV	Dorsal motor nucleus of the vagus
DRL	Differential reinforcement
DVC	Dorsal vagal complex
EEG	Electroencephalogram
FFA	Free fatty acids
FST	Forced swimming test
GABA	Gamma-aminobutyric acid

GI	Gastrointestinal
GLP-1	Glucagon-like peptide-1
HCET	Home cage emergence test
ICV	Intracerebroventricular (injection into the cerebral ventricle)
IDO	Indoleamine 2,3-dioxygenase
IP	Intraperitoneal
LAT1	Large neutral amino acid transporter
LiCl	Lithium chloride
LH	Lateral hypothalamus
LNAA	Large neutral amino acids
MAO	Monoamine oxidase
MDD	Major depressive disorder
mRNA	messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NAD^+	Nicotinamide adenine dinucleotide
NMDA	N-methyl-D-aspartate
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract

OCD	Obsessive compulsive disorder
OECD	Organisation for Economic Cooperation and Development
OFT	Open field test
OT	Oxytocin
otr	Optic tract
PFA	Paraformaldehyde
POMC	Pro-opiomelanocortin
PPD	Postpartum depression
PVN	Paraventricular nucleus of the hypothalamus
PYY	Peptide YY
REM	Rapid eye movement
RNA	Ribonucleic acid
SCTD	Sub-chronic tryptophan depletion
SERT	Serotonin transporter
SON	Supraoptic nucleus
SSRI	Selective serotonin reuptake inhibitors
TAT1	Aromatic amino acid transporter
TBS	Tris-buffered saline

TCA	Tricyclic antidepressants
TDO	Tryptophan 2,3-dioxygenase
TPH	Tryptophan hydroxylase
TRP	Tryptophan
TSOI	Tryptophan side chain oxidase I
VMH	Ventromedial hypothalamic nucleus
VP	Vasopressin
VTA	Ventral tegmental area

1 Chapter One – Introduction and Aims

Despite developments over the past few decades in the prevention, management and treatment of obesity, New Zealand has the third highest prevalence of this health problem amongst the Organisation for Economic Cooperation and Development (OECD) countries. The New Zealand clinical obesity rate is currently 1 in 3 adults – with a further third being deemed overweight [1]. The condition is often associated with multiple co-morbidities, including cardiovascular (CVS) disease, diabetes and certain cancers [2-5]. Obesity develops when an individual's energy intake habitually exceeds energy expenditure, resulting in weight gain [6-9]. Excessive weight gain can often be attributed to the dysregulation of mechanisms that control hunger and promote satiety [10].

However, humans and laboratory animals eat not only to satisfy calorie needs, but also for reward [11]. To a large extent, feeding reward stems from palatability of tastants, and that is derived from – among others – specific macronutrient composition and pleasant taste. While it is unquestionable that high-fat/high-sugar diets have propelled the obesity 'epidemic', this phenomenon has also generated hope that certain dietary manipulations might have an opposite effect, paving a way toward developing 'functional foods' whose composition will curb excessive consumption. One of the early strategies has been to increase protein content in diets. As high protein content impairs palatability and acceptability of food item [12-18], this approach has had only a limited success due to patients' poor adherence to therapies. Later it was hypothesised that certain free amino acids might be able to produce a satiating effect [19-24], which could eliminate the need of

adding whole protein. Indeed, though evidence is still relatively scarce, the early findings imply that certain single amino acids (including the one selected for this project L-tryptophan (TRP) may act as feeding inhibitors [19, 23, 25-29]. Therefore, the current thesis focused on defining a potential hypophagic action of TRP and examining whether brain circuits that promote satiety are activated by this essential amino acid.

1.1 Protein intake and appetite

Initial studies in the 1970's by Booth and colleagues compared satiety responses in human subjects receiving either protein-rich (40%) or protein-poor (9.1%) isocaloric crispbread lunches. When an identical supplementary meal was provided three hours later, the total caloric intake in that meal was less for those provided with the higher protein option [30].

Since then, several decades of research, in both humans and laboratory animals, have found suppression of food intake and body weight loss to be associated with high-protein diets [12-18]. For humans, the recommended level of dietary protein intake is 10-15% of total energy, when an individual is in energy balance and at a stable weight [31]. A human study compared caloric intake and bodyweight change achieved through a 15% protein diet and a 30% protein diet. Participants received a fixed volume baseline diet (15% protein, 35% fat and 50% carbohydrate), then a high protein, isocaloric, fixed volume diet (30% protein, 20% fat and 50% carbohydrate) each for two weeks. For subsequent 12 weeks, participants were given ad libitum access to the high protein diet [14]. A sustained reduction in ad libitum caloric intake and significant weight loss were reported [12-14]. Among the

three macronutrients (proteins, carbohydrates and fats), protein has been identified as the strongest inhibitor of food intake [12, 15, 18, 32-36]. In a cohort of young adults, provision of either an isocaloric (240-kcal) high-protein (77% of energy from protein), high-carbohydrate (84%) or high-fat (58%) snack promoted satiety as observed in the increased latency to request for dinner by 60, 34 and 25 minutes respectively [33]. In rats, delivery of isoenergetic loads of protein (35% and 50% gluten load) via intraoral cannula induced a greater reduction in appetite, compared to animals given an isocaloric starch (carbohydrate) 0% gluten load [15].

However, these aforementioned high-protein diets tend to have diminished palatability, greatly reducing consumption of food for reward [37]. McArthur and colleagues trained rats (over two-weeks) to eat daily for a 3-hour period, first utilising a basal 20% casein-based diet, followed by a low protein diet (6% casein) for 5 weeks. A higher protein (75% casein) diet was then given for a day which prompted a significant decrease in food intake. This suppression was evident after one minute – too short a time period for the metabolic effects of the diet to be seen – thereby indicating the early depression of food intake being due to poor initial palatability [37]. Importantly, Bensaid and colleagues demonstrated that rats rapidly reduced intake of a high-protein diet (50% milk protein). Over time this consumption increased, though not to the consistently high level previously achieved with a low-protein diet (14% milk protein). This was not due to an acquired conditioned taste aversion (CTA) but the initial lower palatability of the diet [38]. As a CTA transpires when an animal associates the given novel flavour with a gastrointestinal (GI) discomfort, this effect could not have been attributed to malaise [39].

The past decades of research have sparked interest in identifying the physiological mechanisms that are involved in protein-induced hypophagia. There is possible epidemiological value of these protein-based strategies for overcoming obesity. The intake of protein triggers postoral and postabsorptive mechanisms at both the central and peripheral level [12-14]. Within the GI tract, endocrine processes begin with the release of cholecystokinin (CCK), glucagon-like peptide (GLP-1) and peptide YY (PYY) (Figure 1.1) [15, 40-45]. Along with the suppression of the hormone ghrelin, altogether it results in an overall reduction in appetite and food consumption (promote early satiation) [46, 47]. Satiation signals are within-meal signals that lead to meal termination [48, 49]. Some of these satiation signalling molecules can act directly in the brain by crossing the blood brain barrier (BBB) [47], whereas others such as CCK, affect the brain via the vagus nerve terminating in the brainstem (the nucleus of the solitary tract (NTS) and area postrema (AP)) [48, 50, 51]. These hormones then trigger activation of a broader network of sites involved in appetite regulation (discussed further in Section 1.5). The cessation of eating in its most fundamental and basic integrative form is an interaction between both the hypothalamic and hindbrain nuclei to control satiation and satiety [52].

Once in the GI tract, proteins are digested to amino acids, which act directly at the gut mucosa, eventually affecting motility and endocrine signals, via interaction with ubiquitous G-protein coupled receptors and transporters [22]. Ultimately, the amino acids can directly enter the bloodstream to act peripherally and – in some cases – also centrally [22, 44].

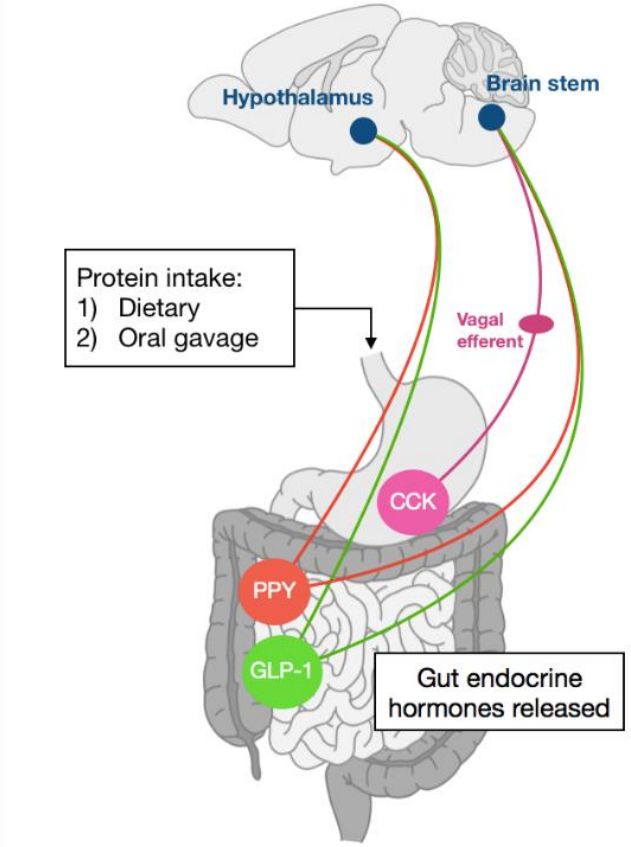

Figure 1.1 Examples of endocrine pathways involved in central network responses following dietary protein consumption

The appetite suppressant action of high-protein diets has been well documented in both humans and animal models. Proteins once consumed are digested to release individual amino acids, whose role in appetite regulation is becoming increasingly recognised.

1.1.1 Amino acids and food intake

Amino acids are now known to be involved in the central regulation of food intake, energy homeostasis and body weight [19-24]. Research has identified a number of individual amino acids (e.g. L-leucine, L-arginine, L-lysine, L-cysteine, TRP and L-glutamic acid), that can act as potent anorexigens [19, 23, 25-29]. One of the most extensively studied is leucine – which has consistently caused a reduction of food intake when administered centrally [19, 26, 53, 54]. Cota and colleagues (2006) demonstrated that central infusions of leucine into the third ventricle suppressed food intake via the regulation of mammalian target of rapamycin signalling (mTOR) in mice [19]. Moreover, leucine-induced anorexia could be reversed by the intracerebroventricular (ICV) administration of rapamycin [19, 26, 53, 54], a specific inhibitor of mTOR [55, 56]. Interestingly, leucine has a dual effect on food intake as – unlike centrally acting amino acid - peripheral administration increases food consumption [57-60], though the underlying mechanism is unclear.

Of late, an array of studies further identified individual amino acids with anorexigenic potential [19, 23, 25-29]. Following either intragastric or intraperitoneal (IP) administration of L-cysteine, in both rat and obese mouse models a dose-dependent reduction in food intake was reported [27]. L-cysteine showed increased neuronal activation in the AP, a brainstem region known to be associated with energy homeostasis [23, 27].

Alamshah et al. (2016) discovered that L-arginine when given to rodents via oral gavage, IP and ICV infusions, all reduced 1-hour food intake. In diet-induced obese mice, administration of L-arginine (16 mmol/kg) via oral gavage reduced the

cumulative 24-hour intake. The reported reduction in food intake was determined to not be mediated via either GLP-1/PYY or via the vagus, thereby indicating that L-arginine may be directly acting on the brain to influence feeding [25]. L-arginine's anorectic potential had previously been suggested by Jordi et al., (2013) [23]. That study analysed all 20 amino acids by giving rats an intragastric isomolar dose of 6.7mmol/kg of selected amino acids. L-arginine and L-lysine were identified as the most potent anorectic amino acids. The suggested common mechanism of action for these amino acids is the activation of brainstem circuitry (through the AP and/or vagus-facilitated processing) [23].

The essential amino acid TRP is thought to impact appetite, but the exact nature of such changes in feeding remains to be elucidated. Some initial studies have demonstrated moderate hypophagia following both TRP supplementation and administration [23, 28, 29]. The daily dietary supplementation of TRP at 3.0 g/kg body weight decreased food intake in ad libitum-fed rats by approximately 25%. The reduction was due to changes in the observed meal pattern, most notably an increase in intermeal intervals and decreased meal number [29]. The intragastric administration of 200-600 mg/kg TRP has been found to cause a similar reduction in intake in rats maintained on the 12-h/day availability schedule of both standard and high-carbohydrate (protein-free) food [28]. However, Jordi and colleagues reported that although rats treated with intragastric TRP (6.7 mmol/kg) consumed less upon the refeeding of standard laboratory chow after 12 h of fasting, the decrease did not reach the level of statistical significance which the authors attributed to multiple comparisons performed in the analysis [23]. The above studies provide support for the general hypothesis that individual amino acids may

produce an anorexigenic effect and indicate that TRP appears to be a candidate molecule to promote hypophagia.

1.2 Tryptophan

TRP is a branched-chain large neutral amino acid (LNAA). The molecule contains an α-amino group, an α-carboxylic acid group, and a side chain indole ring thereby classifying it as a non-polar, aromatic amino acid (Figure 1.2) [61]. TRP is an essential amino acid, hence it must be supplied via the consumption of dietary proteins as it cannot be synthesised by the organism itself [62]. The typical role for TRP is protein synthesis but it also serves as a precursor in multiple biochemical and functional pathways in the periphery and in central nervous system (CNS) [63-65]. This includes the production of kynurenine, serotonin (5-HT), melatonin and niacin [63, 66-70]. These substances affect several physiological processes, for example, sleep/wake cycle, mood, circadian rhythms, cognition, memory and the central regulation of appetite [64, 65, 71-75].

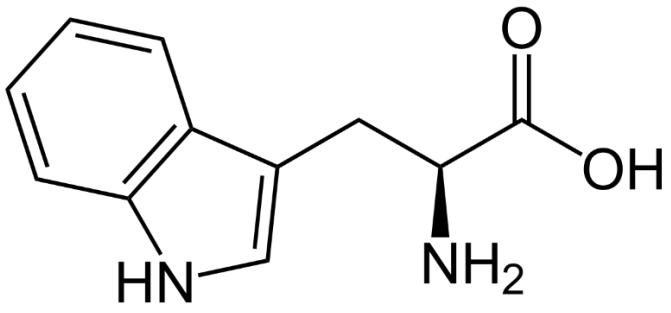

Figure 1.2. Molecular structure of tryptophan

After consumption, proteins are digested and broken into their constituent amino acids. The absorption of TRP occurs quickly, and TRP enters the general circulation from the GI tract in a dose-dependent manner in minutes [28, 29, 76]. Ng and Anderson (1992) showed that both plasma and brain TRP increased for 30 minutes after delivery of 100 mg/kg dose via IP injection and, to a lesser degree, after intragastric infusion [28]. The uptake of TRP and other neutral amino acids occurs via the apical membrane of the intestinal absorptive cells using the epithelial transporter B^0AT1 [77]. Within the enterocytes' basolateral membrane is the aromatic amino acid transporter (TAT1), responsible for the transportation across the membrane and into the adjacent cells [77]. Eventually, TRP will directly enter the circulation where it is commonly bound to serum albumin via its indole ring (Figure 1.2) [78]. This contrasts with the other amino acids which typically remain free [61, 79]. Interestingly, the binding site of albumin has poor affinity for TRP and, due to competition from free fatty acids (FFA) and other drugs for the same site, some will remain unbound in the general circulation [76, 79, 80]. In fact, the binding of TRP to albumin may also have a minor influence on the transportation of TRP via large neutral amino acid (LAT1) transporter across the BBB [81].

From the general circulation, TRP can directly cross the BBB [82, 83]. TRP is competitively transported across the BBB by LAT1 that is located on the capillary endothelial cells. LAT1 is highly expressed on the luminal side of the BBB endothelium [84]. This transporter binds other LNAA with similar affinities as TRP, therefore, the uptake of TRP by LAT1 is influenced by the plasma ratio of TRP to these other LNAA/BCAA [85-90]. Plasma concentrations of TRP, and BBB transport, have been shown to be influenced by alterations in dietary availability

[91]. Simply increasing the TRP dietary content exhibits increased uptake of TRP across the BBB [83, 92]. The chronic ingestion of different protein sources, for example, gluten, soy, casein, or α-lactalbumin, induced altered brain TRP levels in rats [93].

Research has revealed that BBB transporters of amino acids act selectively [94]. Administration of radiolabelled amino acids in rats demonstrated preferential uptake (5-10 times greater) for essential over non-essential amino acids [95, 96]. The LAT1 transporter is the predominant amino acid transporter in the BBB and is responsible for the transport of most essential amino acids from the luminal side [84, 97, 98]. Abluminally expressed transporters export amino acids across the BBB back into general circulation [97, 99]. Therefore, the BBB can control both the import and export of amino acids to regulate central levels [94].

1.2.1 Metabolic fates and key pathways of tryptophan

The predominant proportion of ingested dietary TRP is utilised via peripheral pathways as compared to central routes [63]. Additional to its role in the periphery, TRP is a biochemical precursor in the CNS for, among others, neuroactive metabolites that are key in appetite regulation. Dietary TRP contributes to several metabolic and functional pathways; protein synthesis, kynurenine and serotonergic pathways – as exemplified below (Figure 1.3) [63-65].

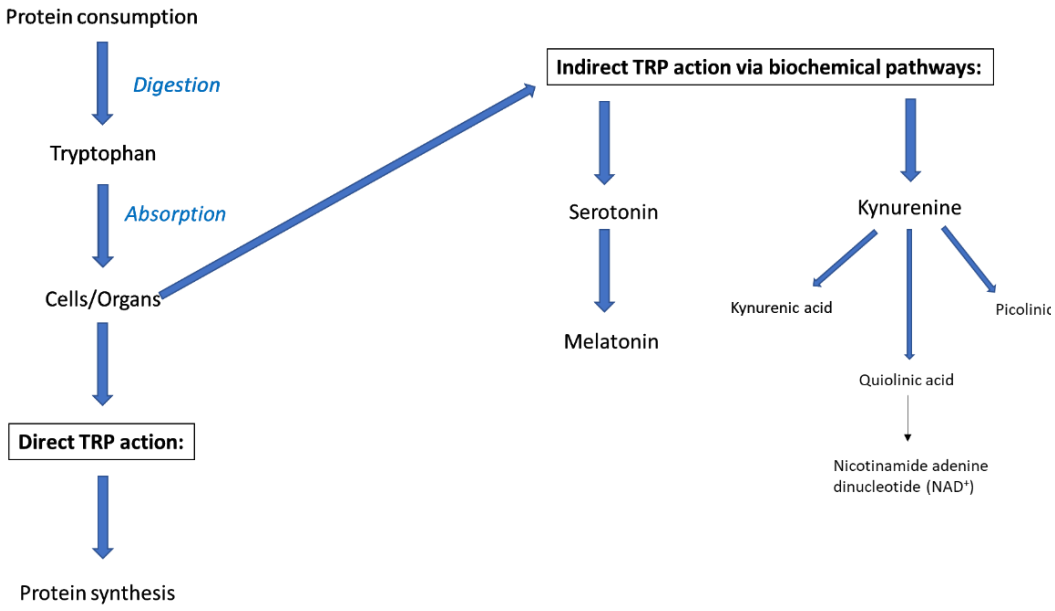

Figure 1.3 Key metabolic and functional pathways of tryptophan

1.2.1.1 Protein synthesis

As an essential amino acid, TRP must be provided by dietary protein sources [62-65]. While no consensus has been reached on the exact proportion of dietary TRP utilised for protein synthesis [63, 100], Allegri and associates used *in vitro* enzyme determination in mammals to provide a 30% estimate [101]. At low levels, TRP acts as a rate-limiting amino acid in protein synthesis [102]. Interestingly, mice fed a sole TRP diet, when compared to those on a balanced amino acid diet, displayed enhanced hepatic protein synthesis. This effect was not observed with other amino acids, such as isoleucine, threonine or methionine [103].

The proportion of dietary TRP required for protein synthesis also varies with the specific physiological status of an individual at the time [64]. For example, young

growing animals obviously have higher TRP requirements for protein growth and accretion compared to adults of the same species [104, 105].

1.2.2 Kynurenine pathway

The predominant metabolic fate for TRP is entry into the kynurenine pathway, some authors suggesting that it applies up to 90% of dietary TRP [63, 68, 100]. This pathway is the primary route by which TRP levels are regulated catabolically [68, 100]. TRP is an intermediary product in the complex kynurenine pathway, producing many metabolites (nicotinamide, carbon dioxide, kynurenic, picolinic, quinolinic and xanthurenic acids) which are active within both peripheral and central nervous systems [106-110]. These metabolites are involved in numerous physiological functions, including GI motility, neuroprotective and neurotoxic actions [111].

Circulatory TRP is the precursor of N-formylkynurenine and gets converted to kynurenine by the enzyme kynurenine formadase, which is a rate limiting process [112]. There are two enzymes that catalyse the reaction – tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO). These two enzymes differ in various characteristics (summarised in Table 1), of note their organ distribution [64]. TDO is primarily found in the liver [113, 114] whereas IDO is found in both central and peripheral tissues, especially the small intestine [64]. Central expression is localised in astrocytes, microglia, brain-infiltrating macrophages and dendritic cells [115, 116], mediating the kynurenine pathway in the brain.

Table 1 Comparison of Tryptophan 2,3-dioxygenase (TDO) and Indoleamine 2,3-dioxygenase (IDO) charactersitics – two key enzymes of the kynurenine pathway. Adapted from Le Floc'h, Otten & Merlot, 2011 [64].

	Tryptophan 2,3-dioxygenase (TDO)	Indoleamine 2,3-dioxygenase (IDO)
Substrates	L-tryptophan	L- & D-tryptophan 5-Hydroxytryptophan 5-Hydroxytryptamine (serotonin)
Cofactors	Molecular oxygen (O_2)	Superoxide anion
Tissue distribution	Liver	Ubiquitous
Function	Degradation tryptophan	Immune regulation

TDO has very low affinity for TRP, therefore, the enzyme is only active when TRP levels exceed those required for protein and 5-HT synthesis [117]. The breakdown of TRP occurs in the liver to regulate TRP homeostasis [64]. This prevents the accumulation of TRP in the plasma and tissues to toxic levels. But TDO action is suppressed when IDO is induced during an inflammatory event [118]. The IDO pathway plays a vital role in the modulation of the peripheral immune response

during infections, pregnancy and inflammations [119-121]. Evidence suggests that the enzyme possesses peripheral and CNS immunosuppressive properties [122].

Only 40% of CNS kynurenine is locally synthesised [123]. It is otherwise sourced from the general circulation, imported across the BBB via LAT1 [124]. As previously mentioned, this pathway provides both neuroprotective and neurotoxic effects [108-110, 125] in the CNS and is implicated with various pathologies, such as brain injuries, Alzheimer's disease (AD), dementia and depression [126]. The over-activation of the kynurenine pathway was described in an AD mouse model brain, where TDO was found to be very highly expressed, thereby indicating a possible link with the neurodegenerative progression of the disease [127]. The kynurenine pathway has also been investigated as a potential new therapeutic option in the treatment of depression. Unfortunately, currently 20% of individuals with major depressive disorder (MDD) are resistant to available medicines [128]. Some propose that depression is linked with inflammation, as many autoimmune or inflammatory disease sufferers exhibit depression [129]. However, as the kynurenine pathway plays a role in peripheral inflammation it may be a potential target for further research into depression therapeutics [130].

Kynurenine metabolites play roles beyond those of inflammation, neuroprotection and neurotoxicity [108-110, 126]. The metabolite nicotinamide, formed in the liver, is a precursor for the enzyme nicotinamide adenine dinucleotide (NAD^+), that is important for redox reactions [131]. Metabolites also have GI functions related to appetite control. Kynurenic acid is an antagonist of N-methyl-d-aspartate (NMDA)

glutamate receptors affecting GI motility [63]. Its administration in dogs with mechanical colonic obstruction caused reduced GI motility in those animals [132].

Kynurenine has also been correlated with body mass index (BMI) and obesity, but to date, no link has been firmly established with food intake or appetite regulation [83, 133]. In a recent human cohort, the analysis of the circulatory levels of kynurenine, as well as, the expression of key kynurenine pathway enzymes in the adipose tissues revealed a direct association with BMI [133].

1.2.3 Serotonergic Pathway

Serotonin (5-HT) is a monoamine neurotransmitter whose synthesis requires TRP as a precursor. Approximately 1-2% of total dietary TRP is utilised for 5-HT synthesis [68, 134]. Of that proportion, the majority is utilised via the peripheral system (approximately 95%) [135]. Most of the peripheral 5-HT is located within the enterochromaffin cells of the gut and in the serotonergic neurons of the enteric nervous system [135, 136]. The remaining proportion is dedicated to 5-HT synthesis within the CNS. With synthesis localised in both gut and the brain, 5-HT is involved in a range of physiological functions, including regulation of the GI system, mood and appetite [64, 71, 74, 75, 135].

The conversion of TRP to 5-HT in the brain is a two-step process (Figure 1.4). TRP first is hydroxylated into 5-hydroxytryptophan by tryptophan hydroxylase (TPH) – in the rate-limiting step [65, 137]. It then undergoes decarboxylation to form 5-HT using the enzyme aromatic acid decarboxylase [91]. Interestingly, the enzyme TPH is not saturated at normal physiological concentrations of TRP in the brain [65, 115],

therefore, 5-HT production is directly proportional to the amount of TRP crossing the BBB [138]. This has been demonstrated by the dose-dependent increase of central 5-HT in response to increases in dietary TRP or changes in the LNAA ratio [137, 139].

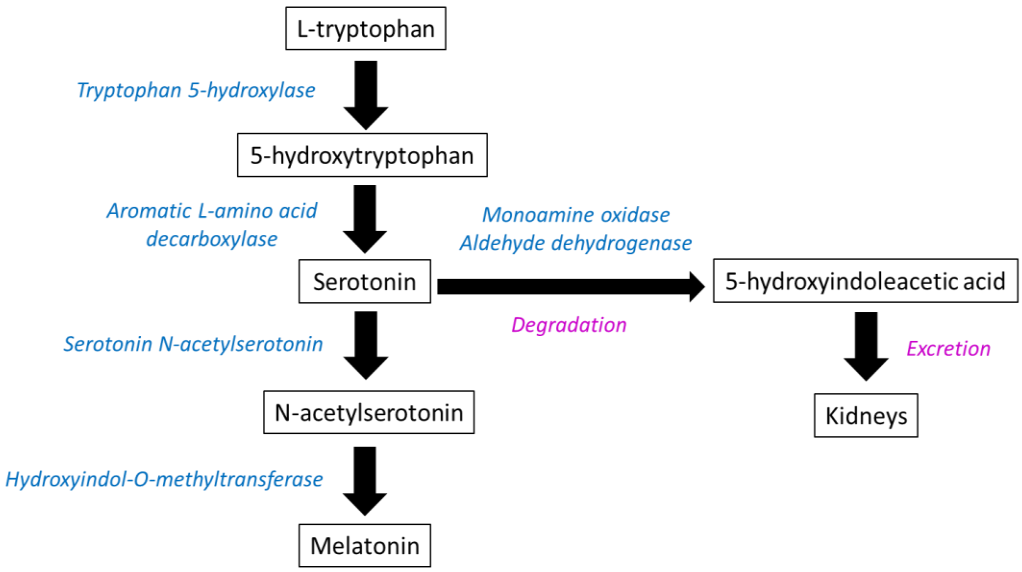

Figure 1.4 The synthesis of serotonin and melatonin within the central nervous system from the biochemical precursor tryptophan. Adapted from Fernstrom, 2016 [70].

There are two different TPH isoforms [140]. The two distinct enzymes were first identified using tph1 knockout mouse models. It was initially assumed that all 5-HT synthesis would be inhibited, but this was not the case as the knockouts displayed typical 5-HT activity as the tph2 gene was additionally present [140]. TPH1 is typically located in peripheral tissues, such as lung epithelial cells and predominantly in the enterochromaffin cells of the GI tract [63, 141-143]. Enterochromaffin intestinal cells synthesise and store 5-HT, activation of these cells by perturbations within the GI tract causes the release of extracellular 5-HT, e.g.

physical, changes in intraluminal pressure due to movement of food or chemical, such as pH, glucose and bile acid [141, 144, 145]. Peripheral 5-HT can then stimulate the enteric nervous system to control GI motility and secretion [145]. Extracellular 5-HT can also directly act upon the CNS via the vagal afferents which project to the hindbrain [146].

TPH2 however is located in neurons, as well as, a small amount within the enteric nervous system, allowing both central and peripheral TPH2 function [135, 147]. Therefore, 5-HT can be synthesised not only in the gut, but also the CNS specifically by neurons in the raphe nucleus [148]. The raphe axons project to numerous brain regions [149, 150]. The rostral group sends axons to the forebrain e.g. cortex, amygdala (AMY), hippocampus and hypothalamus [151]. Many of these brain regions (especially, the hypothalamus) are involved in energy homeostasis and appetite regulation [152].

These regions express various 5-HT receptor subtypes [152-154]. Since the first discovery of 5-HT receptors made in 1950's in the ileum of guinea pigs [155], multiple 5-HT receptor subtypes have been identified e.g. 5-HT1A/5-HT1B/5-HT1C/5-HT1D in the CNS as well as a peripheral 5-HT3 receptors [156]. Most of the 5-HT receptors are located within the nervous system (including substantia nigra, hippocampus, AMY, striatum and the frontal cortex) [151, 157, 158] as well as the GI tract [159].

The reuptake of 5-HT occurs from the synaptic cleft back into the presynaptic neuron via the 5-HT transporter (SERT) [64, 160]. It is metabolised to 5-

hydroxylindoleacetic acid (5HIAA) by monoamine oxidase (MAO) and aldehyde dehydrogenase, then finally is excreted via the kidneys (Figure 1.4) [64].

From the point of the presumed role of TRP itself in appetite control, it is important to note that 5-HT administration reduces food intake via central and peripheral neuroendocrine pathways involving, among others, ghrelin and agouti-related protein (AGRP) [161]. 5-HT agonists suppress carbohydrate intake [162]. Changes in 5-HT synthesis or availability can affect food intake [152].

1.2.3.1 Melatonin

Melatonin entrains circadian rhythms, including those related to appetite [163]. Its synthesis relies on the pineal metabolism of 5-HT [66, 67]. The reaction is mediated by N-acetyltransferase to form N-acetylserotonin, and then, melatonin, via the enzyme hydroxyindol-O-methyltranferase (Figure 1.4) [164].

Melatonin is synthesised during the dark phase [66, 164], but perturbations in plasma TRP availability can have long-term consequences on melatonin synthesis [165, 166]. In rats, 150mg/kg TRP generated an increase in circulating melatonin levels [167]. Dietary TRP fed to chickens in the light-phase caused enhanced melatonin synthesis for approximately 3 hours [166].

In energy intake/metabolism studies, supplementation of non-diabetic rats with melatonin for four weeks showed a satiating effect [168]. Subcutaneous administration of melatonin, daily for 12 days in rats, lowered food consumption by 8.8% [169]. Human studies, however, have not yet provided a clear link between melatonin levels and food intake [170, 171].

1.3 Key behavioural and physiological effects of tryptophan

The physiological and behavioural effects of TRP arise from engaging a combination of the aforementioned peripheral and central processes. These TRP utilising pathways (e.g. kynurenine, serotonergic and melatonin pathways), when dysregulated, have been implicated in an array of complex disorders, including depression, sleep regression, anxiety, stress and social deficits [64, 71-75, 107, 126, 163].

The two common approaches to study the involvement of TRP in behavioural or physiological processes involve either TRP loading/supplementation (methods that increase TRP availability) or acute tryptophan depletion (ATD) [65]. ATD involves suppression of TRP levels through, e.g., biochemical and dietary manipulation [172, 173]. Over the past 50 years a substantial number of studies have been published that analyse the beneficial effects of TRP supplementation in humans and animals that are suffering from various behavioural and psychiatric conditions.

1.3.1 Sleep

In the sleep-wake cycle, rapid eye movement (REM) and non-REM sleep are crucial phases of sleep. The REM constitutes approximately 20-25%, and the remainder is non-REM sleep, which is often described as “deep” sleep [174, 175]. Within a typical sleep cycle (average period of 90-120 minutes), non-REM occurs first (90 minutes) and is made up of four stages which go from progressively light (stage one) to deep (stage four) sleep. Then a brief period of REM sleep occurs. This alternation continues throughout the night, with increases in REM sleep and

the depth of non-REM sleep declining [69]. Any sleep pattern disruptions or disorders (i.e. insomnia) can have multiple health effects, including an increased risk of CVS, diabetes, and possibly, mood disorders [176].

The direct influence of TRP ingestion on brain 5-HT levels suggests a potential role of TRP supplementation in enhancing sleep [71, 72]. 5-HT can be further metabolised in the pineal gland to the hormone melatonin, as described above in section 1.2.3.1 [70]. Administration of TRP enhances melatonin synthesis and its plasma concentration [167]. Treatments that decrease 5-HT concentration interrupt the sleep cycle, whereas sleep improvement is observed when 5-HT levels rise [73, 177-179]. For example, healthy human volunteers receiving TRP (7.5g in milk drink) once daily for 10 days, showed an increase in both non-REM sleep and delta wave sleep, as well as a decrease in REM sleep, compared to those receiving the placebo [178].

TRP supplementation in healthy participants with no sleep problems caused a mild hypnotic effect with reduced sleep latency [175, 180-184]. A range of oral doses seem effective in humans including 500 mg or 1 g mixed with 30 mg of the carbohydrate maltodextrin [180], 1.2 g or 2.4 g tablet [181], and 4 g tablet [182]. George and colleagues analysed sleep latency and sleepiness using the Standford Sleepiness Scale in 10 healthy humans. Participants were randomly assigned on separate days either placebo, 1.2 g or 2.4 g TRP orally. Both TRP doses were associated with reduced sleep latency, and the latency highly correlated with plasma TRP levels. The higher TRP dose was also associated with greater overall

sleepiness [181]. Doses between 1-5 g TRP given orally in mild insomniacs caused an improvement in sleep latency [175, 183, 184]. TRP loading, therefore, may have a beneficial effect on sleep patterns.

The relationship between TRP and the regulation of the sleep-wake cycle extends upon critical CNS developmental phases in early childhood [185]. Breastmilk traditionally has higher levels of TRP than that of infant formulations [186, 187]. Heine and colleagues showed that the plasma TRP levels in breast-fed infants is typically higher than in those maintained on formula [185]. Fluctuations in TRP levels of breastmilk paralleled urinary levels of 6-sulfatoxymelatonin (a melatonin metabolite) in infants [188], suggesting a link between TRP intake and melatonin synthesis. Others have shown that maternal milk plays a role in the regulation of circadian rhythms in infants [189, 190]. In fact, Steinberg and collaborators used the measure of sleep latency and TRP plasma levels to assess the effect of TRP-loading (0, 294, 588 or 882 μmol/L) on infant sleep. Sleep latency was defined as the time from the completion of the formula feed to the first stage of REM sleep. The highest TRP-fortified formula reduced sleep latency from 27.7 to 18.7 minutes compared to breastfed counterparts. Interestingly, the reduced sleep latency correlated with the plasma TRP:LNAA ratio [186].

TRP depletion is oftentimes used to obtain information about the functional effects of lower brain 5-HT levels [65]. Nakamaru-Ogiso et al. induced 5-HT deficiency in rats by IP administration of the enzyme tryptophan side chain oxidase I (TSOI). This TRP-degrading enzyme reduces TRP plasma levels, by 1-2% within two hours. In the following six hours, brain 5-HT levels continued to decline, reaching a 30%

reduction compared to controls. In this rat model, both the circadian rhythmicity of sleep cycle and overall locomotor activity were reduced [173].

TRP depletion to suppress 5-HT synthesis has also been used in human studies. In 12 healthy participants, TRP depletion was achieved through diet manipulation that led to altering the TRP:LNAA plasma ratio [191]. This influenced 5-HT level due to competitive transport of LNAA across the BBB via LAT1 (refer to section 1.2) [192]. The initial two nights established a baseline and on days three and four patients were given a low-protein diet. The last night of the study participants were given either a drink of amino acid mixture without TRP or one containing also 2.3 g TRP (control group). Compared to controls, the TRP-depletion strategy caused an 85% reduction in plasma TRP levels. The use of sleep EEG revealed changes in sleep features, including, reduced REM sleep and increased awakeness [191]. The manipulation of the serotonergic system, via the depletion of its precursor, influences sleep disorders and patterns.

1.3.2 Tryptophan and mood control

Tryptophan (via the 5-HT circuit) has a profound influence on mood parameters and - consequently – on behaviours/physiological functions intertwined with mood. Therefore, it is not surprising that dysregulation within the TRP → 5-HT biochemical pathway (including cellular and intercellular fate of 5-HT) underlies depression, anxiety, and obsessive-compulsive disorder (OCD) and their comorbidities (such as aberrant appetite, sleep and sociality, to name a few) [64, 65, 193]. One therapeutic strategy is the use of pro-serotonergic drugs (e.g. selective

serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants (TCA)), that increase 5-HT signalling [65].

As with sleep, ATD studies via dietary manipulation are useful in depression studies for assessing the direct effects of TRP deficiency [194, 195]. In humans, Delgado and colleagues (1989) analysed the behavioural effects of gradual dietary TRP depletion in healthy individuals. A 10-day TRP restriction diet was employed, with one group maintained on 700 mg/day and the other on 200 mg/day. Both of those diets produced a decline in total plasma TRP of 15-20% but no significant behavioural changes were reported [194]. A later study by the same group of investigators analysed ATD in depressed patients who were currently not receiving treatment. Rapid dietary depletion of TRP was induced in a double-blind, placebo-controlled crossover study with one group receiving a 15-amino acid drink minus TRP, and the other, the same drink but inclusive of TRP. This caused a brief depression relapse in 67% of the TRP depletion group [195]. If TRP depletion induces depression, then it is expected that the opposite will occur with TRP loading [65].

Indeed, the beneficial effects of increased TRP levels have been observed in patients suffering from mild to moderate depression [196, 197]. In a clinical trial of 115 depressed patients a 12-week double-blind study assessed the effectiveness of TRP compared to a placebo. All participants received the placebo for the first week – then the experimental group received 1 g of TRP, three times daily for up to 12 weeks. Chronic TRP supplementation showed improvements according to analysis

with the Hamilton Depression Scale [197]. TRP supplementation in healthy individuals shows little to no effect on mood [65].

TRP supplementation in animal depression models has been controversial with some reporting no effect and others reporting mild improvement [172, 198]. Brown and colleagues (1998) demonstrated that when rats were orally gavaged with TRP-containing amino acid mixture, an amino acid load without TRP or water, there were no acute differences in behaviour between the groups in an operant schedule of differential reinforcement (DRL) and locomotor activity [172], both of which are changed after central 5-HT [199-203]. On the other hand, Blokland and associates utilised a moderate TRP depletion animal model to reduce central 5-HT levels. Rats were orally infused with either a TRP-positive amino acid load or a TRP-negative amino acid load. Five hours after infusion, the home cage emergence test (HCET) was performed, in which the animal was placed in its home cage in the arena with the lid removed and latency till the animal leaves its home cage was recorded. The TRP depleted rats exhibited increased latency indicating more anxiety-related behaviour [204]. Other methodologies used to assess depressive-like behaviours include sucrose preference test, open field test (OFT) and forced swim test (FST). Typically, depressed rats exhibit reduced sucrose intake; reduced distance travelled and increased immobility in OFT; and a decreased latency to immobility during FST [205]. In sub-chronic TRP depletion (SCTD) studies, Sprague-Dawley rats were supplemented with low TRP diets (0.04%) compared to control (0.2% TRP) for either 0, 7 or 14 days, followed by assessment of depression-like behaviours via FST. Those on chronic sub-TRP showed increased floating time and reduced swimming time, and it was reversed with the administration of the SSRI paroxetine.

This study also notes an increase in the metabolite kynurenine, suggesting a role for the kynurenine pathway in depression [198].

Kynurenine pathway metabolites exhibit various neuroprotective and neurotoxic effects [108-110], implicated in various pathologies, including depression [126]. For example, during pregnancy, immune-inflammatory activity generates more kynurenine pathway metabolites [130, 206-208]. Conversely, high levels of kynurenine pathway metabolites (e.g. kynurenic acid and quinolinic acid) have neuroregulatory effects [209].

1.4 Tryptophan and appetite regulation: a possible mediator of satiety

Evidence providing support for a role of TRP in termination of food intake, is still very limited [23, 28, 29]. Daily dietary supplementation of TRP at 3.0 g/kg bodyweight reduced food intake in ad libitum-fed rats by approximately 25% and it led to an overall reduction in bodyweight. This was the result of alterations in meal pattern – most noticeably increases in meal size, intermeal intervals, meal time and a decrease in meal number [29].

In 1992, Ng and Anderson infused TRP in rats maintained on a 12-h/day availability schedule of both standard and high-carbohydrate (protein-free) diet. These intragastric infusions (200-600 mg/kg TRP) induced a decrease in episodic consumption [28]. Jordi and colleagues (2013) also utilised the intragastric route of administration. Food-deprived rats received isomolar doses (6.7 mmol/kg) of one of the 20 amino acids, including TRP. At refeeding, animals gavaged with TRP

demonstrated a 25% reduction in chow intake, when compared to the vehicle. TRP was ranked as the 4th most potent anorexigen when compared to the other amino acids. Unfortunately, statistical significance was not achieved when corrected for multiple comparisons [23].

Importantly in that study, c-Fos immunoreactivity (commonly used as a measure of neuronal activation [210]) analysis revealed alterations in neuronal activity following exposure to anorexigenic amino acids (albeit, due to the lack of significance, TRP was excluded from the c-Fos mapping experiment). Increase in the number of Fos positive nuclear profiles in the brainstem, particularly in the dorsal vagal complex (DVC) components, including, the NTS and the AP, suggested a likely involvement of vagal afferents and potential influence of biochemical changes in plasma (since the AP is virtually devoid of the BBB) [23]. Axons from the DVC innervate other brain sites, notably, the hypothalamus [211].

1.5 Mechanisms controlling food intake: basic information

Energy balance, crucial for the survival of an organism and for the maintenance of fundamental physiological functions, relies on mechanisms that ensure that energy intake equals or exceeds energy expenditure. In the obesogenic environment, excessive consumption maintained for a long-time period can cause weight gain and obesity [6-9]. Feeding regulation is critical to enable an organism to meet both energy and nutrient requirements, without being at risk of jeopardising internal milieu (e.g., through excessive stomach distension, presence of toxins or high osmotic load) [212, 213]. These integrated regulatory mechanisms are required to constantly enable the adaptation of consummatory behaviour to changes in both the

internal (e.g., neurohormones, osmolality, food derived signals, chemo- and mechanoreceptors) and external environment (e.g., circadian rhythms and social behaviours). Thus, the control of food intake is an interplay between both the homeostatic (that protects internal milieu) and non-homeostatic (e.g. hedonic) systems [11].

Satiation is the process relating to the termination of food consumption due to a feeling of fullness, ensuring that maximum gastric capacity is not breached [52, 213]. There are mechanoreceptors in the stomach itself that sense gastric distension and relay this information via the vagal afferents to the hindbrain [214]. Upon ingestion of protein, satiety-inducing hormones (e.g. CCK, GLP-1, and PYY) are released from the enteroendocrine cells of the GI tract. These can directly act centrally by crossing the BBB from the general circulation or indirectly via vagal afferents [47]. The hindbrain structures relay information to the hypothalamus including peripheral signals, such as neural (vagal), hormonal (e.g. CCK) and biochemical (e.g. plasma ion/toxins) inputs [47, 52, 214]. These relay centres include the DVC which encompasses the nucleus of the NTS, the dorsal motor nucleus of the vagus (DMNV) and AP [47, 211].

The hypothalamic network integrates numerous endocrine signals (Figure 1.5), for example, leptin and insulin – leptin reports on the levels of energy stored in fat [215]. Decreased leptin signalling from adipocytes promotes increased caloric intake and fat accumulation [216-218]. Leptin modulates intake via leptin receptors expressed in the hypothalamus e.g., arcuate nucleus (ARC), supraoptic nucleus (SON), paraventricular nucleus (PVN), ventromedial hypothalamic nuclei (VMH)

and the lateral hypothalamic area (LH) [216]. The ARC contains vast populations of neurons expressing orexigenic and anorexigenic peptides. These neurons are capable of sensing energy related signals and activate feeding-relevant pathways in order to generate behavioural changes conducive to maintaining energy balance. For example, ARC neurons are stimulated by both leptin and insulin, express proopiomelacortin (POMC), which – after posttranslational processing – gives rise to alpha-melanocyte-stimulating hormone (α-MSH) [52, 216]. α-MSH is released in the PVN where it binds the melanocortin-4 receptors to reduce food intake [219-221]. Conversely, ARC orexigenic neurons express neuropeptide Y (NPY) and AGRP. Activity of these cells is inhibited by leptin [52, 216]. Orexigenic action of AGRP involves competitive antagonism of PVN melanocortin-4 receptors, whereas NPY influences PVN activity to enhance appetite via interactions with its Y1/Y5 receptors [52, 216]. To add to this complexity of appetite stimulating processes elicited by ARC NPY/AGRP neurons is the inhibition of POMC neurons by co-release of gamma-aminobutyric acid (GABA) [222, 223]. Hence, aside from activating relevant PVN target cells that promote appetite, NPY/AGRP neurons simultaneously block activation of the ARC POMC cells, thereby further ensuring hyperphagia by reducing satiety [216].

Appetite regulation is not simply dependent on energy homeostasis; food ingestion is greatly motivated by palatable foods, even when such diets are overabundant and readily available (hence, energy deficit is not the main drive to eat). This has been reliably shown in studies involving a cafeteria diet paradigm, in which animals presented with a choice of nutritionally adequate yet bland versus highly palatable yet nutritionally poor foods, overconsume the palatable ones [224]. Remarkably,

reward circuitry promotes hedonic feeding despite aversive conditions such as foot shocks or extreme cold, even when animals are not deprived [225, 226].

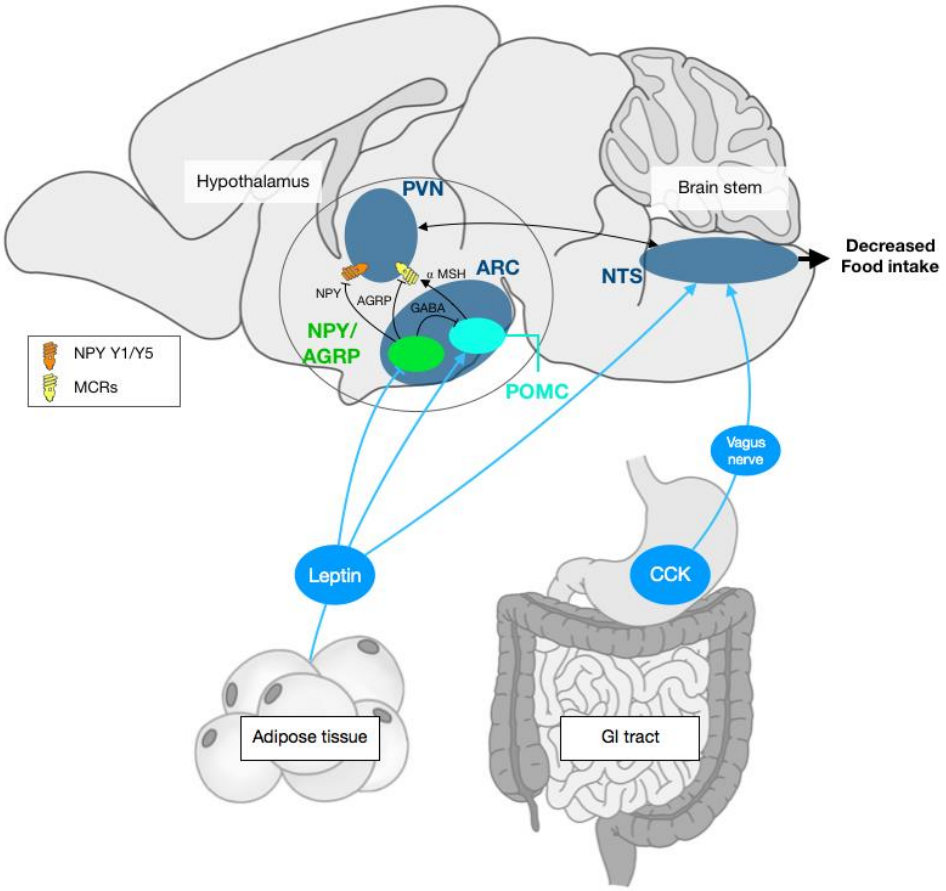

Figure 1.5 A simplified schematic of food intake control by the central nervous system with a focus on anorexigenic peripheral signals. Cholecystokinin (CCK) is released from the gastrointestinal (GI) tract and acts upon nucleus of the solitary tract (NTS) via vagal afferents. The adiposity signalling hormone leptin affects neuropeptide Y (NPY) and agouti-related protein (AGRP) expressing neurons as well as proopiomelanocortin (POMC) expressing neurons located in the arcuate nucleus (ARC) of the hypothalamus. POMC is processed into alpha-melanocyte-stimulating hormone (α-MSH) which binds to melanocortin receptors (MCRs) in the paraventricular nucleus (PVN) to suppress appetite. NPY/AGRP neurons inhibit POMC production via gamma-aminobutyric acid (GABA) co-release. They also act on the PVN with AGRP being a competitive antagonist of MCRs and NPY interacting with Y1/Y5 receptors. Figure adapted from Morton GJ et al., 2014 [215].

A multitude of reward-related pre- and post-absorptive peripheral processes [227] affect activation of various brain regions in the mesocorticolimbic, hypothalamic and midbrain circuitry and stimulate neural processing conducive to reinforcement of the rewarding aspect of food [228, 229]. Consumption of palatable food enhances neuronal activity in reward-related areas and changes gene expression of reward-related genes, especially those related to the dopamine, opioid and endocannabinoid systems [230].

Two brain regions crucial for reward processing are: the ventral tegmental area (VTA) and nucleus accumbens (Acb), and they are part of a network that relies on opioid, dopamine, 5-HT, and endocannabinoid signalling [231-237]. Dopaminergic activity is associated with the motivational aspect of reward [238]. In rats, consumption of 0.3M sucrose increased dopamine metabolite levels in the Acb by three-fold compared to the consumption of water [239]. The concentration-dependent effect of sucrose on dopamine levels in the Acb have been shown in rats given sucrose concentrations (0.03M, 0.1M and 0.3M) via a gastric cannula (bypassing taste receptors) [240]. Opioids are involved with processing the hedonic value or palatability aspects of the diet [241, 242]. Opioid receptor agonists promote the consumption of palatable food by increasing their hedonic value, whereas antagonists have the opposite effect [243, 244].

1.6 Role of oxytocin in appetite regulation

The last decade of feeding research has brought a tremendous leap in our understanding of neuropeptidergic systems that bridge control of ingestive behaviour driven by homeostatic needs with that driven by reward. One of the most

crucial sets of discoveries pertains to the neurohormone oxytocin (OT), a nine-amino acid anorexigenic peptide synthesised mainly in the hypothalamic PVN and SON [252-256]. OT cells reciprocally communicate with DVC neurons (Figure 1.6), thus serving as a key element of pathways that ensure integration of peripheral information relevant to the energy/feeding status of the organism [245-247].

While the majority of magnocellular OT neurons in the PVN and SON terminate in the neurohypophysis, parvocellular PVN OT cells target the aforementioned brainstem areas (thus being a part of the 'homeostatic' circuit) as well as sites involved in reward [212, 248-251]. The combination of the two general CNS projection targets serves as a neuroanatomical foundation implicating OT in mediation of satiety to protect internal milieu and to decrease feeding-derived hedonics [212]. Early experiments showed that lesioning of the PVN in rats disturbs the PVN to hindbrain projections and resulted in greater food consumption and body weight [252-254].

The release of OT and enhanced OT neuronal activity have been observed to coincide with termination of feeding [255-258]. Arletti et al. demonstrated that ICV OT greatly decreased chow intake in deprived rats [256]. Ho and colleagues determined that activation of NTS neurons mediated this effect of OT [259], strengthening the hypothesis for the role of the hindbrain in OT-driven feeding inhibition.

Both hyperphagia and obesity have been observed in mutations that result in abnormalities in PVN neuronal development, leading to OT deficiencies [260-262]. Kublaoui and colleagues utilised the single-minded (sim-1) mouse model which

lacks sim-1, a transcription factor responsible for the development of the PVN. Hyperphagic obesity was observed in these individuals, and it was later reversed by OT treatment [260].

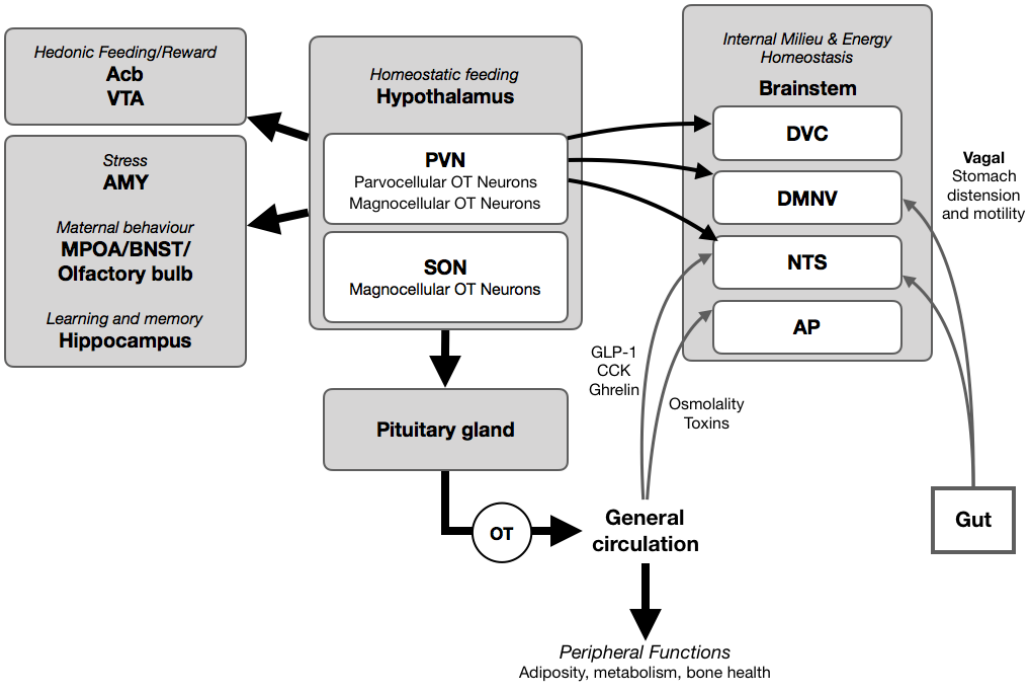

Figure 1.6 Central functional OT pathways with a focus on those involved in food intake regulation. The parvocellular OT neurons within the PVN project to the brainstem particularly the NTS, AP, and DMNV, all known to be involved in appetite regulation. The magnocellular OT neurons project to the pituitary gland where OT gets released into the general circulation. OT is also involved in anorexigenic inputs from the periphery, for example, GI tract distension and osmolality of the blood. Other feeding related sites known to contain OT neurons and receptors include those involved in reward (ventral tegmental area (VTA), nucleus accumbens (Acb), bed nucleus of the stria terminalis (BST)), energy homeostasis (ventromedial hypothalamic nucleus (VMH)) and stress (AMY). Adapted from Olszewski et al., 2010 [263].

Changes in other calorie-independent 'homeostatic' parameters induce OT neuronal activation and termination of feeding [212]. For example, excessive stomach distension and elevated plasma osmolality (ion imbalance due to salt loading) lead to OT release [264-266]. OT also inhibits intake of toxin-tainted food and generates long-term avoidance of those by acting through both the AMY and brainstem [267].

Whilst the role of OT in energy homeostasis is now widely recognised, the notion for the role of central OT in feeding reward and macronutrient preference has only begun to emerge over the past several years [212, 268-270]. The OT receptor is highly expressed in brain regions associated with food reward, particularly the VTA and Acb, with PVN OT neurons innervating both of these key regions [248-251]. In fact, OT terminals have been found near mesolimbic neurons where they form both somatic and axodendritic synapses [248, 249].

The cross-link between the OT and the reward system appears reciprocal [245-247]. The existence of opioid receptors on hypothalamic OT neurons provides evidence for this relationship, and so does the fact that orexigenic opioid agonists silence activation of OT cells [271-274]. This interaction also appears to involve non-feeding rewards e.g. social or reproductive behaviour and alcohol/drug intake [212, 268]. Cocaine usage in female rats altered OT receptor binding density in the bed nucleus of the stria terminalis (BNST) [275]. The intraparenchymal infusion of OT removes conditioned place preference induced by methamphetamine and ethanol, as well as, overcoming methamphetamine seeking behaviour [276-278]. Female mice exhibit conditioned social preference after administration of ICV OT [279].

Knockout models further support the role of OT in feeding reward. Amico and et al. demonstrated that the deletion of the OT gene resulted in excessive intake of palatable sucrose [269]. This effect was independent of the 24-hour light cycle, with high sucrose intake maintained over both light and dark phases [270]. When the OT-null knockouts were provided a two-choice test between water and sucrose, animals consistently displayed a stronger preference for the sucrose solution. They also drank more of a highly palatable and non-caloric saccharin solution [280]. Increased consumption of this particular solution by OT-null mice provides evidence for OTs role in hedonic, rather than homeostatic, feeding.

To follow up on the knockout findings, rodent injection studies in wild-type animals utilising the BBB penetrant OT antagonist, L-368,899, in both choice and no-choice feeding paradigms were performed [281]. These have consistently found elevated carbohydrate intake, with no effect on fat consumption [282, 283]. Mullis and colleagues demonstrated that direct VTA OT administration reduced reward-driven sucrose consumption which was reversible with pre-treatment of L-368,899. They also identified that a blockade of the OT receptor in the VTA leads to overconsumption of sugar [284]. OT injection into the Acb core (but not the shell) reduced intake of palatable carbohydrates and, to a greater extent, the intake of saccharin [285]. Some evidence also exists for the role of OT in fat intake regulation. Zhang and colleagues provided infusions of OT receptor antagonist into the third ventricle of mice fed a high-fat diet. They observed increased food intake and body weight [286, 287].

Lastly, consideration needs to be made for the fact that OT influences a multitude of functions that are intertwined with ingestive behaviour. Consequently, the orexigenic response to L-368,899, depends on the place within social hierarchy in group-fed mice [288], whereas individually housed mice consistently increased sugar intake following administration of L-368,899 [282, 283, 289].

Despite the great interest in OT's involvement in feeding termination, to date a possible relationship between this key hypothalamic satiety mediator that is part of both homeostatic and hedonic pathways and a presumably anorexigenic amino acid, TRP, has not been investigated. It leaves a significant gap in our knowledge of both how TRP affects consumption and whether circuits encompassing OT neurons can respond to changes in TRP availability. Therefore, I hypothesised that TRP will exhibit an anorexigenic effect and that this TRP-induced hypophagia will be mediated via central circuitry encompassing OT neurons.

1.7 Aims

The **overarching aim** of this doctoral research was t**o examine whether tryptophan suppresses food intake and whether this potential anorexigenic effect is mediated via central circuits that regulate consumption**. This project, utilising mice and rats as model organisms, encompassed three specific aims:

Specific Aim 1: To determine whether intragastric administration of TRP affects intake of tastants that differ in energy density and in palatability (palatable, non-caloric and caloric). To examine whether the changes in appetite observed after a TRP intragastric preload are accompanied by changes in neuronal activation (c-Fos), in brain areas that regulate food intake (Chapter 2).

Specific Aim 2: To determine whether the IP administration of TRP (thereby, preventing the direct action of TRP on gut mucosa) affects intake of tastants that differ in energy density and in palatability (palatable, non-caloric and caloric). To examine whether the changes in appetite observed after a TRP IP preload are accompanied by changes in neuronal activation (c-Fos), in brain areas that regulate food intake (Chapter 3).

Specific Aim 3: To determine whether the anorexigenic effect of TRP is accompanied by changes in OT neuronal activation. To assess whether OT receptor blockade with an OT antagonist alleviates the anorexigenic effect of TRP on consumption (Chapter 4).

1.8 References

1. of, H.M., *Annual Update of Key Results 2015/2016: New Zealand Health Survey*, M.o. Health, Editor. 2015: Wellington.
2. Halton, T.L. and F.B. Hu, *The effects of high protein diets on thermogenesis, satiety and weight loss: a critical review.* J Am Coll Nutr, 2004. **23**(5): p. 373-85.
3. Catenacci, V.A., J.O. Hill, and H.R. Wyatt, *The obesity epidemic.* Clin Chest Med, 2009. **30**(3): p. 415-44, vii.
4. Caballero, B., *The global epidemic of obesity: an overview*. Epidemiol Rev, 2007. **29**: p. 1-5.
5. Khaodhiar, L., K.C. McCowen, and G.L. Blackburn, *Obesity and its comorbid conditions.* Clin Cornerstone, 1999. **2**(3): p. 17-31.
6. Bray, G.A., *Food intake, sympathetic activity, and adrenal steroids.* Brain Res Bull, 1993. **32**(5): p. 537-41.
7. Allen, M.S. and B.J. Bradford, *Control of food intake by metabolism of fuels: a comparison across species.* Proc Nutr Soc, 2012. **71**(3): p. 401-9.
8. French, S.A., L.H. Epstein, R.W. Jeffery, J.E. Blundell, and J. Wardle, *Eating behavior dimensions. Associations with energy intake and body weight. A review.* Appetite, 2012. **59**(2): p. 541-9.
9. Hill, J.O., H.R. Wyatt, and J.C. Peters, *Energy balance and obesity*. Circulation, 2012. **126**(1): p. 126-32.
10. Camerino, C., *Low sympathetic tone and obese phenotype in oxytocin-deficient mice.* Obesity (Silver Spring), 2009. **17**(5): p. 980-4.
11. Zheng, H., N.R. Lenard, A.C. Shin, and H.R. Berthoud, *Appetite control and energy balance regulation in the modern world: reward-driven brain overrides repletion signals.* Int J Obes (Lond), 2009. **33 Suppl 2**: p. S8-13.
12. Poppitt, S.D., D. McCormack, and R. Buffenstein, *Short-term effects of macronutrient preloads on appetite and energy intake in lean women.* Physiol Behav, 1998. **64**(3): p. 279-85.
13. Westerterp-Plantenga, M.S., M.P. Lejeune, I. Nijs, M. van Ooijen, and E.M. Kovacs, *High protein intake sustains weight maintenance after body weight loss in humans.* Int J Obes Relat Metab Disord, 2004. **28**(1): p. 57-64.

14. Weigle, D.S., P.A. Breen, C.C. Matthys, H.S. Callahan, K.E. Meeuws, V.R. Burden, and J.Q. Purnell, *A high-protein diet induces sustained reductions in appetite, ad libitum caloric intake, and body weight despite compensatory changes in diurnal plasma leptin and ghrelin concentrations.* Am J Clin Nutr, 2005. **82**(1): p. 41-8.
15. Bensaid, A., D. Tome, D. Gietzen, P. Even, C. Morens, N. Gausseres, and G. Fromentin, *Protein is more potent than carbohydrate for reducing appetite in rats.* Physiol Behav, 2002. **75**(4): p. 577-82.
16. Jean, C., S. Rome, V. Mathe, J.F. Huneau, N. Aattouri, G. Fromentin, C.L. Achagiotis, and D. Tome, *Metabolic evidence for adaptation to a high protein diet in rats.* J Nutr, 2001. **131**(1): p. 91-8.
17. Lacroix, M., C. Gaudichon, A. Martin, C. Morens, V. Mathe, D. Tome, and J.F. Huneau, *A long-term high-protein diet markedly reduces adipose tissue without major side effects in Wistar male rats.* Am J Physiol Regul Integr Comp Physiol, 2004. **287**(4): p. R934-42.
18. Burton-Freeman, B., D.W. Gietzen, and B.O. Schneeman, *Meal pattern analysis to investigate the satiating potential of fat, carbohydrate, and protein in rats.* Am J Physiol, 1997. **273**(6 Pt 2): p. R1916-22.
19. Cota, D., K. Proulx, K.A. Smith, S.C. Kozma, G. Thomas, S.C. Woods, and R.J. Seeley, *Hypothalamic mTOR signaling regulates food intake.* Science, 2006. **312**(5775): p. 927-30.
20. Anderson, G.H., *Control of protein and energy intake: role of plasma amino acids and brain neurotransmitters.* Can J Physiol Pharmacol, 1979. **57**(10): p. 1043-57.
21. Seve, B., M.C. Meunier-Salaun, M. Monnier, Y. Colleaux, and Y. Henry, *Impact of dietary tryptophan and behavioral type on growth performance and plasma amino acids of young pigs.* J Anim Sci, 1991. **69**(9): p. 3679-88.
22. Wellendorph, P. and H. Brauner-Osborne, *Molecular basis for amino acid sensing by family C G-protein-coupled receptors.* Br J Pharmacol, 2009. **156**(6): p. 869-84.

23. Jordi, J., B. Herzog, S.M. Camargo, C.N. Boyle, T.A. Lutz, and F. Verrey, *Specific amino acids inhibit food intake via the area postrema or vagal afferents.* J Physiol, 2013. **591**(22): p. 5611-21.
24. Koehnle, T.J., M.C. Russell, and D.W. Gietzen, *Rats rapidly reject diets deficient in essential amino acids.* J Nutr, 2003. **133**(7): p. 2331-5.
25. Alamshah, A., A.K. McGavigan, E. Spreckley, J.S. Kinsey-Jones, A. Amin, I.R. Tough, H.C. O'Hara, A. Moolla, K. Banks, R. France, G. Hyberg, M. Norton, W. Cheong, A. Lehmann, S.R. Bloom, H.M. Cox, and K.G. Murphy, *L-arginine promotes gut hormone release and reduces food intake in rodents.* Diabetes Obes Metab, 2016. **18**(5): p. 508-18.
26. Morrison, C.D., X. Xi, C.L. White, J. Ye, and R.J. Martin, *Amino acids inhibit Agrp gene expression via an mTOR-dependent mechanism.* Am J Physiol Endocrinol Metab, 2007. **293**(1): p. E165-71.
27. McGavigan, A.K., H.C. O'Hara, A. Amin, J. Kinsey-Jones, E. Spreckley, A. Alamshah, A. Agahi, K. Banks, R. France, G. Hyberg, C. Wong, G.A. Bewick, J.V. Gardiner, A. Lehmann, N.M. Martin, M.A. Ghatei, S.R. Bloom, and K.G. Murphy, *L-cysteine suppresses ghrelin and reduces appetite in rodents and humans.* Int J Obes (Lond), 2015. **39**(3): p. 447-55.
28. Ng, L.T. and G.H. Anderson, *Route of administration of tryptophan and tyrosine affects short-term food intake and plasma and brain amino acid concentrations in rats.* J Nutr, 1992. **122**(2): p. 283-93.
29. Ayaso, R., H. Ghattas, M. Abiad, and O. Obeid, *Meal pattern of male rats maintained on amino acid supplemented diets: the effect of tryptophan, lysine, arginine, proline and threonine.* Nutrients, 2014. **6**(7): p. 2509-22.
30. Booth, D.A., A. Chase, and A.T. Campbell, *Relative effectiveness of protein in the late stages of appetite suppression in man.* Physiol Behav, 1970. **5**(11): p. 1299-302.
31. Westerterp-Plantenga, M.S., S.G. Lemmens, and K.R. Westerterp, *Dietary protein - its role in satiety, energetics, weight loss and health.* Br J Nutr, 2012. **108 Suppl 2**: p. S105-12.
32. Anderson, G.H. and S.E. Moore, *Dietary proteins in the regulation of food intake and body weight in humans.* J Nutr, 2004. **134**(4): p. 974S-9S.

33. Marmonier, C., D. Chapelot, and J. Louis-Sylvestre, *Effects of macronutrient content and energy density of snacks consumed in a satiety state on the onset of the next meal.* Appetite, 2000. **34**(2): p. 161-8.
34. Bellissimo, N. and T. Akhavan, *Effect of macronutrient composition on short-term food intake and weight loss.* Adv Nutr, 2015. **6**(3): p. 302S-8S.
35. Beasley, J.M., B.A. Ange, C.A. Anderson, E.R. Miller, 3rd, T.P. Erlinger, J.T. Holbrook, F.M. Sacks, and L.J. Appel, *Associations between macronutrient intake and self-reported appetite and fasting levels of appetite hormones: results from the Optimal Macronutrient Intake Trial to Prevent Heart Disease.* Am J Epidemiol, 2009. **169**(7): p. 893-900.
36. Stubbs, R.J., M.C. van Wyk, A.M. Johnstone, and C.G. Harbron, *Breakfasts high in protein, fat or carbohydrate: effect on within-day appetite and energy balance.* Eur J Clin Nutr, 1996. **50**(7): p. 409-17.
37. McArthur, L.H., W.F. Kelly, D.W. Gietzen, and Q.R. Rogers, *The role of palatability in the food intake response of rats fed high-protein diets.* Appetite, 1993. **20**(3): p. 181-96.
38. Bensaid, A., D. Tome, D. L'Heureux-Bourdon, P. Even, D. Gietzen, C. Morens, C. Gaudichon, C. Larue-Achagiotis, and G. Fromentin, *A high-protein diet enhances satiety without conditioned taste aversion in the rat.* Physiol Behav, 2003. **78**(2): p. 311-20.
39. Green, K.F., L.S. Holmstrom, and M.A. Wollman, *Relation of cue to consequence in rats: effect of recuperation from illness.* Behav Biol, 1974. **10**(4): p. 491-503.
40. Batterham, R.L., H. Heffron, S. Kapoor, J.E. Chivers, K. Chandarana, H. Herzog, C.W. Le Roux, E.L. Thomas, J.D. Bell, and D.J. Withers, *Critical role for peptide YY in protein-mediated satiation and body-weight regulation.* Cell Metab, 2006. **4**(3): p. 223-33.
41. van der Klaauw, A.A., J.M. Keogh, E. Henning, V.M. Trowse, W.S. Dhillo, M.A. Ghatei, and I.S. Farooqi, *High protein intake stimulates postprandial GLP1 and PYY release.* Obesity (Silver Spring), 2013. **21**(8): p. 1602-7.

42. Blom, W.A., A. Lluch, A. Stafleu, S. Vinoy, J.J. Holst, G. Schaafsma, and H.F. Hendriks, *Effect of a high-protein breakfast on the postprandial ghrelin response.* Am J Clin Nutr, 2006. **83**(2): p. 211-20.
43. Tome, D., J. Schwarz, N. Darcel, and G. Fromentin, *Protein, amino acids, vagus nerve signaling, and the brain.* Am J Clin Nutr, 2009. **90**(3): p. 838S-843S.
44. Fromentin, G., N. Darcel, C. Chaumontet, A. Marsset-Baglieri, N. Nadkarni, and D. Tome, *Peripheral and central mechanisms involved in the control of food intake by dietary amino acids and proteins.* Nutr Res Rev, 2012. **25**(1): p. 29-39.
45. Bowen, J., M. Noakes, and P.M. Clifton, *Appetite hormones and energy intake in obese men after consumption of fructose, glucose and whey protein beverages.* Int J Obes (Lond), 2007. **31**(11): p. 1696-703.
46. Cummings, D.E., J.Q. Purnell, R.S. Frayo, K. Schmidova, B.E. Wisse, and D.S. Weigle, *A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans.* Diabetes, 2001. **50**(8): p. 1714-9.
47. Cummings, D.E. and J. Overduin, *Gastrointestinal regulation of food intake.* J Clin Invest, 2007. **117**(1): p. 13-23.
48. Moran, T.H., *Gut peptides in the control of food intake.* Int J Obes (Lond), 2009. **33 Suppl 1**: p. S7-10.
49. Woods, S.C. and D.A. D'Alessio, *Central Control of Body Weight and Appetite.* The Journal of Clinical Endocrinology & Metabolism, 2008. **93**(11_supplement_1): p. s37-s50.
50. Woods, S.C., *Gastrointestinal satiety signals I. An overview of gastrointestinal signals that influence food intake.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2004. **286**(1): p. G7-G13.
51. Grill, H.J. and M.R. Hayes, *Hindbrain neurons as an essential hub in the neuroanatomically distributed control of energy balance.* Cell Metab, 2012. **16**(3): p. 296-309.
52. Guyenet, S.J. and M.W. Schwartz, *Clinical review: Regulation of food intake, energy balance, and body fat mass: implications for the*

pathogenesis and treatment of obesity. J Clin Endocrinol Metab, 2012. **97**(3): p. 745-55.

53. Blouet, C. and G.J. Schwartz, *Brainstem nutrient sensing in the nucleus of the solitary tract inhibits feeding.* Cell Metab, 2012. **16**(5): p. 579-87.
54. Blouet, C., Y.H. Jo, X. Li, and G.J. Schwartz, *Mediobasal hypothalamic leucine sensing regulates food intake through activation of a hypothalamus-brainstem circuit.* J Neurosci, 2009. **29**(26): p. 8302-11.
55. Guertin, D.A. and D.M. Sabatini, *The pharmacology of mTOR inhibition.* Sci Signal, 2009. **2**(67): p. pe24.
56. Bjornsti, M.A. and P.J. Houghton, *The TOR pathway: a target for cancer therapy.* Nat Rev Cancer, 2004. **4**(5): p. 335-48.
57. Morrison, C.D., S.D. Reed, and T.M. Henagan, *Homeostatic regulation of protein intake: in search of a mechanism.* Am J Physiol Regul Integr Comp Physiol, 2012. **302**(8): p. R917-28.
58. Laviano, A., M.M. Meguid, A. Inui, and F. Rossi-Fanelli, *Role of leucine in regulating food intake.* Science, 2006. **313**(5791): p. 1236-8; author reply 1236-8.
59. Wang, S.B., P. Khondowe, S.F. Chen, J.J. Yu, G. Shu, X.T. Zhu, L.N. Wang, P. Gao, Q.Y. Xi, Y.L. Zhang, and Q.Y. Jiang, *Effects of "Bioactive" amino acids leucine, glutamate, arginine and tryptophan on feed intake and mRNA expression of relative neuropeptides in broiler chicks.* Journal of Animal Science and Biotechnology, 2012. **3**.
60. Purpera, M.N., L. Shen, M. Taghavi, H. Munzberg, R.J. Martin, S.M. Hutson, and C.D. Morrison, *Impaired branched chain amino acid metabolism alters feeding behavior and increases orexigenic neuropeptide expression in the hypothalamus.* J Endocrinol, 2012. **212**(1): p. 85-94.
61. Mc, M.R. and J.L. Oncley, *The specific binding of L-tryptophan to serum albumin.* J Biol Chem, 1958. **233**(6): p. 1436-47.
62. Radwanski, E.R. and R.L. Last, *Tryptophan biosynthesis and metabolism: biochemical and molecular genetics.* Plant Cell, 1995. **7**(7): p. 921-34.

63. Keszthelyi, D., F.J. Troost, and A.A. Masclee, *Understanding the role of tryptophan and serotonin metabolism in gastrointestinal function.* Neurogastroenterol Motil, 2009. **21**(12): p. 1239-49.
64. Le Floc'h, N., W. Otten, and E. Merlot, *Tryptophan metabolism, from nutrition to potential therapeutic applications.* Amino Acids, 2011. **41**(5): p. 1195-1205.
65. Silber, B.Y. and J.A. Schmitt, *Effects of tryptophan loading on human cognition, mood, and sleep.* Neurosci Biobehav Rev, 2010. **34**(3): p. 387-407.
66. Acuna-Castroviejo, D., G. Escames, C. Venegas, M.E. Diaz-Casado, E. Lima-Cabello, L.C. Lopez, S. Rosales-Corral, D.X. Tan, and R.J. Reiter, *Extrapineal melatonin: sources, regulation, and potential functions.* Cell Mol Life Sci, 2014. **71**(16): p. 2997-3025.
67. Reiter, R.J., I. Sabry, M. Nordio, M.K. Vaughan, and S. Migliaccio, *Rate of reproductive involution following either exposure to short days or daily administration of melatonin is faster in inbred than in random-bred female Syrian hamsters.* J Endocrinol, 1989. **120**(3): p. 489-96.
68. Bender, D.A., *Biochemistry of tryptophan in health and disease.* Mol Aspects Med, 1983. **6**(2): p. 101-97.
69. Fernstrom, J.D., *Effects and side effects associated with the non-nutritional use of tryptophan by humans.* J Nutr, 2012. **142**(12): p. 2236S-2244S.
70. Fernstrom, J.D., *A Perspective on the Safety of Supplemental Tryptophan Based on Its Metabolic Fates.* J Nutr, 2016. **146**(12): p. 2601S-2608S.
71. Eccleston, D., G.W. Ashcroft, and T.B. Crawford, *Effect of tryptophan administration on 5HIAA in cerebrospinal fluid in man.* J Neurol Neurosurg Psychiatry, 1970. **33**(2): p. 269-72.
72. Oswald, I., G.W. Ashcroft, R.J. Berger, D. Eccleston, J.I. Evans, and V.R. Thacore, *Some experiments in the chemistry of normal sleep.* Br J Psychiatry, 1966. **112**(485): p. 391-9.
73. Jouvet, M., *Biogenic amines and the states of sleep.* Science, 1969. **163**(3862): p. 32-41.

74. Meltzer, H.Y., *Role of serotonin in depression.* Ann N Y Acad Sci, 1990. **600**: p. 486-99; discussion 499-500.
75. Voigt, J.P. and H. Fink, *Serotonin controlling feeding and satiety.* Behav Brain Res, 2015. **277**: p. 14-31.
76. Sainio, E.L., K. Pulkki, and S.N. Young, *L-Tryptophan: Biochemical, nutritional and pharmacological aspects.* Amino Acids, 1996. **10**(1): p. 21-47.
77. Ramadan, T., S.M. Camargo, V. Summa, P. Hunziker, S. Chesnov, K.M. Pos, and F. Verrey, *Basolateral aromatic amino acid transporter TAT1 (Slc16a10) functions as an efflux pathway.* J Cell Physiol, 2006. **206**(3): p. 771-9.
78. Pardridge, W.M., *Tryptophan transport through the blood-brain barrier: in vivo measurement of free and albumin-bound amino acid.* Life Sci, 1979. **25**(17): p. 1519-28.
79. Madras, B.K., E.L. Cohen, H.N. Munro, and R.J. Wurtman, *Elevation of serum free tryptophan, but not brain tryptophan, by serum nonesterified fatty acids.* Adv Biochem Psychopharmacol, 1974. **11**(0): p. 143-51.
80. McMenamy, R.H., *The binding of indole analogues to defatted human serum albumin at different chloride concentrations.* J Biol Chem, 1964. **239**: p. 2835-41.
81. Pardridge, W.M., *Brain metabolism: a perspective from the blood-brain barrier.* Physiol Rev, 1983. **63**(4): p. 1481-535.
82. Pardridge, W.M. and W.H. Oldendorf, *Kinetic analysis of blood-brain barrier transport of amino acids.* Biochim Biophys Acta, 1975. **401**(1): p. 128-36.
83. Richard, D.M., M.A. Dawes, C.W. Mathias, A. Acheson, N. Hill-Kapturczak, and D.M. Dougherty, *L-Tryptophan: Basic Metabolic Functions, Behavioral Research and Therapeutic Indications.* Int J Tryptophan Res, 2009. **2**: p. 45-60.
84. Boado, R.J., J.Y. Li, M. Nagaya, C. Zhang, and W.M. Pardridge, *Selective expression of the large neutral amino acid transporter at the blood-brain barrier.* Proc Natl Acad Sci U S A, 1999. **96**(21): p. 12079-84.

85. Shulkin, B.L., A.L. Betz, R.A. Koeppe, and B.W. Agranoff, *Inhibition of neutral amino acid transport across the human blood-brain barrier by phenylalanine.* J Neurochem, 1995. **64**(3): p. 1252-7.
86. Pardridge, W.M., *The role of blood-brain barrier transport of tryptophan and other neutral amino acids in the regulation of substrate-limited pathways of brain amino acid metabolism.* J Neural Transm Suppl, 1979(15): p. 43-54.
87. Fernstrom, J.D., *Large neutral amino acids: dietary effects on brain neurochemistry and function.* Amino Acids, 2013. **45**(3): p. 419-30.
88. Fernstrom, J.D., M.J. Hirsch, and D.V. Faller, *Tryptophan concentrations in rat brain. Failure to correlate with free serum tryptophan or its ratio to the sum of other serum neutral amino acids.* Biochem J, 1976. **160**(3): p. 589-95.
89. Fernstrom, J.D. and D.V. Faller, *Neutral amino acids in the brain: changes in response to food ingestion.* J Neurochem, 1978. **30**(6): p. 1531-8.
90. Leathwood, P.D. and J.D. Fernstrom, *Effect of an oral tryptophan carbohydrate load on tryptophan, large neutral amino-acid, and serotonin and 5-hydroxyindoleacetic acid levels in monkey brain.* Journal of Neural Transmission-General Section, 1990. **79**(1-2): p. 25-34.
91. Palego, L., L. Betti, A. Rossi, and G. Giannaccini, *Tryptophan Biochemistry: Structural, Nutritional, Metabolic, and Medical Aspects in Humans.* J Amino Acids, 2016. **2016**: p. 8952520.
92. Shabbir, F., A. Patel, C. Mattison, S. Bose, R. Krishnamohan, E. Sweeney, S. Sandhu, W. Nel, A. Rais, R. Sandhu, N. Ngu, and S. Sharma, *Effect of diet on serotonergic neurotransmission in depression.* Neurochem Int, 2013. **62**(3): p. 324-9.
93. Choi, S., B. DiSilvio, M.H. Fernstrom, and J.D. Fernstrom, *Effect of chronic protein ingestion on tyrosine and tryptophan levels and catecholamine and serotonin synthesis in rat brain.* Nutr Neurosci, 2011. **14**(6): p. 260-7.
94. Heeley, N. and C. Blouet, *Central Amino Acid Sensing in the Control of Feeding Behavior.* Front Endocrinol (Lausanne), 2016. **7**: p. 148.

95. Battistin, L., A. Grynbaum, and A. Lajtha, *The uptake of various amino acids by the mouse brain in vivo.* Brain Res, 1971. **29**(1): p. 85-99.
96. Oldendorf, W.H., *Brain uptake of radiolabeled amino acids, amines, and hexoses after arterial injection.* Am J Physiol, 1971. **221**(6): p. 1629-39.
97. Hawkins, R.A., R.L. O'Kane, I.A. Simpson, and J.R. Vina, *Structure of the blood-brain barrier and its role in the transport of amino acids.* J Nutr, 2006. **136**(1 Suppl): p. 218S-26S.
98. Killian, D.M. and P.J. Chikhale, *Predominant functional activity of the large, neutral amino acid transporter (LAT1) isoform at the cerebrovasculature.* Neurosci Lett, 2001. **306**(1-2): p. 1-4.
99. Betz, A.L. and G.W. Goldstein, *Polarity of the blood-brain barrier: neutral amino acid transport into isolated brain capillaries.* Science, 1978. **202**(4364): p. 225-7.
100. Peters, J.C., *Tryptophan nutrition and metabolism: an overview.* Adv Exp Med Biol, 1991. **294**: p. 345-58.
101. Allegri, G., C.V. Costa, A. Bertazzo, M. Biasiolo, and E. Ragazzi, *Enzyme activities of tryptophan metabolism along the kynurenine pathway in various species of animals.* Farmaco, 2003. **58**(9): p. 829-36.
102. Fleck, A., J. Shepherd, and H.N. Munro, *Protein synthesis in rat liver: influence of amino acids in diet on microsomes and polysomes.* Science, 1965. **150**(3696): p. 628-9.
103. Sidransky, H., D.S. Sarma, M. Bongiorno, and E. Verney, *Effect of dietary tryptophan on hepatic polyribosomes and protein synthesis in fasted mice.* J Biol Chem, 1968. **243**(6): p. 1123-32.
104. Henry, Y., B. Seve, Y. Colleaux, P. Ganier, C. Saligaut, and P. Jego, *Interactive effects of dietary levels of tryptophan and protein on voluntary feed intake and growth performance in pigs, in relation to plasma free amino acids and hypothalamic serotonin.* J Anim Sci, 1992. **70**(6): p. 1873-87.
105. Henry, Y., B. Seve, A. Mounier, and P. Ganier, *Growth performance and brain neurotransmitters in pigs as affected by tryptophan, protein, and sex.* J Anim Sci, 1996. **74**(11): p. 2700-10.

106. Moroni, F., *Tryptophan metabolism and brain function: focus on kynurenine and other indole metabolites.* Eur J Pharmacol, 1999. **375**(1-3): p. 87-100.
107. Stone, T.W. and L.G. Darlington, *Endogenous kynurenines as targets for drug discovery and development.* Nat Rev Drug Discov, 2002. **1**(8): p. 609-20.
108. Stone, T.W., *Neuropharmacology of quinolinic and kynurenic acids.* Pharmacol Rev, 1993. **45**(3): p. 309-79.
109. Vecsei, L., L. Szalardy, F. Fulop, and J. Toldi, *Kynurenines in the CNS: recent advances and new questions.* Nat Rev Drug Discov, 2013. **12**(1): p. 64-82.
110. Heyes, M.P., K. Saito, E.O. Major, S. Milstien, S.P. Markey, and J.H. Vickers, *A mechanism of quinolinic acid formation by brain in inflammatory neurological disease. Attenuation of synthesis from L-tryptophan by 6-chlorotryptophan and 4-chloro-3-hydroxyanthranilate.* Brain, 1993. **116 (Pt 6)**: p. 1425-50.
111. de Jong, W.H., R. Smit, S.J. Bakker, E.G. de Vries, and I.P. Kema, *Plasma tryptophan, kynurenine and 3-hydroxykynurenine measurement using automated on-line solid-phase extraction HPLC-tandem mass spectrometry.* J Chromatogr B Analyt Technol Biomed Life Sci, 2009. **877**(7): p. 603-9.
112. Kotake, Y. and I. Masayama, *The intermediary metabolism of tryptophan. XVIII. The mechanism of formation of kynurenine from tryptophan.* Z. physiol. Chem, 1936. **243**: p. 237-244.
113. Bertazzo, A., E. Ragazzi, M. Biasiolo, C.V. Costa, and G. Allegri, *Enzyme activities involved in tryptophan metabolism along the kynurenine pathway in rabbits.* Biochim Biophys Acta, 2001. **1527**(3): p. 167-75.
114. Knox, W., V. Auerbach, and E. Lin, *Enzymatic and metabolic adaptations in animals.* Physiological reviews, 1956. **36**(2): p. 164-254.
115. Ruddick, J.P., A.K. Evans, D.J. Nutt, S.L. Lightman, G.A. Rook, and C.A. Lowry, *Tryptophan metabolism in the central nervous system: medical implications.* Expert Rev Mol Med, 2006. **8**(20): p. 1-27.

116. Yamazaki, F., T. Kuroiwa, O. Takikawa, and R. Kido, *Human indolylamine 2,3-dioxygenase. Its tissue distribution, and characterization of the placental enzyme.* Biochem J, 1985. **230**(3): p. 635-8.

117. Murray, M.F., *Tryptophan depletion and HIV infection: a metabolic link to pathogenesis.* Lancet Infect Dis, 2003. **3**(10): p. 644-52.

118. Takikawa, O., R. Yoshida, R. Kido, and O. Hayaishi, *Tryptophan degradation in mice initiated by indoleamine 2,3-dioxygenase.* J Biol Chem, 1986. **261**(8): p. 3648-53.

119. Grohmann, U., F. Fallarino, and P. Puccetti, *Tolerance, DCs and tryptophan: much ado about IDO.* Trends Immunol, 2003. **24**(5): p. 242-8.

120. Moffett, J.R. and M.A. Namboodiri, *Tryptophan and the immune response.* Immunol Cell Biol, 2003. **81**(4): p. 247-65.

121. Gonzalez, E.M., L.A. Penedo, P. Oliveira-Silva, P. Campello-Costa, R.C. Guedes, and C.A. Serfaty, *Neonatal tryptophan dietary restriction alters development of retinotectal projections in rats.* Exp Neurol, 2008. **211**(2): p. 441-8.

122. Mandi, Y. and L. Vecsei, *The kynurenine system and immunoregulation.* J Neural Transm (Vienna), 2012. **119**(2): p. 197-209.

123. Gal, E.M. and A.D. Sherman, *Synthesis and metabolism of L-kynurenine in rat brain.* J Neurochem, 1978. **30**(3): p. 607-13.

124. Slattery, J.A., A.J. Page, C.L. Dorian, S.M. Brierley, and L.A. Blackshaw, *Potentiation of mouse vagal afferent mechanosensitivity by ionotropic and metabotropic glutamate receptors.* J Physiol, 2006. **577**(Pt 1): p. 295-306.

125. Smith, A.J., T.W. Stone, and R.A. Smith, *Neurotoxicity of tryptophan metabolites.* Biochem Soc Trans, 2007. **35**(Pt 5): p. 1287-9.

126. Nemeth, H., J. Toldi, and L. Vecsei, *Role of kynurenines in the central and peripheral nervous systems.* Curr Neurovasc Res, 2005. **2**(3): p. 249-60.

127. Wu, W., J.A. Nicolazzo, L. Wen, R. Chung, R. Stankovic, S.S. Bao, C.K. Lim, B.J. Brew, K.M. Cullen, and G.J. Guillemin, *Expression of tryptophan 2,3-dioxygenase and production of kynurenine pathway metabolites in triple transgenic mice and human Alzheimer's disease brain.* PLoS One, 2013. **8**(4): p. e59749.

128. Berlim, M.T. and G. Turecki, *Definition, assessment, and staging of treatment-resistant refractory major depression: a review of current concepts and methods.* Can J Psychiatry, 2007. **52**(1): p. 46-54.
129. McInnis, C.M., M.V. Thoma, D. Gianferante, L. Hanlin, X. Chen, J.G. Breines, S. Hong, and N. Rohleder, *Measures of adiposity predict interleukin-6 responses to repeated psychosocial stress.* Brain, behavior, and immunity, 2014. **42**: p. 33-40.
130. Reus, G.Z., K. Jansen, S. Titus, A.F. Carvalho, V. Gabbay, and J. Quevedo, *Kynurenine pathway dysfunction in the pathophysiology and treatment of depression: Evidences from animal and human studies.* J Psychiatr Res, 2015. **68**: p. 316-28.
131. Ikeda, M., H. Tsuji, S. Nakamura, A. Ichiyama, Y. Nishizuka, and O. Hayaishi, *Studies on the biosynthesis of nicotinamide adenine dinucleotide. Ii. A role of picolinic carboxylase in the biosynthesis of nicotinamide adenine dinucleotide from tryptophan in mammals.* J Biol Chem, 1965. **240**: p. 1395-401.
132. Kaszaki, J., Z. Palasthy, D. Erczes, A. Racz, C. Torday, G. Varga, L. Vecsei, and M. Boros, *Kynurenic acid inhibits intestinal hypermotility and xanthine oxidase activity during experimental colon obstruction in dogs.* Neurogastroenterol Motil, 2008. **20**(1): p. 53-62.
133. Favennec, M., B. Hennart, R. Caiazzo, A. Leloire, L. Yengo, M. Verbanck, A. Arredouani, M. Marre, M. Pigeyre, A. Bessede, G.J. Guillemin, G. Chinetti, B. Staels, F. Pattou, B. Balkau, D. Allorge, P. Froguel, and O. Poulain-Godefroy, *The kynurenine pathway is activated in human obesity and shifted toward kynurenine monooxygenase activation.* Obesity (Silver Spring), 2015. **23**(10): p. 2066-74.
134. Wolf, H., *The effect of hormones and vitamin B6 on urinary excretion of metabolites of the kynurenine pathway.* Scand J Clin Lab Invest Suppl, 1974. **136**: p. 1-186.
135. Gershon, M.D. and J. Tack, *The serotonin signaling system: from basic understanding to drug development for functional GI disorders.* Gastroenterology, 2007. **132**(1): p. 397-414.

136. Racke, K. and H. Schworer, *Regulation of serotonin release from the intestinal mucosa.* Pharmacol Res, 1991. **23**(1): p. 13-25.
137. Young, S.N. and S. Gauthier, *Tryptophan availability and the control of 5-hydroxytryptamine and tryptamine synthesis in human CNS.* Adv Exp Med Biol, 1981. **133**: p. 221-30.
138. Pardridge, W.M., *Blood-brain barrier carrier-mediated transport and brain metabolism of amino acids.* Neurochem Res, 1998. **23**(5): p. 635-44.
139. Sarwar, G. and H.G. Botting, *Liquid concentrates are lower in bioavailable tryptophan than powdered infant formulas, and tryptophan supplementation of formulas increases brain tryptophan and serotonin in rats.* J Nutr, 1999. **129**(9): p. 1692-7.
140. Cote, F., E. Thevenot, C. Fligny, Y. Fromes, M. Darmon, M.A. Ripoche, E. Bayard, N. Hanoun, F. Saurini, P. Lechat, L. Dandolo, M. Hamon, J. Mallet, and G. Vodjdani, *Disruption of the nonneuronal tph1 gene demonstrates the importance of peripheral serotonin in cardiac function.* Proc Natl Acad Sci U S A, 2003. **100**(23): p. 13525-30.
141. Rindi, G., A.B. Leiter, A.S. Kopin, C. Bordi, and E. Solcia, *The "normal" endocrine cell of the gut: changing concepts and new evidences.* Ann N Y Acad Sci, 2004. **1014**: p. 1-12.
142. Gershon, M.D., *5-Hydroxytryptamine (serotonin) in the gastrointestinal tract.* Curr Opin Endocrinol Diabetes Obes, 2013. **20**(1): p. 14-21.
143. Sikander, A., S.V. Rana, and K.K. Prasad, *Role of serotonin in gastrointestinal motility and irritable bowel syndrome.* Clin Chim Acta, 2009. **403**(1-2): p. 47-55.
144. Hansen, M.B. and A.B. Witte, *The role of serotonin in intestinal luminal sensing and secretion.* Acta Physiol (Oxf), 2008. **193**(4): p. 311-23.
145. Mawe, G.M. and J.M. Hoffman, *Serotonin signalling in the gut--functions, dysfunctions and therapeutic targets.* Nat Rev Gastroenterol Hepatol, 2013. **10**(8): p. 473-86.
146. Brookes, S.J., N.J. Spencer, M. Costa, and V.P. Zagorodnyuk, *Extrinsic primary afferent signalling in the gut.* Nat Rev Gastroenterol Hepatol, 2013. **10**(5): p. 286-96.

147. Walther, D.J., J.U. Peter, S. Bashammakh, H. Hortnagl, M. Voits, H. Fink, and M. Bader, *Synthesis of serotonin by a second tryptophan hydroxylase isoform.* Science, 2003. **299**(5603): p. 76.

148. Hornung, J.P., *The human raphe nuclei and the serotonergic system.* J Chem Neuroanat, 2003. **26**(4): p. 331-43.

149. Nobin, A. and A. Bjorklund, *Topography of the monoamine neuron systems in the human brain as revealed in fetuses.* Acta Physiol Scand Suppl, 1973. **388**: p. 1-40.

150. Takahashi, H., S. Nakashima, E. Ohama, S. Takeda, and F. Ikuta, *Distribution of serotonin-containing cell bodies in the brainstem of the human fetus determined with immunohistochemistry using antiserotonin serum.* Brain Dev, 1986. **8**(4): p. 355-65.

151. Charnay, Y. and L. Leger, *Brain serotonergic circuitries.* Dialogues Clin Neurosci, 2010. **12**(4): p. 471-87.

152. Harrold, J.A., T.M. Dovey, J.E. Blundell, and J.C. Halford, *CNS regulation of appetite.* Neuropharmacology, 2012. **63**(1): p. 3-17.

153. Halford, J.C. and J.A. Harrold, *5-HT 2C receptor agonists and the control of appetite*, in *Appetite Control*. 2012, Springer. p. 349-356.

154. Donovan, M.H. and L.H. Tecott, *Serotonin and the regulation of mammalian energy balance.* Front Neurosci, 2013. **7**: p. 36.

155. Gaddum, J.H. and Z.P. Picarelli, *Two kinds of tryptamine receptor.* Br J Pharmacol Chemother, 1957. **12**(3): p. 323-8.

156. Gothert, M. and E. Schlicker, *Classification of serotonin receptors.* J Cardiovasc Pharmacol, 1987. **10 Suppl 3**: p. S3-7.

157. Hannon, J. and D. Hoyer, *Molecular biology of 5-HT receptors.* Behav Brain Res, 2008. **195**(1): p. 198-213.

158. Fink, K.B. and M. Gothert, *5-HT receptor regulation of neurotransmitter release.* Pharmacol Rev, 2007. **59**(4): p. 360-417.

159. Lesurtel, M., C. Soll, R. Graf, and P.A. Clavien, *Role of serotonin in the hepato-gastroIntestinal tract: an old molecule for new perspectives.* Cell Mol Life Sci, 2008. **65**(6): p. 940-52.

160. Sanchez, M.G., M. Morissette, and T. Di Paolo, *Oestradiol modulation of serotonin reuptake transporter and serotonin metabolism in the brain of monkeys.* J Neuroendocrinol, 2013. **25**(6): p. 560-9.
161. Smitka, K., H. Papezova, K. Vondra, M. Hill, V. Hainer, and J. Nedvidkova, *The role of "mixed" orexigenic and anorexigenic signals and autoantibodies reacting with appetite-regulating neuropeptides and peptides of the adipose tissue-gut-brain axis: relevance to food intake and nutritional status in patients with anorexia nervosa and bulimia nervosa.* Int J Endocrinol, 2013. **2013**: p. 483145.
162. Wurtman, J.J. and R.J. Wurtman, *Drugs that enhance central serotoninergic transmission diminish elective carbohydrate consumption by rats.* Life Sci, 1979. **24**(10): p. 895-903.
163. Cajochen, C., K. Krauchi, and A. Wirz-Justice, *Role of melatonin in the regulation of human circadian rhythms and sleep.* J Neuroendocrinol, 2003. **15**(4): p. 432-7.
164. Hardeland, R., *Melatonin metabolism in the central nervous system.* Curr Neuropharmacol, 2010. **8**(3): p. 168-81.
165. Sanchez, S., S.D. Paredes, C.L. Sanchez, C. Barriga, R.J. Reiter, and A.B. Rodriguez, *Tryptophan administration in rats enhances phagocytic function and reduces oxidative metabolism.* Neuro Endocrinol Lett, 2008. **29**(6): p. 1026-32.
166. Herichova, I., M. Zeman, and J. Veselovský, *Effect of Tryptophan Administration of Melatonin Concentrations in the Pineal Gland, Plasma and Gastrointestinal Tract of Chickens.* Acta Veterinaria Brno, 1998. **67**(2): p. 89-95.
167. Huether, G., B. Poeggeler, A. Reimer, and A. George, *Effect of tryptophan administration on circulating melatonin levels in chicks and rats: evidence for stimulation of melatonin synthesis and release in the gastrointestinal tract.* Life Sci, 1992. **51**(12): p. 945-53.
168. Montano, M.E., V. Molpeceres, J.L. Mauriz, E. Garzo, I.B. Cruz, P. Gonzalez, and J.P. Barrio, *Effect of melatonin supplementation on food and*

water intake in streptozotocin-diabetic and non-diabetic male Wistar rats. Nutr Hosp, 2010. **25**(6): p. 931-8.

169. Narang, G.D., D.V. Singh, and C.W. Turner, *Effect of melatonin on thyroid hormone secretion rate and feed consumption of female rats.* Proc Soc Exp Biol Med, 1967. **125**(1): p. 184-8.

170. Del Fabbro, E., R. Dev, D. Hui, L. Palmer, and E. Bruera, *Effects of melatonin on appetite and other symptoms in patients with advanced cancer and cachexia: a double-blind placebo-controlled trial.* J Clin Oncol, 2013. **31**(10): p. 1271-6.

171. Gonnissen, H.K., R. Hursel, F. Rutters, E.A. Martens, and M.S. Westerterp-Plantenga, *Effects of sleep fragmentation on appetite and related hormone concentrations over 24 h in healthy men.* Br J Nutr, 2013. **109**(4): p. 748-56.

172. Brown, C.M., P.J. Fletcher, and D.V. Coscina, *Acute amino acid loads that deplete brain serotonin fail to alter behavior.* Pharmacol Biochem Behav, 1998. **59**(1): p. 115-21.

173. Nakamaru‐Ogiso, E., H. Miyamoto, K. Hamada, K. Tsukada, and K. Takai, *Novel biochemical manipulation of brain serotonin reveals a role of serotonin in the circadian rhythm of sleep–wake cycles.* European Journal of Neuroscience, 2012. **35**(11): p. 1762-1770.

174. Markov, D. and M. Goldman, *Normal sleep and circadian rhythms: neurobiologic mechanisms underlying sleep and wakefulness.* Psychiatr Clin North Am, 2006. **29**(4): p. 841-53; abstract vii.

175. Hartmann, E., J. Cravens, and S. List, *Hypnotic effects of L-tryptophan.* Arch Gen Psychiatry, 1974. **31**(3): p. 394-7.

176. Bravo, R., S. Matito, J. Cubero, S.D. Paredes, L. Franco, M. Rivero, A.B. Rodriguez, and C. Barriga, *Tryptophan-enriched cereal intake improves nocturnal sleep, melatonin, serotonin, and total antioxidant capacity levels and mood in elderly humans.* Age (Dordr), 2013. **35**(4): p. 1277-85.

177. Wyatt, R.J., T.N. Chase, D.J. Kupfer, J. Scott, and F. Snyder, *Brain catecholamines and human sleep.* Nature, 1971. **233**(5314): p. 63-5.

178. Wyatt, R.J., K. Engelman, D.J. Kupfer, D.H. Fram, A. Sjoerdsma, and F. Snyder, *Effects of L-tryptophan (a natural sedative) on human sleep.* Lancet, 1970. **2**(7678): p. 842-6.

179. Wyatt, R.J., K. Engelman, D.J. Kupfer, J. Scott, A. Sjoerdsma, and F. Snyder, *Effects of para-chlorophenylalanine on sleep in man.* Electroencephalogr Clin Neurophysiol, 1969. **27**(5): p. 529-32.

180. Chauffard-Alboucq, F.A., P.D. Leathwood, and C.A. Dormond, *Changes in plasma amino acid and subjective sleepiness ratings in humans after consumingL-tryptophan/maltodextrin mixes.* Amino Acids, 1991. **1**(1): p. 37-45.

181. George, C.F., T.W. Millar, P.J. Hanly, and M.H. Kryger, *The effect of L-tryptophan on daytime sleep latency in normals: correlation with blood levels.* Sleep, 1989. **12**(4): p. 345-53.

182. Spinweber, C.L., R. Ursin, R.P. Hilbert, and R.L. Hilderbrand, *L-tryptophan: effects on daytime sleep latency and the waking EEG.* Electroencephalogr Clin Neurophysiol, 1983. **55**(6): p. 652-61.

183. Hartmann, E. and C.L. Spinweber, *Sleep induced by L-tryptophan. Effect of dosages within the normal dietary intake.* J Nerv Ment Dis, 1979. **167**(8): p. 497-9.

184. Hartmann, E., *L-Tryptophan: effects on sleep.* Monogr Neural Sci, 1976. **3**: p. 26-32.

185. Heine, W.E., *The significance of tryptophan in infant nutrition.* Adv Exp Med Biol, 1999. **467**: p. 705-10.

186. Steinberg, L.A., N.C. O'Connell, T.F. Hatch, M.F. Picciano, and L.L. Birch, *Tryptophan intake influences infants' sleep latency.* J Nutr, 1992. **122**(9): p. 1781-91.

187. Janas, L.M., M.F. Picciano, and T.F. Hatch, *Indices of protein metabolism in term infants fed either human milk or formulas with reduced protein concentration and various whey/casein ratios.* J Pediatr, 1987. **110**(6): p. 838-48.

188. Cubero, J., V. Valero, J. Sanchez, M. Rivero, H. Parvez, A.B. Rodriguez, and C. Barriga, *The circadian rhythm of tryptophan in breast milk affects*

the rhythms of 6-sulfatoxymelatonin and sleep in newborn. Neuro Endocrinol Lett, 2005. **26**(6): p. 657-61.

189. Lucas, A. and I. St James-Roberts, *Crying, fussing and colic behaviour in breast- and bottle-fed infants.* Early Hum Dev, 1998. **53**(1): p. 9-18.
190. Horne, R.S., P.M. Parslow, D. Ferens, A.M. Watts, and T.M. Adamson, *Comparison of evoked arousability in breast and formula fed infants.* Arch Dis Child, 2004. **89**(1): p. 22-5.
191. Voderholzer, U., M. Hornyak, B. Thiel, C. Huwig-Poppe, A. Kiemen, A. Konig, J. Backhaus, D. Riemann, M. Berger, and F. Hohagen, *Impact of experimentally induced serotonin deficiency by tryptophan depletion on sleep EEG in healthy subjects.* Neuropsychopharmacology, 1998. **18**(2): p. 112-24.
192. Fernstrom, J.D., M.H. Fernstrom, and M.A. Gillis, *Acute effects of aspartame on large neutral amino acids and monoamines in rat brain.* Life Sci, 1983. **32**(14): p. 1651-8.
193. Bhagya, V., B.N. Srikumar, T.R. Raju, and B.S. Shankaranarayana Rao, *The selective noradrenergic reuptake inhibitor reboxetine restores spatial learning deficits, biochemical changes, and hippocampal synaptic plasticity in an animal model of depression.* J Neurosci Res, 2015. **93**(1): p. 104-20.
194. Delgado, P.L., D.S. Charney, L.H. Price, H. Landis, and G.R. Heninger, *Neuroendocrine and behavioral effects of dietary tryptophan restriction in healthy subjects.* Life Sci, 1989. **45**(24): p. 2323-32.
195. Delgado, P.L., L.H. Price, H.L. Miller, R.M. Salomon, G.K. Aghajanian, G.R. Heninger, and D.S. Charney, *Serotonin and the neurobiology of depression. Effects of tryptophan depletion in drug-free depressed patients.* Arch Gen Psychiatry, 1994. **51**(11): p. 865-74.
196. Young, S.N. and M. Leyton, *The role of serotonin in human mood and social interaction. Insight from altered tryptophan levels.* Pharmacol Biochem Behav, 2002. **71**(4): p. 857-65.
197. Thomson, J., H. Rankin, G.W. Ashcroft, C.M. Yates, J.K. McQueen, and S.W. Cummings, *The treatment of depression in general practice: a comparison of L-tryptophan, amitriptyline, and a combination of L-*

tryptophan and amitriptyline with placebo. Psychol Med, 1982. **12**(4): p. 741-51.

198. Franklin, M., I. Bermudez, H. Murck, N. Singewald, and S. Gaburro, *Sub-chronic dietary tryptophan depletion--an animal model of depression with improved face and good construct validity.* J Psychiatr Res, 2012. **46**(2): p. 239-47.
199. Gerson, S.C. and R.J. Baldessarini, *Motor effects of serotonin in the central nervous system.* Life Sci, 1980. **27**(16): p. 1435-51.
200. Fletcher, P.J., *Effects of 8-OH-DPAT, 5-CT and muscimol on behaviour maintained by a DRL20 schedule of reinforcement, following microinjection into the dorsal or median raphe nuclei.* Behav Pharmacol, 1994. **5**(3): p. 326-336.
201. Fletcher, P.J., *Effects of combined or separate 5,7-dihydroxytryptamine lesions of the dorsal and median raphe nuclei on responding maintained by a DRL 20s schedule of food reinforcement.* Brain Res, 1995. **675**(1-2): p. 45-54.
202. Wogar, M.A., C.M. Bradshaw, and E. Szabadi, *Impaired acquisition of temporal differentiation performance following lesions of the ascending 5-hydroxytryptaminergic pathways.* Psychopharmacology (Berl), 1992. **107**(2-3): p. 373-8.
203. Wogar, M.A., C.M. Bradshaw, and E. Szabadi, *Effect of lesions of the ascending 5-hydroxytryptaminergic pathways on choice between delayed reinforcers.* Psychopharmacology (Berl), 1993. **111**(2): p. 239-43.
204. Blokland, A., C. Lieben, and N.E. Deutz, *Anxiogenic and depressive-like effects, but no cognitive deficits, after repeated moderate tryptophan depletion in the rat.* J Psychopharmacol, 2002. **16**(1): p. 39-49.
205. Lu, X., Y. Wang, C. Liu, and Y. Wang, *Depressive disorder and gastrointestinal dysfunction after myocardial infarct are associated with abnormal tryptophan-5-hydroxytryptamine metabolism in rats.* PLoS One, 2017. **12**(2): p. e0172339.
206. Kohl, C., T. Walch, R. Huber, G. Kemmler, G. Neurauter, D. Fuchs, E. Solder, H. Schrocksnadel, and B. Sperner-Unterweger, *Measurement of*

tryptophan, kynurenine and neopterin in women with and without postpartum blues. J Affect Disord, 2005. **86**(2-3): p. 135-42.

207. Schrocksnadel, K., B. Widner, A. Bergant, G. Neurauter, H. Schennach, H. Schrocksnadel, and D. Fuchs, *Longitudinal study of tryptophan degradation during and after pregnancy.* Life Sci, 2003. **72**(7): p. 785-93.
208. Maes, M. and W. Rief, *Diagnostic classifications in depression and somatization should include biomarkers, such as disorders in the tryptophan catabolite (TRYCAT) pathway.* Psychiatry Res, 2012. **196**(2-3): p. 243-9.
209. Duan, K.M., J.H. Ma, S.Y. Wang, Z. Huang, Y. Zhou, and H. Yu, *The role of tryptophan metabolism in postpartum depression.* Metab Brain Dis, 2018.
210. Kovacs, K.J., *Measurement of immediate-early gene activation- c-fos and beyond.* J Neuroendocrinol, 2008. **20**(6): p. 665-72.
211. Rui, L., *Brain regulation of energy balance and body weight.* Rev Endocr Metab Disord, 2013. **14**(4): p. 387-407.
212. Olszewski, P.K., A. Klockars, and A.S. Levine, *Oxytocin: A Conditional Anorexigen whose Effects on Appetite Depend on the Physiological, Behavioural and Social Contexts.* J Neuroendocrinol, 2016. **28**(4).
213. Davis, J.D. and G.P. Smith, *Learning to sham feed: behavioral adjustments to loss of physiological postingestional stimuli.* Am J Physiol, 1990. **259**(6 Pt 2): p. R1228-35.
214. Ritter, R.C., *Gastrointestinal mechanisms of satiation for food.* Physiol Behav, 2004. **81**(2): p. 249-73.
215. Morton, G.J., T.H. Meek, and M.W. Schwartz, *Neurobiology of food intake in health and disease.* Nat Rev Neurosci, 2014. **15**(6): p. 367-78.
216. Morton, G.J., D.E. Cummings, D.G. Baskin, G.S. Barsh, and M.W. Schwartz, *Central nervous system control of food intake and body weight.* Nature, 2006. **443**(7109): p. 289-95.
217. Cohen, P., C. Zhao, X. Cai, J.M. Montez, S.C. Rohani, P. Feinstein, P. Mombaerts, and J.M. Friedman, *Selective deletion of leptin receptor in neurons leads to obesity.* J Clin Invest, 2001. **108**(8): p. 1113-21.

218. Zhang, Y., R. Proenca, M. Maffei, M. Barone, L. Leopold, and J.M. Friedman, *Positional cloning of the mouse obese gene and its human homologue.* Nature, 1994. **372**(6505): p. 425-32.

219. Rowland, N.E., J.W. Schaub, K.L. Robertson, A. Andreasen, and C. Haskell-Luevano, *Effect of MTII on food intake and brain c-Fos in melanocortin-3, melanocortin-4, and double MC3 and MC4 receptor knockout mice.* Peptides, 2010. **31**(12): p. 2314-7.

220. Balthasar, N., L.T. Dalgaard, C.E. Lee, J. Yu, H. Funahashi, T. Williams, M. Ferreira, V. Tang, R.A. McGovern, C.D. Kenny, L.M. Christiansen, E. Edelstein, B. Choi, O. Boss, C. Aschkenasi, C.Y. Zhang, K. Mountjoy, T. Kishi, J.K. Elmquist, and B.B. Lowell, *Divergence of melanocortin pathways in the control of food intake and energy expenditure.* Cell, 2005. **123**(3): p. 493-505.

221. Giraudo, S.Q., C.J. Billington, and A.S. Levine, *Feeding effects of hypothalamic injection of melanocortin 4 receptor ligands.* Brain Res, 1998. **809**(2): p. 302-6.

222. Tong, Q., C.P. Ye, J.E. Jones, J.K. Elmquist, and B.B. Lowell, *Synaptic release of GABA by AgRP neurons is required for normal regulation of energy balance.* Nat Neurosci, 2008. **11**(9): p. 998-1000.

223. Wu, G., *Amino acids: metabolism, functions, and nutrition.* Amino Acids, 2009. **37**(1): p. 1-17.

224. Sclafani, A. and D. Springer, *Dietary obesity in adult rats: similarities to hypothalamic and human obesity syndromes.* Physiol Behav, 1976. **17**(3): p. 461-71.

225. Cabanac, M. and K. Johnson, *Analysis of a conflict between palatability and cold exposure in rats.* Physiology & behavior, 1983. **31**(2): p. 249-253.

226. Oswald, K.D., D.L. Murdaugh, V.L. King, and M.M. Boggiano, *Motivation for palatable food despite consequences in an animal model of binge eating.* Int J Eat Disord, 2011. **44**(3): p. 203-11.

227. !!! INVALID CITATION !!! {}.

228. Berridge, K.C., C.Y. Ho, J.M. Richard, and A.G. DiFeliceantonio, *The tempted brain eats: pleasure and desire circuits in obesity and eating disorders.* Brain Res, 2010. **1350**: p. 43-64.
229. Egecioglu, E., K.P. Skibicka, C. Hansson, M. Alvarez-Crespo, P.A. Friberg, E. Jerlhag, J.A. Engel, and S.L. Dickson, *Hedonic and incentive signals for body weight control.* Rev Endocr Metab Disord, 2011. **12**(3): p. 141-51.
230. Pecina, S., K.S. Smith, and K.C. Berridge, *Hedonic hot spots in the brain.* Neuroscientist, 2006. **12**(6): p. 500-11.
231. David, V., A. Matifas, S. Gavello-Baudy, L. Decorte, B.L. Kieffer, and P. Cazala, *Brain regional Fos expression elicited by the activation of mu- but not delta-opioid receptors of the ventral tegmental area: evidence for an implication of the ventral thalamus in opiate reward.* Neuropsychopharmacology, 2008. **33**(7): p. 1746-59.
232. Woolley, J.D., B.S. Lee, B. Kim, and H.L. Fields, *Opposing effects of intra-nucleus accumbens mu and kappa opioid agonists on sensory specific satiety.* Neuroscience, 2007. **146**(4): p. 1445-52.
233. Laviolette, S.R., N.M. Lauzon, S.F. Bishop, N. Sun, and H. Tan, *Dopamine signaling through D1-like versus D2-like receptors in the nucleus accumbens core versus shell differentially modulates nicotine reward sensitivity.* J Neurosci, 2008. **28**(32): p. 8025-33.
234. Wise, R.A., *Role of brain dopamine in food reward and reinforcement.* Philos Trans R Soc Lond B Biol Sci, 2006. **361**(1471): p. 1149-58.
235. Pickel, V.M., E.T. Shobin, D.A. Lane, and K. Mackie, *Cannabinoid-1 receptors in the mouse ventral pallidum are targeted to axonal profiles expressing functionally opposed opioid peptides and contacting N-acylphosphatidylethanolamine-hydrolyzing phospholipase D terminals.* Neuroscience, 2012. **227**: p. 10-21.
236. McBride, W.J., B. Bodart, L. Lumeng, and T.K. Li, *Association between low contents of dopamine and serotonin in the nucleus accumbens and high alcohol preference.* Alcohol Clin Exp Res, 1995. **19**(6): p. 1420-2.
237. Neumaier, J.F., E.S. Vincow, A. Arvanitogiannis, R.A. Wise, and W.A. Carlezon, Jr., *Elevated expression of 5-HT1B receptors in nucleus*

accumbens efferents sensitizes animals to cocaine. J Neurosci, 2002. **22**(24): p. 10856-63.

238. Figlewicz, D.P. and A.J. Sipols, *Energy regulatory signals and food reward.* Pharmacol Biochem Behav, 2010. **97**(1): p. 15-24.
239. Hajnal, A. and R. Norgren, *Accumbens dopamine mechanisms in sucrose intake.* Brain Res, 2001. **904**(1): p. 76-84.
240. Hajnal, A. and R. Norgren, *Sucrose sham feeding decreases accumbens norepinephrine in the rat.* Physiol Behav, 2004. **82**(1): p. 43-7.
241. Kenny, P.J., *Reward mechanisms in obesity: new insights and future directions.* Neuron, 2011. **69**(4): p. 664-79.
242. Berridge, K.C., *Food reward: brain substrates of wanting and liking.* Neurosci Biobehav Rev, 1996. **20**(1): p. 1-25.
243. Olszewski, P.K. and A.S. Levine, *Central opioids and consumption of sweet tastants: when reward outweighs homeostasis.* Physiol Behav, 2007. **91**(5): p. 506-12.
244. Giraudo, S.Q., M.K. Grace, C.C. Welch, C.J. Billington, and A.S. Levine, *Naloxone's anorectic effect is dependent upon the relative palatability of food.* Pharmacol Biochem Behav, 1993. **46**(4): p. 917-21.
245. Llewellyn-Smith, I.J., D.O. Kellett, D. Jordan, K.N. Browning, and R.A. Travagli, *Oxytocin-immunoreactive innervation of identified neurons in the rat dorsal vagal complex.* Neurogastroenterol Motil, 2012. **24**(3): p. e136-46.
246. Swanson, L.W. and H.G. Kuypers, *The paraventricular nucleus of the hypothalamus: cytoarchitectonic subdivisions and organization of projections to the pituitary, dorsal vagal complex, and spinal cord as demonstrated by retrograde fluorescence double-labeling methods.* J Comp Neurol, 1980. **194**(3): p. 555-70.
247. Rinaman, L., *Oxytocinergic inputs to the nucleus of the solitary tract and dorsal motor nucleus of the vagus in neonatal rats.* J Comp Neurol, 1998. **399**(1): p. 101-9.
248. Succu, S., F. Sanna, C. Cocco, T. Melis, A. Boi, G.L. Ferri, A. Argiolas, and M.R. Melis, *Oxytocin induces penile erection when injected into the ventral*

tegmental area of male rats: role of nitric oxide and cyclic GMP. Eur J Neurosci, 2008. **28**(4): p. 813-21.

249. Sofroniew, M.V., *Projections from vasopressin, oxytocin, and neurophysin neurons to neural targets in the rat and human.* J Histochem Cytochem, 1980. **28**(5): p. 475-8.
250. Gould, B.R. and H.H. Zingg, *Mapping oxytocin receptor gene expression in the mouse brain and mammary gland using an oxytocin receptor-LacZ reporter mouse.* Neuroscience, 2003. **122**(1): p. 155-67.
251. Qi, J., J.Y. Yang, M. Song, Y. Li, F. Wang, and C.F. Wu, *Inhibition by oxytocin of methamphetamine-induced hyperactivity related to dopamine turnover in the mesolimbic region in mice.* Naunyn Schmiedebergs Arch Pharmacol, 2008. **376**(6): p. 441-8.
252. Leibowitz, S.F., N.J. Hammer, and K. Chang, *Hypothalamic paraventricular nucleus lesions produce overeating and obesity in the rat.* Physiol Behav, 1981. **27**(6): p. 1031-40.
253. Sims, J.S. and J.F. Lorden, *Effect of paraventricular nucleus lesions on body weight, food intake and insulin levels.* Behav Brain Res, 1986. **22**(3): p. 265-81.
254. Shor-Posner, G., A.P. Azar, S. Insinga, and S.F. Leibowitz, *Deficits in the control of food intake after hypothalamic paraventricular nucleus lesions.* Physiol Behav, 1985. **35**(6): p. 883-90.
255. Olson, B.R., M.D. Drutarosky, M.S. Chow, V.J. Hruby, E.M. Stricker, and J.G. Verbalis, *Oxytocin and an oxytocin agonist administered centrally decrease food intake in rats.* Peptides, 1991. **12**(1): p. 113-8.
256. Arletti, R., A. Benelli, and A. Bertolini, *Influence of oxytocin on feeding behavior in the rat.* Peptides, 1989. **10**(1): p. 89-93.
257. Deblon, N., C. Veyrat-Durebex, L. Bourgoin, A. Caillon, A.L. Bussier, S. Petrosino, F. Piscitelli, J.J. Legros, V. Geenen, M. Foti, W. Wahli, V. Di Marzo, and F. Rohner-Jeanrenaud, *Mechanisms of the anti-obesity effects of oxytocin in diet-induced obese rats.* PLoS One, 2011. **6**(9): p. e25565.
258. Sabatier, N., G. Leng, and J. Menzies, *Oxytocin, feeding, and satiety.* Front Endocrinol (Lausanne), 2013. **4**: p. 35.

259. Ho, J.M., V.T. Anekonda, B.W. Thompson, M. Zhu, R.W. Curry, B.H. Hwang, G.J. Morton, M.W. Schwartz, D.G. Baskin, S.M. Appleyard, and J.E. Blevins, *Hindbrain Oxytocin Receptors Contribute to the Effects of Circulating Oxytocin on Food Intake in Male Rats.* Endocrinology, 2014. **155**(8): p. 2845-57.

260. Kublaoui, B.M., T. Gemelli, K.P. Tolson, Y. Wang, and A.R. Zinn, *Oxytocin deficiency mediates hyperphagic obesity of Sim1 haploinsufficient mice.* Mol Endocrinol, 2008. **22**(7): p. 1723-34.

261. Swaab, D.F., J.S. Purba, and M.A. Hofman, *Alterations in the hypothalamic paraventricular nucleus and its oxytocin neurons (putative satiety cells) in Prader-Willi syndrome: a study of five cases.* J Clin Endocrinol Metab, 1995. **80**(2): p. 573-9.

262. Martin, A., M. State, G.M. Anderson, W.M. Kaye, J.M. Hanchett, C.W. McConaha, W.G. North, and J.F. Leckman, *Cerebrospinal fluid levels of oxytocin in Prader-Willi syndrome: a preliminary report.* Biol Psychiatry, 1998. **44**(12): p. 1349-52.

263. Olszewski, P.K., A. Klockars, H.B. Schioth, and A.S. Levine, *Oxytocin as feeding inhibitor: maintaining homeostasis in consummatory behavior.* Pharmacol Biochem Behav, 2010. **97**(1): p. 47-54.

264. Calatayud, S., E. Quintana, J. Esplugues, and M.D. Barrachina, *Role of central oxytocin in the inhibition by endotoxin of distension-stimulated gastric acid secretion.* Naunyn Schmiedebergs Arch Pharmacol, 1999. **360**(6): p. 676-82.

265. Renaud, L.P., M. Tang, M.J. McCann, E.M. Stricker, and J.G. Verbalis, *Cholecystokinin and gastric distension activate oxytocinergic cells in rat hypothalamus.* Am J Physiol, 1987. **253**(4 Pt 2): p. R661-5.

266. Nelson, E.E., J.R. Alberts, Y. Tian, and J.G. Verbalis, *Oxytocin is elevated in plasma of 10-day-old rats following gastric distension.* Brain Res Dev Brain Res, 1998. **111**(2): p. 301-3.

267. Olszewski, P.K., J.R. Waas, L.L. Brooks, F. Herisson, and A.S. Levine, *Oxytocin receptor blockade reduces acquisition but not retrieval of taste*

aversion and blunts responsiveness of amygdala neurons to an aversive stimulus. Peptides, 2013. **50**: p. 36-41.

268. Klockars, A., A.S. Levine, and P.K. Olszewski, *Central oxytocin and food intake: focus on macronutrient-driven reward.* Front Endocrinol (Lausanne), 2015. **6**: p. 65.
269. Amico, J.A., R.R. Vollmer, H.M. Cai, J.A. Miedlar, and L. Rinaman, *Enhanced initial and sustained intake of sucrose solution in mice with an oxytocin gene deletion.* Am J Physiol Regul Integr Comp Physiol, 2005. **289**(6): p. R1798-806.
270. Billings, L.B., J.A. Spero, R.R. Vollmer, and J.A. Amico, *Oxytocin null mice ingest enhanced amounts of sweet solutions during light and dark cycles and during repeated shaker stress.* Behav Brain Res, 2006. **171**(1): p. 134-41.
271. Inenaga, K., H. Imura, N. Yanaihara, and H. Yamashita, *Kappa-selective opioid receptor agonists leumorphin and dynorphin inhibit supraoptic neurons in rat hypothalamic slice preparations.* J Neuroendocrinol, 1990. **2**(3): p. 389-95.
272. Pumford, K.M., G. Leng, and J.A. Russell, *Morphine actions on supraoptic oxytocin neurones in anaesthetized rats: tolerance after i.c.v. morphine infusion.* J Physiol, 1991. **440**: p. 437-54.
273. Glass, M.J., C.J. Billington, and A.S. Levine, *Opioids and food intake: distributed functional neural pathways?* Neuropeptides, 1999. **33**(5): p. 360-8.
274. Le Merrer, J., J.A. Becker, K. Befort, and B.L. Kieffer, *Reward processing by the opioid system in the brain.* Physiol Rev, 2009. **89**(4): p. 1379-412.
275. Jarrett, T.M., M.S. McMurray, C.H. Walker, and J.M. Johns, *Cocaine treatment alters oxytocin receptor binding but not mRNA production in postpartum rat dams.* Neuropeptides, 2006. **40**(3): p. 161-7.
276. Baracz, S.J., N.A. Everett, I.S. McGregor, and J.L. Cornish, *Oxytocin in the nucleus accumbens core reduces reinstatement of methamphetamine-seeking behaviour in rats.* Addict Biol, 2016. **21**(2): p. 316-25.

277. Baracz, S.J., P.I. Rourke, M.C. Pardey, G.E. Hunt, I.S. McGregor, and J.L. Cornish, *Oxytocin directly administered into the nucleus accumbens core or subthalamic nucleus attenuates methamphetamine-induced conditioned place preference.* Behav Brain Res, 2012. **228**(1): p. 185-93.
278. Bahi, A., *The oxytocin receptor impairs ethanol reward in mice.* Physiol Behav, 2015. **139**: p. 321-7.
279. Kent, K., V. Arientyl, M.M. Khachatryan, and R.I. Wood, *Oxytocin induces a conditioned social preference in female mice.* J Neuroendocrinol, 2013. **25**(9): p. 803-10.
280. Heinrichs, M., B. von Dawans, and G. Domes, *Oxytocin, vasopressin, and human social behavior.* Front Neuroendocrinol, 2009. **30**(4): p. 548-57.
281. Singru, P.S., G. Wittmann, E. Farkas, G. Zseli, C. Fekete, and R.M. Lechan, *Refeeding-activated glutamatergic neurons in the hypothalamic paraventricular nucleus (PVN) mediate effects of melanocortin signaling in the nucleus tractus solitarius (NTS).* Endocrinology, 2012. **153**(8): p. 3804-14.
282. Olszewski, P.K., A. Klockars, A.M. Olszewska, R. Fredriksson, H.B. Schioth, and A.S. Levine, *Molecular, immunohistochemical, and pharmacological evidence of oxytocin's role as inhibitor of carbohydrate but not fat intake.* Endocrinology, 2010. **151**(10): p. 4736-44.
283. Herisson, F.M., L.L. Brooks, J.R. Waas, A.S. Levine, and P.K. Olszewski, *Functional relationship between oxytocin and appetite for carbohydrates versus saccharin.* Neuroreport, 2014. **25**(12): p. 909-14.
284. Mullis, K., K. Kay, and D.L. Williams, *Oxytocin action in the ventral tegmental area affects sucrose intake.* Brain Res, 2013. **1513**: p. 85-91.
285. Herisson, F.M., J.R. Waas, R. Fredriksson, H.B. Schioth, A.S. Levine, and P.K. Olszewski, *Oxytocin Acting in the Nucleus Accumbens Core Decreases Food Intake.* J Neuroendocrinol, 2016. **28**(4).
286. Zhang, G. and D. Cai, *Circadian intervention of obesity development via resting-stage feeding manipulation or oxytocin treatment.* Am J Physiol Endocrinol Metab, 2011. **301**(5): p. E1004-12.

287. Zhang, Y., K. Guo, R.E. LeBlanc, D. Loh, G.J. Schwartz, and Y.H. Yu, *Increasing dietary leucine intake reduces diet-induced obesity and improves glucose and cholesterol metabolism in mice via multimechanisms.* Diabetes, 2007. **56**(6): p. 1647-54.

288. Olszewski, P.K., K. Allen, and A.S. Levine, *Effect of oxytocin receptor blockade on appetite for sugar is modified by social context.* Appetite, 2015. **86**: p. 81-7.

289. Huang, W., A.F. Sved, and E.M. Stricker, *Vasopressin and oxytocin release evoked by NaCl loads are selectively blunted by area postrema lesions.* Am J Physiol Regul Integr Comp Physiol, 2000. **278**(3): p. R732-40.

2 Effects of intragastric preloads of L-tryptophan on ingestive behaviour: a link with key central mechanisms that regulate consumption

2.1 Abstract

Human and laboratory animal studies suggest that dietary supplementation of a free essential amino acid, L-tryptophan (TRP), reduces food intake. It is unclear whether an acute gastric preload of TRP decreases consumption and whether central mechanisms underlie TRP-driven hypophagia. I examined the effect of TRP administered via intragastric gavage on both energy- and palatability-induced feeding in mice. Then sought to identify central mechanisms that mediate the anorexigenic effect of TRP. These effects on the consumption of energy-dense and energy-dilute tastants were established in mice stimulated to eat by energy deprivation or palatability. A conditioned taste aversion (CTA) paradigm was used to assess whether hypophagia is unrelated to sickness. c-Fos immunohistochemistry was employed to detect TRP-induced activation of feeding-related brain sites associated with feeding-related circuitry. TRP reduced intake of energy-dense standard chow in deprived animals and energy-dense palatable chow in sated mice. A CTA was not induced by anorexigenic doses of TRP. Intragastric TRP failed to affect intake of palatable yet calorie-dilute or noncaloric solutions (10% sucrose, 4.1% Intralipid or 0.1% saccharin) even for TRP doses that decreased water intake in thirsty mice. Fos immunoreactivity analysis revealed that TRP increases activation of several key feeding-related brain areas, especially in the brainstem and

hypothalamus. In sum, intragastric TRP preloads decrease food and water intake, and that TRP-induced hypophagia is partially mediated via central satiety-related circuits.

2.2 Introduction

Elevated dietary protein reduces food intake by affecting oral, postoral and postabsorptive mechanisms, including consumption-related reward, satiation, and energy metabolism [1-3]. While the gross protein value greatly affects energy balance, it has been suggested that in the process of ensuring that precise nutritional requirements are met - individual amino acids may play a role in regulating a drive to ingest food [4]. The need to control food intake is particularly pressing in relation to the essential amino acids, i.e., those that cannot be synthesised by the mammalian organism [5]. Thus far, an anorexigenic effect has been previously reported following the administration of individual amino acids, for example, L-leucine, L-arginine, L-lysine, L-cysteine and L-glutamic acid [6-12].

One such amino acid is L-tryptophan (TRP) [13, 14]. TRP interacts indirectly via ubiquitous G-protein coupled receptors [15]. Also, it enters the general circulation directly from the GI tract via transporters in a dose-dependent manner within minutes after intragastric delivery [16-18], and ultimately crosses the BBB thereby directly affecting CNS neurons [13, 19]. The LAT1 transporter is the main amino acid transporter in the BBB and is responsible for the transport predominantly of essential amino acids (including TRP) from the luminal side [20-22]. Thus, the physiological and behavioural effects of dietary TRP stem from engaging a combination of peripheral and central processes.

In studies focusing on the regulation of feeding behaviour, TRP has been proposed to promote hypophagia; however, the data remain somewhat inconsistent. For example, daily dietary supplementation of TRP at 3.0 g/kg body weight reduced

food intake in ad libitum-fed rats by ca 25% [16]. Intragastrically administered 200-600 mg/kg TRP has been found to cause a similar decrease in consumption in rats maintained on the 12-h/day availability schedule of both standard and high-carbohydrate (protein-free) food [17]. More recently, the intraduodenal infusion of low-caloric TRP loads (either 0.075 or 0.15 kcal/min for 90 minutes) in humans, suppressed acute energy intake and increased fullness after the post-infusion standardised buffet meal [23]. On the other hand, Jordi et al. (2013) reported only an insignificant reduction in chow intake in rats treated with intragastric TRP after 12 h of fasting, although the experiment may have been statistically underpowered [6].

The current set of studies was focused on providing more systematic evidence related to the presumed role of intragastric TRP in feeding control and the involvement of central circuitry in mediating TRP-induced hypophagia. The intragastric route for administration was selected as it most resembles normal dietary intake of amino acids, thereby allowing the normal uptake of amino acids within the GI tract, whilst bypassing the taste receptors [17].

Using the mouse model, I examined the acute effects of intragastric TRP preload on deprivation-induced intake of energy-dense chow as well as on the intake of energy-dense palatable chow in non-deprived animals. A CTA paradigm was employed to provide evidence that TRP-driven anorexia does not arise from sickness/malaise [24]. Then, I determined whether TRP can diminish palatability-driven consumption of calorie-dilute solutions, and whether the anorexigenic effect extends onto water intake.

Furthermore, I expanded on the aforementioned evidence indicating the involvement of the brain in mediating anorexic properties of amino acids by treating mice with a predetermined hypophagic dose of TRP and mapping c-Fos immunoreactivity (c-Fos-IR, a marker of neuronal activation [25]) in central sites known to control appetite [26].

2.3 Materials and methods

2.3.1 Animals and intragastric administration

Adult male (12 weeks) C57BL/6 mice were housed individually under a 12:12 h light/dark cycle (lights on at 07:00) and maintained at a controlled temperature of 22˚C. The mice had ad libitum access to tap water and standard laboratory chow (Specialty Diets, Australia) unless specified otherwise. All procedures received prior approval from the University of Waikato animal ethics committee.

Animals were acclimatised to the oral gavage procedure prior to the studies. Individuals either received an equal volume (0.2ml) of water or L-TRP solution (Sigma, UK).

2.3.2 Consummatory behaviour studies

2.3.2.1 Effect of TRP on deprivation-induced intake of standard chow

Animals were divided into **four** groups (n=7-9/group) and were food-deprived overnight. They were then given either TRP (**600**, 1200 or 1800 mg/kg TRP in 0.2ml water) or equal volume of vehicle (water) intragastrically 20 minutes prior to re-feeding (food returned to cages at 10:00). The doses were selected based on the

study by Jordi et al. (2013) on amino acid intragastric administration, in which 6.7 mmol/kg molar (equivalent to 1200 mg/kg TRP) was tested as the standard dose for all amino acids [6]. Chow intake was measured at 1 and 4 h. Water was available at all times. To control for the effect of amino acid-derived osmolality, in a separate experiment, alanine intragastric infusions (6.7 mmol/kg, which is a molar equivalent to 1200 mg/kg TRP; the same volume was infused) was used versus water and saw no effect on deprivation-induced food intake (Figure 2.2 D).

2.3.2.2 Effect of TRP on the intake of palatable chow in non-deprived mice

In order to examine whether TRP decreases the consumption of palatable solid food in non-deprived animals, by following our previously published protocol ([27, 28]), then gave mice 2-h access to palatable goat milk-enriched chow (3.5 kcal/g; Plant & Food Research, New Zealand) at 10:00. Twenty minutes earlier, the animals received TRP (600 mg/kg or 1200 mg/kg) or vehicle (n=6/group). Standard chow was removed from the cages from the time of intragastric infusion until the end of the 2-h period of palatable diet availability. The animals had had previous episodic exposure to this chow to avoid neophobia.

2.3.2.3 Effect of TRP on the episodic intake of palatable solutions

The paradigm described herein was based on the protocol used in our earlier studies [28-30]. The palatable solution tests were done in separate crossover tests using different mice. Three cohorts of mice were accustomed to receiving 10% sucrose, 0.1% saccharin or 4.1% Intralipid palatable solutions for 2 h/day on 2 days (10:00-12:00) prior to the experiment to avoid neophobia (sucrose study: n=7-8/group; saccharin study: n=7/group; Intralipid study: n=7/group). Intralipid and sucrose

were isocaloric. On the experimental day, animals were given TRP (saccharin and Intralipid: 600 mg/kg and 1200 mg/kg TRP; sucrose: 1200 mg/kg and 1800 mg/kg TRP) or vehicle 20 minutes before gaining access to the solutions. Chow and water were removed from the cages from the time of intragastric infusion until the end of the 2-h testing period. Palatable solution intake was measured after 2 h by weighing bottles.

2.3.2.4 Effect of TRP on water intake in water-deprived rats

Overnight water-deprived mice were treated with intragastric TRP (600 mg/kg or 1200 mg/kg) or vehicle (n=6/group) 20 minutes prior to regaining access to water. At 10:00, water bottles were returned to the cages and intake was measured 2 h post-injection. Chow was removed from hoppers from the time of intragastric infusion until the end of the 2-h water intake test.

2.3.3 Effect of TRP on the acquisition of a CTA

The standard CTA protocol was applied utilising a novel flavoured solution (see e.g., [30]). Mice had water taken away 16 h before their first exposure to a novel strawberry-flavoured KoolAid solution (2 h; from 10:00 to 12:00; Kraft Foods; prepared fresh according to the manufacturer's instructions) followed by intragastric infusion of 1200 mg/kg TRP (the dose that reliably caused hypophagia in all of the aforementioned models in which TRP was found to be anorexigenic) or saline (n=7/group). One hour after the treatment, water was returned to the cages to avoid dehydration. Two days later, animals were deprived of water again and, after 18 h, they were subjected to a 2-h two-bottle test in which a choice between the strawberry-flavoured KoolAid and water were given. Intakes of water and

KoolAid were measured after 2 h, and preference was expressed as the percentage of KoolAid intake in the total volume of consumed fluid (KoolAid+water).

2.3.4 Immunohistochemistry

The mice received either a single intragastric infusion of 1200 mg/kg TRP (the dose that reliably caused hypophagia in all of the aforementioned models in which TRP was found to be anorexigenic) or water vehicle (n=6/group for single Fos staining). Immediately after the treatment, which was performed between 10:00-11:00, both food and water were removed from the cages. Animals were anaesthetised (35% urethane) and perfused with saline (20mL) followed by 50mL of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) between 80-90 minutes after TRP/vehicle infusions. Brains were removed and postfixed overnight in PFA at 4˚C. Coronal 60 – um vibratome (Leica, Germany) sections were processed for c-Fos immunostaining. The tissue was treated for 10 minutes in 3% H2O2 in 10% methanol (in Tris-buffered saline (TBS); pH 7.4) and incubated overnight at 4˚C in the rabbit anti-Fos antibody (1:1400; Synaptic Systems, Goettingen, Germany). The next day, sections were incubated for 1 h in the secondary goat-anti-rabbit antibody (1:400; Vector Laboratories, USA), then rinsed in the buffer before incubating in the avidin-biotin complex (1 h; Vector Laboratories). Peroxidase was visualised with 0.05% DAB, 0.01 % H2O2 and 0.2% nickel sulphate (Sigma, USA). All incubations were conducted in a mixture of 0.25% gelatin and 0.5% Triton X-100 (Sigma, USA) in TBS. Intermediate rinsing was performed with TBS. Sections were mounted on gelatinised slides, dried, dehydrated in ascending concentrations of ethanol, soaked in xylene and embedded in Entellan (Merck, Germany). The number of c-Fos immunoreactive nuclei was

counted bilaterally in all regions of interest (4-5 sections containing a given site per animal) by a person blinded to the experimental group allocation. Densities of c-Fos positive nuclei (per mm2) were averaged per mouse and then per group.

2.3.5 Statistical analyses

Data from all consummatory behaviour studies utilising single TRP administration were processed with a Student's t-test (two-group comparisons) or a one-way ANOVA followed by Dunnet's post-hoc analysis (multiple-group comparisons). Correction for multiple comparisons was applied. Values are presented as means ± S.E.M and they were deemed significantly different when $p \leq 0.05$.

2.4 Results

Intragastric administration of TRP caused a statistically significant reduction in the amount of standard chow eaten after overnight energy deprivation (Figure 2.1 A). This decrease was evident for 1200 and 1800 mg/kg doses at 1 h ($F(3, 26)=12.96$; 1200 mg/kg $P=0.004$; 1800 mg/kg, $P=0.004$) and 4 h ($F(3, 26)=13.6$; 1200 mg/kg $P=0.002$; 1800 mg/kg, $P=0.012$) of re-feeding, but there was no effect at 24 h. In sated mice that were stimulated to eat by brief, 2-h, access to palatable chow, not only 1200 mg/kg TRP, but also a 600-mg/kg dose decreased consumption ($F(2, 15)=5.24$; $P=0.028$ and 0.022, respectively; Figure 2.1 B). Importantly, 1200 mg/kg TRP did not promote the development of a CTA to a novel KoolAid solution (Figure 2.2 A). Alanine, used in this study as an osmotic control, did not cause a reduction in deprivation-induced chow intake (Figure 2.2 B).

1200 mg/kg TRP significantly reduced water intake induced by water deprivation ($F(2, 14)=7.73$; $P=0.003$; Figure 2.3 A). TRP failed to affect consumption of palatable non-caloric saccharin (Figure 2.3 B) and calorie-dilute (0.4 kcal/g) 10% sucrose and 4.1% Intralipid solutions in non-deprived mice (Figure 2.4 A, B). Even a dose as high as 1800 mg/kg, TRP did not modify sucrose solution intake.

Administration of TRP at 1200 mg/kg, the dose that caused the most reliable anorexigenic effect in all consumption paradigms in which TRP generated a statistically significant change, affected c-Fos immunoreactivity in 8 out of 13 specific brain regions involved in the regulation of ingestive behaviour (Figure 2.5 A). An increase was observed in the dorsal medial nucleus of the vagus (DMNV; $P=0.049$) and nucleus of the solitary tract (NTS; $P=0.006$) in the brainstem (Figure 2.6 C), in the hypothalamic PVN ($P=0.001$), SON ($P=0.024$) (Figure 2.5 B, C) and dorsomedial (DMH; $P=0.001$) nuclei (Figure 2.6 A), as well as in the central nucleus of the amygdala (CeA; $P=0.001$) (Figure 2.6 B). A decrease in the density of c-Fos positive was noted in the core and shell subdivisions of the nucleus accumbens (AcbC, $P=0.019$; AcbSh, $P=0.027$) (Figure 2.6 D).

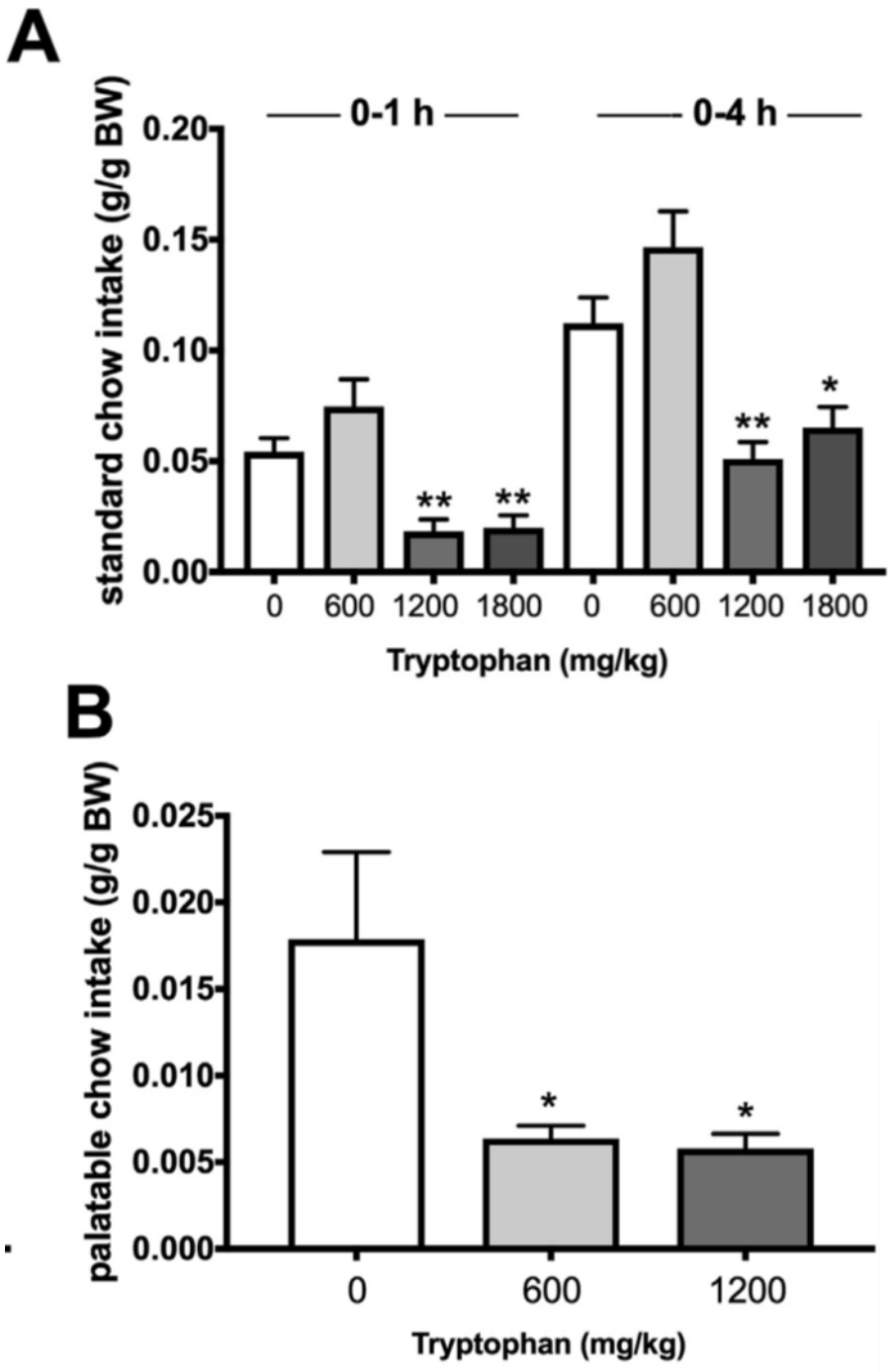

Figure 2.1 The effect of intragastric TRP preload on (A) deprivation-induced intake of standard chow at 1h and 4 h of re-feeding; and (B) intake of palatable chow in non-deprived animals. Doses are in mg/kg BW. Water served as vehicle (0 mg). * - P≤0.05; ** - P≤0.01; * - P≤0.001.**

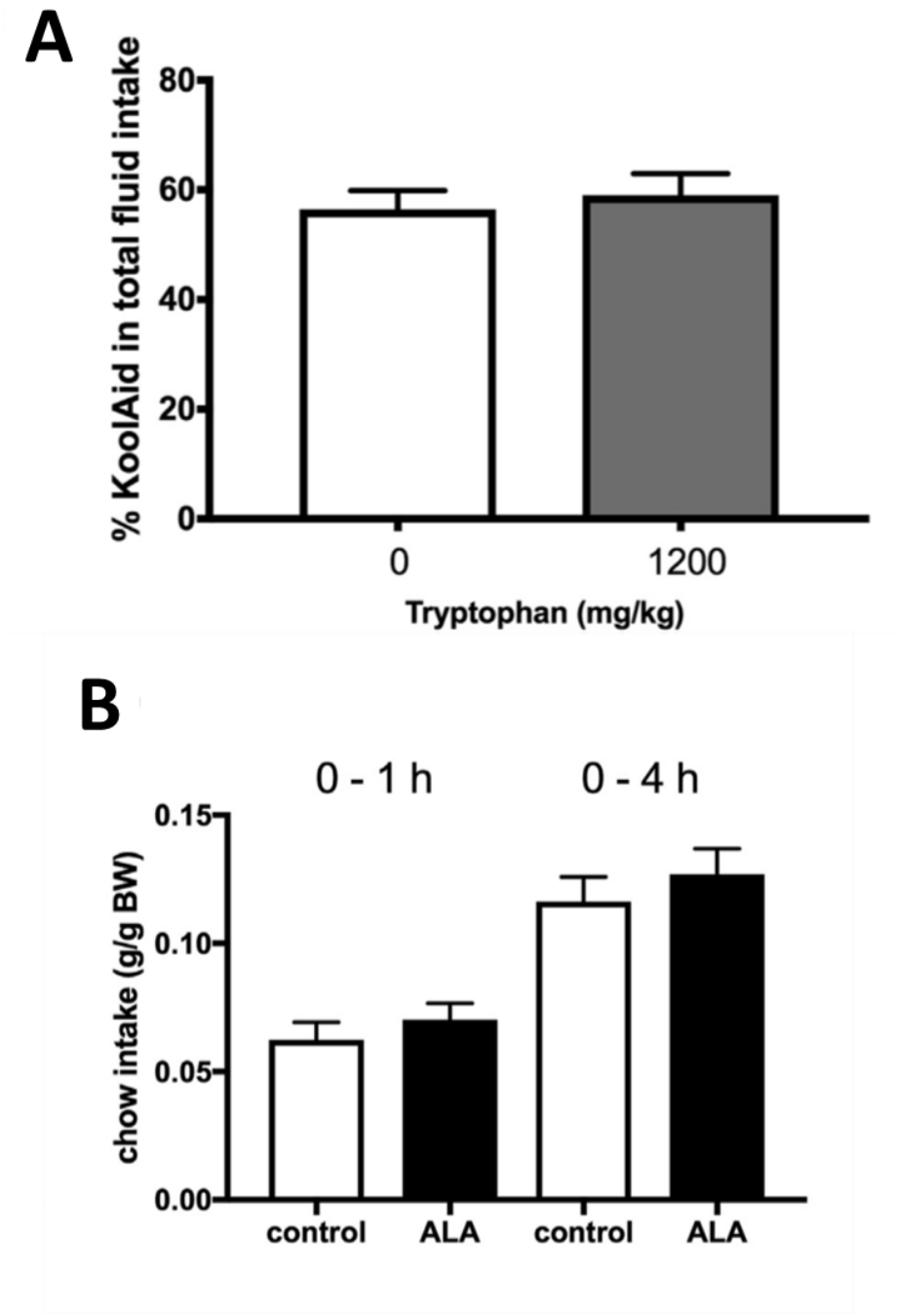

Figure 2.2 The effect of intragastric TRP preload on (A) acquisition of a conditioned taste aversion (CTA) to a novel KoolAid solution (expressed as a percentage of KoolAid intake in the total KoolAid+water consumption assessed in a two-bottle choice test performed 48 h after the initial exposure to KoolAid). Panel (B) shows deprivation-induced chow intake after intragastric alanine (as an osmotic control for TRP). Doses are in mg/kg BW. Water served as vehicle (0 mg). * - P≤0.05; ** - P≤0.01; * - P≤0.001.**

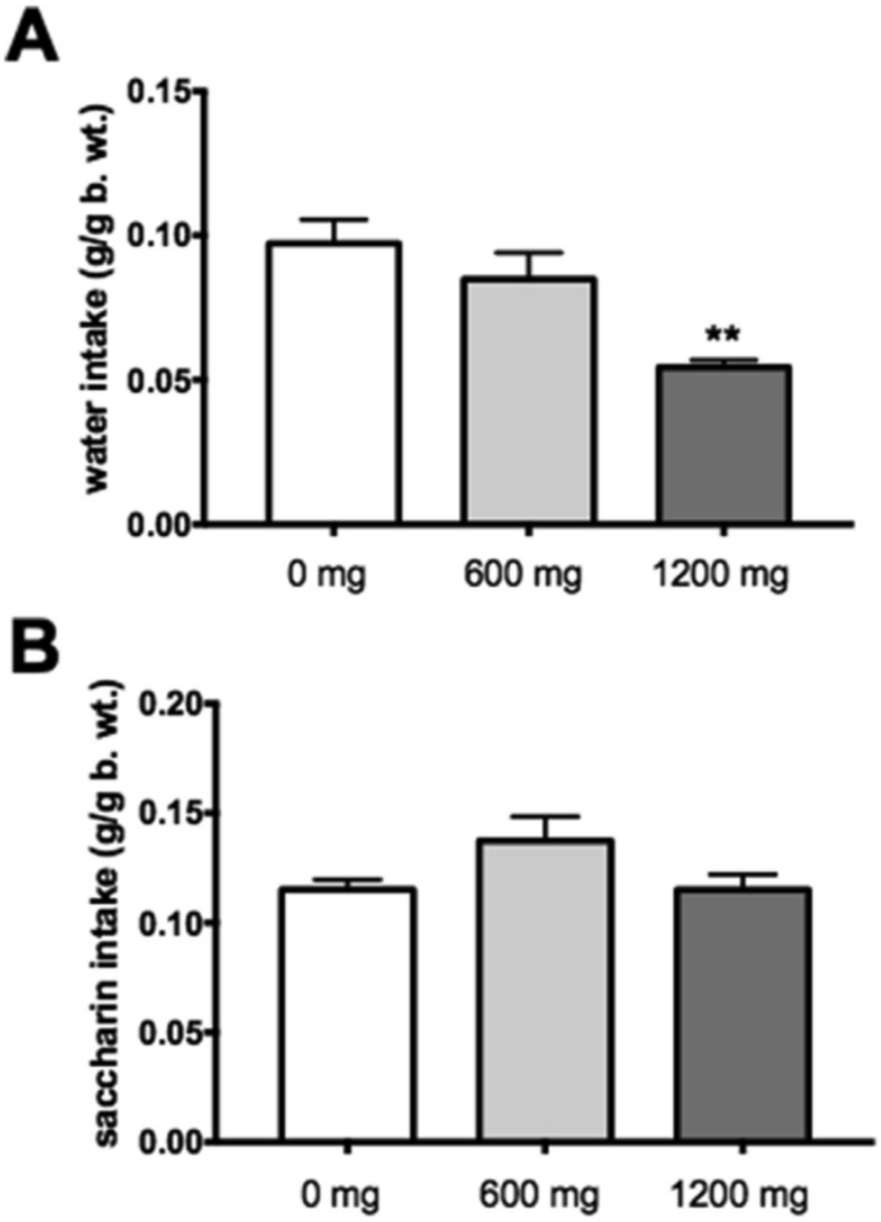

Figure 2.3 The effect of intragastric TRP preload on (A) water intake induced by water deprivation; and (B) palatability-induced intake of a 0.1% saccharin solution. TRP doses are in mg/kg BW. Water served as vehicle (0 mg). * - P≤0.05; ** - P≤0.01.

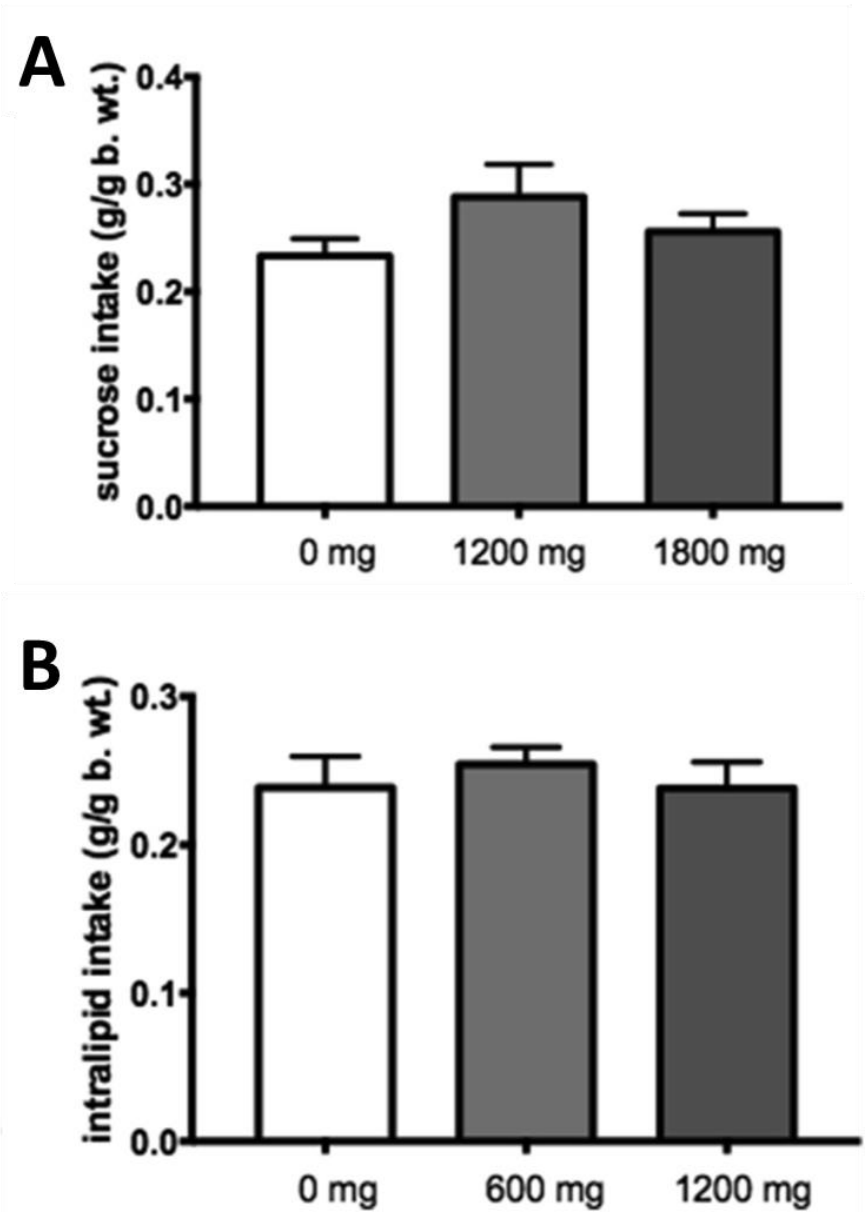

Figure 2.4 The effect of intragastric TRP preload on (C) palatability-induced intake of a 10% sucrose solution, and (D) palatability-induced intake of a 4.1% Intralipid solution. TRP doses are in mg/kg BW. Water served as vehicle (0 mg). * - P≤0.05; ** - P≤0.01.

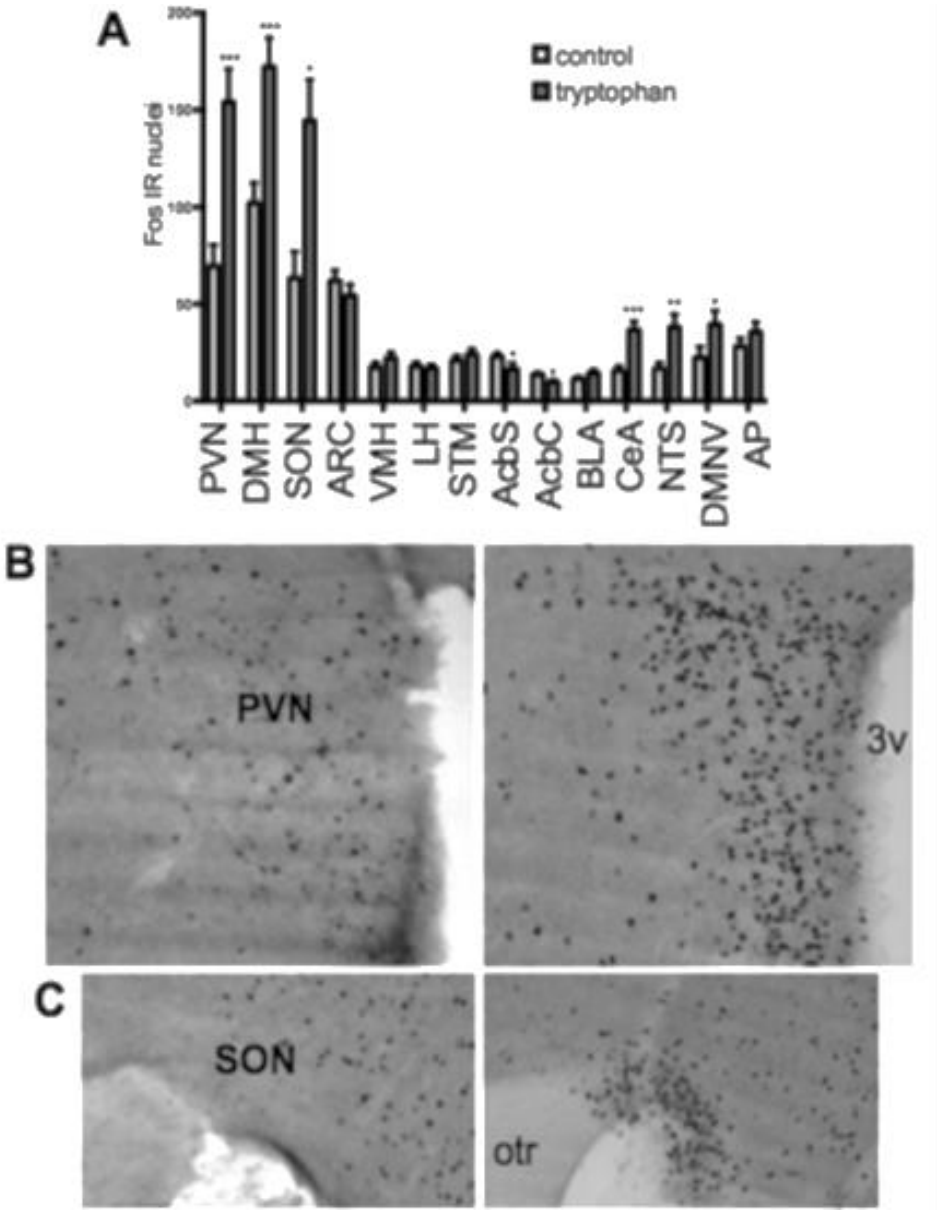

Figure 2.5 c-Fos immunoreactivity in select feeding-related brain sites following intragastric administration of 1200 mg/kg TRP versus vehicle. The graph (A) shows densities of Fos positive nuclear profiles per mm2 of each area of interest. Photomicrographs (B-C) depict c-Fos in some brain areas in which a significant change was noted (control: left panels; TRP: right panel). 3v – third ventricle; 4v – fourth ventricle; aca – anterior commissure; AcbC – nucleus accumbens core; AcbS – nucleus accumbens shell; AP – area postrema; ARC – arcuate nucleus; BLA – basolateral amygdala; CeA – central nucleus of the amygdala; DMH – dorsomedial hypothalamic nucleus; DMNV – dorsal motor nucleus of the vagus; LH – lateral hypothalamus; NTS – nucleus of the solitary tract; otr – optic tract; PVN – paraventricular hypothalamic nucleus; SON – supraoptic nucleus; VMH – ventromedial hypothalamic nucleus; * - P≤0.05; ** - P≤0.01; * - P≤0.001.**

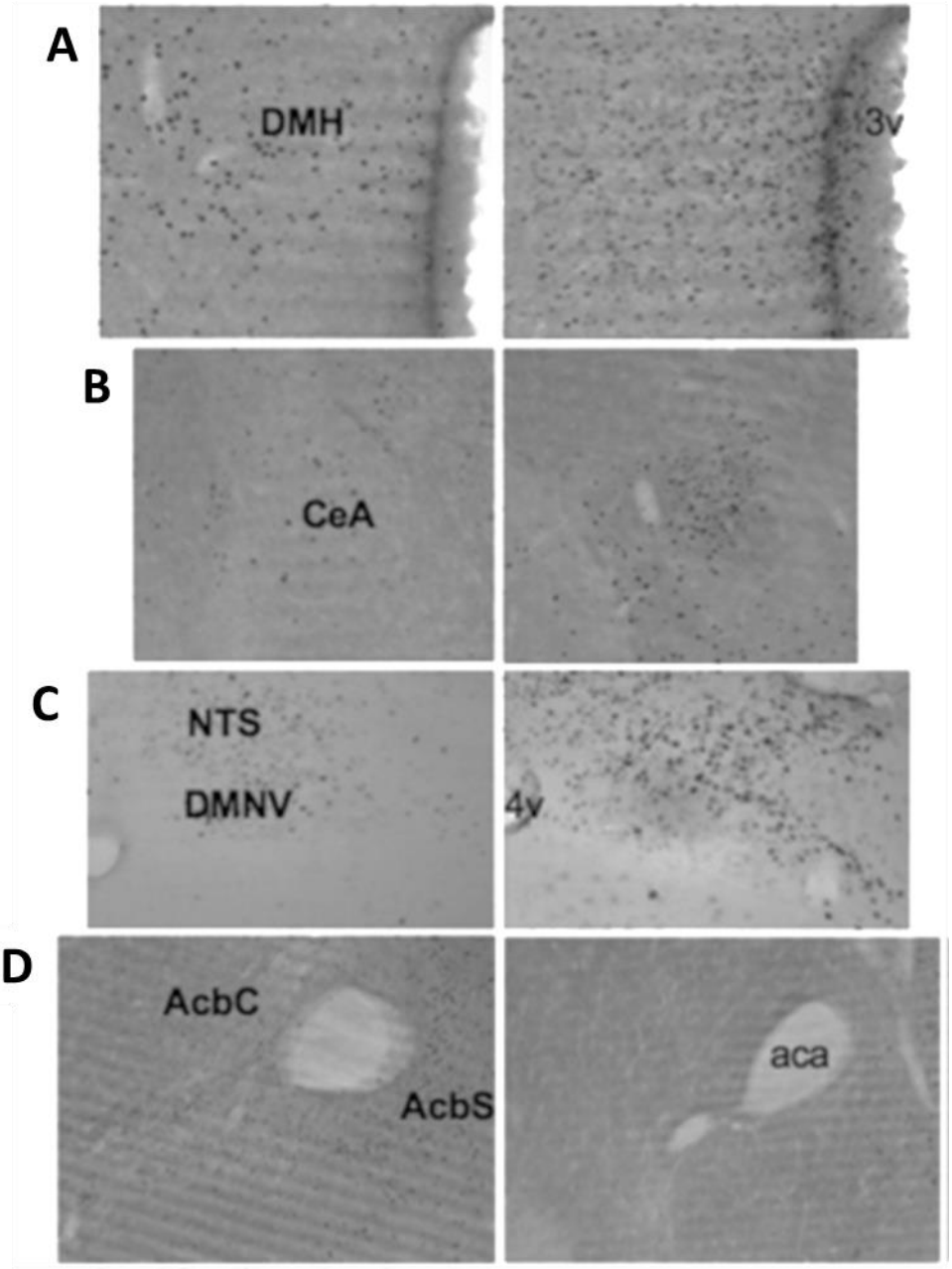

Figure 2.6 c-Fos immunoreactivity in select feeding-related brain sites following intragastric administration of 1200 mg/kg TRP versus vehicle. Photomicrographs (A-D) depict c-Fos in brain areas in which a significant change was noted (control: left panels; TRP: right panel). 3v – third ventricle; 4v – fourth ventricle; aca – anterior commissure; AcbC – nucleus accumbens core; AcbS – nucleus accumbens shell; CeA – central nucleus of the amygdala; DMH – dorsomedial hypothalamic nucleus; DMNV – dorsal motor nucleus of the vagus; NTS – nucleus of the solitary tract; * - P≤0.05; ** - P≤0.01; * - P≤0.001.**

2.5 Discussion

Enrichment of food with protein promotes a decrease in consumption, as consistently observed in human and laboratory animal studies [1-3]. A variety of factors have been implicated in this anorexigenic effect. High-protein diets tend to have diminished palatability, resulting in a reduced drive for consumption. Protein intake triggers endocrine processes conducive to early satiation, such as the release of GLP-1 and PYY, as reported for both humans and mice [31, 32]. A reduction in appetite occurs during protein consumption, but also during a subsequent meal, which reflects sustained satiety in protein-derived hypophagia [33]. The epidemiological potential of mechanisms mediating protein-derived satiety for overcoming the current obesity 'epidemic', has been increasingly recognised over the past years.

Research has discovered that not only complex proteins, but specific individual amino acids, can act as potent anorexigens e.g., L-leucine [9, 12], L-cysteine [8], L-arginine, L-lysine and L-glutamic acid [6, 7]. Unlike those aforementioned amino acids, prior data on feeding-related effects of TRP are inconsistent, with some authors reporting moderate hypophagia [16, 17], whereas others failing to observe a decrease in appetite in rats [6]. The current findings provide evidence for the role of TRP in the regulation of consumption suggesting that a TRP preload reduces ingestive behaviour and this effect is potentially mediated via central appetite-related circuits.

In line with the proposed role in promoting satiation thereby reducing meal size [16], intragastric TRP in deprived mice decreased standard chow intake by 30% of

the baseline levels during the first hour of re-feeding, and the hypophagic effect continued at 4 h. The intraduodenal infusion of low-caloric TRP loads (either 0.075 or 0.15 kcal/min for 90 minutes) in humans, demonstrated a comparable suppression of episodic energy intake and increased fullness after the post-infusion standardised buffet meal [23]. In deprived animals, motivated to eat by hunger, 1200 mg/kg TRP was necessary to reduce consumption. However, in sated mice given 2-h access to palatable chow, a 600-mg/kg dose was observed to effectively decrease intake. Thus, an effective anorexigenic dose of TRP depends on the nutritional and feeding motivational status stemming from hunger.

Of note, in the previously mentioned scenarios, both the standard and palatable chow were calorie-dense, complex and stable diets, meaning they contain required macro- and micronutrients. Therefore, the question then arose as to whether TRP would reduce consumption of nutritionally inadequate non- and low-caloric solutions, whose intake is driven by palatability. Those experimental models are particularly important in identifying central mechanisms responsible for TRP-induced hypophagia. Many neuroactive agents affect either only eating for energy (e.g., neuropeptide Y [34-39] and AGRP can motivate the consumption of calorie-dense ingestants regardless of their palatability [40-43]) or eating for reward (e.g. opioids modify intake of energy-dilute palatable solutions [44-49]) or eating stimulated by both of these variables (e.g. OT decreases intake for both palatability and energy, by acting through disperse circuitry [30, 50]).

TRP failed to reduce the consumption of all other solutions sweetened with sugar as well as with a non-carbohydrate, non-caloric sweetener, saccharin. Likewise, TRP was ineffective at decreasing intake of a palatable Intralipid emulsion.

Therefore, in this context, regardless of whether the pleasant taste and/or postingestive effects are associated with fat or carbohydrate content or saccharin-derived sweet flavour, TRP does not suppress palatability-driven intake. The absence of anorexigenic effects in these paradigms is striking when considering the fact that 1200 mg/kg TRP does reduce water consumption in thirsty mice, whereas the even higher 1800 mg/kg dose utilised in the sugar solution study had no effect. This emphasises the fact that the previously observed TRP-induced hypophagia, on palatable chow intake, is not associated with reward processing. Previous research demonstrated that intragastric TRP in rats was equally effective in reducing the intake of high-carbohydrate (and protein-free), as well as standard chow available in 12-h daily sessions [17]. Therefore, based on those findings and the data shown in this thesis, one can speculate that the mechanisms behind TRP's anorexigenic effects are related to the hunger-satiation continuum rather than palatability-driven feeding. Notably, the effect of TRP on thirst may be a contributing factor to generalised hypophagia.

The fact that intragastric administration of TRP does not always lead to inhibition of ingestive behaviour suggests that TRP-driven hypophagia is unrelated to sickness/malaise. The data obtained in our CTA experiment further underline this notion. Toxin-treated mice subjected to an aversion conditioning paradigm develop avoidance of a novel tastant when presented concurrently with a toxin [51-53]. The current data show that TRP-infused mice do not decrease preference towards the novel KoolAid solution in the CTA model. This instils greater confidence in allowing us to speculate that TRP engages appetite-regulatory processes unrelated to sickness/malaise.

The pattern of c-Fos immunoreactivity evoked by 1200 mg/kg TRP (the dose effective in all our feeding paradigms where intragastric TRP reduced consumption) was consistent with the earlier reports in rats, suggesting the significant role of brainstem circuitry in mediating amino acid-driven hypophagia [6]. Elevated NTS and DMNV neuronal activity indicates the importance of vagal afferents (for review, see [54]), whereas a minor (albeit, non-significant) increase in AP c-Fos levels suggests the involvement of this sensory circumventricular organ. c-Fos changes were observed outside of the brainstem, in other known feeding-related brain regions, which suggests that TRP engages more widespread networks (either in a direct or indirect manner). The most robust increase in neuronal activation was discovered in the hypothalamic PVN, DMH and SON. These three areas receive input from the brainstem [54] and characteristically show increased activity at meal termination, as well as with physiological changes that signify the need to cease consumption (e.g. stomach distension or elevated plasma osmolality) [55, 56]. This concurrent TRP-induced change in c-Fos immunoreactivity in the brainstem-hypothalamic circuit is consistent with the proposed role for TRP in the regulation of feeding for energy, as suggested in an earlier study in rats [16, 17].

Some aspects of the Fos pattern aligns well with the observed effect of TRP on thirst-driven water intake, as a subset of the activated sites mediates not just feeding, but also drinking behaviour [57]. For instance, the SON where vasopressin (VP) neurons are accumulated in its ventral portion, displays high c-Fos activation in the TRP group [58]; also the central nucleus of the amygdala (CeA), which, aside from mediating multiple anorexigenic signals, is involved in the regulation of osmotic balance and water intake via NTS-descending pathways [59-61]. Remarkably, the

AcbC and AcbS, regions usually associated with reward, showed a slight decline in c-Fos levels below control values. Therefore, this might indicate a connection between TRP and Acb-based mediating energy consumption, since meal size in energy-deprived rats is controlled via the accumbal circuitry too; as seen in the recent AcbC OT injection studies [30].

In sum, intragastric TRP reduces both food and water intake, and TRP-induced hypophagia appears to be potentially mediated via dispersed central circuits, most prominently, the brainstem and hypothalamus.

2.6 References

1. Poppitt, S.D., D. McCormack, and R. Buffenstein, *Short-term effects of macronutrient preloads on appetite and energy intake in lean women.* Physiol Behav, 1998. **64**(3): p. 279-85.
2. Weigle, D.S., P.A. Breen, C.C. Matthys, H.S. Callahan, K.E. Meeuws, V.R. Burden, and J.Q. Purnell, *A high-protein diet induces sustained reductions in appetite, ad libitum caloric intake, and body weight despite compensatory changes in diurnal plasma leptin and ghrelin concentrations.* Am J Clin Nutr, 2005. **82**(1): p. 41-8.
3. Westerterp-Plantenga, M.S., M.P. Lejeune, I. Nijs, M. van Ooijen, and E.M. Kovacs, *High protein intake sustains weight maintenance after body weight loss in humans.* Int J Obes Relat Metab Disord, 2004. **28**(1): p. 57-64.
4. Koehnle, T.J., M.C. Russell, and D.W. Gietzen, *Rats rapidly reject diets deficient in essential amino acids.* J Nutr, 2003. **133**(7): p. 2331-5.
5. Le Floc'h, N., W. Otten, and E. Merlot, *Tryptophan metabolism, from nutrition to potential therapeutic applications.* Amino Acids, 2011. **41**(5): p. 1195-1205.
6. Jordi, J., B. Herzog, S.M. Camargo, C.N. Boyle, T.A. Lutz, and F. Verrey, *Specific amino acids inhibit food intake via the area postrema or vagal afferents.* J Physiol, 2013. **591**(22): p. 5611-21.
7. Alamshah, A., A.K. McGavigan, E. Spreckley, J.S. Kinsey-Jones, A. Amin, I.R. Tough, H.C. O'Hara, A. Moolla, K. Banks, R. France, G. Hyberg, M. Norton, W. Cheong, A. Lehmann, S.R. Bloom, H.M. Cox, and K.G. Murphy, *L-arginine promotes gut hormone release and reduces food intake in rodents.* Diabetes Obes Metab, 2016. **18**(5): p. 508-18.
8. McGavigan, A.K., H.C. O'Hara, A. Amin, J. Kinsey-Jones, E. Spreckley, A. Alamshah, A. Agahi, K. Banks, R. France, G. Hyberg, C. Wong, G.A. Bewick, J.V. Gardiner, A. Lehmann, N.M. Martin, M.A. Ghatei, S.R. Bloom, and K.G. Murphy, *L-cysteine suppresses ghrelin and reduces appetite in rodents and humans.* Int J Obes (Lond), 2015. **39**(3): p. 447-55.

9. Cota, D., K. Proulx, K.A. Smith, S.C. Kozma, G. Thomas, S.C. Woods, and R.J. Seeley, *Hypothalamic mTOR signaling regulates food intake.* Science, 2006. **312**(5775): p. 927-30.
10. Morrison, C.D., X. Xi, C.L. White, J. Ye, and R.J. Martin, *Amino acids inhibit Agrp gene expression via an mTOR-dependent mechanism.* Am J Physiol Endocrinol Metab, 2007. **293**(1): p. E165-71.
11. Blouet, C., Y.H. Jo, X. Li, and G.J. Schwartz, *Mediobasal hypothalamic leucine sensing regulates food intake through activation of a hypothalamus-brainstem circuit.* J Neurosci, 2009. **29**(26): p. 8302-11.
12. Blouet, C. and G.J. Schwartz, *Brainstem nutrient sensing in the nucleus of the solitary tract inhibits feeding.* Cell Metab, 2012. **16**(5): p. 579-87.
13. Richard, D.M., M.A. Dawes, C.W. Mathias, A. Acheson, N. Hill-Kapturczak, and D.M. Dougherty, *L-Tryptophan: Basic Metabolic Functions, Behavioral Research and Therapeutic Indications.* Int J Tryptophan Res, 2009. **2**: p. 45-60.
14. Wurtman, R.J., F. Hefti, and E. Melamed, *Precursor control of neurotransmitter synthesis.* Pharmacol Rev, 1980. **32**(4): p. 315-35.
15. Wellendorph, P. and H. Brauner-Osborne, *Molecular basis for amino acid sensing by family C G-protein-coupled receptors.* Br J Pharmacol, 2009. **156**(6): p. 869-84.
16. Ayaso, R., H. Ghattas, M. Abiad, and O. Obeid, *Meal pattern of male rats maintained on amino acid supplemented diets: the effect of tryptophan, lysine, arginine, proline and threonine.* Nutrients, 2014. **6**(7): p. 2509-22.
17. Ng, L.T. and G.H. Anderson, *Route of administration of tryptophan and tyrosine affects short-term food intake and plasma and brain amino acid concentrations in rats.* J Nutr, 1992. **122**(2): p. 283-93.
18. Sainio, E.L., K. Pulkki, and S.N. Young, *L-Tryptophan: Biochemical, nutritional and pharmacological aspects.* Amino Acids, 1996. **10**(1): p. 21-47.
19. Pardridge, W.M. and W.H. Oldendorf, *Kinetic analysis of blood-brain barrier transport of amino acids.* Biochim Biophys Acta, 1975. **401**(1): p. 128-36.

20. Boado, R.J., J.Y. Li, M. Nagaya, C. Zhang, and W.M. Pardridge, *Selective expression of the large neutral amino acid transporter at the blood-brain barrier.* Proc Natl Acad Sci U S A, 1999. **96**(21): p. 12079-84.
21. Hawkins, R.A., R.L. O'Kane, I.A. Simpson, and J.R. Vina, *Structure of the blood-brain barrier and its role in the transport of amino acids.* J Nutr, 2006. **136**(1 Suppl): p. 218S-26S.
22. Killian, D.M. and P.J. Chikhale, *Predominant functional activity of the large, neutral amino acid transporter (LAT1) isoform at the cerebrovasculature.* Neurosci Lett, 2001. **306**(1-2): p. 1-4.
23. Steinert, R.E., N.D. Luscombe-Marsh, T.J. Little, S. Standfield, B. Otto, M. Horowitz, and C. Feinle-Bisset, *Effects of intraduodenal infusion of L-tryptophan on ad libitum eating, antropyloroduodenal motility, glycemia, insulinemia, and gut peptide secretion in healthy men.* J Clin Endocrinol Metab, 2014. **99**(9): p. 3275-84.
24. Green, K.F., L.S. Holmstrom, and M.A. Wollman, *Relation of cue to consequence in rats: effect of recuperation from illness.* Behav Biol, 1974. **10**(4): p. 491-503.
25. Kovacs, K.J., *Measurement of immediate-early gene activation- c-fos and beyond.* J Neuroendocrinol, 2008. **20**(6): p. 665-72.
26. Olszewski, P.K., A. Klockars, and A.S. Levine, *Oxytocin: A Conditional Anorexigen whose Effects on Appetite Depend on the Physiological, Behavioural and Social Contexts.* J Neuroendocrinol, 2016. **28**(4).
27. Klockars, A., C. Brunton, L. Li, A.S. Levine, and P.K. Olszewski, *Intravenous administration of oxytocin in rats acutely decreases deprivation-induced chow intake, but it fails to affect consumption of palatable solutions.* Peptides, 2017. **93**: p. 13-19.
28. Olszewski, P.K., A. Klockars, A.M. Olszewska, R. Fredriksson, H.B. Schioth, and A.S. Levine, *Molecular, immunohistochemical, and pharmacological evidence of oxytocin's role as inhibitor of carbohydrate but not fat intake.* Endocrinology, 2010. **151**(10): p. 4736-44.

29. Herisson, F.M., L.L. Brooks, J.R. Waas, A.S. Levine, and P.K. Olszewski, *Functional relationship between oxytocin and appetite for carbohydrates versus saccharin.* Neuroreport, 2014. **25**(12): p. 909-14.
30. Herisson, F.M., J.R. Waas, R. Fredriksson, H.B. Schioth, A.S. Levine, and P.K. Olszewski, *Oxytocin Acting in the Nucleus Accumbens Core Decreases Food Intake.* J Neuroendocrinol, 2016. **28**(4).
31. Batterham, R.L., H. Heffron, S. Kapoor, J.E. Chivers, K. Chandarana, H. Herzog, C.W. Le Roux, E.L. Thomas, J.D. Bell, and D.J. Withers, *Critical role for peptide YY in protein-mediated satiation and body-weight regulation.* Cell Metab, 2006. **4**(3): p. 223-33.
32. van der Klaauw, A.A., J.M. Keogh, E. Henning, V.M. Trowse, W.S. Dhillo, M.A. Ghatei, and I.S. Farooqi, *High protein intake stimulates postprandial GLP1 and PYY release.* Obesity (Silver Spring), 2013. **21**(8): p. 1602-7.
33. Burton-Freeman, B., D.W. Gietzen, and B.O. Schneeman, *Meal pattern analysis to investigate the satiating potential of fat, carbohydrate, and protein in rats.* Am J Physiol, 1997. **273**(6 Pt 2): p. R1916-22.
34. Henry, M., L. Ghibaudi, J. Gao, and J.J. Hwa, *Energy metabolic profile of mice after chronic activation of central NPY Y1, Y2, or Y5 receptors.* Obes Res, 2005. **13**(1): p. 36-47.
35. Mashiko, S., A. Ishihara, H. Iwaasa, H. Sano, Z. Oda, J. Ito, M. Yumoto, M. Okawa, J. Suzuki, T. Fukuroda, M. Jitsuoka, N.R. Morin, D.J. MacNeil, L.H. Van der Ploeg, M. Ihara, T. Fukami, and A. Kanatani, *Characterization of neuropeptide Y (NPY) Y5 receptor-mediated obesity in mice: chronic intracerebroventricular infusion of D-Trp(34)NPY.* Endocrinology, 2003. **144**(5): p. 1793-801.
36. Morley, J.E., A.S. Levine, B.A. Gosnell, J. Kneip, and M. Grace, *Effect of neuropeptide Y on ingestive behaviors in the rat.* Am J Physiol, 1987. **252**(3 Pt 2): p. R599-609.
37. Raposinho, P.D., D.D. Pierroz, P. Broqua, R.B. White, T. Pedrazzini, and M.L. Aubert, *Chronic administration of neuropeptide Y into the lateral ventricle of C57BL/6J male mice produces an obesity syndrome including*

hyperphagia, hyperleptinemia, insulin resistance, and hypogonadism. Mol Cell Endocrinol, 2001. **185**(1-2): p. 195-204.

38. Sainsbury, A., I. Cusin, F. Rohner-Jeanrenaud, and B. Jeanrenaud, *Adrenalectomy prevents the obesity syndrome produced by chronic central neuropeptide Y infusion in normal rats.* Diabetes, 1997. **46**(2): p. 209-14.
39. Stanley, B.G., S.E. Kyrkouli, S. Lampert, and S.F. Leibowitz, *Neuropeptide Y chronically injected into the hypothalamus: a powerful neurochemical inducer of hyperphagia and obesity.* Peptides, 1986. **7**(6): p. 1189-92.
40. Graham, M., J.R. Shutter, U. Sarmiento, I. Sarosi, and K.L. Stark, *Overexpression of Agrt leads to obesity in transgenic mice.* Nat Genet, 1997. **17**(3): p. 273-4.
41. Hagan, M.M., P.A. Rushing, L.M. Pritchard, M.W. Schwartz, A.M. Strack, L.H. Van Der Ploeg, S.C. Woods, and R.J. Seeley, *Long-term orexigenic effects of AgRP-(83---132) involve mechanisms other than melanocortin receptor blockade.* Am J Physiol Regul Integr Comp Physiol, 2000. **279**(1): p. R47-52.
42. Hagan, M.M., S.C. Benoit, P.A. Rushing, L.M. Pritchard, S.C. Woods, and R.J. Seeley, *Immediate and prolonged patterns of Agouti-related peptide-(83--132)-induced c-Fos activation in hypothalamic and extrahypothalamic sites.* Endocrinology, 2001. **142**(3): p. 1050-6.
43. Rossi, M., M.S. Kim, D.G. Morgan, C.J. Small, C.M. Edwards, D. Sunter, S. Abusnana, A.P. Goldstone, S.H. Russell, S.A. Stanley, D.M. Smith, K. Yagaloff, M.A. Ghatei, and S.R. Bloom, *A C-terminal fragment of Agouti-related protein increases feeding and antagonizes the effect of alpha-melanocyte stimulating hormone in vivo.* Endocrinology, 1998. **139**(10): p. 4428-31.
44. Kelley, A.E., E.P. Bless, and C.J. Swanson, *Investigation of the effects of opiate antagonists infused into the nucleus accumbens on feeding and sucrose drinking in rats.* J Pharmacol Exp Ther, 1996. **278**(3): p. 1499-507.
45. Levine, A.S., D.T. Weldon, M. Grace, J.P. Cleary, and C.J. Billington, *Naloxone blocks that portion of feeding driven by sweet taste in food-restricted rats.* Am J Physiol, 1995. **268**(1 Pt 2): p. R248-52.

46. Ragnauth, A., H. Ruegg, and R.J. Bodnar, *Evaluation of opioid receptor subtype antagonist effects in the ventral tegmental area upon food intake under deprivation, glucoprivic and palatable conditions.* Brain Res, 1997. **767**(1): p. 8-16.
47. Rockwood, G.A. and L.D. Reid, *Naloxone modifies sugar-water intake in rats drinking with open gastric fistulas.* Physiol Behav, 1982. **29**(6): p. 1175-8.
48. Ruegg, H., W.Z. Yu, and R.J. Bodnar, *Opioid-receptor subtype agonist-induced enhancements of sucrose intake are dependent upon sucrose concentration.* Physiol Behav, 1997. **62**(1): p. 121-8.
49. Weldon, D.T., E. O'Hare, J. Cleary, C.J. Billington, and A.S. Levine, *Effect of naloxone on intake of cornstarch, sucrose, and polycose diets in restricted and nonrestricted rats.* Am J Physiol, 1996. **270**(6 Pt 2): p. R1183-8.
50. Mullis, K., K. Kay, and D.L. Williams, *Oxytocin action in the ventral tegmental area affects sucrose intake.* Brain Res, 2013. **1513**: p. 85-91.
51. Mickley, G.A., Z. Hoxha, S. Bacik, C.L. Kenmuir, J.A. Wellman, J.M. Biada, and A. DiSorbo, *Spontaneous recovery of a conditioned taste aversion differentially alters extinction-induced changes in c-Fos protein expression in rat amygdala and neocortex.* Brain Res, 2007. **1152**: p. 139-57.
52. Thiele, T.E., M.F. Roitman, and I.L. Bernstein, *c-Fos induction in rat brainstem in response to ethanol- and lithium chloride-induced conditioned taste aversions.* Alcohol Clin Exp Res, 1996. **20**(6): p. 1023-8.
53. Yamamoto, T., T. Shimura, N. Sako, Y. Yasoshima, and N. Sakai, *Neural substrates for conditioned taste aversion in the rat.* Behav Brain Res, 1994. **65**(2): p. 123-37.
54. Travagli, R.A., G.E. Hermann, K.N. Browning, and R.C. Rogers, *Brainstem circuits regulating gastric function.* Annu Rev Physiol, 2006. **68**: p. 279-305.

55. Huang, W., A.F. Sved, and E.M. Stricker, *Vasopressin and oxytocin release evoked by NaCl loads are selectively blunted by area postrema lesions.* Am J Physiol Regul Integr Comp Physiol, 2000. **278**(3): p. R732-40.
56. McCann, M.J. and R.C. Rogers, *Oxytocin excites gastric-related neurones in rat dorsal vagal complex.* J Physiol, 1990. **428**: p. 95-108.
57. Antunes-Rodrigues, J., M. de Castro, L.L. Elias, M.M. Valenca, and S.M. McCann, *Neuroendocrine control of body fluid metabolism.* Physiol Rev, 2004. **84**(1): p. 169-208.
58. Hurbin, A., H. Orcel, G. Alonso, F. Moos, and A. Rabie, *The vasopressin receptors colocalize with vasopressin in the magnocellular neurons of the rat supraoptic nucleus and are modulated by water balance.* Endocrinology, 2002. **143**(2): p. 456-66.
59. Andrade-Franze, G.M., C.A. Andrade, L.A. De Luca, Jr., P.M. De Paula, D.S. Colombari, and J.V. Menani, *Lesions in the central amygdala impair sodium intake induced by the blockade of the lateral parabrachial nucleus.* Brain Res, 2010. **1332**: p. 57-64.
60. Cai, H., W. Haubensak, T.E. Anthony, and D.J. Anderson, *Central amygdala PKC-delta(+) neurons mediate the influence of multiple anorexigenic signals.* Nat Neurosci, 2014. **17**(9): p. 1240-8.
61. Saha, S., *Role of the central nucleus of the amygdala in the control of blood pressure: descending pathways to medullary cardiovascular nuclei.* Clin Exp Pharmacol Physiol, 2005. **32**(5-6): p. 450-6.

3 Effects of intraperitoneal L-tryptophan on ingestive behaviour and neuronal activation in feeding-related brain sites

3.1 Abstract

In the previous chapter, I found that intragastric TRP moderately decreases feeding for energy. The analysis of c-Fos immunoreactivity after a hypophagic intragastric TRP dose showed increased activation of feeding-related hypothalamic and brainstem sites. In the current set of experiments, I investigated whether anorexigenic and brain activation effects persist when TRP administration bypasses the gut, i.e., TRP is injected directly in the intraperitoneal (IP) cavity in rats. The effect of IP TRP was examined in both energy- and palatability-induced feeding. 30 and 100 mg/kg IP TRP suppressed chow intake in energy deprived rats. Only a higher 100 mg/kg IP TRP dose reduced consumption of palatable chow and palatable sucrose, saccharin and Intralipid solutions in sated animals. Thirst-driven water intake was reduced after both 30 and 100 mg/kg IP TRP. Neither 30 nor 100 mg/kg IP TRP promoted the development of a conditioned taste aversion, which indicates a lack of treatment-induced sickness/malaise. The analysis of c-Fos in feeding-related brain sites showed the most pronounced effect of IP TRP on hypothalamic paraventricular and supraoptic nuclei and the nucleus of the solitary tract (NTS) in the brainstem. It can be concluded that IP TRP suppresses energy deprivation-induced intake of standard chow and thirst-induced water intake within

the same dose range, whereas higher doses are necessary to reduce consumption of palatable solutions in sated animals. IP TRP's hypophagic effect is accompanied by enhanced activation of central circuitry that encompasses brainstem and hypothalamic sites, but changes are not as prominent as with intragastric TRP.

3.2 Introduction

Data presented in Chapter 2 characterising feeding effects of intragastric TRP strongly suggest that this essential amino acid generates moderate hypophagia. This outcome is in concert with the observations reported by Jordi and co-workers who found that gavaging animals with TRP prior to a standard meal produces a decrease in consumption that nears significance (only a trend was detected, though, as correction for multiple comparison was applied, because the effects of as many as 20 amino acids were analysed simultaneously) [1]. Similar to that study, our c-Fos immunoreactivity mapping presented in Chapter 2 showed the involvement of the brainstem circuit, particularly the dorsal vagal complex (DVC).

TRP levels have been found to rise in the general circulation within minutes after intragastric infusion [2]; TRP also crosses the BBB [3, 4]. Hence, it can be presumed that one way through which TRP exerts its anorexigenic action (especially, its portion likely mediated by the brain) is by directly interacting with CNS neurons. However, the few reports on the consequences of intragastric infusion of TRP published to date emphasise the concomitant release of select gut hormones (including glucagon-like peptide-1 (GLP-1) and cholecystokinin (CCK)) in response to the TRP treatment [5, 6]. It raises a question as to whether TRP-driven anorexia observed after intragastric delivery may stem from the action of this amino acid on gut mucosa and submucosa. If so, this could be that the potential interaction with gut cells may be an underlying cause leading to changes in digestive functions, hormone release and/or GI motility, having all these factors affect numerous endocrine and neuroendocrine pathways reflected by changes in

brain activity after TRP. This is particularly pressing taking into account that hormones, such as CCK and GLP-1, decrease food intake, change gastric motility, stimulate c-Fos expression in the brainstem and affect activation of a wide brain circuitry encompassing, among others, the hypothalamus [7-10]. In order to address this dilemma, the current chapter is dedicated to studying feeding and brain activation effects of TRP delivered via a route that bypasses a direct contact with the gut mucosa, i.e., via an IP injection.

Similar to the scarcity of data related to intragastric TRP, literature on feeding-related effects of IP TRP is very limited. Early studies revealed a decrease in consumption in response to 75 mg/kg IP TRP in rats maintained on a choice of 10% or 60% casein diet, with a noticeable reduction in carbohydrate intake [11]. The IP TRP injection of 50 mg/kg and 100 mg/kg in 24 h-deprived rats reduced food intake by 20% and 30% respectively, after one hour of re-feeding [12, 13]. Furthermore, 100 mg/kg TRP suppressed food intake by 33-45% in rats maintained on the 12-h/day availability schedule of both standard and high-carbohydrate (protein-free) food. Interestingly, in that study noticeably higher plasma and brain TRP levels were observed after a 30-minute period when an equivalent TRP dose was administered IP rather than intragastrically [2]; this finding was further supported by [14]. On the other hand, Peters et al. failed to observe any effect of 100 mg/kg TRP on food intake in rats [15], although the cross-sectional design might have led to false negatives. Thus far, there have been no studies investigating effectiveness of IP TRP in reducing intake of palatable solutions. Theref

ore, the current set of studies was aimed to provide systematic characterisation of potential anorexigenic effects of IP TRP in the context of eating for energy, eating for palatability and drinking for thirst, as well as concurrent changes in brain activation.

First, I examined the acute effects of IP TRP first on deprivation-induced intake of energy-dense standard chow, and then on the intake of energy-dense palatable chow in sated rats. A conditioned taste aversion (CTA) paradigm was utilised to exclude a possibility that TRP-driven anorexia arises from sickness/malaise [16]. Then, determined whether TRP is capable of diminishing palatability-driven consumption of calorie-dilute solutions; whose intake was unaffected in our previous intragastric TRP study (Chapter 2). Furthermore, IP TRP's effect on water intake in water-deprived rats was analysed. Lastly, I expanded on the prior evidence indicating the involvement of the brain in mediating anorexigenic properties of TRP (Chapter 2). This was achieved by treating rats with a hypophagic dose of IP TRP and mapping c-Fos immunoreactivity in central sites known to control appetite, including the ones that were found to be affected by intragastric TRP as shown in the previous chapter.

3.3 Materials and methods

3.3.1 Animals and intraperitoneal administration

Adult male Sprague–Dawley rats (AgResearch, Hamilton, NZ) weighing 250-260 g at the beginning of the studies were housed individually in Plexiglas cages (LD 12:12, lights on at 07:00; 21°C). Rats had ad libitum access to water and

standard chow (Specialty Diets, Australia) unless specified otherwise. All procedures received prior approval from the University of Waikato animal ethics committee, and they are compliant with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publ., no. 80–23, rev. 1996).

Drugs and injections: TRP (Sigma, UK) were administered in isotonic saline. Animals were acclimatised to handling and receiving IP injections prior to the studies.

3.3.2 Consummatory behaviour studies

3.3.2.1 Effect of IP TRP on chow intake after overnight food deprivation

Overnight food-deprived rats were injected IP with 0 (saline), 10 mg/kg, 30 mg/kg or 100 mg/kg (n=7-11/group). The 100 mg/kg dose has been demonstrated to be effective in reducing food intake in rats [2]. Pre-weighed chow pellets were placed in food hoppers immediately after the treatment. Food intake was measured 1, 4 and 24 h post-injection and corrected for spillage.

3.3.2.2 Effect of IP TRP on deprivation-induced water intake

Rats were water-deprived overnight and injected IP with either 0 (saline), 10, 30 or 100 mg/kg TRP (n=10-14/group) prior to regaining access to water. At 10:00, water bottles were returned to the cages and intake was measured 1 h post-injection. Chow was removed during the time of the drinking test.

3.3.2.3 Effect of TRP on the intake of palatable chow in non-deprived rats

In order to examine whether TRP decreases consumption of palatable solid food in non-deprived animals, following a previously published protocol ([9, 15]), and gave rats 2-h access to palatable goat milk-enriched chow (3.5 kcal/g; PFR, New Zealand) at 10:00. Five minutes earlier, the animals received IP TRP at 0 (saline), 30 or 100 mg/kg (n=7-8/group). Standard chow was removed during the 2-h period of palatable diet availability. The animals had previous exposure to the palatable chow to avoid neophobia.

3.3.2.4 Effect of TRP on the episodic intake of palatable solutions

The paradigm described herein was based on the protocol used in our earlier studies [17-19]. Three cohorts of rats were accustomed to receiving 10% sucrose, 0.1% saccharin or 4.1% Intralipid palatable solutions for 1 h/day for 2 days (10:00-11:00) prior to the experiment to avoid neophobia (sucrose study: n=6-7/group; saccharin study: n=10/group; goat skim milk study: n=10/group; intralipid study: n=8-9/group). On the experimental day, animals were given IP TRP (at 0 (saline), 30 and 100 mg/kg TRP), 5 minutes before gaining access to the solutions. Chow and water were removed during the 2-h testing period. Palatable solution intake was measured after 1 h by weighing bottles.

3.3.2.5 Effect of IP TRP on the acquisition of a conditioned taste aversion (CTA)

In this standard CTA protocol (see e.g., [19]), rats had water taken away 16 h before their first exposure to a novel strawberry-flavoured KoolAid solution (1 h; from 10:00 to 11:00; Kraft Foods; prepared fresh according to the manufacturer's

instructions). Chow was removed at the time of fluid presentation. Afterwards they received an IP injection of 0 (saline), 30 or 100 mg/kg TRP (effective anorexigenic doses based on the aforementioned experiments; n=10/group). Rats treated with IP 6 mEq LiCl (vs saline) served as a positive CTA control (n=7/group). One hour after the injections, water and chow were returned to the cages. Two days later, animals were deprived of water again for 16 h, however, on the subsequent day, they were given a two-bottle preference test between KoolAid and water for 2 h (chow removed during the test). Bottles were weighed and percentages of KoolAid intake (out of cumulative intake, i.e. KoolAid + water) were calculated to assess acquisition of a CTA to the flavoured solution.

3.3.3 Immunohistochemistry

The rats received either a single IP injection of 30 mg/kg TRP (this dose caused a decrease in deprivation-reduced chow intake) or vehicle (n=6/group). Immediately after the treatment, which was performed between 10:00-11:00, both food and water were removed from the cages. Animals were anaesthetised (35% urethane) and perfused with saline (50mL) followed by 500mL of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) between 80-90 minutes after TRP/vehicle infusions. Brains were removed and postfixed overnight in PFA at 4˚C. Coronal 60 – um vibratome (Leica, Germany) sections were processed for c-Fos immunostaining. The tissue was treated for 10 minutes in 3% H2O2 in 10% methanol (in TBS; pH 7.4) and incubated overnight at 4˚C in the rabbit anti-Fos antibody (1:14000; Synaptic Systems, Goettingen, Germany). Subsequently, sections were incubated for 1 h in the goat-anti-rabbit antibody (1:400; Vector Laboratories, USA) and then in the avidin-biotin complex (1 h; Vector

Laboratories). Peroxidase was visualised with 0.05% DAB, 0.01 % H_2O_2 and 0.2% nickel sulphate (Sigma, USA). All incubations were conducted in a mixture of 0.25% gelatin and 0.5% Triton X-100 (Sigma, USA) in TBS. Intermediate rinsing was performed with TBS. Sections were mounted on gelatinised slides, dried, dehydrated in ascending concentrations of ethanol, soaked in xylene and embedded in Entellan (Merck, Germany). The number of c-Fos immunoreactive nuclei was counted bilaterally in all regions of interest (4-5 sections containing a given site per animal). Densities of c-Fos positive nuclei (per mm2) were averaged per rat and then per group.

3.3.4 Statistical analyses

Data from all consummatory behaviour studies utilising single TRP administration were processed with a Student's t-test (two-group comparisons) or a one-way ANOVA followed by Dunnet's post-hoc analysis (multiple-group comparisons). Correction for multiple comparisons was applied. Immunohistochemistry were analysed with a Student's t-test. Values are presented as means ± S.E.M and they were deemed significantly different when $p \leq 0.05$.

3.4 Results

IP TRP decreased deprivation-induced chow intake at 1 and 4 h post-injection (Figure 3.1A). At 1 h, 30 and 100 mg/kg were effective ($F(3, 30)=24.9$; $p=0.001$ and $p<0.001$, respectively), whereas at 4 h, only animals treated with 100 mg/kg ate less ($F(3, 31)=8.281$; $p<0.001$). Thirst-driven intake of water was also suppressed

by 30 mg/kg and 100 mg/kg TRP ($F(3, 41)=5.053$; $p=0.043$ and $p=0.042$, respectively; Figure 3.1 B).

In non-deprived animals given episodic access to either palatable solid or liquid tastants, 30 mg/kg TRP was ineffective (Figure 3.1 C/Figure 3.2 A-C). However, the 100-mg/kg dose decreased intake of palatable chow ($F(2, 19)=3.46$, $p=0.031$) (Figure 3.1 C), as well as of 10% sucrose ($F(2, 17)=0.0278$, $p=0.0155$) and 0.1% saccharin ($F(2, 27)=4.389$, $p=0.0139$) (Figure 3.2 D,E). The mean values for Intralipid intake after 100 mg/kg TRP were ca. 20% lower, but the difference did not reach significance.

Neither 30 nor 100 mg/kg TRP supported the development of a CTA to a novel KoolAid solution, unlike the IP injection of LiCl ($p<0.001$), which caused a 60% decline in preference for this ingestant (Figure 3.3 A,B).

The lowest anorexigenic dose of TRP (30 mg/kg) elevated c-Fos immunoreactivity in the NTS ($p=0.006$), PVN ($p=0.0044$) and SON ($p=0.0015$), and caused a significant decline in the density of c-Fos positive nuclei in the basolateral amygdala (BLA) ($p=0.0223$) (Figure 3.5 A-D). Also a decrease approaching significance ($p=0.0698$) in the AcbS was noted (Figure 3.4 A).

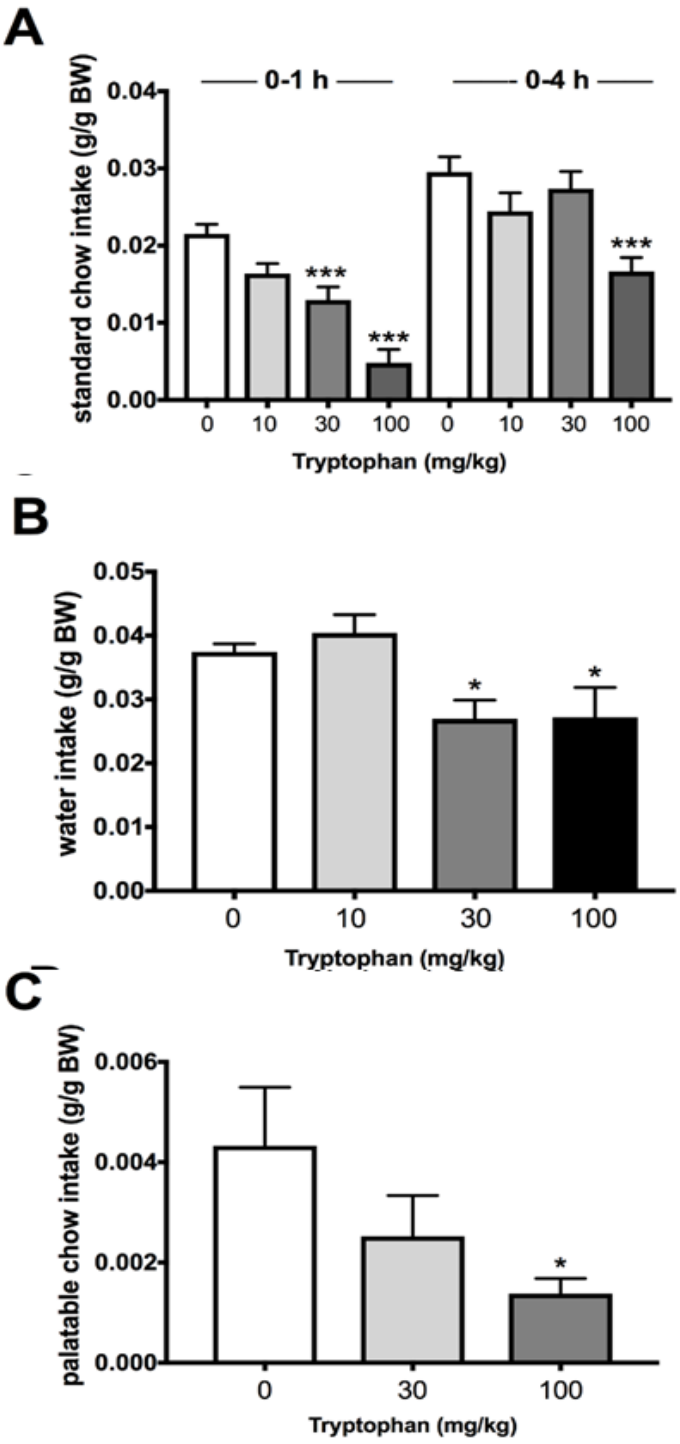

Figure 3.1 Effect of IP TRP on standard chow intake induced by overnight energy deprivation (A), water intake induced by overnight water deprivation (B) and on consumption of palatable chow (C) in non-deprived animals. TRP doses shown in mg/kg (0 indicates saline control). */* - significantly different from saline controls, P<0.05 and P<0.001, respectively.**

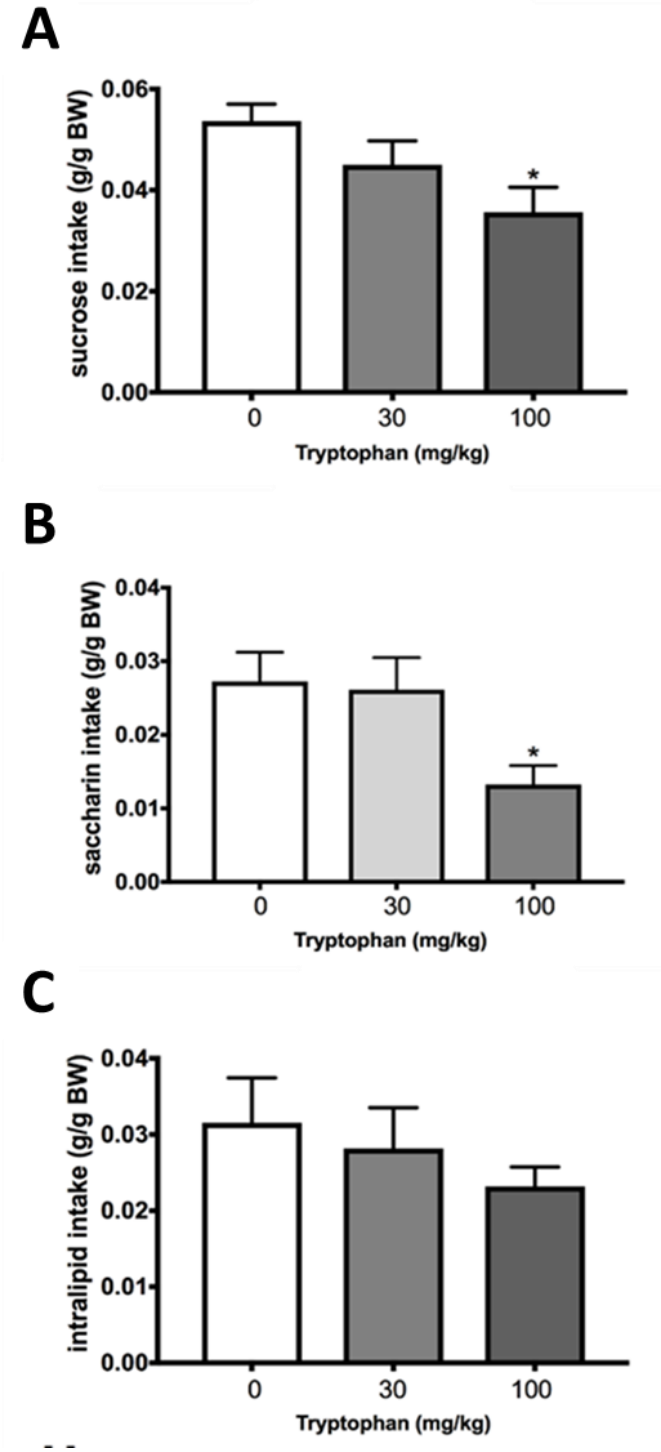

Figure 3.2 Effect of IP TRP on 10% sucrose (A), 0.1% saccharin (B) and 4.1% Intralipid (C) in non-deprived animals. TRP doses shown in mg/kg (0 indicates saline control). */* - significantly different from saline controls, P<0.05 and P<0.001, respectively.**

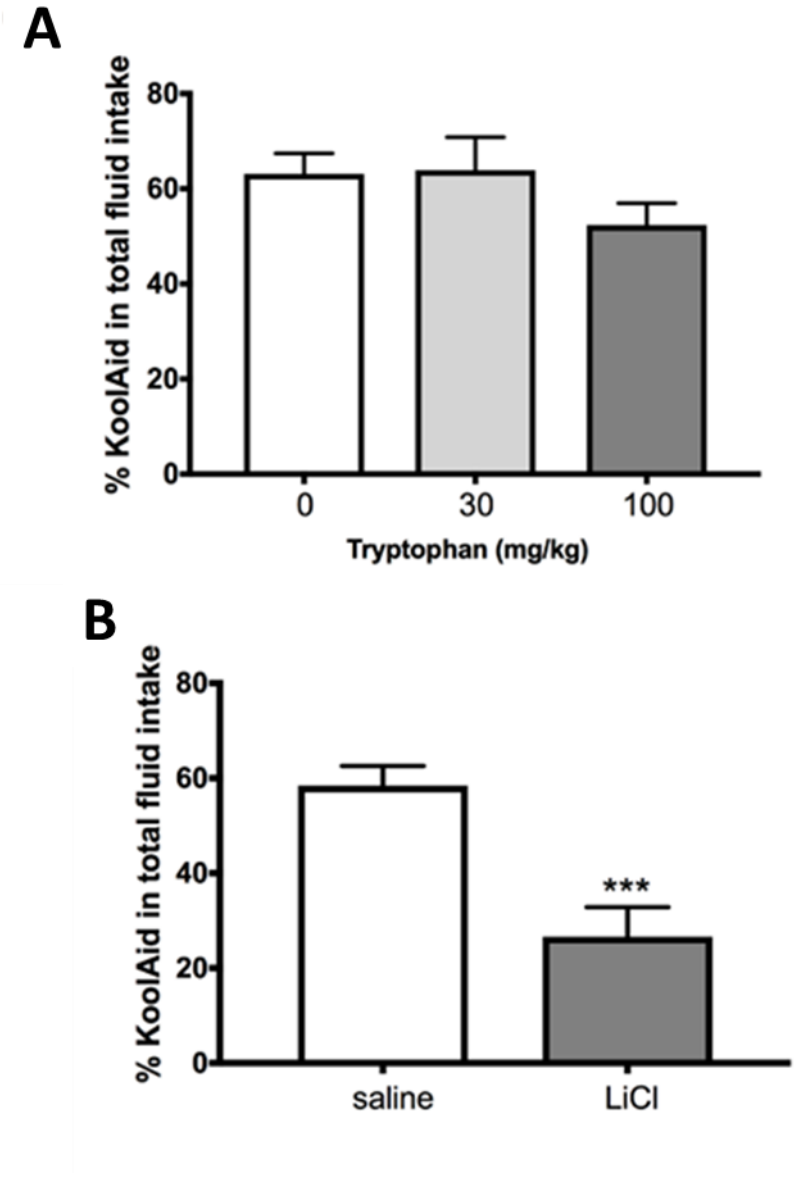

Figure 3.3 Effect of IP TRP on the acquisition of a CTA to a KoolAid solution (A). (B) shows a positive control for a CTA facilitated by an IP injection of LiCl. TRP doses shown in mg/kg (0 indicates saline control). */* - significantly different from saline controls, P<0.05 and P<0.001, respectively.**

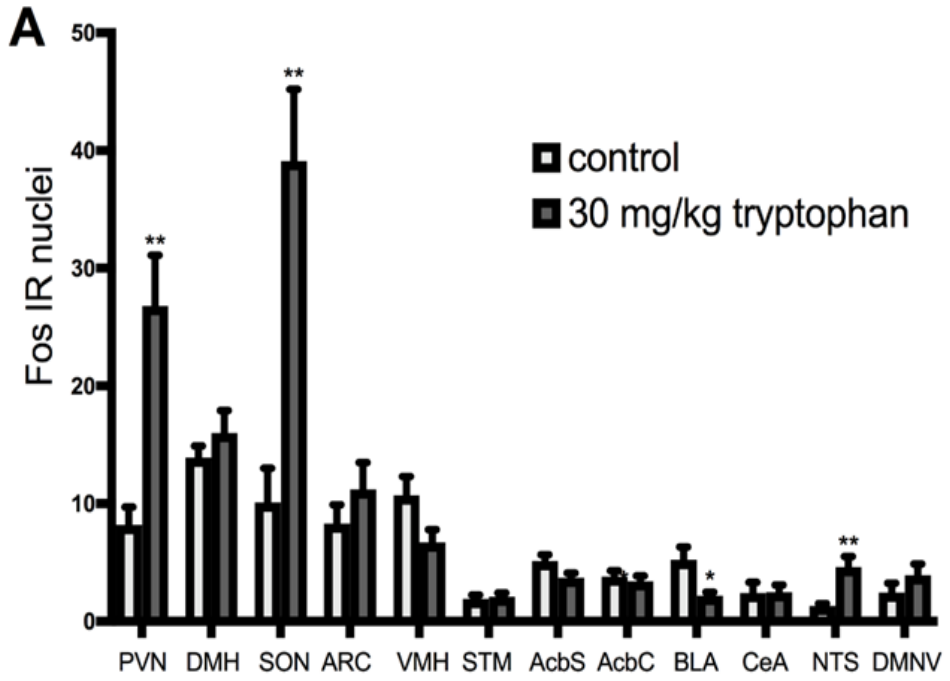

Figure 3.4 c-Fos immunoreactivity in select feeding-related brain sites following IP 30 mg/kg TRP vs saline. The graph (A) shows densities of Fos positive nuclei per mm^2 of each area of interest. AcbC – nucleus accumbens core; AcbS – nucleus accumbens shell; ARC – arcuate nucleus; BLA – basolateral amygdala; CeA – central nucleus of the amygdala; DMH – dorsomedial hypothalamic nucleus; DMNV – dorsal motor nucleus of the vagus; NTS – nucleus of the solitary tract; PVN – paraventricular hypothalamic nucleus; SON – supraoptic nucleus; STM – bed nucleus of the stria terminalis; VMH – ventromedial hypothalamic nucleus; * - P≤0.05; ** - P≤0.01; * - P≤0.001.**

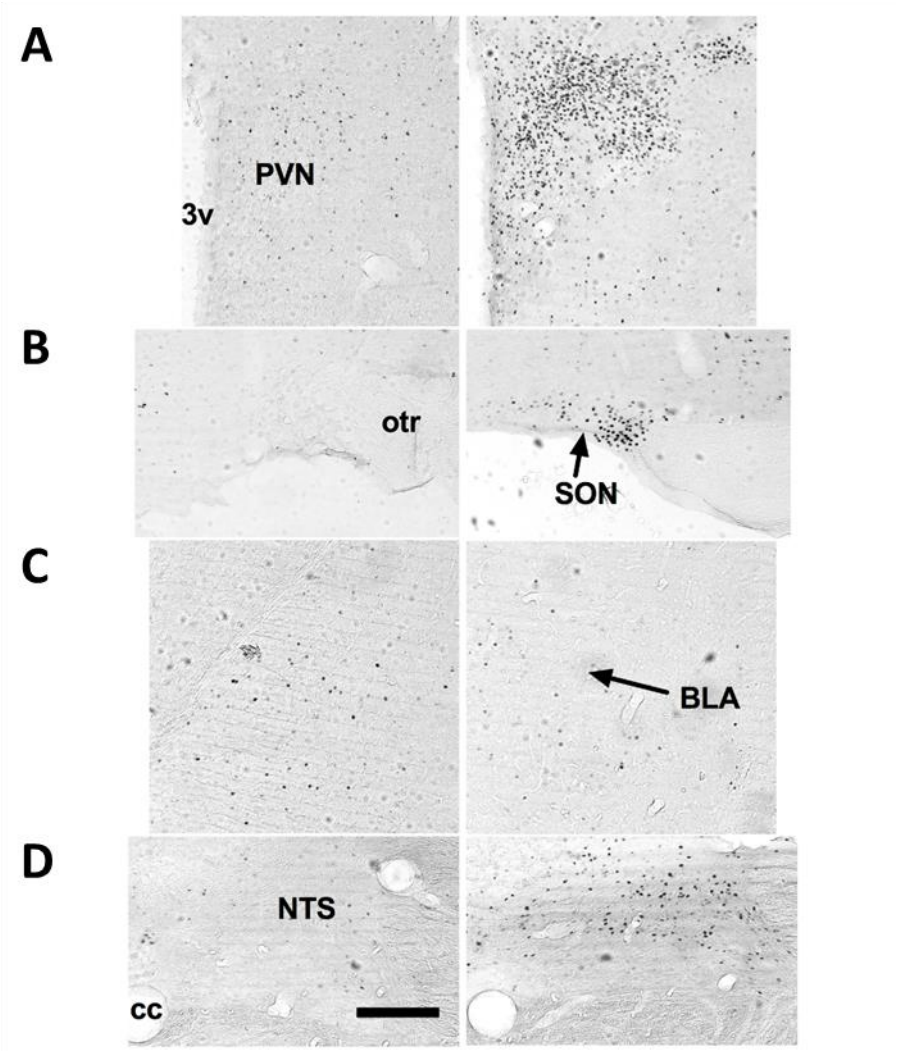

Figure 3.5 c-Fos immunoreactivity in select feeding-related brain sites following IP 30 mg/kg TRP vs saline. Photomicrographs (A-D) depict c-Fos in brain areas in which a significant change was noted (control: left panels; TRP: right panel). 3v – third ventricle; BLA – basolateral amygdala; cc – central canal; NTS – nucleus of the solitary tract; otr – optic tract; PVN – paraventricular hypothalamic nucleus; SON – supraoptic nucleus; * - P≤0.05; ** - P≤0.01; * - P≤0.001.**

3.5 Discussion

As mentioned in the introductory chapter to this thesis, a high-protein content of a diet suppresses appetite [20-26]. The notion that some individual amino acids act as anorexigens [1, 2, 27-31] seems particularly attractive as it offers an avenue of practical approaches in which a reduction in appetite is achieved by – instead of complex and not too palatable protein – by some individual amino acid(s). As TRP has been proposed as a candidate molecule promoting hypophagia, it is critical to assess the range of its anorexigenic effects as well as mechanisms that it engages in order to reduce food intake.

In Chapter 2 it was shown that intragastric TRP decreases deprivation-induced food intake and, at higher doses, thirst-induced drinking, but it is ineffective in changing consumption of palatable solutions. The data presented in this chapter indicate that IP TRP, thus TRP that does not come into contact with the gut mucosa, retains its anorexigenic properties. It should be noted, however, that the lowest dose of IP TRP that suppresses deprivation-induced chow intake is the same as the dose that decreases thirst-induced water drinking behaviour. This is in contrast to intragastric TRP, which induced effects on drinking at a dose that was 1/2log higher than that necessary to reduce hunger-driven intake of chow. Hence, while in the case of intragastric TRP, the effects on consummatory behaviour were more related to intake of calories, IP TRP appears to have a generalised suppressing effect on deprivation-induced intake of both food and water. Although it is a purely speculative statement, one can envision that the direct contact of TRP with gut mucosa might be engaging a broader array of, e.g., hormonal responses that

originate in the GI tract (as mentioned earlier, GLP-1 and CCK belong to this category ([7-10])) that later become relayed either directly or via vagus to brain circuitry to produce changes in ingestive behaviour.

IP TRP produced a very short-lasting hypophagia in energy-deprived rats. The reduction in chow intake was observed only in the first hour post-injection and it was compensated during the next 3 hours of re-feeding. It was therefore even a faster compensatory response than that noted after intragastric TRP, which maintained consumption at a lower level after 4 h (but not after 24 h) (Chapter 2). In the studies presented herein, 30 mg/kg was the lowest dose that decreased deprivation-induced intake of chow. It is only somewhat lower than the 50-100 mg/kg range reported earlier for energy deprivation [12, 13] as well as for 12h-12h daily food access restriction [2]. Therefore, an anorexigenic IP TRP appears to have a greater effect on food intake related to energy status and hunger.

While in animals having energy deficit, 30 mg/kg TRP was sufficient to decrease standard chow intake, in sated rats offered palatable chow, thus, motivated to eat by rewarding value of food, only the higher dose (100 mg/kg) reduced consumption. The same 100 mg/kg dose reduced intake of palatable solutions (sweet energy-dilute sucrose and non-caloric saccharin, and Intralipid) in non-deprived rats, whereas 30 mg/kg remained ineffective, which underscores the relationship between TRP and deprivation. It is possible that composition of this liquid diet may have contributed to the heightened responsiveness to TRP, however, this should be treated more as a hypothesis than an evidence-based claim.

Importantly, both 30 mg/kg and 100 mg/kg IP TRP reduced water intake in thirsty rats. Therefore, the same dose of IP TRP affected chow and water intake after deprivation. This is in contrast to the findings with intragastric TRP (Chapter 2) which had to be infused at a higher dose to reduce water than chow intake. This outcome calls into question whether the effect of IP TRP on consumption of palatable solutions in non-deprived animals is a direct consequence of TRP's action on palatability-driven appetite or a secondary effect related to changes in drinking behaviour. The fact that 100 mg/kg IP TRP also diminished intake of palatable energy-dense chow in sated animals gives us greater confidence in that the observed effect on intake of rewarding tastants cannot be completely attributed to changes in thirst. Importantly, our CTA study utilising a standard protocol [19] demonstrated that peripheral administration of 30 mg/kg TRP (lowest anorexigenic dose) did not shift animals' preference for a novel KoolAid solution, strongly indicating that this observed IP TRP-driven hypophagia is not related to sickness/malaise.

The fact that distribution of c-Fos immunoreactivity induced by 30 mg/kg TRP was fairly consistent with the effect of intragastric TRP (Chapter 2), suggests that regardless of an administration route, TRP engages an overlapping set of sites. This was particularly apparent in relation to enhanced neuronal activation in the brainstem and hypothalamus. It underpins the importance of vagal input into the brainstem in mediating hypophagic properties of TRP, whereas c-Fos changes observed again in forebrain areas indicate the involvement of broader pathways interconnected with the brainstem (including those that encompass the hypothalamus, a key energy homeostasis controlling region) [32]. In previous studies, these regions have typically shown increased activation coinciding with the

termination of meal and, with physiological changes that signify the need to cease consumption (e.g. stomach distension or high plasma osmolality) [33, 34].

It should be noted that some of those brain sites also influence drinking behaviour and again could offer insight into neural basis of the IP TRP-induced decline in water intake [35]. For example, the SON which showed increased c-Fos immunoreactivity in the IP TRP group, is involved in the regulation of osmotic balance and water intake via NTS-descending pathways and has a large number of VP neurons [36-39]. A slight c-Fos decrease was observed in the AcbS (not AcbC as with intragastric TRP), the area associated with reward. This provides further evidence for a possible link between TRP and accumbal-based regulation of consumption for energy: in food-deprived animals meal size is regulated via accumbal circuitry too, as observed in a recent AcbC OT injection studies [19]. Interestingly, an observed significant decrease in neuronal activation was detected in the BLA, one of the subdivisions of the AMY. The AMY is typically associated with the emotional aspects of feeding regulation and hunger-driven consummatory behaviour [40]. Additionally, it expresses a range of both hyperphagia and hypophagia mediating receptors, including those for opioids, OT and ghrelin [40-42].

Alterations in brainstem neuronal activity are consistent with earlier reports implicating brainstem circuitry with amino-acid driven hypophagia [1]. Analysis of c-Fos after hypophagic IP TRP dose demonstrated somewhat less pronounced neuronal activation than following intragastric TRP. Thereby, one can speculate that the effect of TRP on gastric mucosa and its potential link with the release of GI

tract hormones (e.g. GLP-1 or CCK) [23, 43-48] might make a difference in the ability of the brain to respond to the presence of TRP. This notion aligns well with Steinert et al. (2014) study who detected higher levels of CCK, glucagon and GLP-1, alongside increased fullness, following the intraduodenal infusion of TRP [5].

In sum, it can be concluded that IP TRP suppresses energy deprivation-induced intake of standard chow and thirst-induced water intake. Higher doses are necessary to reduce consumption of palatable solutions; this hypophagic effect is facilitated via dispersed central circuitry that encompasses brainstem and hypothalamic sites.

3.6 References

1. Jordi, J., B. Herzog, S.M. Camargo, C.N. Boyle, T.A. Lutz, and F. Verrey, *Specific amino acids inhibit food intake via the area postrema or vagal afferents.* J Physiol, 2013. **591**(22): p. 5611-21.
2. Ng, L.T. and G.H. Anderson, *Route of administration of tryptophan and tyrosine affects short-term food intake and plasma and brain amino acid concentrations in rats.* J Nutr, 1992. **122**(2): p. 283-93.
3. Pardridge, W.M. and W.H. Oldendorf, *Kinetic analysis of blood-brain barrier transport of amino acids.* Biochim Biophys Acta, 1975. **401**(1): p. 128-36.
4. Richard, D.M., M.A. Dawes, C.W. Mathias, A. Acheson, N. Hill-Kapturczak, and D.M. Dougherty, *L-Tryptophan: Basic Metabolic Functions, Behavioral Research and Therapeutic Indications.* Int J Tryptophan Res, 2009. **2**: p. 45-60.
5. Steinert, R.E., N.D. Luscombe-Marsh, T.J. Little, S. Standfield, B. Otto, M. Horowitz, and C. Feinle-Bisset, *Effects of intraduodenal infusion of L-tryptophan on ad libitum eating, antropyloroduodenal motility, glycemia, insulinemia, and gut peptide secretion in healthy men.* J Clin Endocrinol Metab, 2014. **99**(9): p. 3275-84.
6. Meyer-Gerspach, A.C., S. Hafliger, J. Meili, A. Doody, J.F. Rehfeld, J. Drewe, C. Beglinger, and B. Wolnerhanssen, *Effect of L-Tryptophan and L-Leucine on Gut Hormone Secretion, Appetite Feelings and Gastric Emptying Rates in Lean and Non-Diabetic Obese Participants: A Randomized, Double-Blind, Parallel-Group Trial.* PLoS One, 2016. **11**(11): p. e0166758.
7. Morton, G.J., D.E. Cummings, D.G. Baskin, G.S. Barsh, and M.W. Schwartz, *Central nervous system control of food intake and body weight.* Nature, 2006. **443**(7109): p. 289-95.
8. Cummings, D.E. and J. Overduin, *Gastrointestinal regulation of food intake.* J Clin Invest, 2007. **117**(1): p. 13-23.
9. Guyenet, S.J. and M.W. Schwartz, *Clinical review: Regulation of food intake, energy balance, and body fat mass: implications for the*

pathogenesis and treatment of obesity. J Clin Endocrinol Metab, 2012. **97**(3): p. 745-55.

10. Ritter, R.C., *Gastrointestinal mechanisms of satiation for food.* Physiol Behav, 2004. **81**(2): p. 249-73.
11. Morris, P., E.T. Li, M.L. MacMillan, and G.H. Anderson, *Food intake and selection after peripheral tryptophan.* Physiol Behav, 1987. **40**(2): p. 155-63.
12. Latham, C.J. and J.E. Blundell, *Evidence for the effect of tryptophan on the pattern of food consumption in free feeding and food deprived rats.* Life Sci, 1979. **24**(21): p. 1971-8.
13. Gibbons, J.L., G.A. Barr, W.H. Bridger, and S.F. Leibowitz, *L-Tryptophan's effects on mouse killing, feeding, drinking, locomotion, and brain serotonin.* Pharmacol Biochem Behav, 1981. **15**(2): p. 201-6.
14. Yuwiler, A., G.L. Brammer, J.E. Morley, M.J. Raleigh, J.W. Flannery, and E. Geller, *Short-term and repetitive administration of oral tryptophan in normal men. Effects on blood tryptophan, serotonin, and kynurenine concentrations.* Arch Gen Psychiatry, 1981. **38**(6): p. 619-26.
15. Peters, J.C., D.B. Bellissimo, and A.E. Harper, *L-tryptophan injection fails to alter nutrient selection by rats.* Physiol Behav, 1984. **32**(2): p. 253-9.
16. Green, K.F., L.S. Holmstrom, and M.A. Wollman, *Relation of cue to consequence in rats: effect of recuperation from illness.* Behav Biol, 1974. **10**(4): p. 491-503.
17. Olszewski, P.K., A. Klockars, A.M. Olszewska, R. Fredriksson, H.B. Schioth, and A.S. Levine, *Molecular, immunohistochemical, and pharmacological evidence of oxytocin's role as inhibitor of carbohydrate but not fat intake.* Endocrinology, 2010. **151**(10): p. 4736-44.
18. Herisson, F.M., L.L. Brooks, J.R. Waas, A.S. Levine, and P.K. Olszewski, *Functional relationship between oxytocin and appetite for carbohydrates versus saccharin.* Neuroreport, 2014. **25**(12): p. 909-14.
19. Herisson, F.M., J.R. Waas, R. Fredriksson, H.B. Schioth, A.S. Levine, and P.K. Olszewski, *Oxytocin Acting in the Nucleus Accumbens Core Decreases Food Intake.* J Neuroendocrinol, 2016. **28**(4).

20. Poppitt, S.D., D. McCormack, and R. Buffenstein, *Short-term effects of macronutrient preloads on appetite and energy intake in lean women.* Physiol Behav, 1998. **64**(3): p. 279-85.
21. Westerterp-Plantenga, M.S., M.P. Lejeune, I. Nijs, M. van Ooijen, and E.M. Kovacs, *High protein intake sustains weight maintenance after body weight loss in humans.* Int J Obes Relat Metab Disord, 2004. **28**(1): p. 57-64.
22. Weigle, D.S., P.A. Breen, C.C. Matthys, H.S. Callahan, K.E. Meeuws, V.R. Burden, and J.Q. Purnell, *A high-protein diet induces sustained reductions in appetite, ad libitum caloric intake, and body weight despite compensatory changes in diurnal plasma leptin and ghrelin concentrations.* Am J Clin Nutr, 2005. **82**(1): p. 41-8.
23. Bensaid, A., D. Tome, D. Gietzen, P. Even, C. Morens, N. Gausseres, and G. Fromentin, *Protein is more potent than carbohydrate for reducing appetite in rats.* Physiol Behav, 2002. **75**(4): p. 577-82.
24. Jean, C., S. Rome, V. Mathe, J.F. Huneau, N. Aattouri, G. Fromentin, C.L. Achagiotis, and D. Tome, *Metabolic evidence for adaptation to a high protein diet in rats.* J Nutr, 2001. **131**(1): p. 91-8.
25. Lacroix, M., C. Gaudichon, A. Martin, C. Morens, V. Mathe, D. Tome, and J.F. Huneau, *A long-term high-protein diet markedly reduces adipose tissue without major side effects in Wistar male rats.* Am J Physiol Regul Integr Comp Physiol, 2004. **287**(4): p. R934-42.
26. Burton-Freeman, B., D.W. Gietzen, and B.O. Schneeman, *Meal pattern analysis to investigate the satiating potential of fat, carbohydrate, and protein in rats.* Am J Physiol, 1997. **273**(6 Pt 2): p. R1916-22.
27. Cota, D., K. Proulx, K.A. Smith, S.C. Kozma, G. Thomas, S.C. Woods, and R.J. Seeley, *Hypothalamic mTOR signaling regulates food intake.* Science, 2006. **312**(5775): p. 927-30.
28. Blouet, C. and G.J. Schwartz, *Brainstem nutrient sensing in the nucleus of the solitary tract inhibits feeding.* Cell Metab, 2012. **16**(5): p. 579-87.
29. Alamshah, A., A.K. McGavigan, E. Spreckley, J.S. Kinsey-Jones, A. Amin, I.R. Tough, H.C. O'Hara, A. Moolla, K. Banks, R. France, G. Hyberg, M. Norton, W. Cheong, A. Lehmann, S.R. Bloom, H.M. Cox, and K.G. Murphy,

L-arginine promotes gut hormone release and reduces food intake in rodents. Diabetes Obes Metab, 2016. **18**(5): p. 508-18.

30. McGavigan, A.K., H.C. O'Hara, A. Amin, J. Kinsey-Jones, E. Spreckley, A. Alamshah, A. Agahi, K. Banks, R. France, G. Hyberg, C. Wong, G.A. Bewick, J.V. Gardiner, A. Lehmann, N.M. Martin, M.A. Ghatei, S.R. Bloom, and K.G. Murphy, *L-cysteine suppresses ghrelin and reduces appetite in rodents and humans.* Int J Obes (Lond), 2015. **39**(3): p. 447-55.
31. Ayaso, R., H. Ghattas, M. Abiad, and O. Obeid, *Meal pattern of male rats maintained on amino acid supplemented diets: the effect of tryptophan, lysine, arginine, proline and threonine.* Nutrients, 2014. **6**(7): p. 2509-22.
32. Travagli, R.A., G.E. Hermann, K.N. Browning, and R.C. Rogers, *Brainstem circuits regulating gastric function.* Annu Rev Physiol, 2006. **68**: p. 279-305.
33. Huang, W., A.F. Sved, and E.M. Stricker, *Vasopressin and oxytocin release evoked by NaCl loads are selectively blunted by area postrema lesions.* Am J Physiol Regul Integr Comp Physiol, 2000. **278**(3): p. R732-40.
34. McCann, M.J. and R.C. Rogers, *Oxytocin excites gastric-related neurones in rat dorsal vagal complex.* J Physiol, 1990. **428**: p. 95-108.
35. Antunes-Rodrigues, J., M. de Castro, L.L. Elias, M.M. Valenca, and S.M. McCann, *Neuroendocrine control of body fluid metabolism.* Physiol Rev, 2004. **84**(1): p. 169-208.
36. Cai, H., W. Haubensak, T.E. Anthony, and D.J. Anderson, *Central amygdala PKC-delta(+) neurons mediate the influence of multiple anorexigenic signals.* Nat Neurosci, 2014. **17**(9): p. 1240-8.
37. Saha, S., *Role of the central nucleus of the amygdala in the control of blood pressure: descending pathways to medullary cardiovascular nuclei.* Clin Exp Pharmacol Physiol, 2005. **32**(5-6): p. 450-6.
38. Andrade-Franze, G.M., C.A. Andrade, L.A. De Luca, Jr., P.M. De Paula, D.S. Colombari, and J.V. Menani, *Lesions in the central amygdala impair sodium intake induced by the blockade of the lateral parabrachial nucleus.* Brain Res, 2010. **1332**: p. 57-64.

39. Hurbin, A., H. Orcel, G. Alonso, F. Moos, and A. Rabie, *The vasopressin receptors colocalize with vasopressin in the magnocellular neurons of the rat supraoptic nucleus and are modulated by water balance.* Endocrinology, 2002. **143**(2): p. 456-66.

40. Klockars, O.A., A. Klockars, A.S. Levine, and P.K. Olszewski, *Oxytocin administration in the basolateral and central nuclei of amygdala moderately suppresses food intake.* Neuroreport, 2018. **29**(6): p. 504-510.

41. Beckman, T.R., Q. Shi, A.S. Levine, and C.J. Billington, *Amygdalar opioids modulate hypothalamic melanocortin-induced anorexia.* Physiol Behav, 2009. **96**(4-5): p. 568-73.

42. Alvarez-Crespo, M., K.P. Skibicka, I. Farkas, C.S. Molnar, E. Egecioglu, E. Hrabovszky, Z. Liposits, and S.L. Dickson, *The amygdala as a neurobiological target for ghrelin in rats: neuroanatomical, electrophysiological and behavioral evidence.* PLoS One, 2012. **7**(10): p. e46321.

43. Batterham, R.L., H. Heffron, S. Kapoor, J.E. Chivers, K. Chandarana, H. Herzog, C.W. Le Roux, E.L. Thomas, J.D. Bell, and D.J. Withers, *Critical role for peptide YY in protein-mediated satiation and body-weight regulation.* Cell Metab, 2006. **4**(3): p. 223-33.

44. van der Klaauw, A.A., J.M. Keogh, E. Henning, V.M. Trowse, W.S. Dhillo, M.A. Ghatei, and I.S. Farooqi, *High protein intake stimulates postprandial GLP1 and PYY release.* Obesity (Silver Spring), 2013. **21**(8): p. 1602-7.

45. Blom, W.A., A. Lluch, A. Stafleu, S. Vinoy, J.J. Holst, G. Schaafsma, and H.F. Hendriks, *Effect of a high-protein breakfast on the postprandial ghrelin response.* Am J Clin Nutr, 2006. **83**(2): p. 211-20.

46. Tome, D., J. Schwarz, N. Darcel, and G. Fromentin, *Protein, amino acids, vagus nerve signaling, and the brain.* Am J Clin Nutr, 2009. **90**(3): p. 838S-843S.

47. Fromentin, G., N. Darcel, C. Chaumontet, A. Marsset-Baglieri, N. Nadkarni, and D. Tome, *Peripheral and central mechanisms involved in the control of food intake by dietary amino acids and proteins.* Nutr Res Rev, 2012. **25**(1): p. 29-39.

48. Bowen, J., M. Noakes, and P.M. Clifton, *Appetite hormones and energy intake in obese men after consumption of fructose, glucose and whey protein beverages.* Int J Obes (Lond), 2007. **31**(11): p. 1696-703.

4 Tryptophan and Oxytocin

4.1 Abstract

Neural circuitry involved in mediating TRP-induced hypophagia has been poorly defined. The studies presented by other authors and in the earlier chapters of this thesis suggest the involvement of the brainstem-hypothalamic pathway. Interestingly, both intragastric and intraperitoneal (IP) TRP administered at hypophagic doses increase c-Fos immunoreactivity in the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei. These sites host neurons that synthesise a key satiety mediator, oxytocin (OT). Therefore, this chapter explored a hypothesis that TRP's anorexigenic action relies on enhanced activity of the OT system. By employing double-immunohistochemistry, I determined that lowest effective doses of intragastric and IP TRP increased the percentage of Fos-positive OT neurons in the PVN and SON, although the effect was more pronounced after intragastric administration. Intragastric TRP also upregulated OT mRNA in the hypothalamus. A blood-brain barrier-penetrant OT receptor antagonist, L-368,899, reversed hypophagia induced by TRP administered via both routes. Unlike the lowest effective dose of intragastric TRP which decreases only feeding, IP TRP at the lowest effective dose diminishes both food and water intake. Hence it was confirmed that L-368,899 pre-treatment does not affect TRP-driven reduction in drinking behaviour. Overall, the findings strongly suggest a functional relationship between TRP and OT in the regulation of energy-driven food intake.

4.2 Introduction

The anorexigenic properties of the essential amino acid TRP have been well documented in an array of paradigms in earlier studies [1-4] as well as in the experiments shown in the previous sections of this thesis. Although evidence suggests that TRP-induced hypophagia is mediated via dispersed central circuitry (Chapter 2 & 3), the exact involvement of specific neural pathways and processes is unclear. This scarcity of precise information regarding the nature of central action of free amino acids is not unique to TRP. In fact, the only anorexigenic amino acid whose neural effects have been well identified is leucine [5-8]: in rats, infusions of leucine into the third ventricle directly suppresses food intake via mTOR signalling and engages a network of hypothalamic sites that regulate appetite [5] (nota bene, peripheral hyperphagic rather than hypophagic action of leucine remains a largely unexplained phenomenon) [9-12].

Jordi and colleagues, who investigated the effect of isomolar intragastric doses of various individual amino acids, established that those that caused hypophagia (L-arginine, L-lysine and L-glutamate) activate brainstem circuitry (most likely, through the AP and/or vagus-facilitated processing). In that report, however, a potential link between TRP and brainstem circuitry was not studied [1]. Sensory mechanisms at the CNS level for all other 19 amino acids (excluding leucine) and their involvement in food intake regulation have yet to be fully elucidated [1, 13]. Importantly, the results of the c-Fos experiments from both the intragastric and IP administration experiments, presented in Chapters 2 and 3 of this thesis, indicated that increased activation of the brainstem occurs also in response to anorexigenic

doses of TRP. As the analysis of c-Fos throughout a host of feeding-related central sites revealed that the PVN and SON of the hypothalamus reliably show a significant increase in the number of c-Fos positive neurons, special attention was directed toward a key satiety mediator synthesised in these hypothalamic sites, oxytocin. I have postulated that a potential mechanism for TRP-driven hypophagia might involve circuitry that encompasses OT neurons.

Oxytocin (OT) is a 9-amino acid neurohormone [14, 15] that inhibits feeding in order to protect internal milieu (e.g. energy balance, stomach distension and plasma osmolality) [16-20]. The increased activation of OT neurons and release of OT, correspond with the cessation of ingestive behaviour [21-26].

Centrally acting OT stimulates suppression of food intake [24, 27, 28]. Arletti and colleagues, demonstrated that ICV OT infusions markedly reduced deprivation-induced food intake in rats [24]. Since then, OT's anorexigenic effect has been well established in relation to energy intake and, more recently, to palatability-driven consumption [23, 27, 29-31]. For example, OT-null knockout mice excessively consume palatable sweet solutions compared to wildtypes [32, 33]. The pharmacological blockade of the OT receptor by the BBB penetrant OT receptor antagonist, L-368,899, in sated mice prevents the anorexigenic effect of OT on the consumption of sweet tastants, and increases intake of such ingestants when the antagonist is administered alone [34].

OT appears to act as a cross-link for feeding termination between energy homeostasis and palatability-driven satiety, by acting through disperse central circuitry [35, 36]. The PVN, whose lesioning in rats results in overeating [37-39],

contains parvocellular cells that release OT centrally, by innervating diverse areas including the DVC [21, 40-46]. Other OT-expressing cells found in the PVN and the SON, belong to the magnocellular subpopulations which supply OT via somatodendritic projections into both periphery and the CNS [21, 40-43, 47-49]. The OT receptor has been found in high densities in regions associated with food reward (i.e. VTA and Acb), both of which are reciprocally interconnected with the PVN [50-53]. The intra-VTA and Acb infusions of OT dose-dependently decreased deprivation-induced chow intake and sucrose consumption in rats [35, 36].

Consequently, the current set of experiments focused on examining whether TRP-driven anorexia is mediated via OT-encompassing circuitry. Double immunohistochemistry (c-Fos + OT) was employed following the lowest hypophagic doses of intraperitoneally and intragastrically administered TRP, to assess TRP-induced activation of OT neurons [54]. The effect of intragastric TRP on hypothalamic OT gene expression was assessed with real-time PCR (rtPCR). The functional importance of CNS circuits encompassing OT in mediating TRP-driven hypophagia was further substantiated by showing the ability of OT receptor blockade, with an OT receptor antagonist L-368,899, to abolish intragastric and IP TRP-induced decrease in consumption.

4.3 Material and methods

4.3.1 Animals

4.3.1.1 Intragastric Model

Adult male C57BL/6 mice were housed individually under a 12:12 h light/dark cycle (lights on at 07:00) and maintained at a controlled temperature of 22˚C. The mice had ad libitum access to tap water and standard laboratory chow (Specialty Diets, Australia) unless specified otherwise. All procedures received prior approval from the University of Waikato animal ethics committee.

4.3.1.2 Intraperitoneal Model

Adult male Sprague–Dawley rats (AgResearch, Hamilton, NZ) weighing 250-260 g at the beginning of the studies were housed individually in Plexiglas cages (LD 12:12, lights on at 07:00; 21°C). Rats had ad libitum access to water and standard chow (Specialty Diets, Australia) unless specified otherwise. All procedures received prior approval from the University of Waikato animal ethics committee, and they are compliant with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publ., no. 80–23, rev. 1996).

4.3.2 Drugs and injections

4.3.2.1 Intragastric Model

Mice were acclimatised to the oral gavage procedure prior to the studies. Intragastric TRP (Sigma, UK) were administered in water (volume=0.2ml). IP L-

368,899 (Tocris, UK) were administered in isotonic saline. Mice were acclimatised to handling and receiving oral gavage and IP injections prior to the studies.

4.3.2.2 Intraperitoneal Model

TRP (Sigma, UK) and L-368,899 (Tocris, UK) were administered in isotonic saline. Rats were acclimatised to handling and receiving IP injections prior to the studies.

4.3.3 Effect of OT receptor antagonist on the ability of intragastric TRP to reduce deprivation-induced intake of standard chow

As TRP crosses the BBB, for the double-administration studies, the BBB-penetrant OT antagonist molecule, L-368,899 (Tocris) was selected [55]. IP administration of this ligand engages a combination of central and peripheral mechanisms, however at the dose range tested, it does not affect intake of standard chow [34]. Overnight food-deprived mice were double-treated with (a) IP saline- intragastric vehicle, (b) IP saline-intragastric TRP (1200 mg/kg), (c) IP L-368,899 (0.03 mg/kg) – intragastric TRP (1200 mg/kg), (d) IP L-368,899 (0.1 mg/kg) – intragastric TRP (1200 mg/kg), (e) IP L-368,899 (0.3 mg/kg) – intragastric TRP (1200 mg/kg) (n=8-9/group). The two drug treatments were spaced 10-15 minutes apart from each other. Food was returned to cages 20 minutes after the second (i.e., TRP or vehicle) infusion and consumption was measured 1 h later.

4.3.4 Effect of OT receptor blockade on the ability of IP TRP to reduce hunger-induced intake of chow

As TRP crosses the BBB, for the double-administration studies, the BBB-penetrant OT antagonist, L-368,899 (Tocris) was selected [55]. IP administration of this

ligand engages a combination of central and peripheral mechanisms, however at the dose range tested, it does not affect intake of chow or water [10]. Rats were double-treated IP with (a) saline + saline, (b) saline + TRP (30 mg/kg), (c) L-368,899 (1 mg/kg) + TRP (30 mg/kg), (d) L-368,899 (1 mg/0kg) + saline (n=8/group). The two drug treatments were spaced 5 minutes apart from each other. Chow was returned to cages 5 minutes after the second (i.e., TRP/vehicle) injection (10:00-10:30) and consumption was measured 1 h later.

4.3.5 Effect of OT receptor blockade on the ability of IP TRP to reduce thirst-induced intake of water

Rats were water-deprived overnight and double-injected IP with (a) saline + saline, (b) saline + TRP (30 mg/kg), (c) L-368,899 (1 mg/kg) + saline, (d) L-368,899 (1 mg/kg) + TRP (30 mg/kg) (n=8-9/group) (n=9-11/group) prior to regaining access to water. The injections were spaced 5 minutes apart from each other. Water bottles were returned to cages 5 minutes after the second (i.e., TRP or vehicle) injection (10:00-10:30), and intake was measured 1 h later. Chow was removed from hoppers during the drinking test.

4.3.6 Effect of the lowest anorexigenic dose of intragastric TRP on activation of unidentified neurons in feeding-related brain sites and hypothalamic OT neurons

The mice received either a single intragastric infusion of 1200 mg/kg TRP (the dose that decreased deprivation-induced chow intake) or water vehicle (n = 8/group for double staining for Fos + OT). Immediately after the treatment, which was

performed between 10:00-11:00, both food and water were removed from the cages. Animals were anaesthetised (35% urethane) and perfused with saline (20 mL) followed by 50 mL of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) between 80 and 90 min after TRP/vehicle infusions. Brains were removed and postfixed overnight in PFA at 4 ºC. Coronal 60-um vibratome (Leica, Germany) sections were processed for double-staining.

For double immunohistochemistry (c-Fos + OT) with fluorescent staining, c-Fos visualisation was performed first using c-Fos primary antibody incubation and then later followed by OT labelling. First, the sections were treated for 10 min in 3% H2O2 in 10% methanol (in TBS; pH 7.4) and incubated overnight at 4ºC in the primary anti-rabbit Fos antibody (1:1400; Synaptic Systems, Goettingen, Germany). The following day, sections were rinsed and then incubated for 1 h in the secondary anti-rabbit antibody (DyLight 594 Goat Anti-Rabbit, 1:400, Vector Laboratories). These sections were then subject to OT labelling, the same protocol was followed except the 3% H2O2 in 10% methanol treatment was extended to 30 min. Also, a 1 h blocking step with 1% bovine serum albumin (BSA) in TBS was added before 48 h incubation with the primary OT (1:500; Millipore; anti-rabbit). After rinsing four times, sections were incubated for 24 h in the fluorescent secondary antibody (DyLight 488 Horse Anti-Rabbit, 1:500, Vector laboratories), before being rinsed again, mounted on gelatinised microscope slides, and after 2-3 min of drying, being embedded in DPX. All incubations were conducted in a mixture of 0.25% gelatin and 0.5% Triton X-100 (Sigma, USA) in TBS. Intermediate rinsing was performed with TBS.

The sections were examined and photographed using a Nikon microscope equipped with an epifluorescent attachment, narrow-band filters and a camera. Co-localisation of immunoreactivity for c-Fos and OT was ascertained by overlaying the captured images of each labelled product. The number of single and double-labelled neurons was counted from these overlays. The percentage of c-Fos positive OT neurons in the paraventricular (PVN) and supraoptic (SON) nuclei was established per mouse and then per experimental group.

4.3.7 Effect of the lowest anorexigenic dose of IP TRP on activation of unidentified neurons in feeding-related brain sites and hypothalamic OT neurons

Neuronal activation (presence of an immediate-early gene product, c-Fos [56]) and biochemical phenotype of OT cells were determined with immunohistochemistry. The rats received an IP injection of 0 (saline) or 30 mg/kg TRP (the lowest hypophagic dose) or vehicle (n=6/group; injection time: 10:00-11:00), and food and water were removed from the cages. Animals were anaesthetised (35% urethane) and perfused with saline (50mL) followed by 500mL of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) 90 minutes after the treatment. Brains were removed and postfixed overnight in PFA at 4˚C. Coronal 60–µm vibratome (Leica, Germany) sections were immunostained for c-Fos. The tissue was treated for 10 minutes with 3% H_2O_2 in 10% methanol (in TBS; pH 7.4) and incubated overnight at 4˚C in the rabbit anti-Fos antibody (1:14000; Synaptic Systems, Germany). Subsequently, sections were incubated for 1 h in the goat-anti-rabbit antibody (1:400; Vector, USA) and then in the avidin-biotin complex (1 h;

Vector). Peroxidase was visualised with 0.05% DAB, 0.01% H_2O_2 and 0.2% nickel sulphate (Sigma, USA). Incubations were conducted in a mixture of 0.25% gelatin and 0.5% Triton X-100 (Sigma) in TBS. Intermediate rinsing was performed with TBS. Sections were mounted on gelatinised slides, dried, dehydrated in ethanol, soaked in xylene and embedded in Entellan (Merck, Germany). c-Fos IR nuclei were counted bilaterally (4-5 sections per site per animal), their densities (per mm^2) were averaged per rat and then per group.

In the c-Fos-OT double staining protocol, after completing the c-Fos detection process, hypothalamic sections underwent immunostaining again, however, this time, the primary OT antibody was used (1:8000; Millipore). No nickel sulphate was added to DAB, hence, OT immunoreactive cells displayed brown instead of black colour. The percentage of c-Fos positive OT neurons in the paraventricular (PVN) and supraoptic (SON) nuclei was established per rat and then per experimental group.

4.3.8 Effect of intragastric TRP on relative expression of OT mRNA in the hypothalamus

The mice received either a single intragastric infusion of 1200 mg/kg TRP (hypophagic dose) or water vehicle (n=8/group) at 10:00. Food and water were taken away right after the treatment and the mice were decapitated 3 hours later. Hypothalami were dissected and placed in RNAlater (Ambion). A standard protocol of sample preparation and rtPCR was followed and, for brevity, only the main steps are described here (see [5] for details). Samples were homogenised in TRIzol (Ambion); RNA was extracted with chloroform and precipitated in isopropanol.

After centrifuging, the pellet was washed, dried, and dissolved in the DNase buffer (NEB). The samples were treated with RNase-free DNase I (Merck) and the absence of genomic DNA was established by PCR of a 5% template. 100ng/µl genomic DNA served as a positive control, whereas MilliQ H2O as a negative one. The product was analysed by electrophoresis. 5µg RNA samples were diluted with MilliQ H2O. RNA was reverse-transcribed in the master mix (Promega; 20µl). 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 [OT primer sequences: CGGTGGATCTCGGACTGAAC (fwd) and TAGCAGGCGGAGGTCAGAG (rev)]. Expression of four housekeeping genes (glyceraldehyde-3-phosphate-dehydrogenase, β-actin, β-tubulin, and ribosomal protein) was used to calculate normalisation factors (GeNorm). Primer efficiencies were calculated with LinRegPCR (HFRC) and Ct values were corrected for differences in primer efficiencies.

4.3.9 Statistical analyses

Data from all ingestive behaviour studies utilising a single injection were processed with a t-test (two-group comparisons) or a one-way ANOVA followed by Dunnet's post-hoc analysis (multiple-group comparisons). Correction for multiple comparisons was applied. In the double-injection experiment, the t-test was used to establish the effect of IP TRP on deprivation-induced chow/water intake and then a one-way ANOVA followed by Dunnet's test was applied to compare differences between vehicle+TRP and the remaining two groups. Immunohistochemistry

results were analysed with a t-test. Values are presented as means ± SEM and they were deemed significantly different for $p \leq 0.05$.

4.4 Results

4.4.1 Intragastric tryptophan preload

In the hypothalamus, both the PVN and SON OT neurons were activated by TRP compared to vehicle (SON, P=0.004; PVN, P=0.001; Figure 4.2 A, B). Animals given TRP intragastrically showed an increase in hypothalamic OT mRNA levels (P=0.006; Figure 4.2 A). Pharmacological blockade of the OT receptors with the BBB-penetrant antagonist L-368,899 at 0.1 mg/kg ($F(3, 29)=3.71$; P=0.041) and 0.3 mg/kg IP (P=0.048) attenuated TRP-induced hypophagia (P=0.001) in the post-deprivation standard chow intake model (Figure 4.2 B).

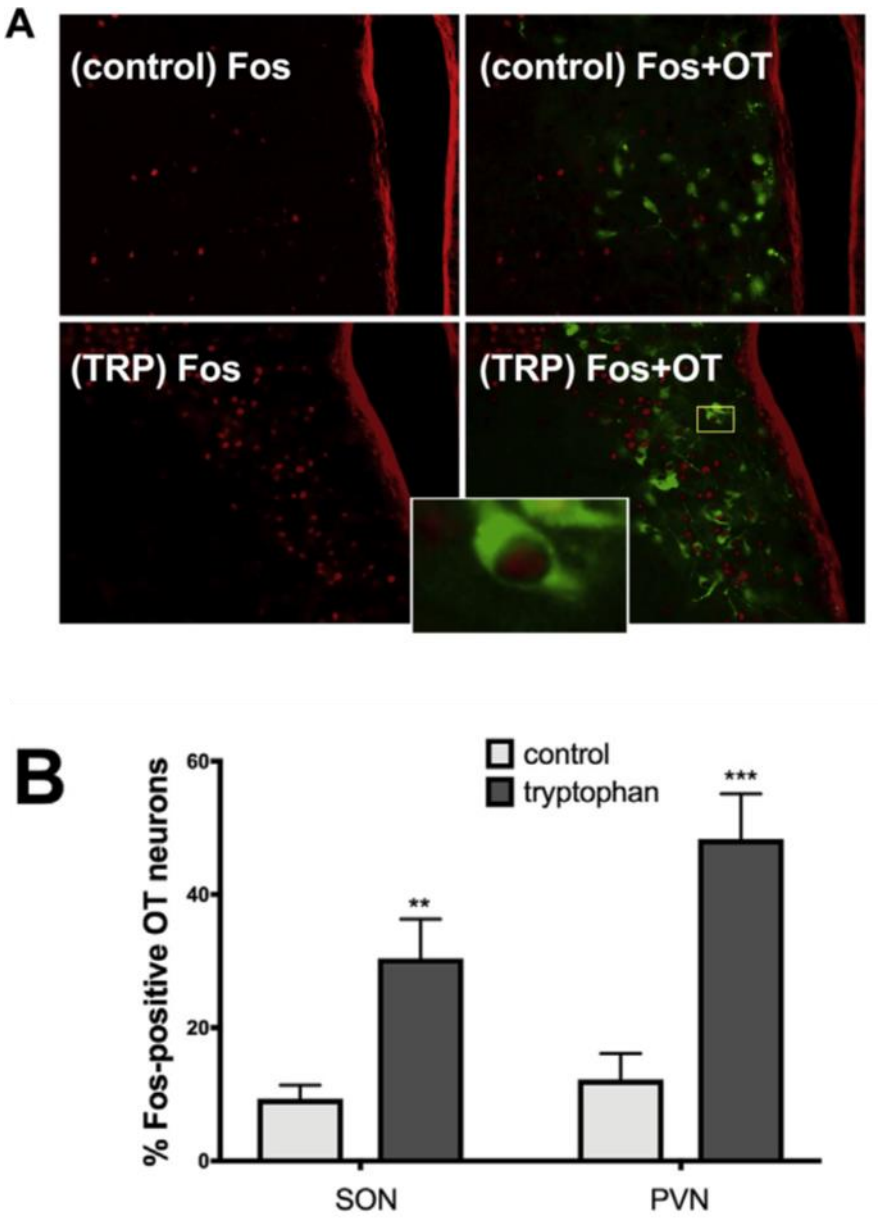

Figure 4.1 Intragastric TRP at 1200 mg/kg increases the percentage of c-Fos positive oxytocin (OT) neurons in the paraventricular (PVN) and supraoptic (SON) nuclei (A, B) Photomicrographs depict hypothalamic PVN sections stained for c-Fos (red) and OT (green). Insert – Fos positive OT neuron; * - P≤0.05; ** - P≤0.01; * - P≤0.001.**

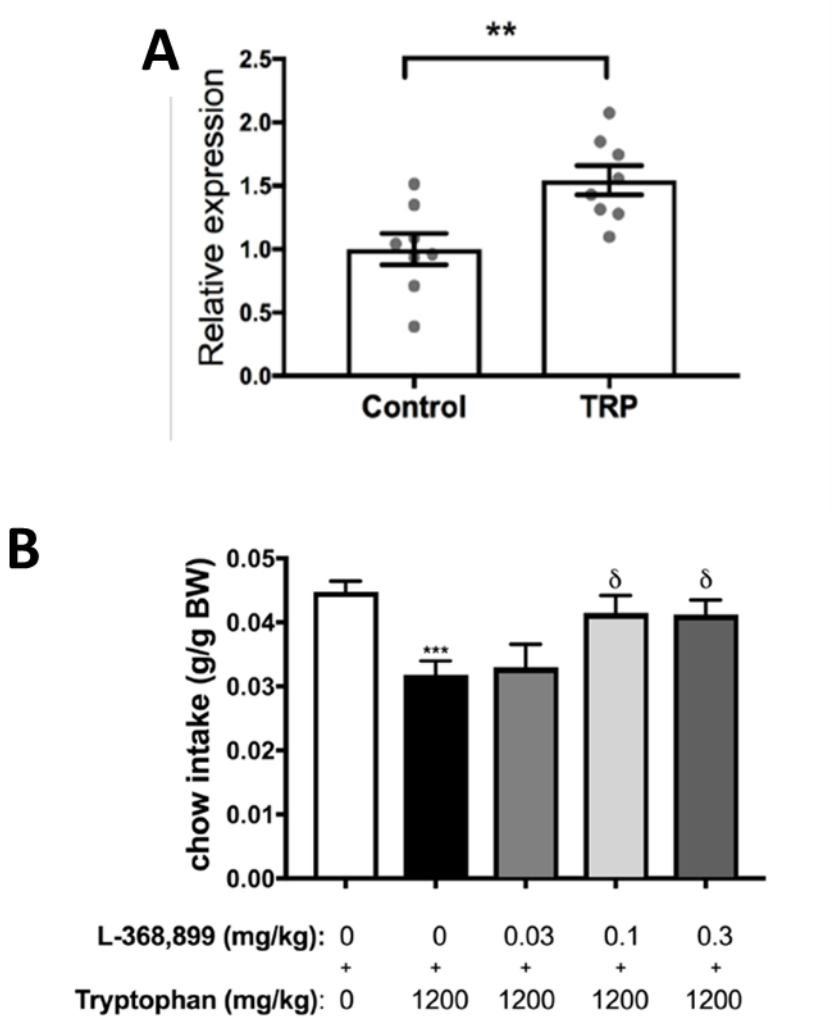

Figure 4.2 Intragastric TRP at 1200 mg/kg elevates relative expression of OT mRNA in the hypothalamus (A), whereas pretreatment with an intraperitoneal OT receptor antagonist, L-368,899, abolishes the anorexigenic effect of TRP in food-deprived mice during refeeding (B). Insert – Fos positive OT neuron; * - P≤0.05; ** - P≤0.01; * - P≤0.001.**

4.4.2 Intraperitoneal tryptophan preload

Double immunostaining revealed an increase in the percentage of activated OT neurons in the PVN ($p<0.001$) and SON ($p=0.0427$) in response to TRP (Figure 4.3 A, B). In feeding studies, only the effect of IP TRP on energy deprivation-induced chow intake ($p=0.042$) could be alleviated by antagonism of the OT receptor with L-368,899 ($F(2, 21)=4.35$, $p=0.0293$; Figure 4.4 A). TRP-driven decrease in water

intake ($p<0.001$) in thirsty rats remained the same after L-368,899 pretreatment (Figure 4.4 B).

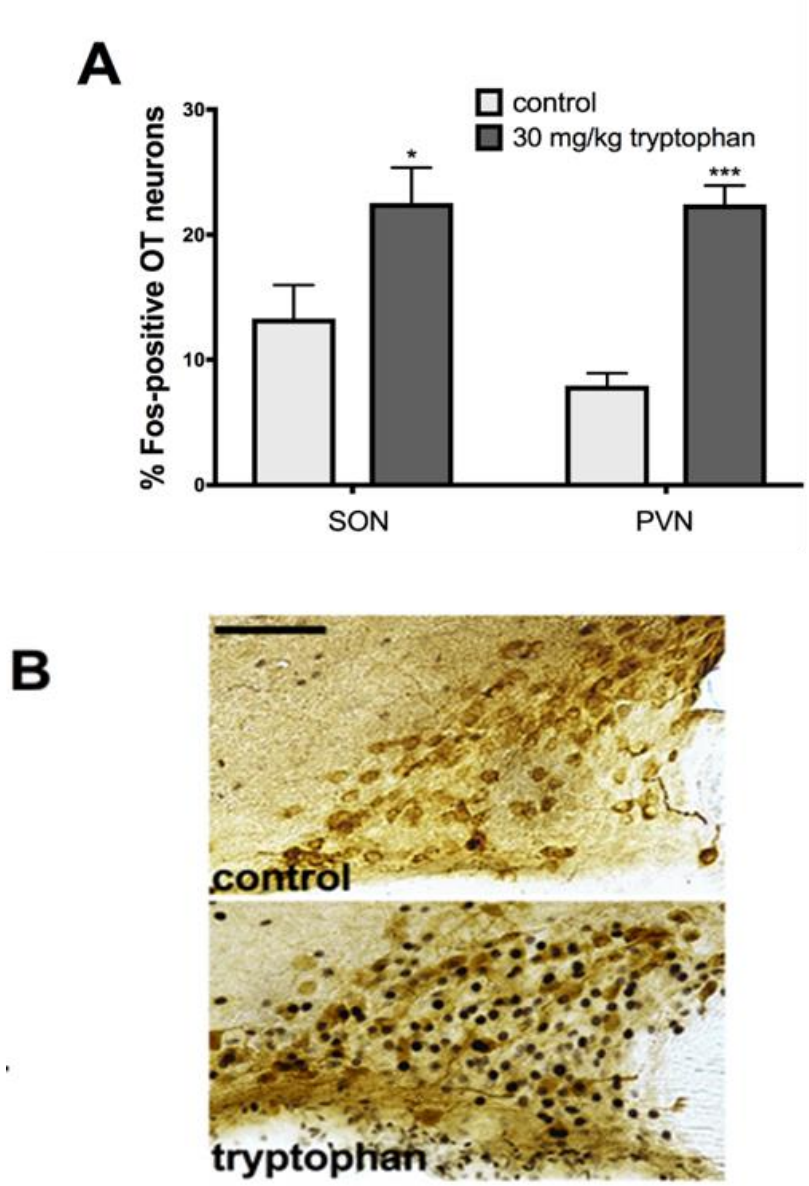

Figure 4.3 Activation (Fos IR) of OT neurons in the PVN and SON in response to 30 mg/kg TRP vs saline control (A, B). Doses shown in mg/kg (0 indicates saline control). */* - significantly different from saline-injected controls, P≤0.05 and P≤0.001, respectively. ∂ - significantly different from the TRP-treated group (P≤0.05).**

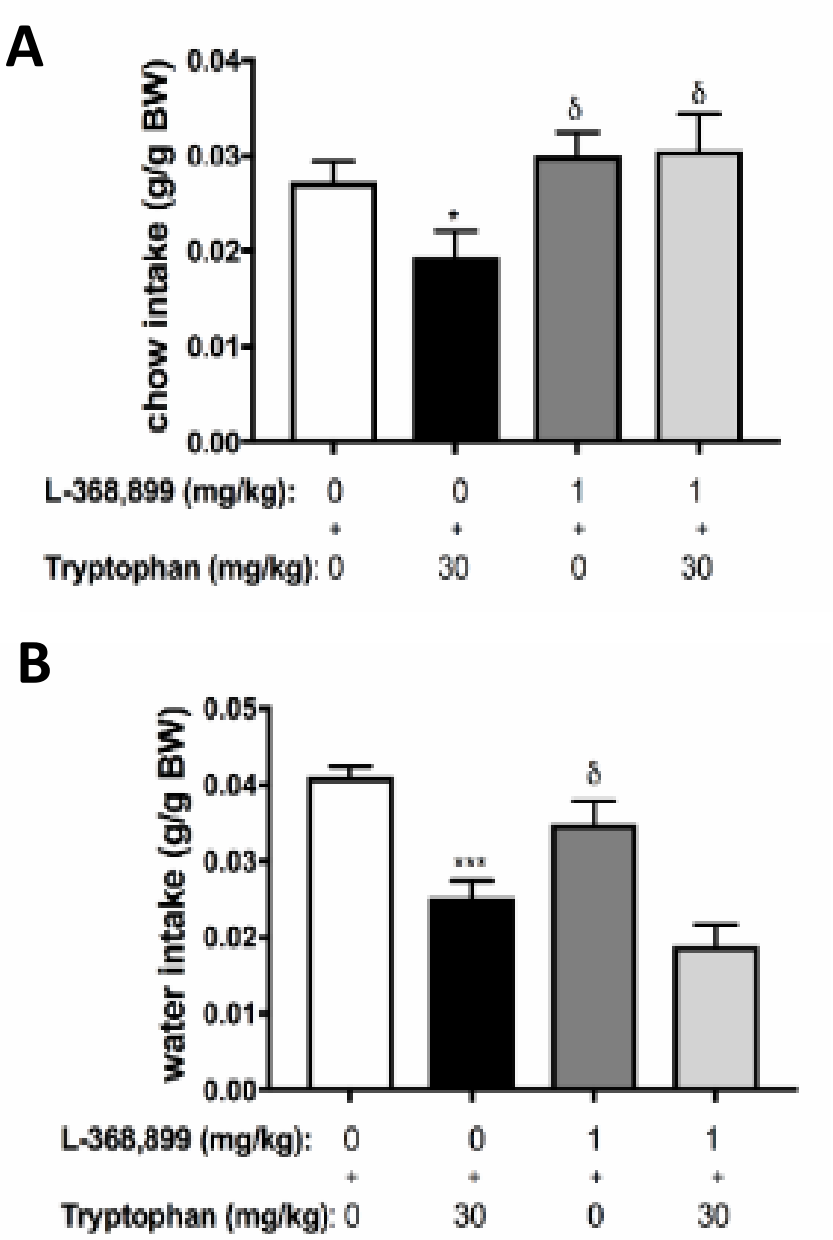

Figure 4.4 The effect of 1 mg/kg OT antagonist, L-369,899, pretreatment on (A) TRP-induced reduction in chow intake after food deprivation, and (B) TRP-induced reduction in water intake after water deprivation. Doses shown in mg/kg (0 indicates saline control). */* - significantly different from saline-injected controls, P≤0.05 and P≤0.001, respectively. ∂ - significantly different from the TRP-treated group (P≤0.05).**

4.5 Discussion

Central nervous system responses to fluctuations in nutrient levels are the most fundamental processes through which activity of neural and neuroendocrine pathways reflects (and responds to) the dynamic energy balance and to immediate changes in availability of key molecules obtained through ingestive behaviour. Most crucially, the presence of glucose sensing neurons in the brain has been well documented and their role in maintaining proper energy balance is unquestionable [57, 58]. It has been also shown that amino acids leucine, L-arginine, L-lysine and L-glutamate [1, 5-8, 59], affect the CNS, but the cellular events that ensue and broader pathways that are engaged by them have been precisely described only for leucine [1, 5-8, 13]. The data presented in this chapter strongly suggest that TRP requires the OT circuit to decrease energy intake, whereas in the case of TRP's effect on water consumption, OT is non-obligatory.

Food and water intake experiments reported in Chapters 2 and 3 of the current thesis indicate that intragastrically administered TRP suppresses energy intake at doses that are lower than those necessary to diminish thirst-driven drinking behaviour. On the other hand, IP TRP suppresses both drinking and eating within the same dose range. Previous injection studies utilising peripheral and central administration of OT have found that while the most consistent effect of OT is termination of food intake, also a decrease in water consumption can be observed [27, 29, 60]. Though the latter is not as frequently reported for all injection routes and pharmacological agents used [28, 35], it is nonetheless considered as a facet of pro-homeostatic action of OT in which regulatory effects of this neurohormone on water intake

might stem from control, for example, of osmolality and/or stomach distension [61-63].

The fact that OT receptor blockade with an OT receptor antagonist, L-368,899, at a dose that does not affect consumption prevented TRP-driven decrease in chow intake strongly suggests that the involvement of the OT circuit is obligatory for satiating effect of TRP to occur. Importantly, this was evident in the case of both intragastric TRP and IP TRP, which indicates that regardless of whether TRP comes in contact with gastric mucosa or its administration mode bypasses the gut, its anorexigenic action relies on activation of OT neurons. L-368,899 being ineffective in alleviating TRP's effect on deprivation-induced water intake suggests that while OT might still participate in this process, it is not essential for drinking behaviour reduction after IP TRP. Different pathways and neuronal populations facilitate feeding and drinking responses to TRP, but it is quite likely that to some extent they overlap (for example in the brainstem component – as evidenced in Chapters 2 and 3 by similarity in c-Fos distribution after intragastric and IP TRP in the DVC, a region that receives vagal input and is partially devoid of the BBB [1, 64]). Considering that IP TRP-induced c-Fos immunoreactivity in the PVN and SON spreads beyond the identified OT neurons, it is quite possible that PVN and SON cells synthesising vasopressin (VP) are involved in the observed thirst effects.

The release of the OT and enhanced OT neuronal activity coincide with satiation-associated termination of feeding in laboratory animals [23-26]. Furthermore, a similar effect on OT system is stimulated by physiologically relevant peptidergic treatments that promote satiation (e.g. α-MSH, CCK, GLP-1) [62, 65-68]. The

present set of data shows that the effect of both intragastric and IP TRP on OT neuronal activation parallels that induced by food loads as well as neuroactive agents that cause termination of consumption. That finding combined with the fact that TRP induces simultaneous activation of the key appetite-related brainstem circuit instils confidence in that TRP-derived hypophagia is indeed of physiological relevance and that the pathways involved in that process encompass OT.

One should note that the OT neuronal activation in animals infused with intragastric TRP was higher than in IP TRP-treated individuals. On the one hand, it may reflect the direct effect of TRP on gastric mucosa and, consequently, a hypothetical link it with, e.g., a release of select gut hormones, such as GLP-1 or CCK [69-72]. It is indeed quite possible taking into account the results of a 2014 study in humans showing that intraduodenal TRP increased levels of CCK, glucagon, and GLP-1, leading to increased fullness and substantially decreased energy intake [4]. However, it is also conceivable that gut stimulation by insertion of the intragastric probe added to the magnitude of the OT response (purely, as a consequence of mechanical stimulation of the GI tract): that vehicle-infused animals have a higher baseline activity than IP controls would support this notion. Nonetheless, the overall increase in both OT neuronal activity (in Fos studies) and OT mRNA expression after TRP signifies a functional relationship between the presence of this amino acid and responsiveness of the OT system.

It should also be mentioned that, although not formally determined due to the limited number of animals per group, TRP affected both magno- and parvocellular subpopulations of OT neurons. The PVN contains both of those subpopulations,

whereas the SON hosts magnocellular OT cells [31]. Parvocellular OT neurons release OT centrally, innervating numerous central areas, including the DVC [21, 40-46]. Other OT magnocellular subpopulations release OT via somatodendritic projections into the periphery (via neurohypophysis) and at central targets [21, 40-43, 47-49]. That TRP seems to affect both elements of the OT circuit, it can be speculated that it might thereby affect CNS-driven processes (via the DVC input) as well as peripheral mechanisms (through circulating OT).

What remains to be elucidated is why TRP (regardless of its route of administration) is such a weak inhibitor of eating for palatability, especially when caloric density is low. It is particularly surprising considering that the OT receptor is highly expressed in brain regions associated with food reward, with PVN OT neurons innervating these key regions [50, 51]. These regions include the VTA, Acb, and BNST [50, 51, 73]. OT receptor blockade by the BBB-penetrant antagonist, L-368,899, prolongs intake of palatable sweet solutions. Mullis and colleagues demonstrated that direct VTA OT administration reduced reward-driven sucrose consumption which was reversible with a pre-treatment with L-368,899 [36]. Intra-Acb and intra-amygdalar infusions of OT have been shown to promote sugar water consumption [35, 74]. Hence, the fact that TRP does not potently affect the portion of feeding driven by pleasure, it can be presumed that only a subset of hypophagia promoting OT neurons is engaged by TRP.

In sum, the findings outlined in this chapter strongly suggest that OT is an important central mediator of TRP-driven decrease in food intake regardless of whether TRP

is delivered intragastrically or its administration directly into the IP cavity bypasses the gut.

4.6 References

1. Jordi, J., B. Herzog, S.M. Camargo, C.N. Boyle, T.A. Lutz, and F. Verrey, *Specific amino acids inhibit food intake via the area postrema or vagal afferents.* J Physiol, 2013. **591**(22): p. 5611-21.
2. Ng, L.T. and G.H. Anderson, *Route of administration of tryptophan and tyrosine affects short-term food intake and plasma and brain amino acid concentrations in rats.* J Nutr, 1992. **122**(2): p. 283-93.
3. Ayaso, R., H. Ghattas, M. Abiad, and O. Obeid, *Meal pattern of male rats maintained on amino acid supplemented diets: the effect of tryptophan, lysine, arginine, proline and threonine.* Nutrients, 2014. **6**(7): p. 2509-22.
4. Steinert, R.E., N.D. Luscombe-Marsh, T.J. Little, S. Standfield, B. Otto, M. Horowitz, and C. Feinle-Bisset, *Effects of intraduodenal infusion of L-tryptophan on ad libitum eating, antropyloroduodenal motility, glycemia, insulinemia, and gut peptide secretion in healthy men.* J Clin Endocrinol Metab, 2014. **99**(9): p. 3275-84.
5. Cota, D., K. Proulx, K.A. Smith, S.C. Kozma, G. Thomas, S.C. Woods, and R.J. Seeley, *Hypothalamic mTOR signaling regulates food intake.* Science, 2006. **312**(5775): p. 927-30.
6. Morrison, C.D., X. Xi, C.L. White, J. Ye, and R.J. Martin, *Amino acids inhibit Agrp gene expression via an mTOR-dependent mechanism.* Am J Physiol Endocrinol Metab, 2007. **293**(1): p. E165-71.
7. Blouet, C. and G.J. Schwartz, *Brainstem nutrient sensing in the nucleus of the solitary tract inhibits feeding.* Cell Metab, 2012. **16**(5): p. 579-87.
8. Blouet, C., Y.H. Jo, X. Li, and G.J. Schwartz, *Mediobasal hypothalamic leucine sensing regulates food intake through activation of a hypothalamus-brainstem circuit.* J Neurosci, 2009. **29**(26): p. 8302-11.
9. Morrison, C.D., S.D. Reed, and T.M. Henagan, *Homeostatic regulation of protein intake: in search of a mechanism.* Am J Physiol Regul Integr Comp Physiol, 2012. **302**(8): p. R917-28.
10. Laviano, A., M.M. Meguid, A. Inui, and F. Rossi-Fanelli, *Role of leucine in regulating food intake.* Science, 2006. **313**(5791): p. 1236-8; author reply 1236-8.

11. Wang, S.B., P. Khondowe, S.F. Chen, J.J. Yu, G. Shu, X.T. Zhu, L.N. Wang, P. Gao, Q.Y. Xi, Y.L. Zhang, and Q.Y. Jiang, *Effects of "Bioactive" amino acids leucine, glutamate, arginine and tryptophan on feed intake and mRNA expression of relative neuropeptides in broiler chicks.* Journal of Animal Science and Biotechnology, 2012. **3**.
12. Purpera, M.N., L. Shen, M. Taghavi, H. Munzberg, R.J. Martin, S.M. Hutson, and C.D. Morrison, *Impaired branched chain amino acid metabolism alters feeding behavior and increases orexigenic neuropeptide expression in the hypothalamus.* J Endocrinol, 2012. **212**(1): p. 85-94.
13. McGavigan, A.K., H.C. O'Hara, A. Amin, J. Kinsey-Jones, E. Spreckley, A. Alamshah, A. Agahi, K. Banks, R. France, G. Hyberg, C. Wong, G.A. Bewick, J.V. Gardiner, A. Lehmann, N.M. Martin, M.A. Ghatei, S.R. Bloom, and K.G. Murphy, *L-cysteine suppresses ghrelin and reduces appetite in rodents and humans.* Int J Obes (Lond), 2015. **39**(3): p. 447-55.
14. Moos, F. and P. Richard, *Paraventricular and supraoptic bursting oxytocin cells in rat are locally regulated by oxytocin and functionally related.* J Physiol, 1989. **408**: p. 1-18.
15. Olszewski, P.K., A. Klockars, H.B. Schioth, and A.S. Levine, *Oxytocin as feeding inhibitor: maintaining homeostasis in consummatory behavior.* Pharmacol Biochem Behav, 2010. **97**(1): p. 47-54.
16. Swanson, L.W. and P.E. Sawchenko, *Hypothalamic integration: organization of the paraventricular and supraoptic nuclei.* Annu Rev Neurosci, 1983. **6**: p. 269-324.
17. Johnstone, L.E., T.M. Fong, and G. Leng, *Neuronal activation in the hypothalamus and brainstem during feeding in rats.* Cell Metab, 2006. **4**(4): p. 313-21.
18. Yamashita, M., Y. Takayanagi, M. Yoshida, K. Nishimori, M. Kusama, and T. Onaka, *Involvement of prolactin-releasing peptide in the activation of oxytocin neurones in response to food intake.* J Neuroendocrinol, 2013. **25**(5): p. 455-65.

19. Lokrantz, C.M., K. Uvnas-Moberg, and J.M. Kaplan, *Effects of central oxytocin administration on intraoral intake of glucose in deprived and nondeprived rats.* Physiol Behav, 1997. **62**(2): p. 347-52.
20. Olszewski, P.K., A. Klockars, A.M. Olszewska, R. Fredriksson, H.B. Schioth, and A.S. Levine, *Molecular, immunohistochemical, and pharmacological evidence of oxytocin's role as inhibitor of carbohydrate but not fat intake.* Endocrinology, 2010. **151**(10): p. 4736-44.
21. Huang, W., A.F. Sved, and E.M. Stricker, *Vasopressin and oxytocin release evoked by NaCl loads are selectively blunted by area postrema lesions.* Am J Physiol Regul Integr Comp Physiol, 2000. **278**(3): p. R732-40.
22. McCann, M.J. and R.C. Rogers, *Oxytocin excites gastric-related neurones in rat dorsal vagal complex.* J Physiol, 1990. **428**: p. 95-108.
23. Olson, B.R., M.D. Drutarosky, M.S. Chow, V.J. Hruby, E.M. Stricker, and J.G. Verbalis, *Oxytocin and an oxytocin agonist administered centrally decrease food intake in rats.* Peptides, 1991. **12**(1): p. 113-8.
24. Arletti, R., A. Benelli, and A. Bertolini, *Influence of oxytocin on feeding behavior in the rat.* Peptides, 1989. **10**(1): p. 89-93.
25. Deblon, N., C. Veyrat-Durebex, L. Bourgoin, A. Caillon, A.L. Bussier, S. Petrosino, F. Piscitelli, J.J. Legros, V. Geenen, M. Foti, W. Wahli, V. Di Marzo, and F. Rohner-Jeanrenaud, *Mechanisms of the anti-obesity effects of oxytocin in diet-induced obese rats.* PLoS One, 2011. **6**(9): p. e25565.
26. Sabatier, N., G. Leng, and J. Menzies, *Oxytocin, feeding, and satiety.* Front Endocrinol (Lausanne), 2013. **4**: p. 35.
27. Arletti, R., A. Benelli, and A. Bertolini, *Oxytocin inhibits food and fluid intake in rats.* Physiol Behav, 1990. **48**(6): p. 825-30.
28. Ho, J.M., V.T. Anekonda, B.W. Thompson, M. Zhu, R.W. Curry, B.H. Hwang, G.J. Morton, M.W. Schwartz, D.G. Baskin, S.M. Appleyard, and J.E. Blevins, *Hindbrain Oxytocin Receptors Contribute to the Effects of Circulating Oxytocin on Food Intake in Male Rats.* Endocrinology, 2014. **155**(8): p. 2845-57.

29. Benelli, A., A. Bertolini, and R. Arletti, *Oxytocin-induced inhibition of feeding and drinking: no sexual dimorphism in rats.* Neuropeptides, 1991. **20**(1): p. 57-62.
30. Klockars, A., C. Brunton, L. Li, A.S. Levine, and P.K. Olszewski, *Intravenous administration of oxytocin in rats acutely decreases deprivation-induced chow intake, but it fails to affect consumption of palatable solutions.* Peptides, 2017. **93**: p. 13-19.
31. Klockars, A., A.S. Levine, and P.K. Olszewski, *Central oxytocin and food intake: focus on macronutrient-driven reward.* Front Endocrinol (Lausanne), 2015. **6**: p. 65.
32. Amico, J.A., R.R. Vollmer, H.M. Cai, J.A. Miedlar, and L. Rinaman, *Enhanced initial and sustained intake of sucrose solution in mice with an oxytocin gene deletion.* Am J Physiol Regul Integr Comp Physiol, 2005. **289**(6): p. R1798-806.
33. Billings, L.B., J.A. Spero, R.R. Vollmer, and J.A. Amico, *Oxytocin null mice ingest enhanced amounts of sweet solutions during light and dark cycles and during repeated shaker stress.* Behav Brain Res, 2006. **171**(1): p. 134-41.
34. Herisson, F.M., L.L. Brooks, J.R. Waas, A.S. Levine, and P.K. Olszewski, *Functional relationship between oxytocin and appetite for carbohydrates versus saccharin.* Neuroreport, 2014. **25**(12): p. 909-14.
35. Herisson, F.M., J.R. Waas, R. Fredriksson, H.B. Schioth, A.S. Levine, and P.K. Olszewski, *Oxytocin Acting in the Nucleus Accumbens Core Decreases Food Intake.* J Neuroendocrinol, 2016. **28**(4).
36. Mullis, K., K. Kay, and D.L. Williams, *Oxytocin action in the ventral tegmental area affects sucrose intake.* Brain Res, 2013. **1513**: p. 85-91.
37. Leibowitz, S.F., N.J. Hammer, and K. Chang, *Hypothalamic paraventricular nucleus lesions produce overeating and obesity in the rat.* Physiol Behav, 1981. **27**(6): p. 1031-40.
38. Sims, J.S. and J.F. Lorden, *Effect of paraventricular nucleus lesions on body weight, food intake and insulin levels.* Behav Brain Res, 1986. **22**(3): p. 265-81.

39. Shor-Posner, G., A.P. Azar, S. Insinga, and S.F. Leibowitz, *Deficits in the control of food intake after hypothalamic paraventricular nucleus lesions.* Physiol Behav, 1985. **35**(6): p. 883-90.
40. Sagar, S.M., K.J. Price, N.W. Kasting, and F.R. Sharp, *Anatomic patterns of Fos immunostaining in rat brain following systemic endotoxin administration.* Brain Res Bull, 1995. **36**(4): p. 381-92.
41. Siaud, P., R. Puech, I. Assenmacher, and G. Alonso, *Microinjection of oxytocin into the dorsal vagal complex decreases pancreatic insulin secretion.* Brain Res, 1991. **546**(2): p. 190-4.
42. Hornby, P.J., *Central neurocircuitry associated with emesis.* Am J Med, 2001. **111 Suppl 8A**: p. 106S-112S.
43. Charpak, S., W.E. Armstrong, M. Muhlethaler, and J.J. Dreifuss, *Stimulatory action of oxytocin on neurones of the dorsal motor nucleus of the vagus nerve.* Brain Res, 1984. **300**(1): p. 83-9.
44. Kirchgessner, A.L. and A. Sclafani, *PVN-hindbrain pathway involved in the hypothalamic hyperphagia-obesity syndrome.* Physiol Behav, 1988. **42**(6): p. 517-28.
45. Blevins, J.E., M.W. Schwartz, and D.G. Baskin, *Evidence that paraventricular nucleus oxytocin neurons link hypothalamic leptin action to caudal brain stem nuclei controlling meal size.* Am J Physiol Regul Integr Comp Physiol, 2004. **287**(1): p. R87-96.
46. Geerling, J.C., J.W. Shin, P.C. Chimenti, and A.D. Loewy, *Paraventricular hypothalamic nucleus: axonal projections to the brainstem.* J Comp Neurol, 2010. **518**(9): p. 1460-99.
47. Pow, D.V. and J.F. Morris, *Dendrites of hypothalamic magnocellular neurons release neurohypophysial peptides by exocytosis.* Neuroscience, 1989. **32**(2): p. 435-9.
48. Miyagawa, A., H. Okamura, and Y. Ibata, *Coexistence of oxytocin and NADPH-diaphorase in magnocellular neurons of the paraventricular and the supraoptic nuclei of the rat hypothalamus.* Neurosci Lett, 1994. **171**(1-2): p. 13-6.

49. de Kock, C.P., K.D. Wierda, L.W. Bosman, R. Min, J.J. Koksma, H.D. Mansvelder, M. Verhage, and A.B. Brussaard, *Somatodendritic secretion in oxytocin neurons is upregulated during the female reproductive cycle.* J Neurosci, 2003. **23**(7): p. 2726-34.
50. Qi, J., J.Y. Yang, M. Song, Y. Li, F. Wang, and C.F. Wu, *Inhibition by oxytocin of methamphetamine-induced hyperactivity related to dopamine turnover in the mesolimbic region in mice.* Naunyn Schmiedebergs Arch Pharmacol, 2008. **376**(6): p. 441-8.
51. Gould, B.R. and H.H. Zingg, *Mapping oxytocin receptor gene expression in the mouse brain and mammary gland using an oxytocin receptor-LacZ reporter mouse.* Neuroscience, 2003. **122**(1): p. 155-67.
52. Sofroniew, M.V., *Projections from vasopressin, oxytocin, and neurophysin neurons to neural targets in the rat and human.* J Histochem Cytochem, 1980. **28**(5): p. 475-8.
53. Succu, S., F. Sanna, C. Cocco, T. Melis, A. Boi, G.L. Ferri, A. Argiolas, and M.R. Melis, *Oxytocin induces penile erection when injected into the ventral tegmental area of male rats: role of nitric oxide and cyclic GMP*. Eur J Neurosci, 2008. **28**(4): p. 813-21.
54. Olszewski, P.K., A. Klockars, and A.S. Levine, *Oxytocin: A Conditional Anorexigen whose Effects on Appetite Depend on the Physiological, Behavioural and Social Contexts.* J Neuroendocrinol, 2016. **28**(4).
55. Boccia, M.L., A.P. Goursaud, J. Bachevalier, K.D. Anderson, and C.A. Pedersen, *Peripherally administered non-peptide oxytocin antagonist, L368,899, accumulates in limbic brain areas: a new pharmacological tool for the study of social motivation in non-human primates.* Horm Behav, 2007. **52**(3): p. 344-51.
56. Kovacs, K.J., *Measurement of immediate-early gene activation- c-fos and beyond.* J Neuroendocrinol, 2008. **20**(6): p. 665-72.
57. Routh, V.H., L. Hao, A.M. Santiago, Z. Sheng, and C. Zhou, *Hypothalamic glucose sensing: making ends meet.* Front Syst Neurosci, 2014. **8**: p. 236.

58. Steinbusch, L., G. Labouebe, and B. Thorens, *Brain glucose sensing in homeostatic and hedonic regulation.* Trends Endocrinol Metab, 2015. **26**(9): p. 455-66.
59. Alamshah, A., A.K. McGavigan, E. Spreckley, J.S. Kinsey-Jones, A. Amin, I.R. Tough, H.C. O'Hara, A. Moolla, K. Banks, R. France, G. Hyberg, M. Norton, W. Cheong, A. Lehmann, S.R. Bloom, H.M. Cox, and K.G. Murphy, *L-arginine promotes gut hormone release and reduces food intake in rodents.* Diabetes Obes Metab, 2016. **18**(5): p. 508-18.
60. Bernal, A., J. Mahia, and A. Puerto, *Oxytocin, water intake, and food sodium availability in male rats.* Horm Behav, 2007. **52**(3): p. 289-96.
61. Calatayud, S., E. Quintana, J. Esplugues, and M.D. Barrachina, *Role of central oxytocin in the inhibition by endotoxin of distension-stimulated gastric acid secretion.* Naunyn Schmiedebergs Arch Pharmacol, 1999. **360**(6): p. 676-82.
62. Renaud, L.P., M. Tang, M.J. McCann, E.M. Stricker, and J.G. Verbalis, *Cholecystokinin and gastric distension activate oxytocinergic cells in rat hypothalamus.* Am J Physiol, 1987. **253**(4 Pt 2): p. R661-5.
63. Nelson, E.E., J.R. Alberts, Y. Tian, and J.G. Verbalis, *Oxytocin is elevated in plasma of 10-day-old rats following gastric distension.* Brain Res Dev Brain Res, 1998. **111**(2): p. 301-3.
64. Zuger, D., K. Forster, T.A. Lutz, and T. Riediger, *Amylin and GLP-1 target different populations of area postrema neurons that are both modulated by nutrient stimuli.* Physiol Behav, 2013. **112-113**: p. 61-9.
65. Bojanowska, E. and B. Stempniak, *tGLP-1 and release of vasopressin and oxytocin from the isolated rat hypothalamo-neurohypophysial system: effects of a tGLP-1 receptor agonist and antagonist.* J Physiol Pharmacol, 2001. **52**(4 Pt 2): p. 781-93.
66. Ladyman, S.R., R.A. Augustine, E. Scherf, H.R. Phillipps, C.H. Brown, and D.R. Grattan, *Attenuated hypothalamic responses to alpha-melanocyte stimulating hormone during pregnancy in the rat.* J Physiol, 2016. **594**(4): p. 1087-101.

67. Ohlsson, B., M.L. Forsling, J.F. Rehfeld, and K. Sjolund, *Cholecystokinin stimulation leads to increased oxytocin secretion in women.* Eur J Surg, 2002. **168**(2): p. 114-8.
68. Verbalis, J.G., M.J. McCann, C.M. McHale, and E.M. Stricker, *Oxytocin secretion in response to cholecystokinin and food: differentiation of nausea from satiety.* Science, 1986. **232**(4756): p. 1417-9.
69. Ritter, R.C., *Gastrointestinal mechanisms of satiation for food.* Physiol Behav, 2004. **81**(2): p. 249-73.
70. Morton, G.J., D.E. Cummings, D.G. Baskin, G.S. Barsh, and M.W. Schwartz, *Central nervous system control of food intake and body weight.* Nature, 2006. **443**(7109): p. 289-95.
71. Cummings, D.E. and J. Overduin, *Gastrointestinal regulation of food intake.* J Clin Invest, 2007. **117**(1): p. 13-23.
72. Guyenet, S.J. and M.W. Schwartz, *Clinical review: Regulation of food intake, energy balance, and body fat mass: implications for the pathogenesis and treatment of obesity.* J Clin Endocrinol Metab, 2012. **97**(3): p. 745-55.
73. Jarrett, T.M., M.S. McMurray, C.H. Walker, and J.M. Johns, *Cocaine treatment alters oxytocin receptor binding but not mRNA production in postpartum rat dams.* Neuropeptides, 2006. **40**(3): p. 161-7.
74. Klockars, O.A., A. Klockars, A.S. Levine, and P.K. Olszewski, *Oxytocin administration in the basolateral and central nuclei of amygdala moderately suppresses food intake.* Neuroreport, 2018. **29**(6): p. 504-510.

5 Discussion and Perspectives

Obesity arises from a dysregulation in energy balance when an individual's energy intake habitually exceeds total energy expenditure [1-4]. Human and laboratory animal studies have consistently demonstrated that individuals eat not only to satisfy energy needs, but also (and, in the obesogenic environment, primarily) for reward [5]. Palatability to a great extent drives this overconsumption of energy by stimulating appetite despite the lack of underlying energy deficit and, importantly, by delaying satiety regardless of the fact that a large amount of energy has been ingested [6, 7]. When these phenomena occur sporadically, i.e., under relative scarcity of palatable food, they prove beneficial for an individual in that they propel search for and avid consumption of diets that are caloric and tasty. However, unrestricted and constant access to palatable foods continues to engage these mechanisms, even though these processes become redundant (and, eventually, harmful, when they lead to obesity) in the evolutionarily unique 'environment of plenty'. Dysregulation of energy homeostasis ensues, at it is accompanied by elements of addictive-like overeating, aberrant peripheral hormonal release and delayed satiety signalling within relevant brain circuits ([8] – to review). Hence, an ongoing quest for developing strategies to overcome excessive food consumption is aimed at identifying molecules and processes that can alleviate the severity of or reverse appetite dysregulation.

One of the fundamental strategies involves manipulating composition of a diet so that it becomes 'satiating' (a broad and colloquial term that encompasses diverse factors, from the volume of a food load to sensory satiety). In line with that notion,

high-protein diets have generated interest for decades as a phenomenon of protein-derived satiety has been well documented in numerous studies [9-15]. The anorectic effect of protein has been linked to oral, postoral and postabsorptive mechanisms, including changes in consumption-related reward, satiation, and energy metabolism [9-11]. However, it was well understood that protein is digested to individual amino acids, thus, at least a portion of the appetite regulatory role of protein could be attributed to them [16]; it is especially likely in relation to essential amino acids, which are not synthesised by the mammalian organisms [17]. This has a significant potential from the therapeutic standpoint as select individual amino acids – should they be used in obesity treatments – might have a better palatability/acceptability profile and their minimum concentrations/frequency of dosing might be easier to determine and control.

Before undertaking the experimental work required for this thesis, it was evident that some amino acids exhibit anorexigenic effects. For example, it has been shown that leucine, L-arginine, L-lysine and L-glutamate [18-21], decrease food intake by utilising CNS mechanisms, but the precise cellular events and broader pathways engaged by them are defined in detail only for leucine [18, 20-24]. Limited studies focused on the essential amino acid, TRP. Some suggested that peripherally administered TRP (via injection or oral/gastric delivery) produces hypophagia, whereas others reported no effect [18, 25-30]. However, considering the ability of ingested TRP to enter not only the general circulation, but also readily cross the BBB, it seemed apparent that role of this key player in neurotransmitter and hormone synthesis pathways (5-HT/melatonin) should be systematically characterised.

The current set of studies shows that free TRP administered before a meal is indeed an anorexigen. It decreases intake of energy by engaging broad feeding-related brain circuitry, including pathways that encompass OT, and it does so without producing undesirable malaise. At the same time, TRP does not seem to be similarly effective in decreasing a drive for palatable solutions, which is critical considering a strong link between palatability and excessive consumption.

Our investigations confirmed effectiveness of TRP, regardless whether intragastric or IP, to reduce the intake of energy-dense diets, especially in response to energy deficit. The intragastric infusion of 600 mg/kg TRP in overnight deprived mice reduced the intake of standard chow, this was in line with the proposed role of dietary amino acids in promoting early satiation thereby decreasing meal size [25]. 30 mg/kg IP TRP, a dose only somewhat lower than in earlier reports [26, 29], reduced the intake of standard chow in hungry rats. In sated mice motivated to eat by palatability, intragastric TRP was necessary to decrease episodic consumption of energy-dense palatable chow, although a higher 1200 mg/kg was needed to produce this effect in the absence of hunger (compared to 600-mg/kg dose in deprivation-induced feeding). In line with that, in sated rats, only the higher dose of 100 mg/kg IP TRP managed to reduce consumption of palatable chow. Thus, it appears that an effective dose of TRP needed to produce food intake termination depends on energy needs.

Importantly, in the aforementioned experiments, both the standard and palatable chow were the calorie-dense, complex and balanced diets containing the essential macro- and micronutrients. Therefore, the question arose as to whether TRP would

also diminish intake of nutritionally inadequate non- and low-calorie solutions in paradigms in which consumption is driven entirely by palatability. This is particularly important for understanding central mechanisms that underlie TRP-driven hypophagia as many neuroactive mediators affect either eating for energy (e.g., NPY [31-36] and AGRP [37-40]) or eating for reward (e.g. opioid receptor ligands preferentially modify intake of energy-dilute palatable sweet and fatty solutions regardless of energy needs [41-46]) or eating stimulated by both of these variables (e.g. OT decreases intake for both palatability and energy, by acting through disperse circuitry [47, 48]. Intragastric TRP even at doses up to 1800 mg/kg, failed to decrease intake of non- and low- caloric solutions including an Intralipid emulsion, sucrose and saccharin solutions. In sated rats motivated to eat by palatability, TRP needed to be administered at a higher, 100-mg/kg dose, to reduce consumption of palatable solutions. Therefore, it seems that in both intragastric and IP TRP delivery routes, palatability-driven feeding mechanisms are not the key regulatory processes affected by this amino acid.

The lack of effect on the intake of the palatable solutions is particularly striking when considering the fact that 1200 mg/kg intragastric TRP suppressed water intake in thirsty mice (in contrast, even the 1800 mg/kg dose employed in the sucrose solution study had no effect). Similarly, IP TRP (both 30 mg/kg and 100 mg/kg) reduced water intake in thirst-driven rats. The analysis of water intake via the intragastric route indicated the suppression of energy intake at doses that are lower than those necessary to diminish thirst-driven behaviour. However, after IP TRP infusion both drinking, and eating were supressed within the equal dose range. This outcome opens a new question as to whether TRP-driven reductions in the intake

of energy-dense chow are intertwined with or are independent from changes in drinking behaviour. This critical issue needs to be elucidated in the future as previous studies have undeniably shown that the amount of ingested fluid affects the amount of ingested food and vice versa. It is noteworthy that the observed anorexigenic effect of TRP is not a result of sickness or malaise. This gives us greater confidence in concluding that intragastric and IP TRP engages actual appetite-regulatory processes rather than producing GI discomfort which then lead to termination of food intake.

The pattern of c-Fos immunoreactivity evoked by 1200 mg/kg intragastric TRP and 30 mg/kg IP TRP was fairly consistent, suggesting the key role of the brainstem circuitry in mediating TRP's effects on feeding. Indeed, intragastric TRP infusion caused elevated NTS and DMNV neuronal activation which underscores the importance of the vagal afferents, whereas a slight (albeit, non-significant) increase in AP c-Fos levels implies a possible involvement of this sensory circumventricular organ [18]. An enhancement of neuronal activation within the NTS was identified following IP TRP. c-Fos changes were observed also in feeding-related forebrain areas, which suggests that TRP engages a broader network of central sites (most notably, the hypothalamus) either in a direct or indirect manner. After intragastric gavage, the most robust increase in neuronal activation occurred in the hypothalamic DMH, PVN and SON, the latter two also exhibited enhanced activity after IP TRP. Those hypothalamic regions receive input from the brainstem [49] and typically show enhanced activation at meal termination as well as upon physiological changes that signify the need to cease consumption (stomach distension, plasma osmolality, etc) [50-52]. The TRP-induced change in c-Fos in

the brainstem-hypothalamic circuit is consistent with the proposed role for TRP in the regulation of feeding for energy [25, 26]. IP TRP, which bypasses the gastric phase, induced a somewhat lower neuronal activation with fewer brain regions affected than intragastric TRP. Possibly, this reflects the effect of TRP on gastric mucosa and its link with the release of GI hormones (e.g. GLP-1 or CCK) [53-58]. Importantly, this Fos pattern is also aligned well with the effect TRP on thirst-driven water intake, as a subset of the activated sites mediates not just feeding, but also drinking behaviour [59]. For example, the SON (with VP) neurons [60] and the CeA, are involved in the regulation of water balance via brainstem-descending pathways [61-63].

What is also important from the point of a functional relationship between TRP and food intake control mechanisms is the fact that both IP and intragastric TRP activate PVN and SON neurons that synthesise OT, a key satiety mediator. Intragastric TRP at an anorexigenic dose of 1200 mg/kg increases OT mRNA expression in the hypothalamus. OT is a 9-amino acid neurohormone [64, 65] that inhibits feeding in order to protect internal milieu (e.g. energy balance, stomach distension and plasma osmolality) [66-70]. Increased activation of OT neurons and release of OT, correspond with the cessation of ingestive behaviour [71-76] and centrally acting OT is known to stimulate the suppression of food intake [74, 77, 78]. The fact that OT receptor blockade with an OT receptor antagonist, L-368,899 at a dose that does not affect consumption, prevented TRP-driven decrease in chow intake strongly suggests that the involvement of the OT circuit is required for satiating effect of TRP to occur. Importantly, this was evident in the case of both intragastric TRP and IP TRP, which indicates that regardless of whether TRP comes in contact with

gastric mucosa or its administration route bypasses the gut, its anorexigenic action relies on activation of OT neurons. L-368,899 being ineffective in alleviating IP TRP's effect on deprivation-induced water intake suggests that while OT might still participate in this process, it is not essential for drinking behaviour reduction after IP TRP. Considering that IP TRP-induced c-Fos immunoreactivity in the PVN and SON spreads beyond the identified OT neurons, it is quite possible that PVN and SON cells synthesising other neuropeptides, such as VP, are involved in the thirst effects [60]. As the release of the OT and OT neuronal activity coincide with satiation-associated termination of feeding [73-76], and a similar effect on OT system is stimulated by administration of peptides that promote satiation (e.g. alpha-MSH, CCK, GLP-1) [51, 79-82]. One should note that the OT neuronal activation (similarly to the general c-Fos profile throughout the brain) in animals infused with intragastric TRP was higher than in IP TRP-treated individuals. It may reflect the direct effect of TRP on gastric mucosa and, consequently, a hypothetical link it with, e.g., a release of select gut hormones, as intraduodenal TRP increases of CCK, glucagon, and GLP-1, levels [83]. Nonetheless, the present data show that the effect of both intragastric and IP TRP on OT neuronal activation parallels that induced by food loads as well as by neuroactive agents that cause termination of consumption. That finding combined with the fact that TRP induces simultaneous activation of the key appetite-related brainstem circuit instils confidence in that TRP-derived hypophagia is indeed of physiological relevance and that the pathways involved in that process encompass OT. Further studies involving modification of central brain circuits, particularly involving genetic ablation of OT neurons or

disruption of brainstem-hypothalamic pathways, would shed more light on the role of central circuitry in TRP-induced hypophagia.

It should be noted that OT receptor is also expressed in the peripheral tissues, for example, in the adipose tissue, uterine smooth muscle and bone cells [84-87]. Studies to date have shown that short-term appetite changes are driven mainly by the central OT receptor, therefore, in short-term feeding studies the use of peripherally administered BBB-penetrant OT receptor ligands is commonly used without the risk of having the effects altered via peripheral mechanisms. The OT receptor antagonist used in the current experiments had been thoroughly studied in the context of its transient effects on appetite, and the outcomes indicate that regardless of whether it is injected centrally or IP, it produces the same consumption profile [48, 88-90]. It should be emphasised, however, that should a long-term treatment be attempted, then central administration of L-368,899 would be preferred, thereby removing the confound of the peripheral pool of the OT receptor.

In retrospect, the analysis of c-Fos immunoreactivity in animals that were treated with the OT receptor antagonist would be useful. One of the expected differences would be a decreased activity within the brainstem areas associated with meal termination that are rich in OT receptor. Combining this experimental approach with analysing the OT plasma levels could serve as a foundation of a future research project that draws upon the findings presented herein. This would enable us to elucidate the contribution between central and peripheral effects of OT relating to TRP's effect.

Importantly, as stress is known to activate both PVN and SON OT neurons [91, 92], one should note that the control groups in our experiments provide an adequate baseline to compare similar administration routes with TRP and vehicle. Attempts were made to try and limit the level of stress experienced by animals, especially following the oral gavage procedure. Animals were always returned to their home cages following infusion and then allowed 20 minutes before tastant given. Rats were avoided for intragastric infusion as individuals demonstrated high levels of stress following the procedure – much lesser response observed in mice.

One of the most striking outcomes of these studies is the fact that TRP is such a poor inhibitor of feeding for reward, especially of calorie-dilute solutions. It is particularly surprising given the functional link between TRP and OT. Especially that the OT receptor is highly expressed in brain regions associated with food reward (e.g. VTA, Acb and BNST), with PVN OT neurons innervating these key regions [93-96]. Also, OT receptor antagonist, L-368,899, prolongs intake of palatable sweet solutions, whereas intra-VTA and intra-Acb OT infusions suppress palatability-driven sucrose consumption, reversible with a pre-treatment of L-368,899 [47, 48]. Hence, the fact that TRP does not potently affect the portion of feeding driven by pleasure suggests that only a subset of hypophagia promoting OT neurons is engaged by this amino acid.

Overall, the findings outlined in this thesis strongly indicate the appetite suppressant role of TRP in energy intake. Also, it is suggested that OT – as part of the brainstem-hypothalamic pathways involved in energy homeostasis - is an

important neural mediator of TRP-driven reduction in food consumption regardless of whether TRP is delivered intragastrically or IP (thus, bypassing the gut).

Importantly intragastric and IP TRP did not cause the development of a CTA, indicating that the observed hypophagia does not stem from aversion. One should note that the mouse model might have benefited from an inclusion of an additional experiment verifying our CTA induction paradigm, in which animals would be injected IP with saline and LiCl. However, previous studies published by our group have verified effectiveness of this particular experimental setup in mice (e.g., [97]) and therefore the institutional ethics regulations prevented us from repeating the experiment yet another time.

Furthermore, the alanine control for osmolality in intragastrically treated mice provided evidence that changes in osmolality that parallel those associated with TRP administration do not by themselves induce hypophagia. Therefore, the anorexigenic effect of TRP was specific to this amino acid, but not to others that produce a similar osmolality profile. Since the anorexigenic dose of IP TRP was more than 10 times lower than after intragastric administration, the likelihood of any significant changes in the osmotic plasma profile after the treatment was so low that it did not warrant including additional amino acid IP controls. One could debate as to whether intragastric alanine or water would serve as a better control for TRP, however, using alanine would add more confounding factors to the analysis (including possibly some unforeseen behavioural changes seemingly unrelated to appetite, but eventually affecting food intake as a secondary outcome). In c-Fos studies, given that alanine acts as an osmotic control, we would expect decreased

c-Fos expression profile in regions associated with osmotic regulation (such as the SON which encompasses VP neurons [59, 60]), in animals that were treated with alanine compared to TRP.

There are some limitations of the studies, and these were minimised where possible. Firstly, one noticeable limitation was the use of two rodent species (mouse – oral gavage and rat – IP injection) alongside differing methods of TRP administration. The model species were selected based on the fact that in our pilot studies, unlike mice, rats did not respond well to the stress of gavaging, as indicated by large discrepancies in food intake within treatment groups and generally lower-than-expected feeding values. Therefore, in order to minimise the risk of stress influencing the outcomes of actual experiments, the intragastric route of administration was matched with a species that was less susceptible to displaying treatment-induced anxiety/discomfort. Furthermore, differences in responsiveness to hypo/hyperphagic treatments between mice and rats are rare. Many orexigens (e.g. ghrelin) and anorexigens (e.g. OT, CCK) are known to be effective in both species. For example, ICV administration of ghrelin in rodents produced a dose-dependent increase in both food intake and bodyweight [98]. Of interest, peripheral OT infusions in diet-induced obese mice/rat models reduces food intake whilst enhancing weightloss [99, 100].

One of the most urgent emerging issues in order to better understand TRP-driven hypophagia is a systematic identification of hormonal, neural and cellular processes that bridge TRP and activation of feeding-related neural networks, including those that encompass OT. Based on the results presented herein and in other reports, it is most plausible that TRP acting peripherally utilises several concurrent processes to induce a reduction in food intake. Initial evidence suggests the importance of the aforementioned GI hormonal route. However, there is also a critical link between TRP and 5-HT and melatonin: both of these affect (directly and indirectly) sleep, mood, anxiety and other processes that translate to altered appetite [17, 101-105]. There is also a possibility of TRP acting at cells directly [57, 106].

Furthermore, the experimental work presented here determined meal-associated changes in appetite following acute administration of TRP. However, in order to mimic therapeutic application of this approach in the basic research setting, one would have to consider the effects of chronic TRP administration. Would a lower subthreshold anorexigenic dose received chronically overtime have an accumulative effect on bodyweight and food intake? Studies to date – also those pertaining to other amino acids – have predominantly focused on the effect of episodic intake after amino acids dosing [18, 24, 26]. Perhaps, amino acids that possess hypophagic tendencies at subthreshold doses could exhibit an enhanced combined effect whilst avoiding the need for high dosages. In regards to TRP, it is well documented that the transporter LAT1 is responsible for the competitive transport of LNAA across the BBB [107-109]. Therefore, by influencing the ratio of other LNAA's to TRP may be able to enhance the uptake of TRP even at a lower dose [110, 111]. In fact, the plasma concentrations of TRP, and BBB transport, have

been shown to be influenced by alterations in dietary availability [112]. One of the next logical long-term steps would be to manipulate TRP levels in the diet.

These questions are of paramount importance, especially in the context of utilising TRP in functional food-based anti-obesity strategies and as a potential 'nutri-pharmaceutical'. Only by answering them will we be able to determine whether and how to supplement TRP in order to maximise its beneficial effects on energy intake, while reducing the risks that stem from the vast complexity of diverse physiological mechanisms that require this essential amino acid.

6 Conclusions

The overarching aim of this doctoral thesis was to examine whether TRP suppresses food intake via energy balance- and/or reward-related mechanisms. The findings of the study are:

- Intragastric and IP TRP pretreatment reduces feeding for energy.
- TRP is a weak inhibitor of palatability-driven consumption: only higher IP TRP doses reduce intake of nil- or low-calorie palatable solutions, whereas intragastric TRP is ineffective.
- Anorexigenic effects of TRP do not stem from sickness/malaise.
- Both intragastric and IP TRP reduces thirst-induced water intake.
- TRP increases activation of several key feeding-related brain areas, especially in the brainstem and hypothalamus.
- TRP-driven reduction in food but not water intake might, at least to some extent, mediated via central circuits that encompass oxytocin.

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8 References

1. Bray, G.A., *Food intake, sympathetic activity, and adrenal steroids.* Brain Res Bull, 1993. **32**(5): p. 537-41.
2. Allen, M.S. and B.J. Bradford, *Control of food intake by metabolism of fuels: a comparison across species.* Proc Nutr Soc, 2012. **71**(3): p. 401-9.
3. French, S.A., L.H. Epstein, R.W. Jeffery, J.E. Blundell, and J. Wardle, *Eating behavior dimensions. Associations with energy intake and body weight. A review.* Appetite, 2012. **59**(2): p. 541-9.
4. Hill, J.O., H.R. Wyatt, and J.C. Peters, *Energy balance and obesity*. Circulation, 2012. **126**(1): p. 126-32.
5. Zheng, H., N.R. Lenard, A.C. Shin, and H.R. Berthoud, *Appetite control and energy balance regulation in the modern world: reward-driven brain overrides repletion signals.* Int J Obes (Lond), 2009. **33 Suppl 2**: p. S8-13.
6. Sclafani, A. and D. Springer, *Dietary obesity in adult rats: similarities to hypothalamic and human obesity syndromes.* Physiol Behav, 1976. **17**(3): p. 461-71.
7. Sampey, B.P., A.M. Vanhoose, H.M. Winfield, A.J. Freemerman, M.J. Muehlbauer, P.T. Fueger, C.B. Newgard, and L. Makowski, *Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation: comparison to high-fat diet.* Obesity (Silver Spring), 2011. **19**(6): p. 1109-17.
8. de Macedo, I.C., J.S. de Freitas, and I.L. da Silva Torres, *The Influence of Palatable Diets in Reward System Activation: A Mini Review.* Adv Pharmacol Sci, 2016. **2016**: p. 7238679.
9. Poppitt, S.D., D. McCormack, and R. Buffenstein, *Short-term effects of macronutrient preloads on appetite and energy intake in lean women.* Physiol Behav, 1998. **64**(3): p. 279-85.
10. Westerterp-Plantenga, M.S., M.P. Lejeune, I. Nijs, M. van Ooijen, and E.M. Kovacs, *High protein intake sustains weight maintenance after body weight loss in humans.* Int J Obes Relat Metab Disord, 2004. **28**(1): p. 57-64.

11. Weigle, D.S., P.A. Breen, C.C. Matthys, H.S. Callahan, K.E. Meeuws, V.R. Burden, and J.Q. Purnell, *A high-protein diet induces sustained reductions in appetite, ad libitum caloric intake, and body weight despite compensatory changes in diurnal plasma leptin and ghrelin concentrations.* Am J Clin Nutr, 2005. **82**(1): p. 41-8.
12. Bensaid, A., D. Tome, D. Gietzen, P. Even, C. Morens, N. Gausseres, and G. Fromentin, *Protein is more potent than carbohydrate for reducing appetite in rats.* Physiol Behav, 2002. **75**(4): p. 577-82.
13. Jean, C., S. Rome, V. Mathe, J.F. Huneau, N. Aattouri, G. Fromentin, C.L. Achagiotis, and D. Tome, *Metabolic evidence for adaptation to a high protein diet in rats.* J Nutr, 2001. **131**(1): p. 91-8.
14. Lacroix, M., C. Gaudichon, A. Martin, C. Morens, V. Mathe, D. Tome, and J.F. Huneau, *A long-term high-protein diet markedly reduces adipose tissue without major side effects in Wistar male rats.* Am J Physiol Regul Integr Comp Physiol, 2004. **287**(4): p. R934-42.
15. Burton-Freeman, B., D.W. Gietzen, and B.O. Schneeman, *Meal pattern analysis to investigate the satiating potential of fat, carbohydrate, and protein in rats.* Am J Physiol, 1997. **273**(6 Pt 2): p. R1916-22.
16. Koehnle, T.J., M.C. Russell, and D.W. Gietzen, *Rats rapidly reject diets deficient in essential amino acids.* J Nutr, 2003. **133**(7): p. 2331-5.
17. Le Floc'h, N., W. Otten, and E. Merlot, *Tryptophan metabolism, from nutrition to potential therapeutic applications.* Amino Acids, 2011. **41**(5): p. 1195-1205.
18. Jordi, J., B. Herzog, S.M. Camargo, C.N. Boyle, T.A. Lutz, and F. Verrey, *Specific amino acids inhibit food intake via the area postrema or vagal afferents.* J Physiol, 2013. **591**(22): p. 5611-21.
19. Alamshah, A., A.K. McGavigan, E. Spreckley, J.S. Kinsey-Jones, A. Amin, I.R. Tough, H.C. O'Hara, A. Moolla, K. Banks, R. France, G. Hyberg, M. Norton, W. Cheong, A. Lehmann, S.R. Bloom, H.M. Cox, and K.G. Murphy, *L-arginine promotes gut hormone release and reduces food intake in rodents.* Diabetes Obes Metab, 2016. **18**(5): p. 508-18.

20. Blouet, C. and G.J. Schwartz, *Brainstem nutrient sensing in the nucleus of the solitary tract inhibits feeding.* Cell Metab, 2012. **16**(5): p. 579-87.
21. Cota, D., K. Proulx, K.A. Smith, S.C. Kozma, G. Thomas, S.C. Woods, and R.J. Seeley, *Hypothalamic mTOR signaling regulates food intake.* Science, 2006. **312**(5775): p. 927-30.
22. Morrison, C.D., X. Xi, C.L. White, J. Ye, and R.J. Martin, *Amino acids inhibit Agrp gene expression via an mTOR-dependent mechanism.* Am J Physiol Endocrinol Metab, 2007. **293**(1): p. E165-71.
23. Blouet, C., Y.H. Jo, X. Li, and G.J. Schwartz, *Mediobasal hypothalamic leucine sensing regulates food intake through activation of a hypothalamus-brainstem circuit.* J Neurosci, 2009. **29**(26): p. 8302-11.
24. McGavigan, A.K., H.C. O'Hara, A. Amin, J. Kinsey-Jones, E. Spreckley, A. Alamshah, A. Agahi, K. Banks, R. France, G. Hyberg, C. Wong, G.A. Bewick, J.V. Gardiner, A. Lehmann, N.M. Martin, M.A. Ghatei, S.R. Bloom, and K.G. Murphy, *L-cysteine suppresses ghrelin and reduces appetite in rodents and humans.* Int J Obes (Lond), 2015. **39**(3): p. 447-55.
25. Ayaso, R., H. Ghattas, M. Abiad, and O. Obeid, *Meal pattern of male rats maintained on amino acid supplemented diets: the effect of tryptophan, lysine, arginine, proline and threonine.* Nutrients, 2014. **6**(7): p. 2509-22.
26. Ng, L.T. and G.H. Anderson, *Route of administration of tryptophan and tyrosine affects short-term food intake and plasma and brain amino acid concentrations in rats.* J Nutr, 1992. **122**(2): p. 283-93.
27. Morris, P., E.T. Li, M.L. MacMillan, and G.H. Anderson, *Food intake and selection after peripheral tryptophan.* Physiol Behav, 1987. **40**(2): p. 155-63.
28. Gibbons, J.L., G.A. Barr, W.H. Bridger, and S.F. Leibowitz, *Manipulations of dietary tryptophan: effects on mouse killing and brain serotonin in the rat.* Brain Res, 1979. **169**(1): p. 139-53.
29. Gibbons, J.L., G.A. Barr, W.H. Bridger, and S.F. Leibowitz, *L-Tryptophan's effects on mouse killing, feeding, drinking, locomotion, and brain serotonin.* Pharmacol Biochem Behav, 1981. **15**(2): p. 201-6.

30. Peters, J.C., D.B. Bellissimo, and A.E. Harper, *L-tryptophan injection fails to alter nutrient selection by rats.* Physiol Behav, 1984. **32**(2): p. 253-9.
31. Henry, M., L. Ghibaudi, J. Gao, and J.J. Hwa, *Energy metabolic profile of mice after chronic activation of central NPY Y1, Y2, or Y5 receptors.* Obes Res, 2005. **13**(1): p. 36-47.
32. Mashiko, S., A. Ishihara, H. Iwaasa, H. Sano, Z. Oda, J. Ito, M. Yumoto, M. Okawa, J. Suzuki, T. Fukuroda, M. Jitsuoka, N.R. Morin, D.J. MacNeil, L.H. Van der Ploeg, M. Ihara, T. Fukami, and A. Kanatani, *Characterization of neuropeptide Y (NPY) Y5 receptor-mediated obesity in mice: chronic intracerebroventricular infusion of D-Trp(34)NPY.* Endocrinology, 2003. **144**(5): p. 1793-801.
33. Morley, J.E., A.S. Levine, B.A. Gosnell, J. Kneip, and M. Grace, *Effect of neuropeptide Y on ingestive behaviors in the rat.* Am J Physiol, 1987. **252**(3 Pt 2): p. R599-609.
34. Raposinho, P.D., D.D. Pierroz, P. Broqua, R.B. White, T. Pedrazzini, and M.L. Aubert, *Chronic administration of neuropeptide Y into the lateral ventricle of C57BL/6J male mice produces an obesity syndrome including hyperphagia, hyperleptinemia, insulin resistance, and hypogonadism.* Mol Cell Endocrinol, 2001. **185**(1-2): p. 195-204.
35. Sainsbury, A., I. Cusin, F. Rohner-Jeanrenaud, and B. Jeanrenaud, *Adrenalectomy prevents the obesity syndrome produced by chronic central neuropeptide Y infusion in normal rats.* Diabetes, 1997. **46**(2): p. 209-14.
36. Stanley, B.G., S.E. Kyrkouli, S. Lampert, and S.F. Leibowitz, *Neuropeptide Y chronically injected into the hypothalamus: a powerful neurochemical inducer of hyperphagia and obesity.* Peptides, 1986. **7**(6): p. 1189-92.
37. Graham, M., J.R. Shutter, U. Sarmiento, I. Sarosi, and K.L. Stark, *Overexpression of Agrt leads to obesity in transgenic mice.* Nat Genet, 1997. **17**(3): p. 273-4.
38. Hagan, M.M., S.C. Benoit, P.A. Rushing, L.M. Pritchard, S.C. Woods, and R.J. Seeley, *Immediate and prolonged patterns of Agouti-related peptide-(83--132)-induced c-Fos activation in hypothalamic and extrahypothalamic sites.* Endocrinology, 2001. **142**(3): p. 1050-6.

39. Hagan, M.M., P.A. Rushing, L.M. Pritchard, M.W. Schwartz, A.M. Strack, L.H. Van Der Ploeg, S.C. Woods, and R.J. Seeley, *Long-term orexigenic effects of AgRP-(83---132) involve mechanisms other than melanocortin receptor blockade.* Am J Physiol Regul Integr Comp Physiol, 2000. **279**(1): p. R47-52.

40. Rossi, M., M.S. Kim, D.G. Morgan, C.J. Small, C.M. Edwards, D. Sunter, S. Abusnana, A.P. Goldstone, S.H. Russell, S.A. Stanley, D.M. Smith, K. Yagaloff, M.A. Ghatei, and S.R. Bloom, *A C-terminal fragment of Agouti-related protein increases feeding and antagonizes the effect of alpha-melanocyte stimulating hormone in vivo.* Endocrinology, 1998. **139**(10): p. 4428-31.

41. Kelley, A.E., E.P. Bless, and C.J. Swanson, *Investigation of the effects of opiate antagonists infused into the nucleus accumbens on feeding and sucrose drinking in rats.* J Pharmacol Exp Ther, 1996. **278**(3): p. 1499-507.

42. Levine, A.S., D.T. Weldon, M. Grace, J.P. Cleary, and C.J. Billington, *Naloxone blocks that portion of feeding driven by sweet taste in food-restricted rats.* Am J Physiol, 1995. **268**(1 Pt 2): p. R248-52.

43. Ragnauth, A., H. Ruegg, and R.J. Bodnar, *Evaluation of opioid receptor subtype antagonist effects in the ventral tegmental area upon food intake under deprivation, glucoprivic and palatable conditions.* Brain Res, 1997. **767**(1): p. 8-16.

44. Rockwood, G.A. and L.D. Reid, *Naloxone modifies sugar-water intake in rats drinking with open gastric fistulas.* Physiol Behav, 1982. **29**(6): p. 1175-8.

45. Ruegg, H., W.Z. Yu, and R.J. Bodnar, *Opioid-receptor subtype agonist-induced enhancements of sucrose intake are dependent upon sucrose concentration.* Physiol Behav, 1997. **62**(1): p. 121-8.

46. Weldon, D.T., E. O'Hare, J. Cleary, C.J. Billington, and A.S. Levine, *Effect of naloxone on intake of cornstarch, sucrose, and polycose diets in restricted and nonrestricted rats.* Am J Physiol, 1996. **270**(6 Pt 2): p. R1183-8.

47. Mullis, K., K. Kay, and D.L. Williams, *Oxytocin action in the ventral tegmental area affects sucrose intake.* Brain Res, 2013. **1513**: p. 85-91.
48. Herisson, F.M., J.R. Waas, R. Fredriksson, H.B. Schioth, A.S. Levine, and P.K. Olszewski, *Oxytocin Acting in the Nucleus Accumbens Core Decreases Food Intake.* J Neuroendocrinol, 2016. **28**(4).
49. Záborszky, L., C.S. Léránth, J. Marton, and M. Palkovits, *Afferent Brainstem Pathways to Hypothalamus and to Limbic System in the Rat.*
50. Calatayud, S., E. Quintana, J. Esplugues, and M.D. Barrachina, *Role of central oxytocin in the inhibition by endotoxin of distension-stimulated gastric acid secretion.* Naunyn Schmiedebergs Arch Pharmacol, 1999. **360**(6): p. 676-82.
51. Renaud, L.P., M. Tang, M.J. McCann, E.M. Stricker, and J.G. Verbalis, *Cholecystokinin and gastric distension activate oxytocinergic cells in rat hypothalamus.* Am J Physiol, 1987. **253**(4 Pt 2): p. R661-5.
52. Nelson, E.E., J.R. Alberts, Y. Tian, and J.G. Verbalis, *Oxytocin is elevated in plasma of 10-day-old rats following gastric distension.* Brain Res Dev Brain Res, 1998. **111**(2): p. 301-3.
53. Batterham, R.L., H. Heffron, S. Kapoor, J.E. Chivers, K. Chandarana, H. Herzog, C.W. Le Roux, E.L. Thomas, J.D. Bell, and D.J. Withers, *Critical role for peptide YY in protein-mediated satiation and body-weight regulation.* Cell Metab, 2006. **4**(3): p. 223-33.
54. van der Klaauw, A.A., J.M. Keogh, E. Henning, V.M. Trowse, W.S. Dhillo, M.A. Ghatei, and I.S. Farooqi, *High protein intake stimulates postprandial GLP1 and PYY release.* Obesity (Silver Spring), 2013. **21**(8): p. 1602-7.
55. Blom, W.A., A. Lluch, A. Stafleu, S. Vinoy, J.J. Holst, G. Schaafsma, and H.F. Hendriks, *Effect of a high-protein breakfast on the postprandial ghrelin response.* Am J Clin Nutr, 2006. **83**(2): p. 211-20.
56. Tome, D., J. Schwarz, N. Darcel, and G. Fromentin, *Protein, amino acids, vagus nerve signaling, and the brain.* Am J Clin Nutr, 2009. **90**(3): p. 838S-843S.
57. Fromentin, G., N. Darcel, C. Chaumontet, A. Marsset-Baglieri, N. Nadkarni, and D. Tome, *Peripheral and central mechanisms involved in the control of*

food intake by dietary amino acids and proteins. Nutr Res Rev, 2012. **25**(1): p. 29-39.

58. Bowen, J., M. Noakes, and P.M. Clifton, *Appetite hormones and energy intake in obese men after consumption of fructose, glucose and whey protein beverages.* Int J Obes (Lond), 2007. **31**(11): p. 1696-703.
59. Antunes-Rodrigues, J., M. de Castro, L.L. Elias, M.M. Valenca, and S.M. McCann, *Neuroendocrine control of body fluid metabolism.* Physiol Rev, 2004. **84**(1): p. 169-208.
60. Hurbin, A., H. Orcel, G. Alonso, F. Moos, and A. Rabie, *The vasopressin receptors colocalize with vasopressin in the magnocellular neurons of the rat supraoptic nucleus and are modulated by water balance.* Endocrinology, 2002. **143**(2): p. 456-66.
61. Andrade-Franze, G.M., C.A. Andrade, L.A. De Luca, Jr., P.M. De Paula, D.S. Colombari, and J.V. Menani, *Lesions in the central amygdala impair sodium intake induced by the blockade of the lateral parabrachial nucleus.* Brain Res, 2010. **1332**: p. 57-64.
62. Saha, S., *Role of the central nucleus of the amygdala in the control of blood pressure: descending pathways to medullary cardiovascular nuclei.* Clin Exp Pharmacol Physiol, 2005. **32**(5-6): p. 450-6.
63. Cai, H., W. Haubensak, T.E. Anthony, and D.J. Anderson, *Central amygdala PKC-delta(+) neurons mediate the influence of multiple anorexigenic signals.* Nat Neurosci, 2014. **17**(9): p. 1240-8.
64. Moos, F. and P. Richard, *Paraventricular and supraoptic bursting oxytocin cells in rat are locally regulated by oxytocin and functionally related.* J Physiol, 1989. **408**: p. 1-18.
65. Olszewski, P.K., A. Klockars, H.B. Schioth, and A.S. Levine, *Oxytocin as feeding inhibitor: maintaining homeostasis in consummatory behavior.* Pharmacol Biochem Behav, 2010. **97**(1): p. 47-54.
66. Swanson, L.W. and P.E. Sawchenko, *Hypothalamic integration: organization of the paraventricular and supraoptic nuclei.* Annu Rev Neurosci, 1983. **6**: p. 269-324.

67. Johnstone, L.E., T.M. Fong, and G. Leng, *Neuronal activation in the hypothalamus and brainstem during feeding in rats.* Cell Metab, 2006. **4**(4): p. 313-21.
68. Yamashita, M., Y. Takayanagi, M. Yoshida, K. Nishimori, M. Kusama, and T. Onaka, *Involvement of prolactin-releasing peptide in the activation of oxytocin neurones in response to food intake.* J Neuroendocrinol, 2013. **25**(5): p. 455-65.
69. Lokrantz, C.M., K. Uvnas-Moberg, and J.M. Kaplan, *Effects of central oxytocin administration on intraoral intake of glucose in deprived and nondeprived rats.* Physiol Behav, 1997. **62**(2): p. 347-52.
70. Olszewski, P.K., A. Klockars, A.M. Olszewska, R. Fredriksson, H.B. Schioth, and A.S. Levine, *Molecular, immunohistochemical, and pharmacological evidence of oxytocin's role as inhibitor of carbohydrate but not fat intake.* Endocrinology, 2010. **151**(10): p. 4736-44.
71. Huang, W., A.F. Sved, and E.M. Stricker, *Vasopressin and oxytocin release evoked by NaCl loads are selectively blunted by area postrema lesions.* Am J Physiol Regul Integr Comp Physiol, 2000. **278**(3): p. R732-40.
72. McCann, M.J. and R.C. Rogers, *Oxytocin excites gastric-related neurones in rat dorsal vagal complex.* J Physiol, 1990. **428**: p. 95-108.
73. Olson, B.R., M.D. Drutarosky, M.S. Chow, V.J. Hruby, E.M. Stricker, and J.G. Verbalis, *Oxytocin and an oxytocin agonist administered centrally decrease food intake in rats.* Peptides, 1991. **12**(1): p. 113-8.
74. Arletti, R., A. Benelli, and A. Bertolini, *Influence of oxytocin on feeding behavior in the rat.* Peptides, 1989. **10**(1): p. 89-93.
75. Deblon, N., C. Veyrat-Durebex, L. Bourgoin, A. Caillon, A.L. Bussier, S. Petrosino, F. Piscitelli, J.J. Legros, V. Geenen, M. Foti, W. Wahli, V. Di Marzo, and F. Rohner-Jeanrenaud, *Mechanisms of the anti-obesity effects of oxytocin in diet-induced obese rats.* PLoS One, 2011. **6**(9): p. e25565.
76. Sabatier, N., G. Leng, and J. Menzies, *Oxytocin, feeding, and satiety.* Front Endocrinol (Lausanne), 2013. **4**: p. 35.
77. Arletti, R., A. Benelli, and A. Bertolini, *Oxytocin inhibits food and fluid intake in rats.* Physiol Behav, 1990. **48**(6): p. 825-30.

78. Ho, J.M., V.T. Anekonda, B.W. Thompson, M. Zhu, R.W. Curry, B.H. Hwang, G.J. Morton, M.W. Schwartz, D.G. Baskin, S.M. Appleyard, and J.E. Blevins, *Hindbrain Oxytocin Receptors Contribute to the Effects of Circulating Oxytocin on Food Intake in Male Rats.* Endocrinology, 2014. **155**(8): p. 2845-57.

79. Bojanowska, E. and B. Stempniak, *tGLP-1 and release of vasopressin and oxytocin from the isolated rat hypothalamo-neurohypophysial system: effects of a tGLP-1 receptor agonist and antagonist.* J Physiol Pharmacol, 2001. **52**(4 Pt 2): p. 781-93.

80. Ladyman, S.R., R.A. Augustine, E. Scherf, H.R. Phillipps, C.H. Brown, and D.R. Grattan, *Attenuated hypothalamic responses to alpha-melanocyte stimulating hormone during pregnancy in the rat.* J Physiol, 2016. **594**(4): p. 1087-101.

81. Ohlsson, B., M.L. Forsling, J.F. Rehfeld, and K. Sjolund, *Cholecystokinin stimulation leads to increased oxytocin secretion in women.* Eur J Surg, 2002. **168**(2): p. 114-8.

82. Verbalis, J.G., M.J. McCann, C.M. McHale, and E.M. Stricker, *Oxytocin secretion in response to cholecystokinin and food: differentiation of nausea from satiety.* Science, 1986. **232**(4756): p. 1417-9.

83. Steinert, R.E., N.D. Luscombe-Marsh, T.J. Little, S. Standfield, B. Otto, M. Horowitz, and C. Feinle-Bisset, *Effects of intraduodenal infusion of L-tryptophan on ad libitum eating, antropyloroduodenal motility, glycemia, insulinemia, and gut peptide secretion in healthy men.* J Clin Endocrinol Metab, 2014. **99**(9): p. 3275-84.

84. Arrowsmith, S. and S. Wray, *Oxytocin: its mechanism of action and receptor signalling in the myometrium.* J Neuroendocrinol, 2014. **26**(6): p. 356-69.

85. Yi, K.J., K.H. So, Y. Hata, Y. Suzuki, D. Kato, K. Watanabe, H. Aso, Y. Kasahara, K. Nishimori, C. Chen, K. Katoh, and S.G. Roh, *The regulation of oxytocin receptor gene expression during adipogenesis.* J Neuroendocrinol, 2015. **27**(5): p. 335-42.

86. Sun, L., R. Tamma, T. Yuen, G. Colaianni, Y. Ji, C. Cuscito, J. Bailey, S. Dhawan, P. Lu, C.D. Calvano, L.L. Zhu, C.G. Zambonin, A. Di Benedetto, A. Stachnik, P. Liu, M. Grano, S. Colucci, T.F. Davies, M.I. New, A. Zallone, and M. Zaidi, *Functions of vasopressin and oxytocin in bone mass regulation.* Proc Natl Acad Sci U S A, 2016. **113**(1): p. 164-9.

87. Zingg, H.H. and S.A. Laporte, *The oxytocin receptor.* Trends Endocrinol Metab, 2003. **14**(5): p. 222-7.

88. Klockars, O.A., J.R. Waas, A. Klockars, A.S. Levine, and P.K. Olszewski, *Neural Basis of Ventromedial Hypothalamic Oxytocin-Driven Decrease in Appetite.* Neuroscience, 2017. **366**: p. 54-61.

89. Olszewski, P.K., K. Allen, and A.S. Levine, *Effect of oxytocin receptor blockade on appetite for sugar is modified by social context.* Appetite, 2015. **86**: p. 81-7.

90. Herisson, F.M., L.L. Brooks, J.R. Waas, A.S. Levine, and P.K. Olszewski, *Functional relationship between oxytocin and appetite for carbohydrates versus saccharin.* Neuroreport, 2014. **25**(12): p. 909-14.

91. Babygirija, R., M. Bulbul, S. Yoshimoto, K. Ludwig, and T. Takahashi, *Central and peripheral release of oxytocin following chronic homotypic stress in rats.* Auton Neurosci, 2012. **167**(1-2): p. 56-60.

92. Minhas, S., C. Liu, J. Galdamez, V.M. So, and R.D. Romeo, *Stress-induced oxytocin release and oxytocin cell number and size in prepubertal and adult male and female rats.* Gen Comp Endocrinol, 2016. **234**: p. 103-9.

93. Qi, J., J.Y. Yang, M. Song, Y. Li, F. Wang, and C.F. Wu, *Inhibition by oxytocin of methamphetamine-induced hyperactivity related to dopamine turnover in the mesolimbic region in mice.* Naunyn Schmiedebergs Arch Pharmacol, 2008. **376**(6): p. 441-8.

94. Gould, B.R. and H.H. Zingg, *Mapping oxytocin receptor gene expression in the mouse brain and mammary gland using an oxytocin receptor-LacZ reporter mouse.* Neuroscience, 2003. **122**(1): p. 155-67.

95. Sofroniew, M.V., *Projections from vasopressin, oxytocin, and neurophysin neurons to neural targets in the rat and human.* J Histochem Cytochem, 1980. **28**(5): p. 475-8.

96. Succu, S., F. Sanna, C. Cocco, T. Melis, A. Boi, G.L. Ferri, A. Argiolas, and M.R. Melis, *Oxytocin induces penile erection when injected into the ventral tegmental area of male rats: role of nitric oxide and cyclic GMP.* Eur J Neurosci, 2008. **28**(4): p. 813-21.

97. Olszewski, P.K., J.R. Waas, L.L. Brooks, F. Herisson, and A.S. Levine, *Oxytocin receptor blockade reduces acquisition but not retrieval of taste aversion and blunts responsiveness of amygdala neurons to an aversive stimulus.* Peptides, 2013. **50**: p. 36-41.

98. Tschop, M., D.L. Smiley, and M.L. Heiman, *Ghrelin induces adiposity in rodents.* Nature, 2000. **407**(6806): p. 908-13.

99. Morton, G.J., B.S. Thatcher, R.D. Reidelberger, K. Ogimoto, T. Wolden-Hanson, D.G. Baskin, M.W. Schwartz, and J.E. Blevins, *Peripheral oxytocin suppresses food intake and causes weight loss in diet-induced obese rats.* Am J Physiol Endocrinol Metab, 2012. **302**(1): p. E134-44.

100. Maejima, Y., Y. Iwasaki, Y. Yamahara, M. Kodaira, U. Sedbazar, and T. Yada, *Peripheral oxytocin treatment ameliorates obesity by reducing food intake and visceral fat mass.* Aging (Albany NY), 2011. **3**(12): p. 1169-77.

101. Silber, B.Y. and J.A. Schmitt, *Effects of tryptophan loading on human cognition, mood, and sleep.* Neurosci Biobehav Rev, 2010. **34**(3): p. 387-407.

102. Oswald, I., G.W. Ashcroft, R.J. Berger, D. Eccleston, J.I. Evans, and V.R. Thacore, *Some experiments in the chemistry of normal sleep.* Br J Psychiatry, 1966. **112**(485): p. 391-9.

103. Jouvet, M., *Biogenic amines and the states of sleep.* Science, 1969. **163**(3862): p. 32-41.

104. Sainio, E.L., K. Pulkki, and S.N. Young, *L-Tryptophan: Biochemical, nutritional and pharmacological aspects.* Amino Acids, 1996. **10**(1): p. 21-47.

105. Cajochen, C., K. Krauchi, and A. Wirz-Justice, *Role of melatonin in the regulation of human circadian rhythms and sleep.* J Neuroendocrinol, 2003. **15**(4): p. 432-7.

106. Wellendorph, P. and H. Brauner-Osborne, *Molecular basis for amino acid sensing by family C G-protein-coupled receptors.* Br J Pharmacol, 2009. **156**(6): p. 869-84.

107. Boado, R.J., J.Y. Li, M. Nagaya, C. Zhang, and W.M. Pardridge, *Selective expression of the large neutral amino acid transporter at the blood-brain barrier.* Proc Natl Acad Sci U S A, 1999. **96**(21): p. 12079-84.

108. Hawkins, R.A., R.L. O'Kane, I.A. Simpson, and J.R. Vina, *Structure of the blood-brain barrier and its role in the transport of amino acids.* J Nutr, 2006. **136**(1 Suppl): p. 218S-26S.

109. Killian, D.M. and P.J. Chikhale, *Predominant functional activity of the large, neutral amino acid transporter (LAT1) isoform at the cerebrovasculature.* Neurosci Lett, 2001. **306**(1-2): p. 1-4.

110. Shulkin, B.L., A.L. Betz, R.A. Koeppe, and B.W. Agranoff, *Inhibition of neutral amino acid transport across the human blood-brain barrier by phenylalanine.* J Neurochem, 1995. **64**(3): p. 1252-7.

111. Pardridge, W.M., *The role of blood-brain barrier transport of tryptophan and other neutral amino acids in the regulation of substrate-limited pathways of brain amino acid metabolism.* J Neural Transm Suppl, 1979(15): p. 43-54.

112. Palego, L., L. Betti, A. Rossi, and G. Giannaccini, *Tryptophan Biochemistry: Structural, Nutritional, Metabolic, and Medical Aspects in Humans.* J Amino Acids, 2016. **2016**: p. 8952520.

Appendix 1

This thesis is partially based on the following paper:

Title: Intragastric preloads of L-tryptophan reduce ingestive behavior via oxytocinergic neural mechanisms in male mice (2018)

Author Contributions:

Gartner SN: data collection, analysis, interpretation and drafted the manuscript

Aidney F: assisted with data collection

Klockars A: assisted with set up/running of experiments, revised the manuscript

Prosser C: helped conceptualised the studies, revision of manuscript

Carpenter EA: helped conceptualised the studies, revision of manuscript

Isgrove K: assisted with data collection

Levine AS: data analysis and critical revision of the article

Olszewski PK: helped conceptualised the studies, helped draft manuscript and approved the final version